Addressing Immunological Challenges to COVID-19 Vaccination

A thesis submitted to the University of Adelaide

in fulfilment of the Degree of

Doctor of Philosophy

by

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August 2022

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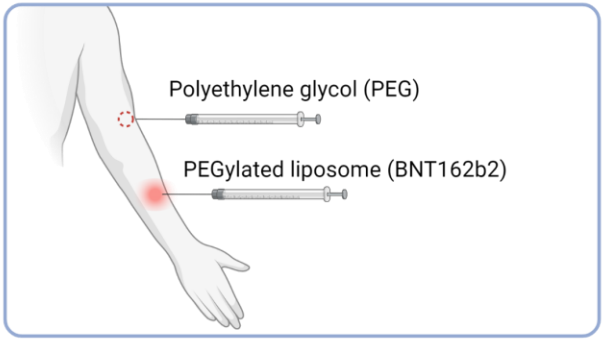
Addressing Immunological Challenges to COVID-19 vaccination

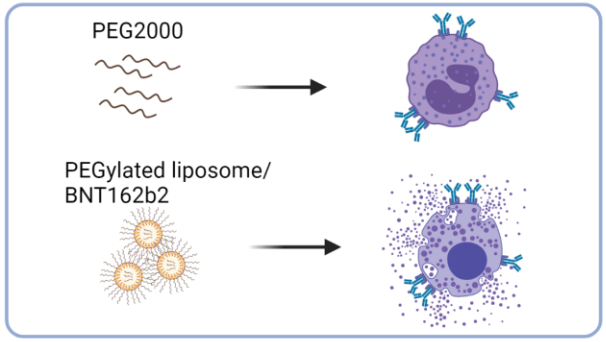
Chapter 2: Allergy to PEGylated Lipid Nanoparticle Vaccines

2.1 Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in patients with PEG allergy



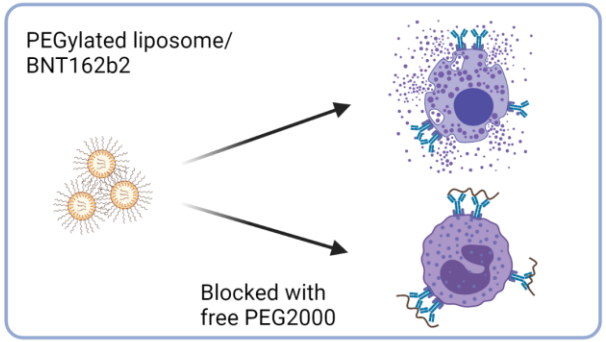
PEG-containing drugs are poor surrogates for allergy testing with BNT162b2 vaccine itself.

2.2 Reply to *In vivo and in vitro* testing with PEGylated nanoparticles



PEG is poorly allergenic in vitro or in vivo unless presented on the surface of a nanoparticle or ingested in high quantities.

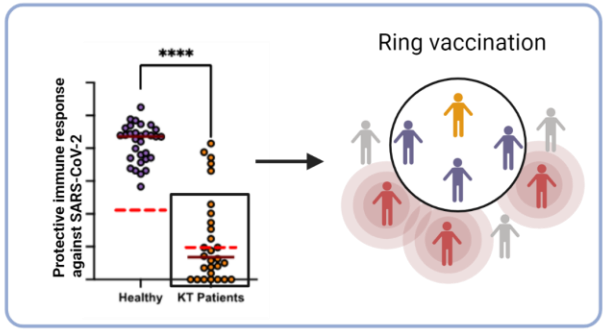
2.3 Chapter 2 extended analysis and discussion



PEG is the inciting allergen in the BNT162b2 vaccine.

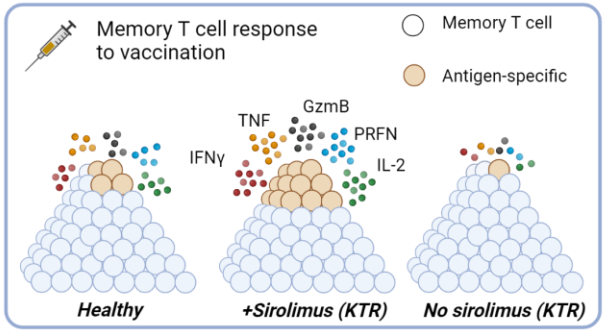
Chapter 3: COVID-19 Vaccination of Kidney Transplant Recipients

3.1 Concurrent vaccination of kidney transplant recipients and close household cohabitants against COVID-19



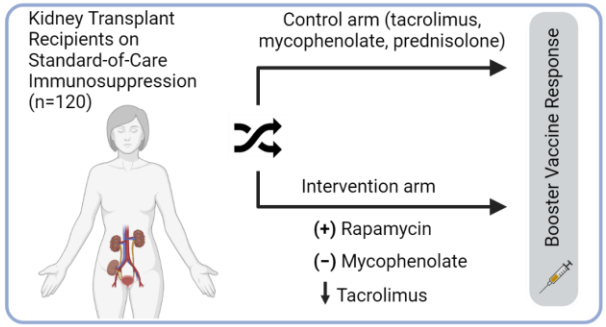
Severely impaired cellular & humoral immunity underlies poor real-world protection afforded KTRs by COVID-19 vaccination.

3.2 mTOR inhibition boosts the formation of functional T cell memory following COVID-19 vaccination of kidney transplant recipients



The first in-human evidence of mTORC1 inhibition as a strategy to boost T cell responses to vaccination.

3.3 Protocol for a randomised controlled trial of immunosuppression modification with rapamycin: the RIVASTIM study



Trial protocol to test rapamycin as an adjuvanting therapy to boost vaccine responses in kidney transplant recipients.

ABSTRACT

The COVID-19 pandemic has spurred the development of several highly effective vaccines, including the BNT162b2 (Pfizer-BioNTech), mRNA-1273 (Moderna), and ChAdOx1 (Oxford-AstraZeneca) vaccines licensed for use in Australia. An unprecedented push to vaccinate the population is underway, however for certain groups that were not represented in clinical trials, further research is needed to inform strategies for safe and effective vaccination. These include immunocompromised patient groups and individuals with allergies to vaccine components.

The BNT162b2 and mRNA-1273 vaccines both consist of an mRNA code delivered in a PEGylated lipid nanoparticle vector. This is a novel format, and mass vaccination has revealed an unusually high incidence of anaphylaxis to these vaccines. Evidence is lacking as to the cause of these reactions, and how to identify and manage at-risk patients. **Chapter 2** outlines the South Australian experience of testing for allergy to COVID-19 vaccines, and defines a cohort of patients with a history of anaphylaxis to polyethylene glycol (PEG) that demonstrate strong hypersensitivity to the BNT162b2 vaccine. Failure of recommended allergy testing protocols, which rely on other PEG-containing medications rather than vaccine itself, to consistently detect vaccine hypersensitivity in these patients led us to investigate the underlying cause. Using a combination of *in vivo* and *ex vivo* testing, we found that unbound PEG is poorly able to induce activation of basophils. However, PEG conjugated to the surface of the lipid nanoparticle is highly allergenic and, as such, *ex vivo* testing with PEGylated liposomal doxorubicin can be used to diagnose PEG allergy and hypersensitivity to the mRNA COVID-19 vaccines in the absence of available vaccine.

Chapter 3 shifts the focus to kidney transplant recipients who have one of the highest COVID-19 mortality rates in the general population. For this reason, transplant recipients were given Phase 1B priority vaccination status in Australia. However, use of

immunosuppressive medications to prevent immune-mediated rejection of their transplanted organ has the potential to reduce vaccine efficacy. This chapter addresses the immunogenicity of the BNT162b2 and ChAdOx1 vaccines in a South Australian cohort of transplant recipients and their close household contacts, and is comprised of three manuscripts. ***Manuscript 3*** highlights profound immune defects in transplant recipients and provides evidence for a ring vaccination strategy to protect this group in the immediate-term. This manuscript is entitled 'Concurrent vaccination of kidney transplant recipients and close household cohabitants against COVID-19'. ***Manuscript 4*** expands the cohort to examine vaccine responses in transplant recipients receiving atypical immunosuppressive drug protocols. We identify and characterise an association between use of the mTOR inhibitor rapamycin and improved vaccine immunogenicity in kidney transplant recipients. This manuscript is entitled 'mTOR inhibitors boost the formation of functional T cell memory following vaccination of kidney transplant recipients'. ***Manuscript 5*** is a protocol paper specifying the design of a multi-centre, randomised, controlled trial to test immunosuppression modification with rapamycin as an intervention to improve vaccine immunogenicity in transplant recipients.

An additional chapter exploring the regulation of IL-10 secretion by B cells is presented as an appendix (Appendix I).

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Griffith Perkins

August 2022

ACKNOWLEDGEMENTS

Firstly, I thank my principal supervisor Professor Toby Coates for his mentorship. Toby has been extraordinarily generous to me during my candidature, and has enthusiastically supported me with opportunity and advocacy. I admire his ethos as a supervisor and hope to carry on these traditions myself in the future.

I would also like to thank my co-supervisors, Dr Christopher Hope and Associate Professor Pravin Hissaria. Chris' infectious fascination with immunology has led to many great conversations, and he has been my go-to counsellor for most things, from experiment design to life advice, for which I am grateful. Although not an official supervisor, Pravin has provided me many interesting opportunities, and I have benefited greatly from his confidence in me.

Above all others I am indebted to my supervisor, and close friend, Dr Plinio Hurtado, for his relentless optimism and patience. Plinio has given me advice every single day (some of it even good advice) and, most importantly, has imparted to me a love for science and research. I consider myself very privileged to have him as a mentor.

I was very lucky to begin my time in the lab with Dr Juewan Kim, Dr Sebastian Stead and Dr Francis Kette, who introduced me to academic life, and who continue to be some of my best friends.

I would like to thank everyone in the Renal Lab, in particular Daniella Penko, Ernesto Hurtado, Svjetlana Kireta, Julie Johnston, Jodie Nitschke, Jacqueline Scaffidi, Denghao Wu, Christopher Drogemuller, and Cheng Sheng Chai. I lucked out with such a kind and generous group.

I have had the opportunity to work with some excellent clinician scientists, including Dr Matthew Tunbridge, Dr Adriana Le, Dr Tania Salehi and Dr Alex Troelnikov, whose input and perspectives have been invaluable.

To my brilliant wife Emily, your unconditional support and encouragement has made the last four years possible.

Lastly, I have the best family that anyone could ask for. Thank you to my parents and my sister, I am grateful for your love and support, and to my in-laws, for your enthusiasm to celebrate every single milestone, no matter how minor.

HONOURS AND AWARDS

2022 Transplant Society of Australia and New Zealand
President's Prize Winner
People's Choice Award
Early Career Researcher Award

2022 The Transplant Society
Travel grant to attend the 29th International Congress of The Transplant Society

2022 Transplant Society of Australia and New Zealand
Travel grant to attend the 13th American Transplant Congress

2021 International Society of Nephrology
Kidney International 2021 Reviewer of the Year

2021 Transplant Society of Australia and New Zealand
Early Career Researcher Award

2019 Transplant Society of Australia and New Zealand
Travel grant to attend the 17th World Congress of the International Pancreas and Islet Transplantation Association

2019 Transplant Society of Australia and New Zealand
Young Investigator Award

2019 American Society of Transplantation
Mentee-Mentor Award

2018 Transplantation Society of Taiwan
Travel grant to attend the Transplantation Science Symposium Asian Regional Meeting

2018 University of Adelaide
Master of Philosophy (No Honours) Scholarship

PUBLICATIONS ARISING

Relating to Chapter 2-

Perkins GB, Troelnikov A, Balouch S, Ahamdie A, Hurtado PR, Hissaria P. Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in patients with PEG allergy. *Journal of Allergy and Clinical Immunology* 2021; 148(1):91-95.
Manuscript 1 of this thesis: a case series identifying the PEGylated lipid nanoparticle as a novel allergen mediating hypersensitivity to the BNT162b2 COVID-19 vaccine and highlighting shortcomings of recommended vaccine allergy testing protocols.

Perkins GB, Troelnikov A, Hissaria P. Reply: *In vivo* and *in vitro* testing with PEGylated nanoparticles. *Journal of Allergy and Clinical Immunology* 2021; 148(3):902-903.
Manuscript 2 of this thesis: a correspondence published in reply to Kelso *et al* evidencing the ineffectiveness of skin testing for the diagnosis of allergy to polyethylene glycol.

Ali S, Perkins GB, Ryoo D, Lee M, Tunbridge M, Yuson C, Smith W, Hissaria P, Le T-T. AstraZeneca ChAdOx1-S COVID-19 vaccine can be safely administered in patients with EDTA allergy. *Allergy, Asthma & Clinical Immunology* 2022; 18(1):1-4.
This study reports on two patients with allergy to ethylenediaminetetracetic acid (EDTA) who tolerated vaccination with the EDTA-containing ChAdOx1 COVID-19 vaccine.

Tunbridge M, Perkins GB, Lee M, Salehi T, Yuson C, Le T-T, Ryoo D, Kette F, Smith W, Gold M, Hissaria P. COVID vaccination can be completed in subjects with a history of allergic reactions to the vaccines or their components – experience from a specialist clinic in South Australia. *Internal Medicine Journal* 2022; 52(11):1884-1890.
A retrospective analysis of the South Australian experience of testing for allergy to COVID-19 vaccines.

Perkins GB, Zuiani J, Tunbridge M, Ryoo D, Ahmadie A, Kette F, Yuson C, Hurtado P, Smith W, Le TT, Hissaria P. PEGylated liposomal medications are highly effective reagents for the diagnosis of polyethylene glycol allergy. Manuscript in preparation.
Assessment of PEGylated lipid nanoparticles for the diagnosis of PEG allergy.

Relating to Chapter 3-

Perkins GB, Tunbridge M, Salehi T, Chai CS, Kireta S, … Hurtado PR, Barry SC, Hissaria P, Grubor-Bauk B, Chadban SJ, Coates PT. Concurrent vaccination of kidney transplant recipients and close household cohabitants against COVID-19. *Kidney International* 2022; 101(5):1077-1080.
Manuscript 3 of this thesis: a prospective study of functional humoral and cellular immune responses to COVID-19 vaccination in kidney transplant recipients and concurrently vaccinated cohabitants.

Perkins GB, Tunbridge M, Salehi T, Chai CS, Kireta S, … Hurtado PR, Chadban SJ, Hissaria P, Barry SC, Grubor-Bauk B, Coates PT. mTOR inhibition boosts the formation of functional T cell memory following COVID-19 vaccination.
Manuscript 4 of this thesis: describes a gain-of-function effect of mTOR inhibition on the formation of functional, antigen-specific T cell memory in response to vaccination, and provides evidence for immunosuppression modification with rapamycin as a strategy to boost vaccine responses in kidney transplant recipients.

Perkins GB, Tunbridge M, Singer J, Salehi T, Ying T, Grubor-Bauk B, Barry SC, Sim B, Hissaria P, Chadban SJ, Coates PT. Rapamycin and inulin for booster vaccine response stimulation (RIVASTIM) – Rapamycin: study protocol for a randomized, controlled trial of immunosuppression modification with rapamycin to improve SARS-CoV-2 vaccine response in kidney transplant recipients. *Trials* 2022; 23(1):1-12.
Manuscript 5 of this thesis: a protocol paper pre-specifying methods, analyses, and outcomes for the RIVASTIM-Rapamycin randomised, controlled trial.

Perkins GB, Tunbridge M, Singer J, Salehi T, Chai CS, Kireta S, Grubor-Bauk B, Hissaria P, Ying T, Chadban SJ, Coates PT. Outcomes of RIVASTIM-Rapamycin: a randomised controlled trial of immunosuppression modification with rapamycin to improve SARS-CoV-2 vaccine response in kidney transplant recipients. Manuscript in preparation.
A randomised, controlled trial of immunosuppression modification as a strategy to boost immunity to a third dose of COVID-19 vaccine in kidney transplant recipients.

Singer J, Tunbridge M, Perkins GB, Salehi T, Ying T, Coates PT, Chadban SJ. Rapamycin and inulin for third dose vaccine response stimulation (RIVASTIM) – Inulin: study protocol for a pilot, multicentre, randomized, double-blinded, controlled trial of dietary inulin to improve SARS-CoV-2 vaccine response in kidney transplant recipients. *BMJ Open* 2022; 12(12):e062747.
A protocol paper pre-specifying methods, analyses, and outcomes for the RIVASTIM-Inulin randomised, controlled trial.

Singer J, Tunbridge M, Perkins GB, Salehi T, Shi B, Chai CS, Kireta S, Hissaria P, Grubor-Bauk B, Ying T, Coates PT, Chadban S. Outcomes of RIVASTIM-Inulin: a randomised, controlled trial of dietary inulin to improve SARS-CoV-2 vaccine response in kidney transplant recipients. Manuscript in preparation.
A randomised, controlled trial of dietary fibre supplementation as a strategy to boost immunity to a third dose of COVID-19 vaccine in kidney transplant recipients.

Relating to COVID-19 vaccination-

Montarello N, Jeffries A, Perkins GB, Wong H, Raith E, Hissaria P, Teo K, Bradley J. Pfizer BNT162b2 COVID-19 vaccine induced fulminant myopericarditis: a case study. Under review by *Critical Care and Resuscitation*.
Immunological workup of a severe case of myopericarditis following COVID-19 vaccination.

Perkins GB, Webb K, Tunbridge M, Chai CS, Kireta S, Yap J, Hurtado P, Coates PT, Hissaria P. Immunogenicity of a third dose of BNT162b2 in multiple sclerosis patients receiving B cell-depleting therapy. Manuscript in preparation.
Analysis of COVID-19 vaccine immunogenicity in patients with multiple sclerosis receiving the B cell-depleting monoclonal antibody ocrelizumab.

Alcheikh A, Perkins GB, Pucar P, Chai CS, Cecchin A, Allen S, Hissaria P, Banovic T, Coates PT, Ross D. Impaired response to COVID-19 vaccination in myelofibrosis patients on JAK inhibitors. Manuscript in preparation.
Investigation of the effect of JAK inhibitors on COVID-19 vaccine responses in myelofibrosis patients.

CONFERENCE PRESENTATIONS

2022 Perkins GB, Tunbridge M, Chai CS, Salehi T, Hope CM, Kireta S, Valtanen P, Hurtado PR, Chadban SJ, Hissaria P, Barry SC, Grubor-Bauk B, Coates PT. mTOR inhibitors promote highly functional T cell immunity in kidney transplant recipients vaccinated against COVID-19. *Oral presentation*, 29th International Congress of The Transplant Society, 10-14 September, Buenos Aires.

2022 Perkins GB, Tunbridge M, Zuiani J, Ryoo D, Ahmadie A, Kette F, Yuson C, Hurtado P, Smith W, Le T-T, Hissaria P. PEGylated liposomal medications are highly effective reagents for the diagnosis of polyethylene glycol allergy. *Highlighted virtual presentation & e-poster*, 32nd Annual Conference of the Australasian Society of Clinical Immunology and Allergy, 30 August – 2 September, Melbourne.

2022 Perkins GB, Tunbridge M, Chai CS, Salehi T, Hope CM, Kireta S, Valtanen P, Hurtado PR, Chadban SJ, Hissaria P, Barry SC, Grubor-Bauk B, Coates PT. mTOR inhibition is associated with an improved immune response to COVID-19 vaccination in kidney transplant recipients. *President's Prize Symposium*, Transplant Society of Australia and New Zealand 40th Annual Scientific Meeting, 19-21 June, Adelaide.

2022 Perkins GB, Tunbridge M, Chai CS, Salehi T, Hope CM, Kireta S, Valtanen P, Hurtado PR, Chadban SJ, Hissaria P, Barry SC, Grubor-Bauk B, Coates PT. Sirolimus use is associated with an improved immune response to COVID-19 vaccination in kidney transplant recipients. *Late Breaking Abstract- Poster*, American Transplant Congress, 4-8 June, Boston.

2021 Perkins GB, Troelnikov A, Tunbridge M, Zuiani J, Ryoo D, Ahmadie A, Salehi T, Hurtado PR, Hissaria P. Presentation of polyethylene glycol (PEG) on lipid nanoparticles increases allergenicity and mediates hypersensitivity to the BNT162b2 COVID-19 vaccine in patients with PEG allergy. *Oral presentation*, Australian and New Zealand Society of Immunology 49th Annual Scientific Meeting, 8-9 December, Virtual meeting.

2021 Perkins GB, Tunbridge M, Chai CS, Salehi T, Yeow AEL, Hope CM, Kireta S, Drogemuller C, Valtanen P, Hurtado P, Chadban S, Barry S, Hissaria P, Grubor-Bauk B, Coates T. Immunogenicity of COVID-19 vaccines in kidney transplant recipients and their close household contacts. *Poster presentation*, 49th Annual Scientific Meeting of the Australia and New Zealand Society of Immunology, 8-9 December, Virtual meeting.

2021 Perkins GB, Troelnikov A, Zuiani J, Tunbridge M, Chai CS, Ryoo D, Ahmadie A, Le T-T, Hurtado PR, Hissaria P. Presentation of polyethylene glycol (PEG) on lipid nanoparticles increases allergenicity and mediates hypersensitivity to the BNT162b2 COVID-19 vaccine in patients with PEG allergy. *Oral presentation*, Adelaide Immunology Retreat, 18-19 October, McLaren Vale.

2021 Perkins GB, Hope CM, Kim J, Coates PT, Hurtado PR. Th1 and Th2 cytokines reciprocally modulate B cell regulatory function. *Oral presentation*, 39th Annual Scientific Meeting of the Transplant Society of Australia and New Zealand, 14-16 March, Virtual Meeting.

2020 Perkins GB, Hope CM, Kim J, Stead SO, Hurtado PR, Coates PT. IL-4 regulates the secretion of IL-10 by human B cells. *Oral presentation & e-poster*, 28th International Congress of The Transplantation Society, 12-16 September, Virtual Meeting.

2019 Perkins GB, Kim J, Coates PT, Hurtado PR. Biphasic IL-10 competence of *in vitro*-expanded human B cells. *Oral presentation,* Adelaide Immunology Retreat, 30-31 August, Handorf.

2019 Perkins GB, Kim J, Hurtado PR, Coates PT. IL-10 competence in human B cells. *Mini-oral presentation*, 17th World Congress of the International Pancreas and Islet Transplantation Association, 2-5 July, Lyon.

2019 Perkins GB, Kim J, Hurtado PR, Coates PT. In vitro expansion of IL-10 competent human B cells. *Oral presentation*, 38th Annual Scientific Meeting of the Transplant Society of Australia and New Zealand, 28-31 July, Sydney.

2019 Perkins GB, Hurtado PR, Coates PT. IL-4 regulates IL-10 secretion by human B cells. *Oral presentation*, International Transplant Science Meeting, 10-13 November, Clearwater Beach.

2019 Perkins GB, Kim J, Coates PT, Hurtado PR. Acquired IL-10 competence in expanded human B cells. *Oral presentation*, 48th Annual Meeting of the Australia and New Zealand Society for Immunology, 8-12 December, Adelaide.

2018 Perkins GB, Hurtado PR, Coates PT. IL-10 production by *in vitro*-expanded, human B cells. *Oral presentation*, Transplantation Science Symposium Asian Regional Meeting, 23-25 November, Taipei.

CHAPTER 1:

LITERATURE REVIEW

1.1. Introduction

Health impact of COVID-19. Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 is thought to have originated by zoonosis in Wuhan, China, in December 2019, and has led to an ongoing global pandemic (1). To-date (February 2022), more than 437 million cases have been reported worldwide, with 5.95 million confirmed COVID-19 deaths (Johns Hopkins Centre for Systems Science and Engineering)(2). These numbers are an underrepresentation as they account only for confirmed cases. Total deaths (any cause) are more reliably reported globally, and sources, including the Economist (using data compiled and curated by Johns Hopkins University), are able to estimate the total number of 'excess deaths' that have occurred since the pandemic began. This is the cumulative number of deaths above that which would be expected in the absence of a pandemic, and accounts for undiagnosed or unreported deaths resulting directly from COVID-19, as well as excess deaths due to the crisis at large, resulting, for example, from increased rates of poverty and overburdening of the health system. As of February 28, 2022, an estimated excess of 19.88 million deaths have occurred globally since January 1, 2020 (3).

Australia has recorded 3.21 million confirmed cases, and 5,210 deaths from COVID-19 (as of February 28, 2022). Up to the end of 2021, excess deaths were negative (3), likely due to effective virus suppression and increased public hygiene measures; deaths from influenza infections dropped from 953 in 2019 (pre-pandemic measures), to 37 in 2020, and no deaths reported (up to November 7) in 2021 (4). Following opening up of borders and relaxation of restrictions towards the end of 2021, excess deaths have risen to 4,262 (3).

COVID-19 disease. Of those diagnosed with symptomatic COVID-19, the most frequent symptoms are fever, cough, tiredness, and loss of taste or smell (5). Severe forms of the disease occur in a small proportion of individuals, most commonly manifesting as acute

respiratory distress syndrome and respiratory failure (6). Severe disease results from damage to the respiratory system caused by a state of hyper-inflammation called a cytokine storm, which is associated with increased age and comorbidities (7, 8). Why this hyperactive immune response occurs in some individuals is the subject of intense research in order to better enable prediction and treatment of those that develop severe disease. Evidence of infected individuals experiencing ongoing, long-term effects of the disease, dubbed 'long COVID', has been building as the pandemic continues (9). Long COVID remains broadly defined and encapsulates a wide range of ongoing symptoms including fatigue, malaise, and cognitive dysfunction, which may have an immune basis (10). Viral infections are well-recognised as triggers for a large number of immune-mediated disorders, and while some of these links are clear, for example acute rheumatic fever following *Streptococcus pyogenes* infection, others required large scale longitudinal studies to confirm (see (11) on the link between Epstein-Barr virus and multiple sclerosis). Pandemics such as this one provide the opportunity to better understand these associations (12).

SARS-CoV-2. There are several coronaviruses that are pathogenic in humans. Most cause common cold symptoms at most, however there have been two notable exceptions in recent history; SARS-CoV-1 and MERS-CoV were the causative agents of the 2002-03 and 2012 SARS and MERS outbreaks, respectively. SARS-CoV-2 shares ~79% and ~50% nucleotide sequence identity with SARS-CoV-1 and MERS-CoV, respectively (13, 14). Upon inhalation, SARS-CoV-2 binds to epithelial cells of the upper respiratory tract via the receptor angiotensin-converting enzyme 2 (ACE2). By comparison with SARS-CoV-1, SARS-CoV-2 has a 4.2 - 20.4% greater affinity for the ACE2 receptor, which is thought to account for its greater transmissibility (15-17). The ACE2 receptor is expressed on many tissues, and SARS-CoV-2 tropism for lung and respiratory tissues is thought to be the result of co-expression of the TMPRSS2 enzyme, which acts to prime the spike glycoprotein for cellular entry (18, 19). If the virus is not cleared from the upper

respiratory tract by the immune system, the virus migrates to the lower airways and undergoes rapid replication in the alveolar epithelial cells of the lungs (20).

Transmission. SARS-CoV-2 is primarily transmitted between humans via respiratory droplets (generated by coughing, sneezing, and to a lesser extent, by singing). The minimum infectious dose required to establish infection has been suggested to be as low as 100 virions, resulting in an approximate infective range of 1.8 metres, although health advice has varied on social distancing recommendations (21). In addition to respiratory droplets, it is now recognised that COVID-19 can be transmitted via exhaled aerosols (22, 23). Aerosols are droplets smaller than 5 μm in diameters, which enables them to persist in air currents rather than fall to the ground with gravity. This potential route of transmission initially went unrecognised by regulatory bodies, resulting in a lack of considered ventilation and several notable transmission events between rooms in hotel quarantine in Australia (24). About one third of those who contract SARS-CoV-2 remain asymptomatic, which has contributed to its spread (25). The proportion of asymptomatic infections is likely to increase with the emergence of the Omicron variant (26).

Variants of concern. The global spread of the SARS-CoV-2 virus has resulted in considerable genetic diversification (27). While the majority of mutations acquired are non-functional or detrimental to the virus, a series of viral variants have emerged over the course of the pandemic due to acquisition of genetic mutations, or combinations of mutations, that increase fitness relative to the predominant strain at the time (27). In order to focus research and public health efforts, the World Health Organisation, in collaboration with national authorities, tracks emerging strains and may designate them as variants of interest (VOI) or variants of concern (VOC). VOI are defined as variants:

> *"with genetic changes that are predicted or known to affect virus characteristics such as transmissibility, disease severity, immune escape, diagnostic or therapeutic escape; AND identified to cause significant community transmission or multiple COVID-19 clusters, in multiple countries with increasing relative prevalence alongside increasing number of*

> *cases over time, or other apparent epidemiological impacts to suggest an emerging risk to global public health."*

VOC are defined as VOI possessing genetic mutations that result in an:

> *"increase in transmissibility or detrimental change in COVID-19 epidemiology; OR increase in virulence or change in clinical disease presentation; OR decrease in effectiveness of public health and social measures or available diagnostics, vaccines, therapeutics."*

In addition to the standard genetic lineage identifiers routinely employed in viral research, VOC are designated letters of the Greek alphabet to assist with public communication and discourse (28). The VOC lineages to-date are presented in Table 1.1.

TABLE 1.1.1. SARS-CoV-2 variants of concern (28)

WHO identifier	Lineage	Earliest documented isolate	Date of VOC designation	Notable mutations	Gain-of-function
Alpha	B.1.1.7	Sep 2020, United Kingdom	18-Dec-20	N501Y*	40-70% increase in transmissibility (29)
Beta	B.1.351	May 2020, South Africa	18-Dec-20	N501Y* E484K# K417N*#	Impaired vaccine-induced neutralisation (30, 31)
Gamma	P.1	Nov 2020, Brazil	11-Jan-21	N501Y* E484K# K417T*# L18F#	Greatly impaired vaccine-induced neutralisation (32)
Delta	B.1.617.2	Oct 2020, India	11-May-21	T478K P681R L452R*#	Increase in transmissibility & impaired vaccine-induced neutralisation (31, 33)
Omicron	B.1.1.529	Nov 2021, multiple	26-Nov-21	Multiple and novel	Greatly impaired vaccine-induced neutralisation (34)

*Increased receptor affinity (35); #Immune escape (36, 37)

Vaccines. The pandemic has been met with the development of several highly effective vaccines against COVID-19. Most vaccines approved to date employ the SARS-CoV-2 spike glycoprotein as the target antigen. The ChAdOx1 vaccine, developed by the University of Oxford and produced by AstraZeneca, was the first vaccine approved for use in Australia by the Therapeutic Goods Administration (TGA), followed by BNT162b2 (Pfizer-BioNTech) and, more recently, mRNA-1273 (Moderna). All three vaccines are novel in that they are principally comprised of nucleic acids, which are translated into spike glycoprotein by the host, rather than delivering the protein directly, as has been done up to now. The ChAdOx1 vaccine is comprised of DNA within a chimpanzee adenovirus vector. The viral vector enters host cells at the site of injection, or in draining lymph nodes, and delivers DNA into the cell nucleus, which is transcribed as mRNA and translated into protein by host machinery (38). By contrast, the BNT162b2 and mRNA-1273 vaccines are comprised of mRNA encapsulated in a lipid nanoparticle. Lipid nanoparticles are endocytosed by host cells, and release mRNA into the cell cytoplasm, which is translated by host ribosomes into spike protein (38). The SARS-CoV-2 spike is a glycoprotein, and is post-translationally glycosylated. While both the lipid nanoparticle and viral vector vaccines likely gain entry to a number of cell types, it is dendritic cells at the site of injection (Langerhans cells) and in draining lymph nodes which are the most significant target cell type (39, 40). As professional phagocytes, dendritic cells are presumed to take up a significant proportion of the vaccine vector, which enables direct antigen-presentation to T cells in secondary lymphoid tissue. Endogenous antigen expression by the dendritic cells likely results in high MHC occupancy by spike-derived peptides, and this has been suggested as the reason for the immunogenicity observed for mRNA vaccines (40). To what extent antigen that is produced by other cell types contributes to the immune response is unknown, however the germinal centre response classically requires B cells to encounter extracellular soluble antigen in the lymph node follicle, suggesting some shedding or release of spike protein from host cells. Interestingly, while the BNT162b2 vaccine encodes a membrane-bound form of the spike glycoprotein, a second candidate, BNT162b1, encoded a secreted trimeric receptor-binding domain construct, and induced similar antibody

responses in phase I trials (41). BNT162b2 was selected over BNT162b1 on the basis of fewer adverse events in phase I trials (41).

Goal of vaccination. The immune response to these vaccines has now been extensively characterised, with an initial focus on antibody response (42-48). The goal of most vaccines against infectious pathogens is to elicit a strong antibody response, and antibody titre is the measure of efficacy used most commonly in early clinical trials and ensuing clinical studies as a correlate for real-world effectiveness. Subsequent Phase 4 trials are able to analyse the effect of vaccination on prevention of transmission, hospitalisation, and death in the community.

TABLE 1.1.2. COVID-19 vaccines available in Australia February 2022*

VACCINE	DEVELOPER/ MANUFACTURER	PLATFORM	VECTOR	ANTIGEN	DATE OF INITIAL APPROVAL	DOSING (PRIMARY SERIES)	APPROVED FOR 3rd DOSE	SERIOUS ADVERSE EFFECTS
ChAdOx1/ AZD1222/ Vaxzevria	Oxford-AstraZeneca	DNA	Replication deficient chimpanzee adenovirus	SARS-CoV-2 Spike	18+ years old: February 2021	2 doses, 4-12 weeks apart***	Yes, if mRNA vaccines contraindicated	Thrombosis with thrombocytopenia
BNT162b2/ Comirnaty	Pfizer-BioNTech	mRNA	PEGylated lipid nanoparticle	SARS-CoV-2 Spike	16+ years old: January 2021; 12+ years old: July 2021; 5+ years old: December 2021**	2 doses, 8 weeks apart	Yes	Anaphylaxis Myo/pericarditis
mRNA-1273/ Spikevax	Moderna	mRNA	PEGylated lipid nanoparticle	SARS-CoV-2 Spike	18+ years old: August 2021; 12+ years old: September 2022; 6+ years old: February 2022**	2 doses, 8 weeks apart	Yes	Anaphylaxis Myo/pericarditis
NVX-CoV2373/ Nuvaxovid	Novavax	Protein subunit	Lipid nanoparticle	SARS-CoV-2 Spike	18+ years old: January 2022	2 doses, 3 weeks apart	Yes, if mRNA vaccines contraindicated	Possible myocarditis

*Janssen received provisional approval in Australia in June 2021, however is not included in Australia's vaccination program; **half-dose (15 μg); ***Initial 4 week interval recommendation adjusted following indications of greater efficacy with 12 weeks interval (enabled greater first dose coverage with limited vaccine supply)(49)

The remarkably high antibody titres elicited by the mRNA-platform vaccines initially suggested the possibility of achieving sterilising immunity (45). Sterilising immunity refers to the suppression of virus transmission within the population; most vaccination programs aim to prevent disease without necessarily preventing virus transmission, and this is the basic criterion used by regulatory bodies such as the Food and Drug Administration in the United States for emergency use authorisation of the COVID-19 vaccines (50). Following vaccination, antibodies passively exist in circulation and at mucosal sites and bind to and neutralise virus before it can enter host cells. As viruses are obligate parasites requiring access to host cells to replicate, an effective neutralising antibody response can prevent the establishment of infection thereby blunting any chain of transmission. Sterilising immunity was considered a plausible goal for SARS-CoV-2 because early trials demonstrated protection against asymptomatic infection and betacoronaviruses do not have high mutation rates that enable other viruses like influenza to evade antibody-mediated immunity generated by past infection or vaccination each year (51). However, sterilising immunity against respiratory viruses is uncommon, even in the absence of high mutation rates. Despite the effective antibody responses to respiratory viruses such as measles and smallpox (variola), past exposure only protects against disease and does not prevent reinfection of mucosal sites (52, 53). These, and other examples, indicate that effective, long-term protection from transmission is rare for mucosal viruses, and may be limited to viruses that rely on blood viremia for their spread (54). Additionally, while SARS-CoV-2 has not demonstrated the plasticity of hyper-mutable viruses like influenza, several variants have emerged with mutations in the spike glycoprotein that reduce the efficacy of vaccine-induced neutralising antibodies (notably beta and omicron)(55). As a result, protection from infection provided by past infection or booster vaccination is limited in duration, and SARS-CoV-2 is likely to persist in the population as an endemic virus. While 'breakthrough' infections have become more common, shading the public perception of the usefulness of vaccination, vaccines continue to be incredibly effective at preventing severe disease and hospitalisation (56).

Immunological hurdles. Our inability to achieve sterilising immunity through vaccination leaves certain sectors of society vulnerable: 1) those who cannot be vaccinated, 2) those for whom the vaccines do not induce a protective immune response, and 3) those who either cannot access a vaccine or refuse vaccination. The first two categories have an immunological basis and will be discussed in this review. Principle amongst these are individuals at-risk of adverse reactions to one or more of the vaccines, and immunocompromised patients who are not able to mount a protective immune response to vaccination. Relevant to this thesis, those with a history of allergy to vaccines or a component of the vaccines under investigation, and transplant recipients and other immunosuppressed groups, were excluded from phase I-III clinical trials of all COVID-19 vaccines. Furthermore, individuals with any allergic history at all (relevant or otherwise) were excluded from the BNT162b2 trials (45, 57, 58). In the face of a pandemic, these minority patient groups are not considered in vaccine clinical trials, and it falls on local medical and regulatory bodies, and often individual treating physicians, to manage these scenarios while research and policy catch up.

1.2. Hypersensitivity to mRNA-platform vaccines

Anaphylaxis to BNT162b2. The BNT162b2 COVID-19 vaccine is the first mRNA-platform vaccine ever approved for human use. Its success opens the doors for a new generation of vaccines against infectious diseases, cancers, gene therapies and beyond. The major advantage of mRNA over protein is the ease and rapidity at which novel targets can be encoded. This will enable the production of personalised cancer vaccines that encode for patient-specific mutated protein antigens, and one group has already applied this technology for the endogenous bioengineering of CAR T cells in mice, removing the need for the isolation, external manipulation, and re-infusion of cells (59). However, since its emergency use approval in August 2020 by the United States Food and Drug Administration (FDA; provisional approval followed in January 2021 by the TGA for use in Australia) a higher-than-expected incidence of systemic anaphylactic

reactions to the vaccine has been reported. The incidence of anaphylaxis meeting the Brighton Collaboration case definition criteria for anaphylaxis, reported to the CDC by clinicians in 2020 via the Vaccine Adverse Event Reporting System (VAERS), was 11.1 cases per million, compared with the standard 1 or fewer cases per million recognised for other common vaccines (60-64). This was revised to 4.7 cases per million in Jan 2021 (65). A prospective, single centre evaluation of employees at Mass General Brigham hospital in March 2021 reported a significantly higher incidence of 247 cases of anaphylaxis per million (66). The cause of these reactions is unknown and has led to uncertainty amongst clinical staff about vaccinating at-risk patient groups, and to vaccine hesitancy amongst the public. Both the CDC VAERS system and the prospective study at Mass General Brigham reported an overrepresentation of females (90% and 94%, respectively) and of individuals with a history of allergy (81% and 63%, respectively) or anaphylaxis (33% and 31%, respectively) amongst those who experienced anaphylaxis after 1 dose of an mRNA-platform vaccine (66).

Mechanism of anaphylaxis. There has been some debate as to the mechanism underlying these reactions. Anaphylaxis is typically a systemic chain reaction that occurs via a type I hypersensitivity reaction ('true allergy'). Type I hypersensitivity results from an inappropriate skewing of the immune response towards a T helper 2-type (Th2) response to an antigen, normally referred to as an allergen. It is unclear what drives this skewing, however many common allergens are enzymes and have been found to directly influence antigen-presentation and cytokine production (67). The Th2 response induced in response to allergens is distinct from the normal Th2 response that evolved to fight helminth infection, in that it lacks negative regulatory elements and favours high levels of interleukin (IL)-13 production (68). IL-13 and IL-4 in germinal centres drive class switch recombination of B cells to IgE. Allergen-specific IgE secreted by antibody-forming cells is taken up by basophils in circulation and mast cells in barrier tissues, and presented on the cell surface bound by the high-affinity receptor for IgE (FcεRI). Upon secondary exposure to the allergen, cross-linking of surface IgE on mast

cells and basophils results in degranulation of preformed cytoplasmic granules containing leukotrienes, histamine and vasoactive factors. The rapid release of these factors drives a local inflammatory response characterised by recruitment and infiltration of immune cells to the exposure site. This response can precipitate the systemic release of inflammatory mediators resulting in anaphylaxis. Anaphylaxis is characterised by urticaria and respiratory difficulty and/or hypotension, and can be fatal (69).

Despite significant atopy (genetic predisposition to allergies) amongst those who have reacted to the BNT162b2 vaccine, the high incidence of reactivity upon first exposure to the vaccine has raised doubts as to the underlying mechanism (61). Type I hypersensitivity classically requires an initial priming exposure, and an alternative mechanism of complement-activation related pseudoallergy (CARPA) has been proposed to account for some or all of the anaphylactic reactions to the vaccine (70). CARPA is a recently described phenomenon that has been proposed to account for other observed infusion reactions that have been reported upon infusion of patients with medications, including lipid nanoparticles (71). While not fully understood, infusion reactions are not immunoglobulin (Ig) E mediated and thus do not require a priming event to sensitise basophils and mast cells. Rather, CARPA reactions likely occur via the stable or semi-stable deposition of IgG on a solid-phase surface (72). This could be the surface of infused red blood cells, or factors deposited on the semi-permeable membrane of dialysis machinery (73). In the case of lipid nanoparticles, antibody deposition on the surface of the nanoparticle may precipitate complement activation (Figure 1.2.1). During the complement cascade, complement components, particularly C3 and C5, are cleaved releasing anaphylatoxins (C3a and C5a). Formation of anaphylatoxins is part of a normal inflammatory response, e.g. at a site of infection, wherein antibodies against conserved or previously encountered antigens trigger the complement cascade. Anaphylatoxins non-specifically induce degranulation of local mast cells via complement receptors, which drives rapid local inflammation. Thus, complement activation could conceivably

account for inflammation in response to vaccination in patients with pre-existing IgG specific to components of the lipid nanoparticle. Notably, intramuscular vaccine administration is quite different to intravenous infusions during which CARPA reactions have been investigated. Other receptors recognising components of the vaccine nanoparticle, such as pattern recognition receptors, could also play a role in the degranulation response. While it is unclear by what mechanism these reactions are occurring in response to the mRNA-platform vaccines, polyethylene glycol is suspected as being the inciting antigen.

Polyethylene glycol. mRNA is highly unstable and has a short half-life in the body (74). Therefore, the BNT162b2, as well as the mRNA-1273, mRNA platform vaccines employ a lipid nanoparticle conduit to transport mRNA. Similar to lipid nanoparticles already in use in the cancer chemotherapy space, these are decorated with polyethylene glycol (PEG) macromolecules to improve pharmaco-dynamic properties and to reduce renal clearance (75). PEGylation of lipid nanoparticles and proteins further prevents aggregation by improving water solubility, and reduces immune activation and opsonisation, potentially via steric hindrance of antibody and receptor binding (75). PEG is a polyether described by the chemical formula $(O\text{-}CH_2\text{-}CH_2)_n\text{-}OH$, where a range of molecular weights can be achieved by polymerisation. As a non-toxic, flexible, water-soluble polymer, PEG is widely used in cosmetics and toothpastes, and PEG-reactive IgM and IgG antibodies are commonly found across the population (76). It is also used in hydrogels for cell encapsulation, plasmid DNA precipitation, to facilitate hybridoma fusion, and in archaeology to preserve wooden underwater salvages by replacing water molecules within the structure (77-79). PEG is also used to protect excavated artefacts from the air including the paint on the famous Terracotta Warriors (80).

PEG allergy. Allergy to PEG-containing medications is not a new concept, however 'PEG allergy' has struggled to achieve widespread recognition due to variability in allergy testing outcomes (81). Patients generally present having had a reaction to a range

of unrelated products that contain PEG. Anaphylactic reactions most commonly occur in response to significant doses of PEG-containing medications, often bowel-prep agents such as MOVICOL® in which PEG is the active, and often sole, ingredient. It is common for patients with a strong history of PEG hypersensitivity to test negative to PEG by skin testing, or to react to some (generally higher) molecular weight PEGs, but not other molecular weights (81). Complexity in PEG allergy diagnosis is further exacerbated by changes in reactivity of patients over time; patients that are skin test positive will commonly become negative following avoidance of PEG-containing products (81). It may be hypothesised that this variability in response is related to PEG not being a protein antigen and, as such, an unequivocal reaction may rely on high epitope density, high concentration of reactive antibodies in tissue or bound to mast cells (which wanes in those who avoid allergen-containing products), and/or other stabilising factors in the body.

Bigini *et al.* recently made the interesting observation that females are overrepresented amongst those with pre-existing PEG IgM and IgG (80% and 100% if considering just those with high titres) (82, 83), which is similar to the representation of females amongst those experiencing anaphylaxis to the BNT162b2 vaccines. While this is a noteworthy association, there is a higher prevalence of anaphylaxis in general in females, including to non-mRNA vaccines, and it might be speculated that increased anti-PEG IgM and IgG could result from increased exposure of females to PEG-containing compounds, such as cosmetics (66).

Evidence for PEG as the inciting allergen in vaccine anaphylaxis comes mainly from a few case reports of patients who had reactions to an mRNA-platform vaccine and were subsequently skin test positive to PEG (84, 85). Therefore, it remains unclear to what extent patients with PEG allergy are representative of those who have had an anaphylactic response upon administration of the vaccine. Similarly, there are reports of patients testing positive to the vaccine but not to PEG, and of patients who have had a

reaction to the first dose of vaccine who are then negative to skin testing with PEG and/or the vaccine itself (61, 85). Therefore, it remains unclear whether PEG is the inciting allergen in cases of anaphylaxis to the vaccines, and it is possible that there is a range of mechanisms underlying the reactions, which may include both IgE-mediated and CARPA reactions. The incidence of vaccine reactions in patients with classical PEG allergy is likely skewed as patients who are skin test positive to PEG, or have a convincing history of PEG allergy, at screening will rarely receive a PEG-containing vaccine.

Notably, cases of cross-reactivity between PEG and polysorbate 80 (PS80; tween-80) have been reported (86). This is of particular significance in Australia, because the only alternative to a PEGylated vaccine is ChAdOx1, which contains PS80 (86).

Testing for hypersensitivity. Due to the immediate nature of type I hypersensitivity reactions, 15 minute monitoring periods after vaccination have been established in case of anaphylaxis. With little known about the cause of these reactions, stratification of those who are at-risk of anaphylaxis to the vaccine has been a challenge for allergists globally. Skin-testing protocols have been suggested to manage patients with a history of atopy resulting in anaphylaxis, particularly with a history of reactivity to PEG-containing substances (87). Skin testing at most centres is based on a range of PEG-containing compounds, as the vaccine is not widely available for this purpose. Patients are first subjected to skin prick testing (SPT), wherein the compounds are introduced into the epidermis by skin prick, and are monitored for the development of wheals over 20-30 minutes. If SPT is negative or equivocal, intradermal testing (IDT) is performed wherein an amount of the substance is injected intradermally.

A laboratory equivalent to *in vivo* testing is a significant goal of clinical allergy research. *Ex vivo* measurement of basophil activation in whole blood in response to allergens is one such possibility. Cross-linking of surface IgE on circulating basophils induces

calcium flux, which results in the rapid fusion of pre-formed granules with the plasma membrane, releasing granule contents into the extracellular environment. Degranulation can be measured by flow cytometry because molecules on the internal face of the bi-lipid granule membrane, including CD63, are exposed on the cell surface when the vesicle fuses with the plasma membrane. Degranulation of a basophil is an all or nothing process, and the percentage of $CD63^{+}$ basophils following stimulation of whole blood with an allergen of interest has been trialled as a diagnostic assay and measure of allergen desensitisation (88).

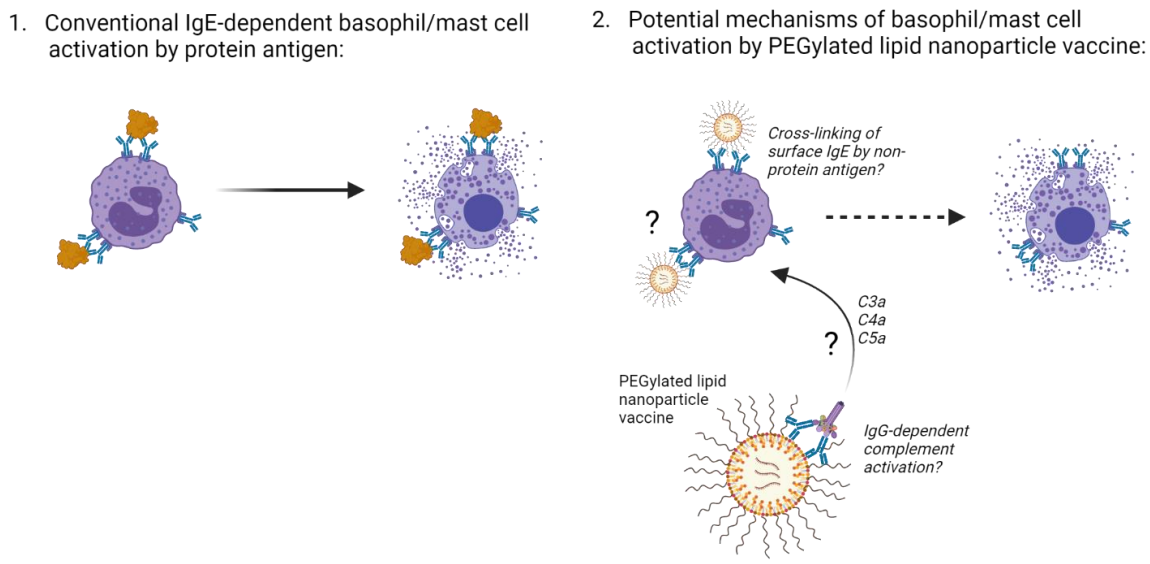


Figure 1.2.1. Potential mechanisms of basophil/mast cell activation in response to PEGylated lipid nanoparticle COVID-19 vaccines. Panel 1: The standard mode of basophil or mast cell activation in response to a protein antigen. Basophils/mast cells express the high-affinity IgE receptor (Fc epsilon RI, $FCER_1$) on the cell surface and sequester polyclonal IgE antibodies on the cell surface. Recognition and binding of multiple epitopes, resulting in IgE cross-linking, triggers an intracellular signalling cascade. Pre-formed cytoplasmic granules are mobilised and fuse with the plasma membrane, releasing the contents of the granules into the extracellular environment in a process called degranulation. A large variety of mediators are released, including vasoactive and chemotactic factors, which drive a local inflammatory response. Panel 2:

Polyethylene glycol (PEG) on the surface of the lipid nanoparticle has been suggested as a vaccine component that may be able to induce degranulation, however it is uncommon for non-protein antigens to cross-link IgE. Mast cell and basophil activation can be triggered by a variety of alternative stimuli, including bi-products of the complement cascade called anaphylatoxins (C3a, C4a, C5a). Deposition of PEG-specific IgG on the nanoparticle surface, and subsequent activation of the complement cascade, may therefore represent an alternative pathway of antigen-specific degranulation. A process similar to this involving complement activation, potentially in concert with ligation of G-protein coupled receptors (GPCRs) and pattern recognition receptors (PRRs), has been described in the setting of infusion reactions and labelled complement-activation related pseudo allergy (CARPA). Created with BioRender.com.

Outstanding questions:

1. How effective are recommended skin testing protocols for detecting hypersensitivity to the COVID-19 vaccines?
2. Is polyethylene glycol the inciting allergen in hypersensitivity to the PEGylated lipid nanoparticle vaccines?
3. By what mechanism do these vaccines induce hypersensitivity/anaphylaxis?

1.3. Vaccination of transplant recipients

Transplant recipients are a particularly vulnerable group to COVID-19. The 28-day case mortality rate for kidney transplant recipients is 24%, increasing significantly with age to almost 50% in those 75 years and older (89). This high mortality is likely related to underlying comorbidities more than to the use of immunosuppressive drugs as similar rates are seen in dialysis patients awaiting transplantation (90, 91). Given this vulnerability, transplant recipients and other immunocompromised groups were prioritised for vaccination in Australia. However, as with allergic individuals, transplant recipients were excluded from vaccine clinical trials given the possibility that the immunosuppressive medications that transplant recipients receive to prevent graft rejection might impair vaccine immunogenicity.

Efficacy of influenza vaccines in KTRs. Transplant recipients will receive most of their vaccines in childhood, prior to receiving their transplant. Flu vaccines however are administered annually, and there is a lack of clarity as to their efficacy in transplant recipients. In 2009/10, Mulley *et al*. measured seroconversion of 151 transplant recipients and 30 healthy controls following vaccination against the H1N1 influenza pandemic (92). They reported detectable vaccine-induced IgG antibodies in just 32% of transplant recipients compared with 77% of healthy controls. Previous studies assessing seroconversion to seasonal influenza vaccines found similar results, with mycophenolate use associated with reduced seroconversion (93-96). However, other studies have found comparable seroconversion to healthy controls (97, 98). This variability may be due to the additive effect of annual vaccinations contributing to improved seroresponse in patients from centres that strongly encourage annual vaccination; studies finding impaired seroconversion tend to be comparing responses to a pandemic strain vaccine to which past annual vaccines would provide less cross-protection. Failure to seroconvert to vaccination is commonly tackled by administration of additional booster doses (classically an issue in hepatitis B vaccination of dialysis

patients). The standard of care at the Royal Adelaide Hospital is a single annual flu vaccination for transplant recipients.

Immune basis of vaccination. The basis of vaccination is to expose the immune system to a harmless form of a pathogen in a way that will generate appropriate immunological memory. Should the immune system then encounter the wild type pathogen, it is equipped to mount a faster, larger and more specific response. The means by which this immunological memory occurs is through antigen receptor diversity and clonal selection. Cells of the adaptive immune system, T and B lymphocytes, possess an antigen receptor generated by the stochastic recombination of gene segments, giving rise to an enormous range of specificities. The theory of clonal selection, first put forward by the Australian immunologist Sir MacFarlane Burnet, submits that productive encounter of an antigen (or antigenic epitope) by a cognate lymphocyte (a T or B cell expressing an antigen receptor specific to that epitope) will result in the outgrowth of a clonal population able to recognise that same, relevant specificity. However, mounting an effective response from a handful of reactive cells takes time. Importantly, this population will not only produce an acute effector response, but will give rise to cellular memory. Memory lymphocytes persist in circulation, or in relevant tissues, at frequencies that are orders of magnitude greater than their naïve precursors (99-101), and are able to respond much more rapidly and robustly (and in the case of B cells, with a higher affinity) to reencounter of their cognate antigen. The defining features of memory lymphocytes are their capacity for self-renewal or long-term persistence, and a lowered requirement for licensing as they possess an antigen receptor specificity that has previously passed necessary immune checkpoints to avoid selection for autoreactive cells.

In the T cell compartment, effector memory and central memory populations form. Effector memory cells (T_{EM}) are pre-programmed to carry out effector functions upon antigen encounter without the need for further differentiation. While effector memory

T cells can be extremely long-lived, central memory (T_{CM}) cells are classically considered to provide life-long memory as they have the capacity for self-renewal at steady-state. Central memory cells are so named because they recirculate back through secondary lymphoid organs, and, as such, are well placed for antigen-encounter and provision of B cell help. The major T cell subpopulations are classically defined based on the markers CD45RA and CD27 or CCR7. This strategy defines a third major population of memory T cells, T effector cells re-expressing CD45RA (T_{EMRA}). Cells of a T_{EMRA} phenotype accumulate with age and associate with chronic infection and, as such, are classically considered to be exhausted antigen-experienced cells (102, 103). However, more recent work has identified considerable functional heterogeneity within the T_{EMRA} compartment, and T_{EMRA} frequency is associated with protection against severe disease from influenza infection (104).

In the B cell compartment, memory is provided by long-lived plasma cells, which reside in specialised niches in the bone marrow and lamina propria, and memory B cells, which recirculate and can migrate into tissues. Similar to memory T cells, memory B cells are less inhibited than naïve cells and rapidly differentiate into antibody-forming cells in response to antigen encounter or pattern recognition receptor (PRR) engagement. Memory B cells in humans express high levels of toll-like receptor 9 (TLR9), and recognition of circulating microbial DNA derived from heterotypic infection or commensal bacteria by memory B cells is thought to contribute to the maintenance of steady-state antibody titres in the serum (105).

While clonal selection provides a mechanism for developing immunity to unknown antigens, deciding whether or not to mount a response (or whether to mount a tolerogenic versus inflammatory response) is equally important. Recognition of a 'foreign' antigen does not necessarily result in an inflammatory response. In fact, most antigens encountered belong to self or to commensal microbiota, and an inflammatory response would result in detrimental outcomes such as autoimmunity. Therefore,

immune checkpoints exist which must be overcome for the licencing of a T or B cell in response to cognate antigen. This need for additional signals was first recognised by Charles Janeway in his now famous introductory address for the Cold Springs Symposium in Immunology in 1974 (106). Janeway pointed out the obvious, but often overlooked, fact that in order for immunisation of an animal against a protein antigen to be productive, the preparation needed to include dissociated bacterial components (106). Janeway hypothesised the existence of germline-encoded receptors that could recognise conserved pathogen-associated molecules in order to discern self from non-self, or dangerous from innocuous. We now know these to be toll-like receptors (TLR), and, more broadly, pattern-recognition receptors (PRR). Recognition of pathogen-associated or damage-associated molecular patterns by antigen-presenting cells results in the expression of co-stimulatory molecules that provide a second signal to T cells in the context of antigen presentation. Microbial and environmental factors at the site of antigen-presenting cell (APC) activation further provide information to direct specialisation of the immune response; this is often provided to T cells in the form of cytokines secreted into the secondary lymphoid organ (SLO) microenvironment, and directs T cell polarisation and chemotaxis. Modern vaccine are therefore administered with adjuvants that provide these additional signals required for a productive immune response. Adjuvants may also promote a more favourable response in other ways, for example by promoting the formation of immune memory in preference to short-lived effector cell formation, which is an unnecessary and potentially detrimental outcome of vaccination. This concept is discussed further under the subheading *Rapamycin*.

While immune memory is classically a feature of the adaptive immune system, non-specific 'trained immunity' occurs within the innate immune system. The best described example of trained immunity is the change in magnitude and polarisation of the cytokine response of monocytes to PRR engagement up to months after infection or vaccination, which is mediated by epigenetic and metabolic changes (107-111). NK cells, which are innate lymphocytes, possess a variety of receptors to recognise healthy versus

infected or cancerous cells (112). Expression of these receptors is stochastic, resulting in a variety of receptor combinations within the population. Particular combinations are thought to better enable detection of particular types of virus, and inheritance of these combinations by daughter cells enables the outgrowth of relevant NK cell 'clones' (112).

T cells. T cells classically fulfil two functions: killing of infected and cancerous cells, which is usually carried out by $CD8^+$ T cells, and orchestration of the immune response, which largely falls to $CD4^+$ T cells and includes providing help to B cells. Unlike B cells, T cells do not recognise antigen directly but via linear epitopes presented in the context of major histocompatibility complex (MHC) molecules on the surface of APCs. In this way, T cells receive information on the context of antigen encounter with the APC and are subjected to the stringent immune checkpoints described previously. Blood-borne antigens filter through the spleen where they can be taken up by spleen-resident APCs and presented to T cells. Antigens present in other tissues may be taken up by sentinel APCs, classically Langerhans cells at barrier tissues, or be caught in the lymphatic system and transported to the lymph nodes that drain that tissue. Upon activation, APCs express high levels of MHCI and MHCII on the cell surface and present a variety of linear peptides derived from endocytosed proteins. Positioning of naïve T cells in draining lymph nodes and other secondary lymphoid organs (SLOs) allows efficient interaction between APCs and T cells in search of cognate T cell receptors (TCRs). A variety of MHC gene alleles exist, and which variant an APC possesses determines the range of linear peptides that can be presented from a given antigen. As there are three distinct MHCI genes and three MHCII genes, a heterozygous individual will likely possess six variants of each; diversity at a population levels is important for achieving herd immunity to a wide variety of pathogens, however becomes a significant problem in transplantation where mismatched MHC molecules (human leukocyte antigens, HLA, in humans) act as key antigens themselves and drive transplant rejection.

$CD8^+$ T cells. $CD4^+$ T cells recognise antigen presented on MHCII, while $CD8^+$ T cells

recognise MHCI complexed antigens. Unlike MHCII, expression of which is restricted to specialised cell types, MHCI is expressed by all nucleated cells. This allows $CD8^+$ T cells to recognise cells presenting pathogen or cancer antigens, however the initial activation of $CD8^+$ T cells requires cross-presentation of antigens on MHCI by APCs. Upon activation, $CD8^+$ T cells undergo successive rounds of proliferation, forming short-lived effector cells (SLECs) and memory-precursors effector cells (SLECs). Effector $CD8^+$ T cells alter their expression of chemokine receptors to enable egress from SLOs and chemotaxis to sites of inflammation where they sample MHCI molecules for presentation of cognate antigen and kill infected cells. Cytotoxicity is commonly achieved by administration of cytotoxic mediators to target cells, including granzyme B and perforin, which are contained in preformed cytosolic granules.

$CD4^+$ T cells. $CD4^+$ T cells are more varied and diverse in phenotype and function. Upon productive antigen encounter, $CD4^+$ T cell classically differentiate into distinct effector subsets that provide specialised 'help' to other cells of the immune system in the form of cytokines and, in the case of B cells, cognate licencing via CD40L.

Potential advantages of promoting the T cell response to vaccination. As discussed in the introduction, the primary goal of most vaccines is (reasonably) to induce a neutralising antibody response rather than a T cell response (although some vaccines, notably the smallpox and yellow fever vaccines, were unwittingly designed with the capacity for both strong humoral and cellular immunogenicity) (113). However, boosting the T cell response to a vaccine will have advantages in some areas:

1. Protection from severe disease. While it is often difficult to isolate the effect of T cells from that of the humoral immune system in clinical studies, T cells are required for clearance of infection and their importance in protection from severe and protracted disease is likely underappreciated. A recent letter to the US Food and Drug Administration, signed by over 60 eminent scientists, stressed the value in measuring T cells in antiviral immunity and

advocated for standardisation of T cell analyses in order to progress our understanding of the role of T cells in protection from infection and disease (114).

2. Protection of immunocompromised patients. While not top-priority for vaccine manufacturers looking to serve the majority population, transplant recipients on immunosuppressive agents, patients with autoimmune diseases or B cell malignancies receiving B cell depleting agents, and those with B cell and antibody-related immunodeficiencies, are unable to mount an effective antibody response and thus may rely on the formation of memory $CD8^+$ T cells to manage and clear infection (115).
3. Long-term immunity. Plasma cells induced by vaccination are secreted in survival niches in the bone marrow where they can continue to produce antibody long-term without an apparent requirement for antigen-encounter. Similarly, it is thought that circulating memory B cells will continue to differentiate into antibody secreting plasmablasts during steady-state in response to TLR agonists such as microbial DNA likely derived from commensal bacterial, thus contributing to the maintenance of circulating antibody titres (105). However, it has become apparent during the course of the COVID-19 pandemic that antibody levels begin to wane after 6-8 months to levels that no longer effectively neutralise virus (particularly immune-evasive variants). As in immunocompromised patients, long-lived memory T cells (which are thought to have a half-life approaching that of their hosts) may provide some protection from disease for a much longer period.
4. Evasive variants. While coronaviruses encode a protein to check for replication errors and thus do not possess the hypermutability characteristic of other RNA viruses such as influenza, the pandemic has seen the emergence of SARS-CoV-2 variants (most notably Beta and Omicron) able to evade antibody neutralisation to alarming extents (31, 116). The T cell repertoire tends to be much broader than the antibody repertoire as it can be directed against intracellular, as well as surface, antigens and tends to focus

less on specific, dominant epitopes. By contrast, the germinal centre machinery hones the B cell repertoire towards a high affinity response to a dominant antigen. The antibody repertoire will continue to focus on the dominant antigen/epitope(s) even if a strain of the virus emerges in which this antigen is no longer important to the infection cycle; this is known as original antigenic sin, and is thought to underlie the limited success of variant specific vaccines (117).

5. Accessing particular niches. While not relevant to the discussion of COVID-19, particular pathogens such as Listeria monocytogenes spread between adjacent cells without being exposed to the extracellular environment and are therefore inaccessible to antibody-mediated immunity. The same concept likely applies to infections in certain tissues, although evidence for this is lacking. Boosting the T cell response is therefore critical for the development of vaccines against diseases such as shingles (118).
6. Pre-existing immunity in the population. While not relevant to vaccination, pre-existing T cell immunity to new infections is common due to cross-reactivity with similar viruses. In the case of SARS-CoV-2, 20-50% of the population has some level of pre-existing immunity due to cross-reactivity of memory T cells generated in response to common-cold coronaviruses (119). In combination with the speed of the T cell response, T cells play an important role early in infection while the B cell/antibody response matures.

B cells. B cells interact with antigens in their native conformation, and B cell receptor specificity is therefore dependent on tertiary antigen structure, not solely on linear amino acid sequence. Where T cells are specialised for dealing with intracellular pathogens, the major function of B cells is to produce antibodies that bind surface antigen on extracellular pathogens. This includes bacterial and parasitic microbes with extracellular lifecycles, but also intracellular pathogens such as viruses that must pass between cells as part of their life cycle. In SLOs, naïve B cells localise to the B cell follicles,

through which soluble antigens in the blood or lymph percolate to be sampled by B cells. A specialised population of stromal cells within the follicle, called follicular dendritic cells (unrelated to the hematopoietic lineage cell), capture antigen on their surface for presentation to B cells. Naïve B cells that encounter cognate antigen will endocytose the antigen:BCR complex, process the antigen for presentation on MHCII, and migrate to the T-B border to present antigen to T cells. CD40 engagement by a cognate T cell results in survival of the B cell and a burst of proliferation. Daughter cells formed at this stage tend to differentiate into short-lived plasmablasts and secrete low affinity IgM antibody. This interaction may further result in migration of the T-B conjugate back into the follicle, seeding a germinal centre. Class switching from IgM to a different class of BCR/antibody is classically thought to occur in the germinal centre (although it may occur more frequently outside of the germinal centre) (120). Choice of isotype is regulated by cytokines received from the T follicular helper cell, which is in turn determined by the microenvironment and potentially the conditions under which the APC encountered the T cell's cognate antigen. Within a germinal centre, B cells undergo massive proliferation and cycle between two distinct zones, the light zone and dark zone. In the dark zone, cells undergo a process of somatic hypermutation, which requires expression of activation-induced cytidine deaminase (AID) and results in the rapid (10^6-fold higher rate than other cells) mutation of the complementarity-determining regions (CDR) of immunoglobulin-coding genes (121). Cells then cycle through the light zone, wherein apoptosis is induced, and cells compete for survival. Survival signals are provided by T_{FH} cells and are dependent on affinity of the BCR for cognate antigen. B cells compete for limited antigen, which is endocytosed and presented to T follicular helper cells. Therefore, in order to receive survival signals and, in this manner, higher affinity BCR are selected (affinity maturation), from which long-lived plasma cells and memory cells are formed.

Immunosuppression. At the time of transplantation, recipients undergo induction therapy. This is typically an infusion with anti-thymocyte globulin (ATG), polyclonal

IgG antibodies raised in goat, rabbit or horse through immunisation with human thymocytes or related cell lines (122). ATG acts to deplete T cells, T cell precursors, NK cells and some subsets of B cells (122). T cell depletion is necessary to prevent acute cellular rejection and the generation of high affinity donor-specific antibodies (DSA) (123). B cells and $CD8^+$ T cells generally reconstitute to a sufficient level within 3 months following induction therapy, while $CD4^+$ T cells can take longer (122). During this time, vaccination is not recommended and patients due to receive a transplant are recommended to be vaccinated as early as possible prior to transplantation (124).

As the immune system reconstitutes, patients commence maintenance therapy. There are four main classes of maintenance immunosuppression in common use: 1) calcineurin inhibitors (CNI), 2) antimetabolites, 3) steroids, and 4) mammalian target of rapamycin inhibitors (mTORi). The standard of care for kidney transplant recipients (KTRs) in Australia is tacrolimus (CNI), mycophenolate (anti-metabolite), and prednisolone (steroid). It is common for patients to develop cancers as a result of impaired immune surveillance; in this instance, treating physicians may elect to taper CNI use in preference of an mTORi due to their anti-cancer properties (125, 126). However, mTORi are ceased prior to tumour resection due to impaired wound healing. mTORi may be used in preference to CNI in some instances due to lower nephrotoxicity, however difficulty with dosing deters most physicians from long term mTORi use. Maintenance immunosuppression is taken for the life of the transplant, and all of these drug classes have the potential to directly impair the vaccine response.

Calcineurin inhibitors include tacrolimus, cyclosporine, and pimecrolimus, all of which aim to blunt T cell activation. Tacrolimus, and to a lesser extent, cyclosporine are used to prevent allotransplant rejection. Tacrolimus and cyclosporine are macrolides that cross into the cell cytoplasm and inhibit the activity of calcineurin (127). To facilitate this activity, CNIs bind to cytoplasmic proteins expressed in lymphocytes known as immunophilins; tacrolimus binds to FKBP12 which complexes with its binding partner

FK506, and cyclosporine binds to cyclophilin (127). These complexes similarly associate with, and inhibit, calcineurin. In T cells, calcineurin is activated in response to antigen receptor signalling, and acts to dephosphorylate the transcription factor NFAT, allowing translocation to the nucleus and transcription of the IL-2 gene (127). IL-2 is an essential autocrine/paracrine growth factor for T cells and, as such, CNIs impair growth and survival of T cells upon antigen encounter.

mTOR inhibitors used in preventing graft rejection include rapamycin (also known as sirolimus) and RAD001 (everolimus). Rapamycin is a macrolide originally isolated from a soil sample obtained on Rapa Nui. Initial interest in rapamycin was as an antifungal, however it was found to inhibit proliferation of T and B lymphocytes and was commercialised as an immunosuppressive treatment for the prevention of renal transplant rejection (128). The anti-proliferative effects of rapamycin were later attributed to its capacity to inhibit the mammalian target of rapamycin (mTOR), which regulates cellular metabolism and cell cycle progression in response to extracellular nutrient availability as part of two complexes, mTORC1 and mTORC2 (129-131). One such growth signal integrated by mTOR is IL-2R signalling, which is required for cell cycle progression in lymphocytes. Recognising this as a potential anticancer treatment, the discoverer of rapamycin, Dr Sehgal, sent the samples to the National Cancer Institute for investigation of its anticancer properties and the rapalog everolimus was approved in 2009 to treat end stage renal cell carcinoma, and in 2012 as a last line treatment for hormone receptor positive HER2 negative breast cancer (128). While the mechanism by which rapamycin and its analogues ('rapalogs') inhibited mTORC1 activation is well established, long-term rapamycin use has been found to also inhibit mTORC2. This underlies the side-effect of insulin resistance associated with long-term rapamycin use, although the mechanism by which inhibition of mTORC2 occurs remains unclear (132).

Mycophenolate mofetil (MMF) and azathioprine (AZA) are antimetabolites used in transplantation. Both drugs are inhibitors of DNA and RNA synthesis. While many cell

types salvage free purine nucleotides for DNA and RNA synthesis, lymphocytes and some other white blood cell types depend strongly on *de novo* purine synthesis (133). This property in B cells is classically utilised in the production of hybridomas. Mycophenolate is metabolised to mycophenolic acid *in vivo*, which is a potent inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH). IMPDH is essential to the purine *de novo* synthesis pathway, converting inosine monophosphate into the nucleotide guanosine monophosphate. Azathioprine interferes with the same pathway. It is metabolised to non-functional nucleotide analogues, thioinosinic acid and thioguanylic acid (analogues of inosine monophosphate and guanosine monophosphate, respectively), giving it a broader range of effects (134). Incorporation of these analogues into DNA results in attenuation of replication, and uptake by enzymes in the purine synthesis pathway results in their inhibition.

The two glucocorticoids typically used to prevent transplant rejection are prednisolone and methylprednisolone. Unlike the other immunosuppressive medications, glucocorticoids are synthetic analogues of endogenous mammalian molecules that activate specific signalling pathways. GCs bind to the glucocorticoid receptor in the cytosol and translocate to the nucleus where the complex acts as a transcriptional regulator to facilitate expression of a suite of genes including anti-inflammatory mediators. The complex can also interact with histone deacetylases to interfere with NF-κB DNA interaction at multiple sites, interfering with a major pro-inflammatory gene expression pathway (135).

mTOR and T cell memory. mTOR inhibitors are associated with reduced viral infections and some cancers, a paradoxical association for an immunosuppressive agent as the immune system is require to fight cancers (126, 136-139). In the transplant community, the reduced incidence of certain cancers and infections in patients receiving mTORi-based protocol relative to CNI-based protocols is often put down as mTORi being a less potent method of immunosuppression, and patients receiving mTORi tending to be on

fewer medications (140). However, in 2009, Araki *et al* published a report in Nature indicating that this might be attributable to a gain-of-function effect on antigen-specific T cells (141). Araki *et al* found that treating mice with low dose rapamycin during lymphocytic choriomeningitis virus (LCMV) infection led to a marked increase in frequency of antigen-specific $CD8^+$ memory T cells in lymphoid and non-lymphoid tissues 20-30 days after infection. Mice that received rapamycin demonstrated significantly better viral control upon reinfection. This work was followed up in 2013 with non-human primates, demonstrating that rapamycin treatment could boost cytotoxic T cell responses to smallpox vaccination when given at the time of the booster dose (142). It is unknown whether this gain-of-function effect of rapamycin on vaccine T cell response is reproduced in humans, however, T cells from individuals with existing immunity to cytomegalovirus (CMV) undergo increased proliferation and cytotoxic mediator-production upon secondary exposure to CMV peptides when treated with rapamycin *in vitro* (137).

From these studies it is apparent that rapamycin increases the frequency of antigen-specific memory $CD8^+$ T cells formed after viral exposure or vaccination, and that this T cell pool is capable of cytokine and cytotoxic mediator production. Beyond T cell frequency however, Araki *et al* found that the memory T cells formed in the presence of rapamycin were functionally superior on a per-cell basis, demonstrating increased expression of markers associated with long-lived memory, greater homeostatic proliferation when transferred into a naïve mouse, and a superior capacity for viral control (141). Notably, the effect of rapamycin on memory cell formation and function had distinct origins, and could be separately achieved depending on the timing of rapamycin treatment. The increased frequency of antigen-specific $CD8^+$ memory cells was the result of reduced contraction of the antigen-specific effector T cell compartment. Following immunisation, the antigen specific $CD8^+$ T cell pool rapidly expands giving rise to an effector population. This population is made up of predominantly short-lived effector cells (SLECs), however also contains memory precursor effector cells (MPECs)

that will give rise to mature, long-lived memory. Expansion of the effector population peaks around Day 8, and then the contraction phase begins, which is characterised by apoptosis of short-lived effector cells. Mice treated with rapamycin throughout the expansion phase only (up to Day 8 after exposure), had reduced contraction of the antigen-specific $CD8^+$ T cell pool, suggesting that increased T cell frequency is the result of a bias towards memory-precursor generation during the expansion phase (141). Conversely, rapamycin exposure only during the effector to memory transition phase (Day 8-35) resulted in the formation of highly functional memory $CD8^+$ T cells, but did not increase their frequency (141). This was a true effect of rapamycin on the cells and not the result of rapamycin favouring the outgrowth of functionally superior cells. Superiority of the antigen-specific $CD8^+$ T cell memory compartment (>10-fold higher frequency vs untreated mice) was maintained out to at least day 165 in rapamycin treated mice (141).

This effect was later demonstrated to be context dependent, as rapamycin had no effect on the T cell response to a viral antigen in the context of a skin graft in mice, but boosted antiviral $CD8^+$ T cell responses in the same mice to the same antigen (143).

The mechanism(s) by which mTORC1 inhibition promotes MPEC formation is/are unknown.

The effector versus memory fate decision. Upon activation (TCR stimulation + co-stimulation), T cells undergo rapid proliferation to generate clonally relevant populations of effector cells to address the immediate threat, and long-lived memory. At its peak, the antigen-specific effector pool is formed of a heterogeneous population of terminally differentiated effector cells, collectively termed SLECs, and < 10% of MPECs with the potential to transition to mature memory and persist long-term. Following multiple rounds of cell division, SLECs and MPECs can be defined in mice by expression of the IL-7R chain CD127 and KLRG1 (144, 145). There is significant

interest in understanding how this fate decision is made in order to develop strategies to bias memory cell formation in response to vaccination.

Multiple factors have been described that influence this decision, including the strength and quality of T cell receptor signalling and APC interaction, and inflammatory signals in the microenvironment (146-148). The majority of studies investigating the signals affecting memory versus effector fate have focused on expression of transcription factors, with multiple axes now described as reciprocally controlling MPEC and SLEC identity, including T-BET/EOMES, ID2/ID3, BLIMP-1/Bcl6, YY1/Nr3c1 and ZEB1/ZEB2 (149-151).

Beyond gene-expression profiles regulated by 'master' transcription factors, MPECs and SLECs fundamentally diverge in other aspects of their cellular identity. Where naïve cells employ oxidative phosphorylation (OXPHOS) for energy production, the rapid cycling of antigen-primed T cells is characterised by a switch to aerobic glycolysis (152). As glycolysis is far less efficient at generating ATP compared with OXPHOS, it is uncommon when oxygen is available. However, aerobic glycolysis is classically employed in particular situations where building biomass is a priority, as carbon molecules derived from glucose and glutamine metabolism, which are largely expelled as carbon dioxide during OXPHOS, can be repurposed. This technique is employed by cancer cells, where it was first described as the Warburg effect. Memory cells, however, return to reliance on OXPHOS. The capacity of precursor cells to form mature memory is dependent on their capacity to reverse their metabolism, engaging catabolic processes that consume biomass to fuel OXPHOS (153). Fatty acid oxidation (FAO) is therein a hallmark of memory cells, and inhibition of this capacity, e.g. by inhibition of cardiolipin synthesis, impairs memory T cell formation *in vivo* (153-155).

The process of differentiation is intimately linked to cell division (156). Tracking of individual antigen-primed T cells has found that MPECs are slower cycling than SLECs,

and this difference is fundamental to their divergent differentiation (156). The importance of cell cycle is likely linked to epigenetic modifications. MPECs can be differentiated from the rapidly cycling bulk of effector subsets at an early stage, due to an elongated G1 phase of their cell cycle (156). During G1, epigenetic modifications are made to the chromatin landscape that define the memory cell identity, which is characterised by a capacity for rapid effector molecule production and differentiation in response to secondary antigen encounter. By contrast, the division speed of the effector precursor populations, which is close to physiological limit of human cells, is thought to restrict the capacity for chromatin modifications resulting in fewer chromatin modifications and global hypomethylation of DNA (157, 158). Epigenomic profiling of antigen-primed CD4 T cells suggests a linear differentiation process wherein the emergence of memory subpopulations occurs as a consequence of increasingly fewer epigenetic modifications from naïve to central memory to effector memory to T_{EMRA} T cells (158).

Also fundamental to the memory T cell identity is the capacity for self-renewal, and for rapid effector responses and further differentiation in response to activation. This is, in part, a feature of their epigenetic landscape. Where SLECs become terminally differentiated and undergo rapid cell death, MPECs possess fewer epigenetic modifications and a more permissive chromatin landscape that enables rapid gene expression and stem cell-like potential for differentiation and homeostatic cell divisions (157, 159).

While dynamic changes in the quality of the immune response, such as antigen-reencounter, can be seen to influence the quality of the resultant memory pool (160), it has been proposed that the fate decision is made early in the clonal expansion process. In fact, a memory versus effector bias can be observed as early as the first cell division (151, 157, 161). Antigen-presentation to a naïve T cell gives rise to two distinct daughter cells through an asymmetric cell division process: one daughter cell remains proximal

to the APC and forms distal to the APC. The proximal daughter cell preferentially inherits surface receptors for the integration of inflammatory signals, including IL-2Rα (CD25) and IFNγR, as well as nutrient sensors and intracellular factors including mTORC1 complex (162-164). The proximal cell is therefore equipped to respond to signals that promote SLEC differentiation, and possesses the machinery to take up nutrients and undergo rapid proliferation. Being proximal to the APC, this daughter cell likely also encounters greater concentrations of IL-2, IFNγ and other inflammatory signalling molecules produced by the APC that favour SLEC formation. Conversely, the distal cell divides more slowly and is more likely to engage autophagy and catabolic processes due to low levels of mTORC1 signalling, which are required for a switch to FAO. Inheritance of these characteristics by progeny cells gives a model wherein a rapidly dividing SLEC-biased lineage would numerically outnumber the slower dividing MPEC-biased lineage, accounting for the > 90% numerical dominance of SLECs at the end of the expansion stage. However, effector-related genes (e.g. granzyme B) have been detected in later cell divisions in cycling cells that give rise to memory populations, leading to the competing hypothesis that all dividing cells begin down the effector cell differentiation pathway and a memory choice must be made later on in the series of cell divisions (160, 165). The current prevailing model for the development of T cell memory is one of dynamic memory development wherein dividing T cells incorporate both division history (beginning from the initial asymmetric division), as well as evolving environmental cues, in the memory versus short-lived effector fate decision.

mTORC1 in the T cell fate decision. As a key signalling node integrating nutrient and cytokine signals, and regulating cell cycle progression and cellular metabolism accordingly, inhibition of mTORC1 activity has the potential to modulate many aspects important to T cell fate (Figure 1.3.1). Unequal partitioning of mTORC1 between daughter cells is the defining feature of asymmetric cell division that regulates the cells' responses to environmental cues, and ultimately their metabolism and proliferative

potential. The influence of mTORC1 partitioning on cellular identity is so significant that, following activation of naïve $CD8^+$ T cells, $mTORC1^{high}$ and $mTORC1^{low}$ cells can be identified by size alone, where smaller cells cycle slower and preferentially develop memory-like characteristics *in vitro* (162, 166). Interestingly, failure of asymmetric cell division underlies reduced heterogeneity of memory cell formation in aged mice, a feature that can be overcome with transient inhibition of mTORC1 (167).

mTORC1 may additionally play a part in regulating transcription factors and cytokine signals related to the T cell fate decision. In 2010, Rao *et al* investigated the mechanism by which IL-12 conditioning of $CD8^+$ T cells leads to the induction of T-BET and type 1 effector cells, and found that it was dependent on STAT4-mediated sustained activation of mTORC1 (168). Inhibition of mTORC1 with rapamycin in IL-12 conditioned $CD8^+$ T cells not only suppressed effector differentiation but promoted expression of EOMES and the formation of memory cells (168). Similar to the findings of Araki *et al*, the memory cells generated possessed all the hallmarks of long-lived memory cells and increased per cell functionality and anti-tumour efficacy (141, 168). Thus, rapamycin may influence $CD8^+$ T cell memory formation by inhibiting T-BET expression in preference of EOMES expression in response to SLEC-biasing factors such as IL-12.

It is also worth noting that, although the impact of mTORC1 inhibition on epigenetic modifications is unclear, changes in epigenetic regulation are likely to occur secondary to altered metabolism and cell cycle progression (144, 169-171).

In sum, high mTORC1 activity in antigen-primed T cells drives the metabolic and transcriptional changes necessary for the differentiation of SLECs, while reduced mTORC1 activity is associated with an alternative cellular state that favours MPEC formation. While some level of mTORC1 signalling is required for productive formation and function of CD8 memory T cells (141, 172), rapamycin treatment peri-vaccination is

likely to induce an mTORC1^{low} state in dividing T cells that favours MPEC differentiation.

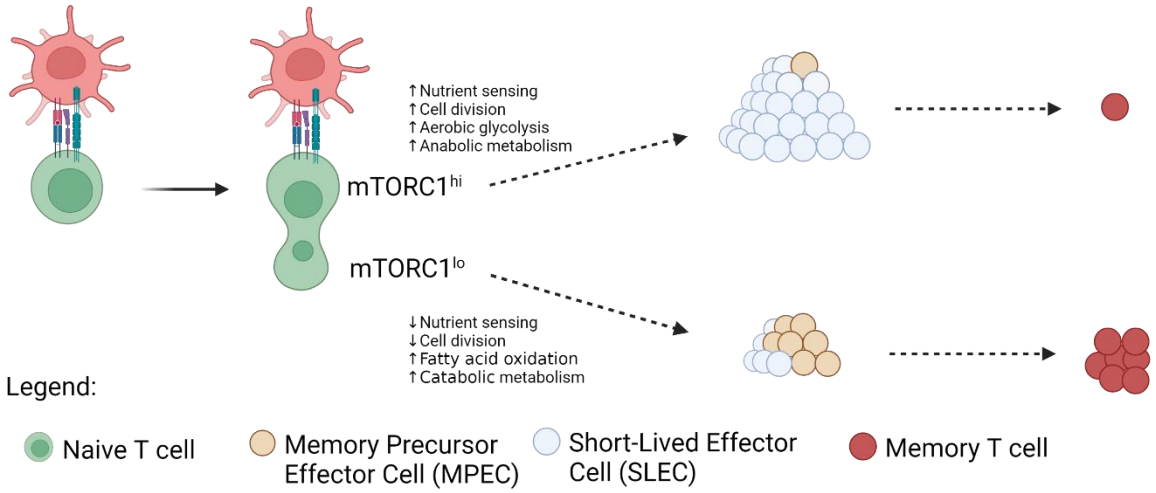


Figure 1.3.1. mTORC1-centric model of CD8$^+$ T cell memory formation. Differentiation is intimately linked with cell division. During the expansion phase, which follows productive antigen-encounter by naïve CD8$^+$ T cells, mTORC1 activity biases dividing cells towards a short-lived effector cell (SLEC) fate by promoting a metabolic and transcriptional/translational program conducive to rapid cell division. mTORC1 activity both promotes cell cycle progression, and engages aerobic glycolysis in order that carbon energy sources (namely glucose and glutamine) may be used to meet the biomass requirements of cell division. In the absence (or inhibition) of mTORC1 activity, cells cycle more slowly and maintain the capacity to switch to oxidative phosphorylation utilising fatty acids as an energy source (fatty acid oxidation, FAO). Nutrients and growth factors (particularly IL-2) in the microenvironment are made scarce by the high demands of the effector expansion and, as such, the capacity to incorporate fatty acids as an alternative energy source is important for the persistence of memory precursor effector cells (MPECs) as the bulk of the effector population undergoes contraction due

to nutrient deprivation. This fate bias is seeded in the very first cell division of an activated naïve T cell, in which mTORC1 complexes are asymmetrically partitioned between daughter cells. This partitioning is governed by proximity to the antigen presenting cell, wherein the proximal daughter cell receives a larger share of mTORC1 complexes in conjunction with growth factor and nutrient receptors required to drive mTORC1 activity and integrate signals that promote a SLEC fate. By contrast, the distal daughter cell lacks the resources for high intensity cell division conducive of the SLEC fate, and is more likely to give rise to progeny biased towards a MPEC fate. Created with BioRender.com.

1.4. Thesis Hypotheses

The goal of this thesis is to tackle immunological hurdles to effective COVID-19 vaccination in South Australia and globally, which is addressed with two specific aims: 1) To understand hypersensitivity to the BNT162b2 vaccine in order to inform identification and management of individuals at-risk of adverse reactions to vaccination, and 2) To assess the immune response of immunosuppressed kidney transplant recipients to BNT162b2 and ChAdOx1 vaccines in order to inform the development and implementation of effective vaccination strategies for this vulnerable population.

Chapter 2 hypotheses:

I. Polyethylene glycol is the inciting allergen in cases of skin test hypersensitivity to the BNT162b2 vaccine.
II. Presentation of polyethylene glycol on lipid nanoparticles improves the capacity of polyethylene glycol to induce activation of sensitised basophils and mast cells *in vivo* and *in vitro*.

Chapter 3 hypotheses:

III. SARS-CoV-2-specific antibody and T cell immunity induced by the BNT162b2 and ChAdOx1 vaccines will be impaired in kidney transplant recipients compared with healthy controls.
IV. Close household contacts of kidney transplant recipients will meet protective thresholds for live virus neutralisation following two doses of COVID-19 vaccine.
V. Kidney transplant recipients receiving mTOR inhibitors will demonstrate better T cell and antibody responses, respectively, to two doses of COVID-19 vaccine compared with patients receiving standard of care therapy.

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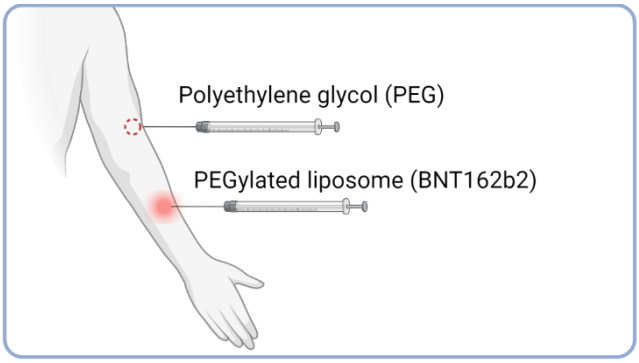
CHAPTER 2:

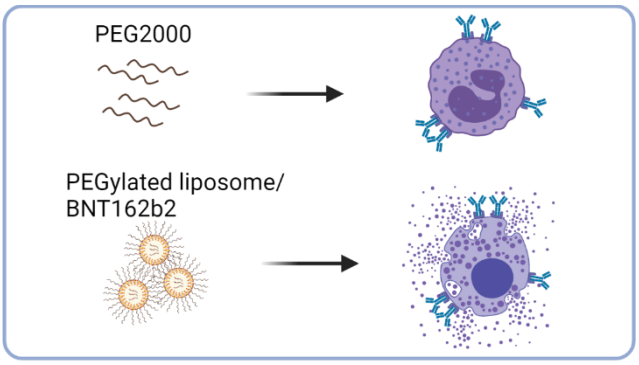
ALLERGY TO PEGYLATED LIPID NANOPARTICLE VACCINES

2.1



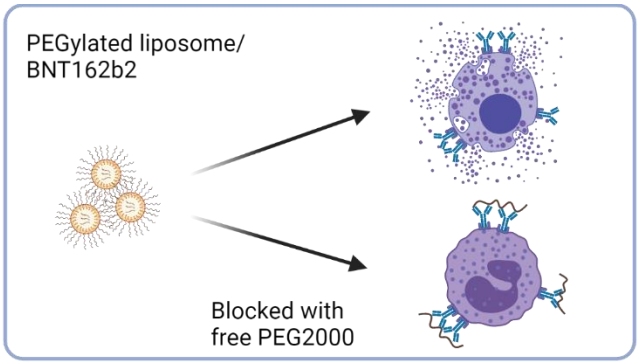
PEG-containing drugs are poor surrogates for allergy testing with BNT162b2 vaccine itself.

2.2



PEG is poorly allergenic in vitro or in vivo unless presented on the surface of a nanoparticle or ingested in high quantities.

2.3



PEG is the inciting allergen in the BNT162b2 vaccine.

2.1. MANUSCRIPT 1-

Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in patients with PEG allergy

Details-

Published in the Journal of Allergy and Clinical Immunology.
Impact factor (2021): 14.290
Submitted April 13, 2021; Revised April 22, 2021; Accepted April 28, 2021; Available online May 12, 2021; Published in print July 01, 2021.

Preface-

This manuscript addresses Hypothesis II: *Presentation of polyethylene glycol on lipid nanoparticles improves the capacity of polyethylene glycol to induce activation of sensitised basophils and mast cells in vivo and in vitro*. This work was done in collaboration with Dr Alexander Troelnikov, clinical immunology registrar. Dr Troelnikov coordinated clinical aspects of the project and is co-first author.

Statement of Authorship

Title of Paper	Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in patients with PEG allergy
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Published by the Journal of Allergy and Clinical Immunology (Mosby) Impact Factor 14.290 (2021) Accepted April 2021

Principal Author

Name of Principal Author (Candidate)	Griffith Perkins		
Contribution to the Paper	Experimental design and optimisation Laboratory work Data analysis Manuscript preparation		
Overall percentage (%)	51%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22-02-2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	22-02-2022

Basophil reactivity to BNT162b2 is mediated by PEGylated lipid nanoparticles in patients with PEG allergy

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Background: The mechanisms underpinning allergic reactions to the BNT162b2 (Pfizer) COVID-19 vaccine remain unknown, with polyethylene glycol (PEG) contained in the lipid nanoparticle suspected as being the cause. **Objective:** Our aim was to evaluate the performance of skin testing and basophil activation testing to PEG, polysorbate 80, and the BNT162b2 (Pfizer) and AZD1222 (AstraZeneca) COVID-19 vaccines in patients with a history of PEG allergy. **Methods:** Three known individuals with PEG allergy and 3 healthy controls were recruited and evaluated for hypersensitivity to the BNT162b2 and AZD1222 vaccines, and to related compounds by skin testing and basophil activation, as measured by CD63 upregulation using flow cytometry. **Results:** We found that the BNT162b2 vaccine induced positive skin test results in patients with PEG allergy, whereas the result of traditional PEG skin testing was negative in 2 of 3 patients. One patient was found to be co-sensitised to both the BNT162b2 and AZD1222 vaccines because of cross-reactive PEG and polysorbate allergy. The BNT162b2 vaccine, but not

PEG alone, induced dose-dependent activation of all patients' basophils *ex vivo*. Similar basophil activation could be induced by PEGylated liposomal doxorubicin, suggesting that PEGylated lipids within nanoparticles, but not PEG in its native state, are able to efficiently induce degranulation**. Conclusions:** Our findings implicate PEG, as covalently modified and arranged on the vaccine lipid nanoparticle, as a potential trigger of anaphylaxis in response to BNT162b2, and highlight shortcomings of current skin testing protocols for allergy to PEGylated liposomal drugs. (J Allergy Clin Immunol 2021;148:91-5.)

Introduction

Early safety monitoring during the mass vaccination campaigns internationally has reported an unexpectedly high rate of immediate hypersensitivity reactions to both the BNT162b2 (Comirnaty, Pfizer, New York, NY) and mRNA-1273 (Moderna, Cambridge, Mass) mRNA lipid nanoparticle vaccines (1, 2).

Although adverse event reporting more recently indicates a lower rate of anaphylaxis of 4 to 5 cases per million for the BNT162b2 vaccine, in a well-characterised cohort of 64,900 health care workers, anaphylaxis was confirmed in 16 patients (or approximately 1 in every 4,000 individuals vaccinated) (3), surpassing the expected rate of 1 case per million (4). Polyethylene glycol (PEG) conjugated to lipids within the lipid nanoparticle encapsulating the mRNA is considered the likely culprit allergen (5). PEG driving the unanticipated frequency of anaphylaxis is surprising, as this is otherwise an infrequent cause of anaphylaxis despite its widespread presence in many other drugs, foods, and cosmetics (6). To assess the risk of allergic reactions to BNT162b2, major authorities have suggested skin testing protocols for patients with a history of anaphylaxis in response to vaccine components such as to PEG and polysorbates (1, 7, 8). There have been reports of cross-reactivity between PEG and polysorbates, and the AZD1222 vaccine (AstraZeneca, Cambridge, United Kingdom), which is the only alternative coronavirus

disease 2019 (COVID-19) vaccine currently available to our patients in Australia, contains polysorbate 80 (1). This study aimed to evaluate a protocol for PEG, polysorbate, and COVID-19 vaccine skin testing and basophil activation testing (BAT) in patients with confirmed PEG hypersensitivity. The testing panel used is based on the presence of PEG 2000 in the BNT162b2 and mRNA-1273 vaccines, and the presence of polysorbate 80 in the AZD1222 vaccine.

Results and Discussion

We recruited 3 adult patients known to our service to have a history of PEG allergy (demographics and details of the index reaction are provided in Table 2.1.1 and Table 2.1.2). In addition to allergy to PEG, Patient 1 had a history of allergy to polysorbates. Skin prick testing and intradermal testing for allergy to PEG and polysorbates was performed by using a panel adapted from Banerji *et al* (1)(Table 2.1.2). The panel consisted of freshly thawed BNT162b2 (Comirnaty) and AZD1222 vaccines that were otherwise destined for discard, as well as readily available and validated skin testing reagents that are related to components of the vaccines licensed for *in vivo* use (the relevant component is specified in Table 2.1.2). For the study, 3 healthy individuals serving as controls were simultaneously tested with the skin testing panel. All patients developed a positive skin test result in relation to the BNT162b2 vaccine, whereas only Patient 1 tested positive for AZD1222, which is in agreement with the previous positive result of a skin test to polysorbate. Surprisingly, Patients 2 and 3 had otherwise negative skin testing results in relation to the panel, including to PEG containing steroid methylprednisolone acetate at a 1:10 dilution, which is the recommended non-irritating concentration (9).

To further explore the results of the skin testing, we performed BAT with a range of PEG molecular weights (200-6000 g/mol) (Tokyo Chemical Industry, Tokyo, Japan) and with both the BNT162b2 vaccine and AZD1222 vaccine. Before skin testing, blood was drawn

for BAT as previously described (10). In brief, IL-3-treated whole blood was incubated with varying concentrations of allergen to induce degranulation and then stained with an antibody cocktail as described. Basophil degranulation following allergen exposure was determined by surface expression of CD63, and the results were compared with a positive (anti-IgE) and negative (PBS) control. We observed no increase in CD63 expression on basophils with PEGs of any molecular weight or with the AZD1222 vaccine (Table 2.1.3). However, we did observe a dose-dependent increase in CD63 expression in the presence of varying concentrations of BNT162b2 in all 3 patients (Figure 2.2.1) that was not observed in vaccinated or unvaccinated, allergy-free controls (n = 3).

TABLE 2.1.1. Patient characteristics

Patient characteristic	Patient 1	Patient 2	Patient 3
Age	51 y	55 y	22 y
Sex	Female	Male	Female
Preceding allergen triggers	Macrogol containing aperients, polysorbate-containing drugs, cosmetics, and foods	Macrogol-containing aperient	Parenteral steroid preparation
Time since last reaction	3 mo	3 mo	5 y

TABLE 2.1.2. Summarised skin test results

Testing	Component allergen	Patient 1	Patient 2	Patient 3
Skin prick testing				
Drug				
Movicol, 100 mg/mL	PEG 3350	Positive, 1 mg/mL	Negative 100 mg/mL	Negative, 100 mg/mL
Optive Advanced	Polysorbate 80 + carmellose	Negative, neat	Negative, neat	Negative, neat
Cellufresh	Carmellose	Negative, neat	Negative, neat	Negative, neat
Methylprednisolone acetate, 40 mg/mL	PEG 3350	Negative, 40 mg/mL	Negative, 40 mg/mL	Negative, 40 mg/mL
Methylprednisolone succinate, 40 mg/mL	Excipient-free	Negative, 40 mg/mL	Negative, 40 mg/mL	Negative, 40 mg/mL
Triamcinolone, 10 mg/mL	Polysorbate 80	Negative, 10 mg/mL	Negative, 10 mg/mL	Negative, 10 mg/mL
BNT162b2, 100 µg/mL	PEGylated nanoparticle	Negative, 100 µg/mL	Negative, 100 µg/mL	Negative, 100 µg/mL
AZD1222, neat	Polysorbate 80	Negative, neat	Negative, neat	Negative, neat
Intradermal testing				
Drug				
Methylprednisolone acetate, 4 mg/mL	PEG 3350	Positive, 0.4 mg/mL	Negative, 4 mg/mL	Negative, 4 mg/mL
Methylprednisolone succinate, 4 mg/mL	Excipient-free	Negative, 4 mg/mL	Negative, 4 mg/mL	Negative, 4 mg/mL
Triamcinolone, 1 mg/mL	Polysorbate 80	Positive, 0.1 mg/mL	—	—
Optive Advanced, 1:10 dilution	Polysorbate 80	—	Negative, 1:10 dilution	Negative, 1:10 dilution
BNT162b	PEGylated nanoparticle	Positive, 1 µg/mL	Positive, 1 µg/mL	Positive, 1 µg/mL
AZD1222	Polysorbate 80	Positive, 1:10	Negative, 1:10	Negative, 1:10

All skin tests were carried out at as a series of 3 dilutions (1:1, 1:10, and 1:100) to a maximal, nonirritating concentration as outlined in the table body.

TABLE 2.1.3. Summary of BAT results

Compound	Range of allergen concentrations tested	BAT results		
		Patient 1	Patient 2	Patient 3
BNT162b2	0.05-10 μg/mL	5 μg/mL	0.05 μg/mL	0.05 μg/mL
PEGylated liposomal doxorubicin	1-10 μg/mL	10 μg/mL	1 μg/mL	10 μg/mL
AZD1222	1:2000 to 1:10 dilution	Negative	Negative	Negative
PEG2000	0.05-5 mg/mL	Negative	Negative	Negative
PEG 200	5 mg/mL	Negative	Negative	Negative
PEG 400	5 mg/mL	Negative	Negative	Negative
PEG 600	5 mg/mL	Negative	Negative	Negative
PEG 6000	5 mg/mL	Negative	Negative	Negative

Results indicate the concentration at which the percentage of $CD63^+$ basophils is greater than 25% of that of the positive control.

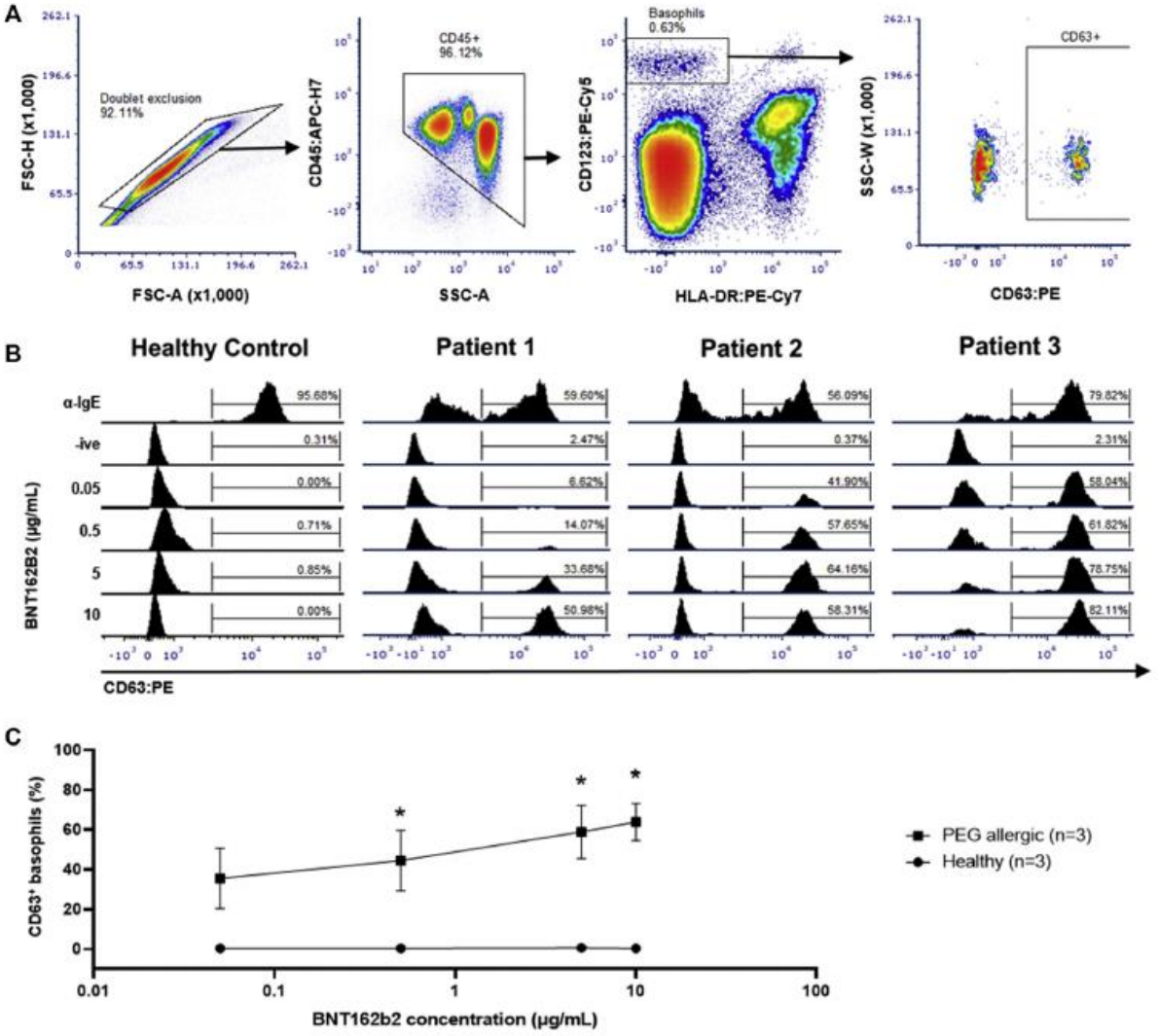

FIGURE 2.1.1. *Ex vivo* basophil activation in response to the BNT162b2 (Comirnaty) vaccine. **A**, Gating strategy for basophils and basophil activation demonstrate for a positive response. CD63+ gating with fluorescence minus one (FMO). **B**, Activation of basophils from 3 patients and a representative healthy control in response to varied concentrations of the BNT162b2 vaccine (or a positive or negative control) presented as histogram. BNT162b2 was diluted in PBS to achieve testing concentrations. **C**, Group comparison of 3 patients with PEG allergy and 3 healthy controls. Statistical significance identified by an unpaired t test for each concentration (*P < 0.05).

Given that the PEG-containing vaccine, but not PEG alone, was able to induce positive BAT and skin testing results, we repeated the BAT with freshly prepared PEGylated liposomal doxorubicin, which contains PEGylated liposomal nanoparticles similar to BNT162b2 (11). We found similar dose-dependent CD63 upregulation, even in the presence of very low concentrations of PEGylated liposomal doxorubicin (Figure 2.1.2 and Table 2.1.3), that was not observed in healthy controls, further supporting the contribution of the PEGylated liposomes to basophil activation.

This is the first study to demonstrate a positive result of COVID-19 vaccine allergy skin testing in a well-characterised cohort of patients with PEG allergy and validate specific vaccine skin testing for detection of PEG allergy. However, our study also demonstrates the limitations of current skin testing panels for macrogol and methylprednisolone acetate in PEG allergy diagnosis and indeed do not substitute for vaccine-specific skin testing. Although all of our patients had a past positive PEG skin testing result, the current skin testing results suggest that in those patients with historical reactions, intradermal testing with drugs containing PEG excipients may yield false-negative results. Intriguingly, BNT162b2 skin testing was able to identify PEG allergy in all 3 patients with PEG allergy despite 2 of them having otherwise tested negative in response to PEG-containing substances. A recent report also highlights this limitation, with a single patient with vaccine allergy demonstrating skin test positivity to only PEG 4000 and not to PEGs and PEG-containing substances with other molecular weights (12). Our results indicate a need to re-evaluate skin testing protocols for suspected PEG allergy and to include PEGylated liposomal compounds.

The current recommendations for patients with PEG allergy suggest the use of an alternative vaccine; however, in Patient 1, who was co-sensitised to polysorbate 80 and PEG, cross-reactivity between BNT162b2 and AZD1222 vaccines was observed after skin testing. Co-sensitisation with polysorbates may be seen in as many as 30% of patients with PEG allergy (13), which would greatly limit COVID-19 vaccine options, given the presence of either excipient in all major vaccines in development (5). Given the urgent need for COVID-19 vaccination, it may be necessary to utilise a desensitisation protocol for co-sensitised patients.

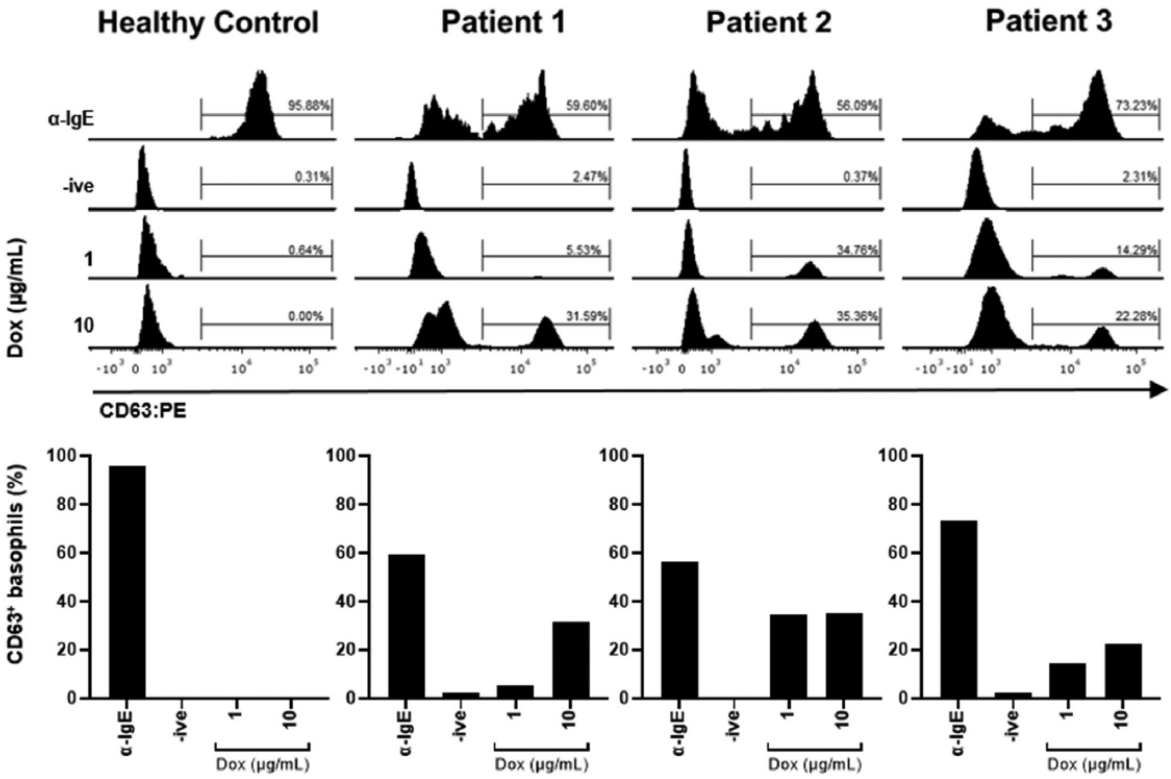

FIGURE 2.1.2. Activation of basophils from 3 patients and a representative healthy control in response to 2 dilutions of PEGylated liposomal doxorubicin (or a positive or negative control) presented as histograms and bar charts.

Ex vivo diagnosis of PEG allergy has historically been challenging and fraught with technical limitations (14, 15), with no clear established protocols. BAT is a powerful diagnostic and prognostic allergy tool, providing complementary diagnostic information to specific IgE and skin testing, with superior diagnostic accuracy for certain drug and food allergies (16). In this study, BAT using PEGylated liposomal nanoparticles was able to identify patients with PEG allergy, even in the absence of a concurrent positive result of skin testing to PEG alone. We were able to show dose-dependent basophil activation with BNT162b2 as well as with PEGylated liposomal doxorubicin, but not to unconjugated PEG. Allergic reactions to PEGylated chemotherapeutics have long been known to cause hypersensitivity-like reactions, although they have been purported to be non–IgE-mediated, complement activation–related pseudoallergy reactions (17). Some authors have suggested that complement activation–related pseudoallergy reactions may be responsible for COVID-19 PEGylated lipid nanoparticle vaccine–related allergy (1, 18). In our study, the concurrent pattern of positive and negative skin and BAT results suggests that the PEG conformation or arrangement on the surface of nanoparticles determines activation of basophils. This could be the result of increased avidity augmenting IgE cross-linking on the surface of basophils, or of by facilitation of oligomerisation-dependent, IgG-mediated complement activation (19). Future studies are required to determine the exact mechanism underlying hypersensitivity in these patients. Our study also demonstrates that PEGylated doxorubicin can effectively activate these patients' basophils, indicating that other PEGylated drugs could be surrogates to detect significant sensitisation to PEGylated lipid nanoparticles. In contrast, AZD1222 did not induce *ex vivo* basophil activation in the patient with a positive skin testing result.

Small sample size is a limitation of our study; however, we believe that our findings emphasise the importance of validating PEG allergy testing protocols in the assessment of suspected allergic reactions to PEGylated drugs, including COVID-19 vaccines.

This is the first report implicating PEGylated lipids as mediators of allergy to BNT162b2 vaccine, as demonstrated by intradermal testing and BAT. Our study indicates the indispensable requirement for specific vaccine skin testing in the evaluation of patients following allergic reactions to BNT162b2. Further studies are required to clarify whether these findings hold true in patients who experience allergic reactions following administration of the COVID-19 vaccines.

Clinical implications: Skin testing with the BNT162b2 (Comirnaty) vaccine itself, and not to PEG-containing surrogates, is required for diagnosis of BNT162b2 hypersensitivity. BAT to PEGylated liposomal drugs may be useful for the assessment of BNT162b2-associated anaphylaxis.

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2.2 MANUSCRIPT 2-

Reply: *In vivo* and *in vitro* testing with PEGylated nanoparticles

Details-

Published in the Journal of Allergy and Clinical Immunology.
Impact Factor (2021): 14.290
Submitted May 30, 2021; Accepted for publication June 02, 2021; Available online June 26, 2021; Published in print September 01, 2021.

Preface-

Manuscript 1 of this thesis attracted a correspondence from Dr John M. Kelso, clinical allergist at the Scripps Clinic in California. The manuscript presented below is our reply, published alongside Dr Kelso's letter in Issue 148, Volume 3 of the Journal of Allergy and Clinical Immunology. Both letters are presented in Appendix III. The additional patient data presented in this manuscript should be considered in sum with that of Manuscript 1 in addressing Hypothesis II of this thesis. To my knowledge, this manuscript was not subject to peer-review.

Statement of Authorship

Title of Paper	Reply to *In vivo* and *in vitro* testing with PEGylated nanoparticles
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published by the Journal of Allergy and Clinical Immunology (Mosby) Impact Factor 14.290 (2021) Accepted June 2021

Principal Author

Name of Principal Author (Candidate)	Griffith Perkins		
Contribution to the Paper	Experimental design and optimisation Laboratory work Data analysis Manuscript preparation		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Alexander Troelnikov		
Contribution to the Paper	Patient recruitment Patient characteristics		
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Name of Co-Author	A/Prof Pravin Hissaria		
Contribution to the Paper	Project conception Manuscript editing Funding		
Signature		Date	18-02-2022

Reply

To the Editor:

We thank Dr Kelso for his correspondence (1) regarding our recent article (2) in which we demonstrated that polyethylene glycol (PEG)-containing substances are not reliable surrogates for skin testing for allergy to the BNT162b2 COVID-19 vaccine. Although PEGylated liposomal drugs induced basophil activation *ex vivo* in 3 patients with PEG allergy, PEG alone did not. Dr Kelso highlights 3 important questions raised by this research that are worth discussing.

The first question relates to why 2 patients who had previously demonstrated a positive skin test result in response to PEG-containing substances appeared to have lost their reactivity. We were also intrigued by this; however, loss of skin test positivity is not uncommon in drug allergy (3), and has recently been documented in patients with PEG allergy (4). As these 2 participants reacted to PEGylated liposomal vaccine, we suggest that the inconsistent skin testing results are a consequence of PEG being poorly allergenic in its native state. In support of this, we present a fourth patient, a 38-year-old female with a history of anaphylaxis following PEG3350-containing bowel prep and PEG excipient-containing drugs. Her skin prick testing was initially performed at another institution, and the results were negative in relation to PEG200, 400, 600, 3350, 2000, and 6000. However, upon oral challenge with 35 mg of PEG3350 (MOVICOL®; Norgine, Sydney, Australia), definite urticaria was observed at multiple sites within 30 minutes. Six weeks later, the patient underwent skin testing panel as we described elsewhere (2) with a positive result in response to the BNT162b2 vaccine. In agreement with our original case series, basophil activation testing demonstrated positivity in response to the vaccine and to PEGylated liposomal doxorubicin (Fig 2.2.1) but not to PEG alone. This case suggests that skin testing with ‘‘free’’ PEG does not necessarily

equate to hypersensitivity, and as such, skin testing with PEGylated liposomal drugs is useful not only for the assessment of vaccine risk but also in the diagnosis of PEG allergy.

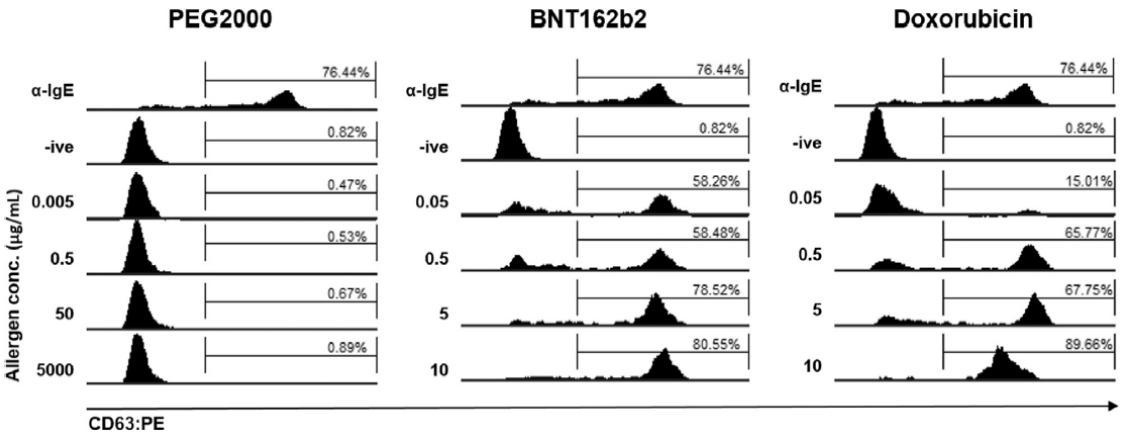

FIGURE 2.2.1. *Ex vivo* basophil activation in response to PEG2000, the PEGylate liposomal COVID-19 vaccine BNT162b2, and PEGylated liposomal doxorubicin in a patient with a positive result of PEG3350 oral challenge with a concurrent negative result of skin prick testing in response to PEG2000, PEG400, PEG600, PEG2000, PEG3350. and PEG6000.

The second question relates to the mechanism by which PEGylated liposomes induce basophil activation. Dr Kelso suggests measuring vaccine-specific IgE level in these patients as an indicator that basophil activation in response to the vaccine is IgE dependent as opposed to involving IgE-independent mechanisms such as complement activation–related pseudoallergy. Although this is valid, there are technical issues with measuring antibodies against PEG or against conformationally intact liposomes (5). The involvement of IgE can, however, be directly tested by selective depletion of IgE in an indirect basophil activation test. This is something that we intend to address.

The third question is whether these patients are representative of those experiencing anaphylaxis following administration of the vaccine. We certainly believe that these

patients are at risk given their history of anaphylaxis in response to PEG and their *in vitro* and *in vivo* reactivity to the vaccine, and we therefore agree with the existing recommendations against using PEGylated liposomal vaccines for vaccination of patients with PEG allergy. Nonetheless, we agree that it is important to now apply basophil activation testing in those who react to administration of the vaccine, and we invite contribution of patients from outside our small local population in South Australia.

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2.3. EXTENDED ANALYSIS AND DISCUSSION

Preface-

The extended data presented here reinforce the findings of Manuscript 1 and 2 in a larger cohort, and address Hypothesis I of this thesis: *Polyethylene glycol is the inciting allergen in cases of skin test hypersensitivity to the BNT162b2 vaccine*. Further discussion is also provided that places the findings of Manuscript 1 and 2 in the broader context of the South Australian experience of allergy testing to COVID-19 vaccines.

Is polyethylene glycol the inciting allergen?

In Manuscripts 1 and 2 we reported four patients with positive intradermal testing to BNT162b2 vaccine, however three out of the four did not react to any other drug containing PEG. Similar findings were made *ex vivo,* with PEG, at a variety of molecular weights, failing to induce measurable basophil activation. Given that all patients had a history of systemic hypersensitivity reactions to free-PEG-containing drugs (which was unequivocally demonstrated for Patient 4 who underwent oral challenge with MOVICOL®), we concluded that, despite its apparent poor allergenicity, PEG must be the inciting component of the BNT162b2 vaccine (and PEGylated liposomal doxorubicin). However, we did not address this directly.

In an extended cohort of nine patients with evidence of *in vivo* or *ex vivo* basophil reactivity to the BNT162b2 vaccine (Table 2.3.1; includes the four published cases), just two out of nine demonstrated skin test reactivity to free-PEG-containing drugs. Basophil reactivity to BNT162b2 vaccine was assessed as previously, and results presented in Figure 2.3.1A.

In order to test whether binding of PEG from the vaccine nanoparticle was required for basophil activation, we performed a competitive inhibition experiment. Whole blood was incubated with the vaccine as previously to induce basophil activation, and varied concentrations of PEG2000 were added concurrently. In this scenario we saw that while PEG2000 alone was unable to induce detectable basophil activation, the addition of PEG2000 at concentrations at and above 50 μg/mL inhibited basophil activation by the vaccine (Figure 2.3.1B). Similar results were observed when samples were pre-incubated with PEG2000 (n = 3, data not shown). To exclude the possibility that PEG2000 was non-specifically suppressing basophil activation at high concentrations, the effect of co-stimulation with PEG2000 (5000 μg/mL) on anti-IgE induced basophil activation was measured and found to be negligible (Figure 2.3.1B).

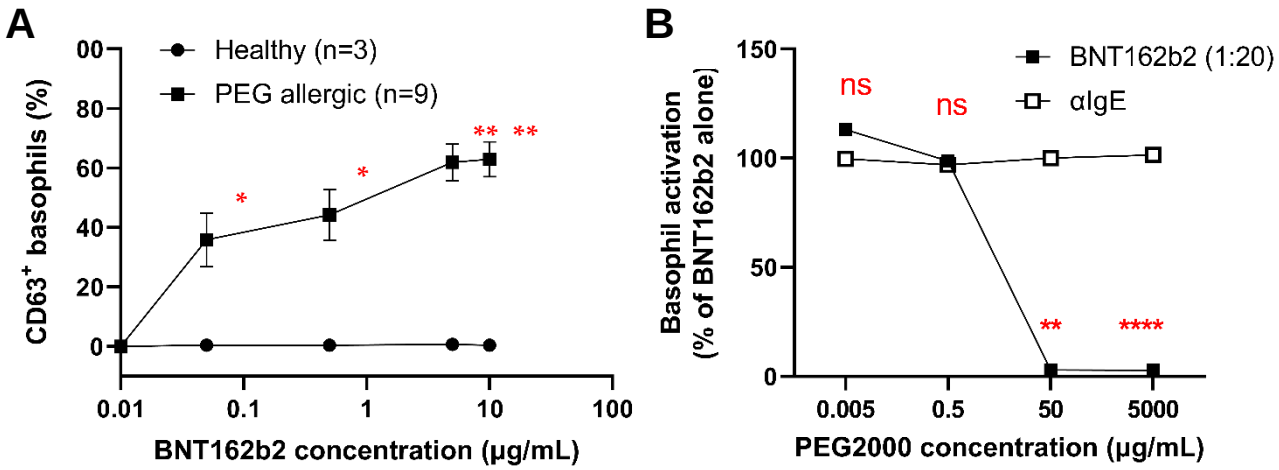

FIGURE 2.3.1. Basophil reactivity to BNT162b2 vaccine is PEG-specific. **(A)** Heparinised whole blood from 9 patients with positive or equivocal skin testing to BNT162b2, and 3 non-allergic individuals, was stimulated for 20 minutes with BNT162b2 vaccine (final dilution 1:20). **(B)** Blood samples were stimulated with BNT162b2 vaccine (final dilution 1:20) or anti-IgE for 20 min to induce basophil activation. PEG2000 was added concurrently at varied concentrations, and inhibition calculated as the percentage basophil activation relative to the uninhibited (vaccine or anti-IgE alone) condition. Statistical significance by paired Student's T test: **$p < 0.01$, *$p < 0.05$, ns=non-significant.

TABLE 2.3.1. Patient characteristics and summary of skin test results*

	AGE	SEX	SENTINEL REACTION	TIME SINCE LAST REACTION	SKIN PRICK TESTING	INTRADERMAL TESTING
Patient 1	51	F	MOVICOL®	3 months	MOVICOL® (PEG3350)	Methylprednisolone (PEG3350), BNT162b2 1:100, Triamcinolone (polysorbate 80) 0.4 mg/mL, ChAdoX1-S 1:100
Patient 2	55	M	MOVICOL®	3 months	Negative	BNT162b2 1:100
Patient 3	22	F	Methylprednisolone acetate	5 years	Negative	BNT162b2 1:100
Patient 4	38	F	MOVICOL®	3 months	Negative	BNT162b2 1:100
Patient 5	51	F	MOVICOL®	22 months	Negative	BNT162b2 1:100
Patient 6	33	F	Intramuscular depo-medrol (PEG-containing)	5 years	Triamcinolone (polysorbate-80), Methylprednisolone acetate (PEG3350)	BNT162b2 1:100, ChAdOx1-S 1:100
Patient 7	54	F	MOVICOL®	3 months	Negative	Equivocal BNT162b2 1:100, 1:10
Patient 8	57	F	Methylprednisolone acetate	14 years	Negative	BNT162b2 1:100
Patient 9	36	F	MOVICOL®	2 months	Negative	BNT162b2 1:10

*Patients 1, 2 and 3 were presented in Manuscript 1. Patient 4 was presented in Manuscript 2.

Clinical context- a summary of the South Australian experience with COVID-19 vaccine allergy

To date, allergy testing has been performed on 55 patients at the Royal Adelaide Hospital Specialty Vaccination Clinic. Twenty one patients were referred following a reaction to the first dose of COVID-19 vaccine (BNT162b2 = 13; ChAdOx1 = 8), 21 for suspected allergy to a relevant excipient, and 13 with a history of reactions to other vaccines. Patients were predominantly female (n = 45, 82%; male n = 10, 18%), and were aged 18 to 83 (median 54) years old.

Following assessment with a combined allergy testing protocol (including skin testing and *ex vivo* basophil testing), 51 patients have now received at least one dose of a COVID-19 vaccine. Nine patients were positive to BNT162b2 by skin test and/or basophil testing. Seven of the nine were positive only for BNT162b2 and have all now received at least one dose of the ChAdOx1 vaccine. The remaining two patients were cross-reactive (skin test positive) to both the BNT162b2 vaccine and ChAdOx1 (or a polysorbate 80 containing drug), however negative basophil activation tests to ChAdOx1 in both cases informed successful challenge with this vaccine.

It is difficult to assess the relationship between the PEG allergic patients characterised in this thesis chapter and those experiencing anaphylaxis to administration of the BNT162b2 vaccine globally. Of the 13 patients referred to the Specialty Vaccination Clinic following reaction to the first dose of BNT162b2, four were considered to meet Brighton criteria for anaphylaxis. Of these four, none demonstrated positive skin test or basophil activation tests to the vaccine.

Of the nine patients that we identified with strong basophil reactivity to BNT162b2, none were challenged with this vaccine and, as such, it remains unclear what reaction the patient group would have to administration of the vaccine. However, given the

outcomes of allergy testing, this group is considered to be at high risk of anaphylaxis to administration of the vaccine. This is supported by a recent assessment of vaccine allergy in the US regional health system that identified 22 patients who had anaphylactic reactions following vaccination with an mRNA COVID-19 vaccine (1). Fifteen out of twenty two had a history of clinical allergy, and 100% of those who were followed up for allergy testing (11/11) demonstrated *ex vivo* basophil activation to their administered vaccine.

Future directions

The mechanism of reaction to the PEGylated nanoparticle vaccines remains unknown. While findings in our patient cohort are consistent with classical IgE-mediated degranulation of basophils and mast cells, the apparent lack of requirement for a priming event and low or undetectable levels of PEG-specific IgE in vaccine reactive patients in other studies support an alternative hypothesis, namely that these reactions are not necessarily IgE-dependent but instead are instances of complement-activation related pseudo allergy (CARPA) (2-5). In support of this, CARPA reactions have been reported following infusions of nanoparticle medicines, and vaccine reactive patients have been reported with high levels of PEG-specific IgM and IgG (1, 6, 7). Given the difficulties associated with measuring antibodies against non-protein antigens like PEG, and the ubiquity of PEG in common products making priming events potentially common, the concerns described above do not constitute strong evidence for an IgE-independent mechanism, particularly in our patient group with known histories of exposure and reactivity to PEG-containing products.

We intend to directly address the mechanism of basophil activation in our patient group, however this remains a future direction due to current restriction relating to COVID-19 in Australia. Dependence on IgE or IgG or complement in these reactions will be evaluated by indirect priming of basophils wherein basophils from a donor will be isolated and acid washed to strip surface IgE. Serum from PEG allergic patients will then

be depleted of IgE or IgG, or heated to inactivate complement, and used to sensitise the stripped basophils. The effect of removing IgE, IgG or complement can then be assessed by basophil reactivity to the vaccine as previously.

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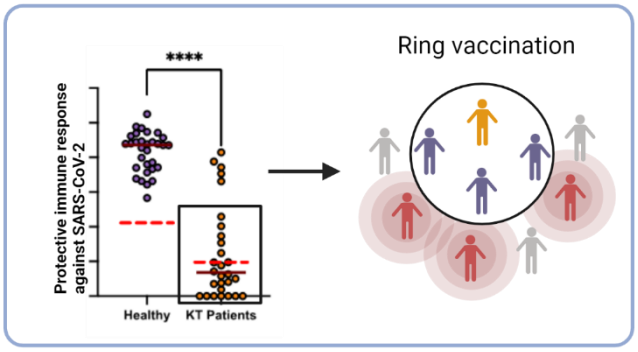
CHAPTER 3:

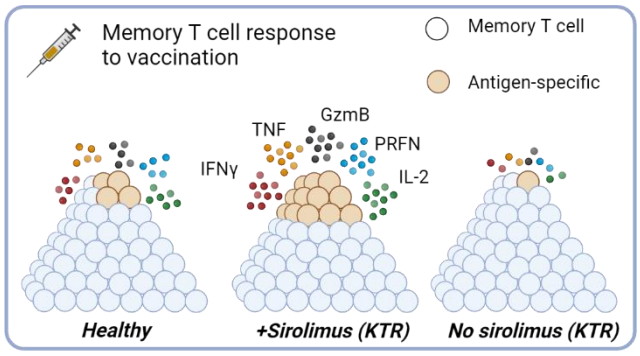
COVID-19 VACCINATION OF KIDNEY TRANSPLANT RECIPIENTS

3.1



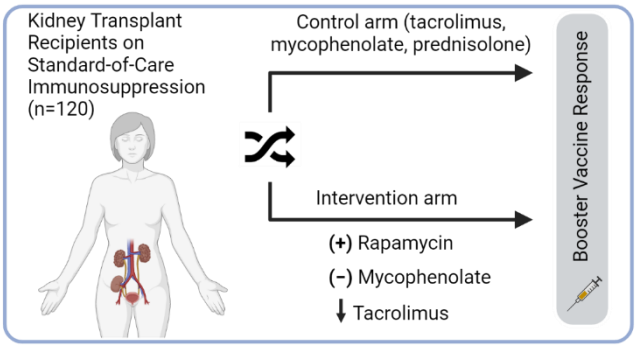
Severely impaired cellular & humoral immunity underlies poor real-world protection afforded KTRs by COVID-19 vaccination.

3.2



The first in-human evidence of mTORC1 inhibition as a strategy to boost T cell responses to vaccination.

3.3



Trial protocol to test rapamycin as an adjuvanting therapy to boost vaccine responses in kidney transplant recipients.

3.1 MANUSCRIPT 3-

Concurrent vaccination of kidney transplant recipients and close household cohabitants against COVID-19

Details-

Published in Kidney International.
Impact factor (2021): 18.998.
Submitted January 15, 2022; Revised February 07, 2022; Accepted February 16, 2022;
Available online March 16, 2022; Published in print May 01, 2022.

Preface-

In Australia, kidney transplant recipients were given priority access to COVID-19 vaccination. In this prospective study, patients were vaccinated concurrently with a close household cohabitant. This study design enabled us to evaluate the level of protection afforded kidney transplant recipients in South Australia by current vaccination strategies (Hypothesis III), and to evaluate ring vaccination as a strategy to protect immunocompromised populations against COVID-19 (Hypothesis IV). In the absence of community transmission, serological neutralisation of live SARS-CoV-2 virus was measured as the gold-standard correlate of protection from SARS-CoV-2 infection and transmission. Ethics approval and trial registration for this study are presented in Appendix VI and Appendix VII, respectively. As vaccination of cohabitants was performed at the same time as that of transplant recipients i.e., during the priority vaccination period (Phase 1B), this study was classed as an interventional trial.

Statement of Authorship

Title of Paper	Concurrent vaccination of kidney transplant recipients and close household cohabitants against COVID-19
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Published in Kidney International (Elsevier) Impact Factor 18.998 (2021) Accepted February 2022

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Contribution to the Paper	Project design Experimental design and optimisation Processing and storage of patients samples Data analysis Manuscript preparation		
Overall percentage (%)	51%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Concurrent Vaccination of Kidney Transplant Recipients and Close Household Cohabitants against COVID-19

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To the Editor:

Kidney transplant recipients (KTR) are highly vulnerable to SARS-CoV-2 infection and severe COVID-19. The 28-day case mortality for KTRs is 24%, and mounting evidence suggests poor immunogenicity and clinical effectiveness of vaccines in this group (1, 2). Priority COVID-19 vaccination and booster dosing of close household contacts (CHC), i.e., ring vaccination, has been suggested as an additional layer of protection for immunocompromised individuals (3). To address this concept, KTRs and their healthy cohabitants were concurrently vaccinated, and their immunity to SARS-CoV-2 compared in a registered observational clinical trial (ACTRN12621000532808).

Forty-six kidney-alone transplant recipients from a South Australian transplant unit currently receiving immunosuppression with a calcineurin inhibitor, antimetabolite (mycophenolate mofetil or azathioprine), and steroid were included (Table 3.1.1). Each patient was paired with a healthy domestic cohabitant of similar age. Participants received two doses of either ChAdOx1 (Oxford-AstraZeneca; n = 26 pairs) or BNT162b2 (Pfizer-BioNTech; n = 20 pairs) vaccine as per Australian Government recommendations. Transplant patients were predominantly male (n = 31, 67%), first-graft recipients (n = 40, 87%), aged 60.8 ± 12.5 years, with transplant age 6.9 ± 7.2 years (see Table 3.1.1).

Circulating anti-RBD IgG antibodies (Elecsys, Roche) and serological neutralisation of live SARS-CoV-2 virus were measured after 2 doses (median 21 days, IQR 21-24) of BNT162b2 or ChAdOx1 vaccines as correlates of protection from symptomatic COVID-19 (4). The study was further designed to enable cohabitants to serve as a healthy control group against which the broader anti-Spike IgG antibody and T cell responses of KTRs over time were compared. There was no COVID-19 community transmission in South Australia at the time of the study and SARS-CoV-2 infection was excluded by serology

in all participants by the accredited pathology service (SA Pathology; SARS-CoV-2 nucleocapsid IgG, Elecsys, Roche).

After the first vaccine dose, anti-Spike IgG (> 2σ above baseline) was detected in only 27.0% of transplant recipients and 83.3% of cohabitants. After two vaccine doses, 100% of cohabitants had detectable titres of anti-Spike IgG compared with only 44% of transplant recipients (Figure 3.1.1A). After the full course of COVID-19 immunisation (2 doses), KTRs had a median anti-Spike IgG titre >1,000-fold lower than that of healthy cohabitants (0.83 vs 1454 AUC, Figure 3.1.1A).

Where anti-Spike IgG levels reflect the magnitude of antibody response to the vaccine, anti-RBD IgG and serum live virus neutralisation are measures of functional immunity to SARS-CoV-2. Seroconversion of anti-RBD IgG (detection limit 0.4 U/mL) and serological neutralisation were both achieved by 100% of cohabitants. In contrast, 10.9% of KTRs showed serum neutralising activity, and 32.6% had detectable titres of anti-RBD IgG (Figure 3.1.1B-C).

To evaluate the level of protection afforded these groups, an anti-RBD IgG titre of 100 U/mL, and serological neutralisation of 40, were used as benchmarks for a protective vaccine response based on similar studies and accepted thresholds for influenza hemagglutination assays (4-6). All cohabitants met these thresholds, achieving anti-RBD IgG titres above 100 U/mL and 50% live virus neutralisation at dilutions of 1/40 or greater. Evidence of protective immunity was rarely achieved by transplant recipients, with 4.3% exceeding 100 U/mL anti-RBD IgG, and 8.7% achieving serological neutralisation of 40 or greater.

Antiviral T cell responses are important in viral clearance and for minimising the severity of COVID-19, particularly in the absence of an effective neutralising antibody

response (7). Conserved SARS-CoV-2 epitopes recognised by T cells may provide cross-protection against viral variants that evade antibody neutralisation (8). Therefore, we evaluated the magnitude of Spike-specific T cell responses (reported by frequency of antigen-induced IFNγ secretion) in KTRs and cohabitants by ELISpot, prior to vaccination and after 2 vaccine doses (Figure 3.1.1D). In line with previous reports (e.g., Anft *et al. (9)*), pre-existing T cell immunity to SARS-CoV-2 Spike was detected in both KTRs and cohabitants (35% and 60%, respectively). T cell responses were increased upon vaccination in 49% of transplant recipients compared to 93% of cohabitants. The median increase from baseline in T cell responses of transplant recipients was 12.6-fold lower than that of cohabitants (22 vs 278 SFU per 10^6 cells).

This study provides the first assessment of vaccine-induced SARS-CoV-2 immunity in kidney transplant recipients compared with synchronously vaccinated controls. KTRs were found to have a profoundly impaired capacity to generate specific IgG after one and two vaccine doses. Recent studies have also observed reduced T cell responses in KTRs (3), however significant pre-existing T cell reactivity has confounded interpretation. Subtracting baseline T cell reactivity allowed us to accurately measure the vaccine-induced cellular response, and revealed significant impairment in the capacity of KTRs to form antiviral T cell immunity.

The majority of SARS-CoV-2 transmission occurs between household contacts (10), and this is likely to be exaggerated amongst transplant recipients due to practised caution at avoiding infection in the community. While epidemiological studies will be important for assessing the effect of vaccination on virus transmission to immunocompromised individuals, live virus neutralisation is the current best *in vitro* correlate of protection from SARS-CoV-2 infection. In the study cohort, all cohabitants met the threshold for effective serological neutralisation, as well as that set for anti-RBD IgG titre. By contrast, only 8.7% of KTRs met at least one of the thresholds. Choice of vaccine did not significantly influence the immune response in KTRs, however superior IgG titres and

serological neutralisation were observed in household controls that received BNT162b2 (Figures S3.1.1 – S3.1.4).

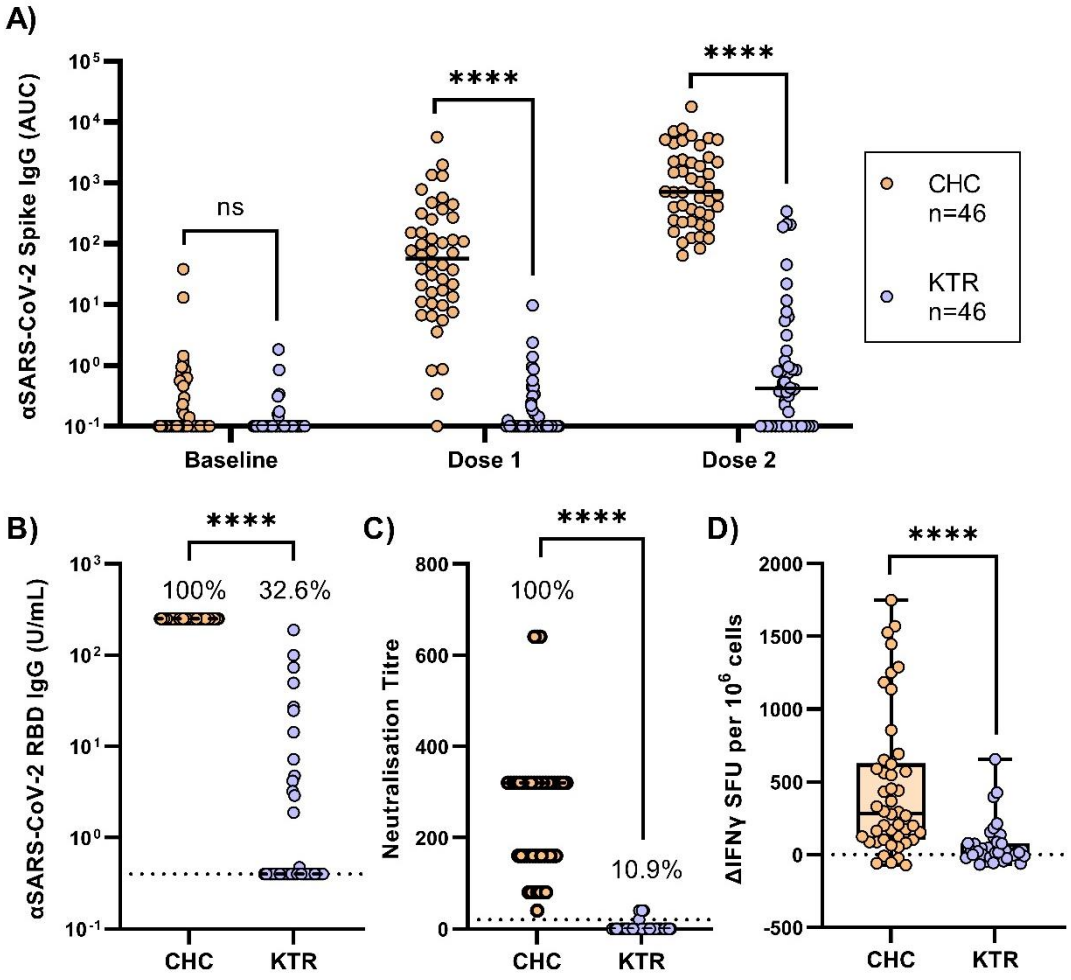


Figure 3.1.1. Immune response of kidney transplant recipients (KTR, n = 46) and cohabitants (CHC, n = 46) to 2 doses of COVID-19 vaccine. KTR and CHC were concurrently vaccinated with the BNT162b2 or ChAdOx1 vaccines. Blood samples collected at baseline, 3 weeks after the first dose, and 3 weeks after the second dose. **(A)** Log-scale longitudinal comparison of serum Spike-specific antibody titres, reported as area under the curve (AUC) units. Circles represent AUC individual participant values, with mean value denoted by black bars. **(B)** Log-scale comparison of serum anti-SARS-CoV-2 RBD IgG titres 3 weeks after dose 2 in KTR and CHC. Results below the detection threshold are plotted as 0.4 U/mL and marked with a dashed line. The percentage of

individuals above the detection limit is indicated. **(C)** Serological neutralisation end-point cut-off titres in KTR and CHC. Data points represent the highest serum dilution factor that yields 50% inhibition of live virus (Wuhan) infection. Twenty was the initial dilution for all samples, and the percentage of individuals achieving neutralisation titres of 20 or above is indicated. Individuals that did not achieve neutralisation at dilution factors of 20 or above were considered negative and ascribed a value of zero, marked with a dashed line. **(D)** Vaccine-induced IFNγ-secreting T cell response. Isolated peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with spike glycoprotein-derived peptides and the frequency of IFNγ-secreting cells measured as spot-forming units (SFU) by ELISpot. SFU at baseline were subtracted from SFU 3 weeks post second dose to determine change in Spike-reactive, IFNγ-secreting T cell frequencies in response to vaccination. Differences between groups tested by two-tailed Mann-Whitney.

In light of recent findings of the poor real-world effectiveness of the BNT162b2 and ChAdOx1 vaccines in solid-organ transplant recipients, these data provide strong support for ring vaccination of cohabitants to reduce the risk of SARS-CoV-2 infection (2). The effectiveness of ring vaccination in the real world is likely to depend on additional factors, including vaccination status of the whole of household, including children as viable vaccination strategies for children are developed, and the emergence and spread of immune-evasive SARS-CoV-2 variants. With no forthcoming strategy to enhance vaccine immunogenicity in KTRs, and efforts underway to develop booster vaccines against the Omicron variant, priority booster vaccination of household contacts should be the preferred vaccination strategy to protect immunocompromised transplant recipients.

TABLE 3.1.1. Participant characteristics

Characteristic	Transplant (n = 46)	Cohabitants (n = 46)
Age	60.8 ± 12.5 years	59.2 ± 12.5 years
Sex	F 15 M 31	F 31 M 15
Vaccine	ChAdOx1-S 26	ChAdOx1-S 25
	BNT162b2 20	BNT162b2 21
Cause of end-stage	Glomerulonephritis (17, 37%)	N/A
kidney disease	Other (12, 26%)	N/A
	Polycystic kidney disease (11, 24%)	N/A
	Diabetes mellitus (3, 6.5%)	N/A
	Hypertension/renovascular (2, 4.5%)	N/A
	Unknown (1, 2%)	N/A
Age of transplanted	0.2 – 34.6 years	N/A
kidney	6.9 ± 7.2 years	N/A
	Median 5.0 years	N/A
Graft number	First graft: 40	N/A
	Second graft: 6	N/A
Graft function	eGFR 54.8 ± 18.5 mL/min/m^2	N/A

Disclosure

The authors declare no conflicts of interest.

Supplementary Methods

Study population

This observational clinical trial (ACTRN12621000532808) was conducted on a single centre population of KTR and their CHC commencing in February 2021. Participants were identified by clinical nephrologists and screened. Inclusion criteria were kidney-only transplant over 18 years old with an available CHC without kidney disease. Exclusion criteria were patients with past COVID-19 infection, those who had already received a COVID-19 vaccine prior to enrolment, those who could not provide informed consent, or those who did not have a non-immunosuppressed and unvaccinated CHC. KTR immunosuppressed with a CNI, antimetabolite, and steroid are presented here. Demographics including gender, age, cause of kidney disease, and graft details were collected and presented as percentages for ordinal variables, and median ± range for continuous variables. See consort diagram below.

Sample processing and PBMC cryopreservation

Forty to fifty millilitres of whole blood was collected from participants by venepuncture prior to vaccination, three weeks after their first dose, and three weeks after their second dose. PBMCs were isolated by density gradient centrifugation within 18 h and cryopreserved in liquid nitrogen prior to analysis.

Anti-SARS-CoV-2 spike IgG

Spike IgG was quantified with an in-house assay, as previously described (Valtanen et al). Prefusion SARS-CoV-2 Spike ectodomain (isolate WHU1, residues 1-1208) with HexaPro mutations (kindly provided by Dr Adam Wheatley) was produced for ELISA. Recombinant proteins were overexpressed in Expi293 cells (Thermo Fisher) and 72 hrs later purified by Ni-NTA affinity and size-exclusion chromatography. Purified proteins

were quantified using the Bradford protein assay (Bio-Rad) and analysed by SDS-PAGE and Western blot before storage at -80°C.

MaxiSorp 96-well plates were coated overnight at 4°C with 5 g/mL of recombinant Spike protein and blocked with 5% w/v skim milk in 0.05% Tween-20/PBS (PBS-T) at room temperature. Heat inactivated patient sera were serially diluted in blocking buffer, added and incubated for 2 h at room temperature, followed by four washes in 0.05% PBST. HRP-conjugated goat anti-human IgG (H+L) (Invitrogen) was diluted 1:30,000 in 5% skim milk in PBST as follows and incubated for 1 hour at room temperature, followed by four washes with PBS-T. Plates were developed with 1-Step™ Ultra TMB Substrate (ThermoFisher Scientific, Waltham, MA) and the reaction stopped with 2M sulphuric acid. Absorbance was measured at 450 nm using Synergy HTX Multi-Mode Microplate Reader. SARS-CoV-2 Spike endpoint titres were calculated for 8 serum dilution (of half log10) and expressed as area under the curve (AUC). The baseline cut-off for seropositivity was defined as 2 standard deviations above the mean pre-vaccination titre for transplant recipients (0.969) and healthy cohabitants (11.63) independently. AUC calculations were performed using GraphPad Prism.

Anti-SARS-CoV-2 receptor binding domain IgG

RBD and nucleocapsid IgG was quantified using the 'Elecsys Anti-SARS-CoV-2 S' and 'Elecsys anti-SARS-CoV-2' assays on the Cobas system (Roche, Basel, Switzerland), as per the manufacturer's instructions. The quantitation range for detection of anti-RBD IgG in this assay is 0.4-250 U/mL.

Live virus neutralisation

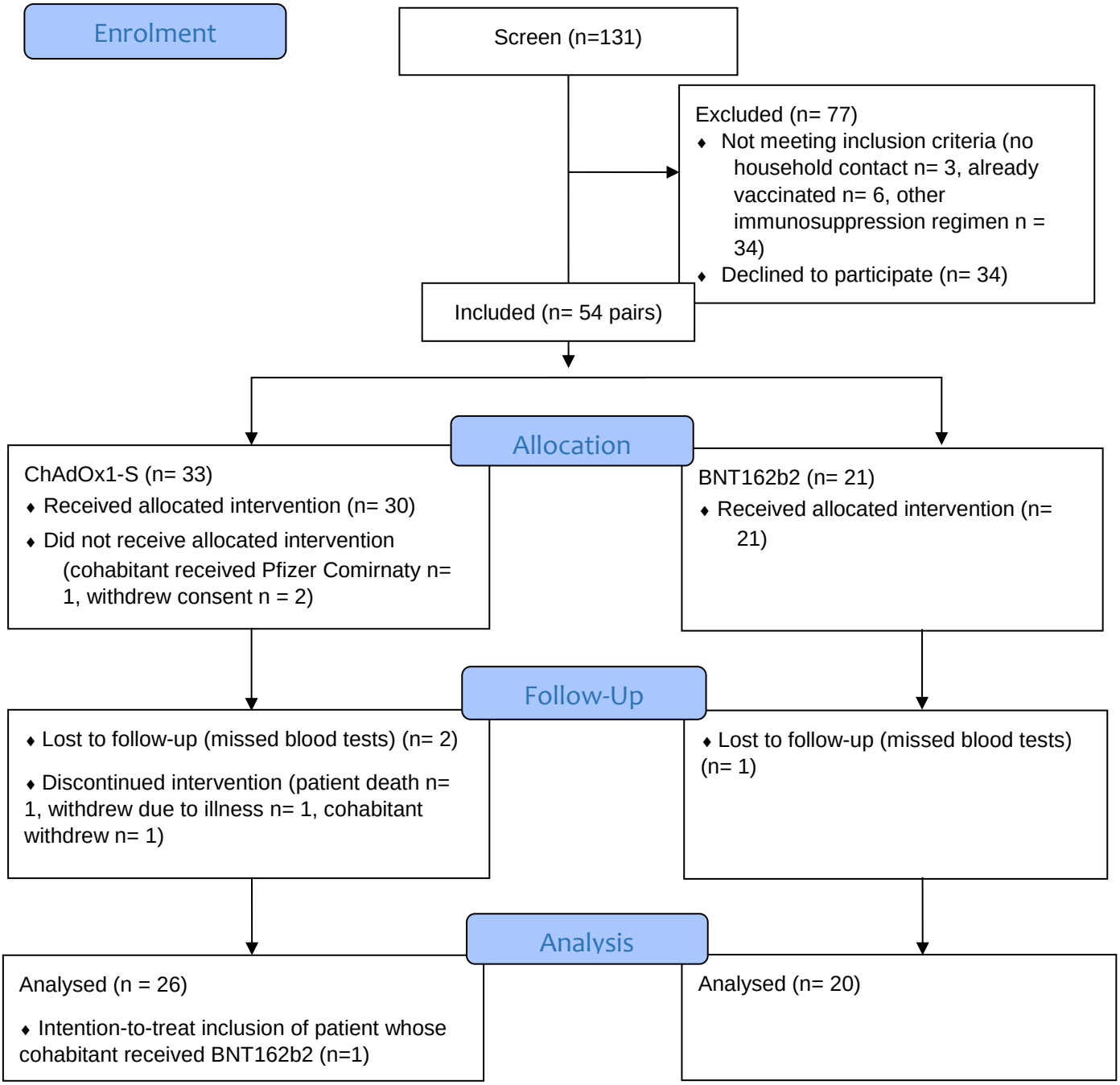
HEK-ACE2/TMPRSS cells (Clone 24; PMID: 34228725) were seeded in 384-well plates at 5×10^3 cells per well in the presence of the live cell nuclear stain Hoechst-33342 dye (NucBlue, Invitrogen, Waltham, MA) at a concentration of 5% v/v. Two-fold dilutions

of patient plasma samples were mixed with an equal volume of SARS-CoV-2 (Wuhan clade B.1) virus solution (1.25×10^4 TCID50/ml) and incubated at 37°C for 1 hour before adding 40 μL in duplicate to the cells (final MOI = 0.05). Plates were incubated for 24 hours post infection and entire wells were imaged by high-content fluorescence microscopy, cell counts obtained with automated image analysis software. Neutralisation was defined as achieving 50% virus neutralisation at serum dilution of 1/20 of greater.

IFNγ ELISpot

Millipore 96-well plates with nitrocellulose membranes (Merck, Branchburg, NJ) were activated with 35% ethanol for 30 s, before washing twice with PBS. Wells were coated with anti-IFNγ capture antibody (Clone 2G1, ThermoFisher, Cambridge, MA) overnight at 4°C, then washed twice with PBS. PBMCs were thawed by dropwise addition of complete medium (RPMI + 20% FCS, glutamate, penicillin, streptomycin) and benzonase (Merck, Kenilworth, NJ) to prevent aggregation, and rested for 2 h before counting. PBMCs were treated with 4 pools of overlapping peptides spanning the length of the spike protein. PHA (7.5 μg/mL; Merck, Branchburg, NJ, USA) and cytomegalovirus pp65 protein derived peptides (PepTivator® CMV pp65, Miltenyi Biotech, Bergisch Gladbach, Germany) were used as positive controls. After 16 h at 37°C, wells were washed five times with PBS, then ten times with PBS + 0.05% tween-20. Captured IFNγ was detected with a biotinylated anti-IFNγ antibody (Clone B133.5; ThermoFisher, Cambridge, MA) at 4 °C overnight. Unbound detection antibody was removed by washing with PBS + 0.05% tween-20, and a streptavidin:HRP conjugate (BD Biosciences, NJ) was added for four hours at 4 °C. AEC substrate (BD Biosciences, New Jersey, USA) was added for 10 min at room temperature, before rinsing with deionized water and enumeration of spots using an ImmunoSpot analyzer and software (Cellular Technology Ltd., Bonn, Germany). All washing steps were performed using an automated plate washer.

Consort Diagram



Supplementary Figures

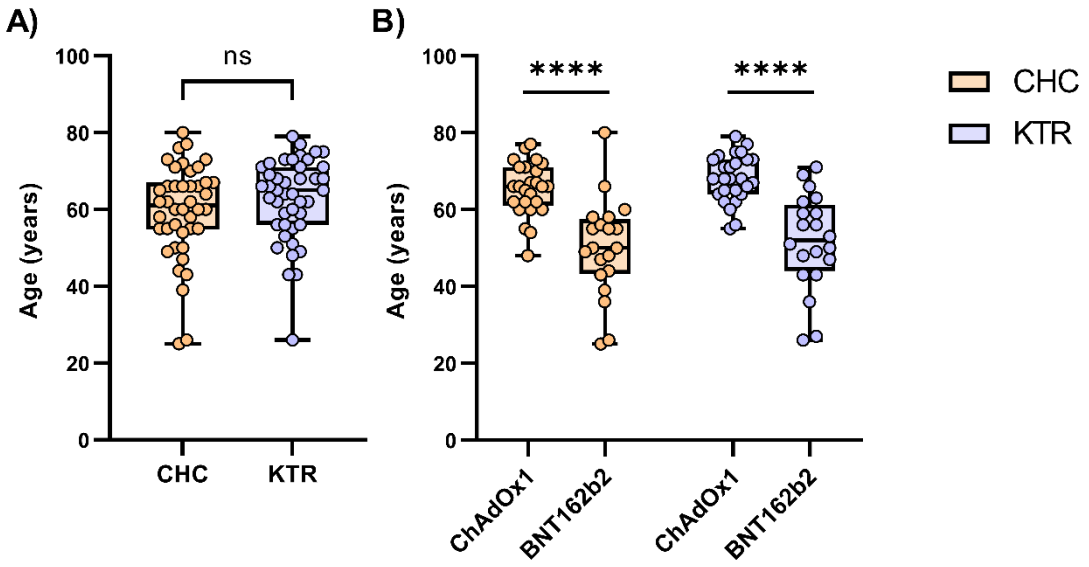


Figure S3.1.1. Participant age by treatment and vaccine. Differences between groups tested by two-tailed Mann-Whitney test.

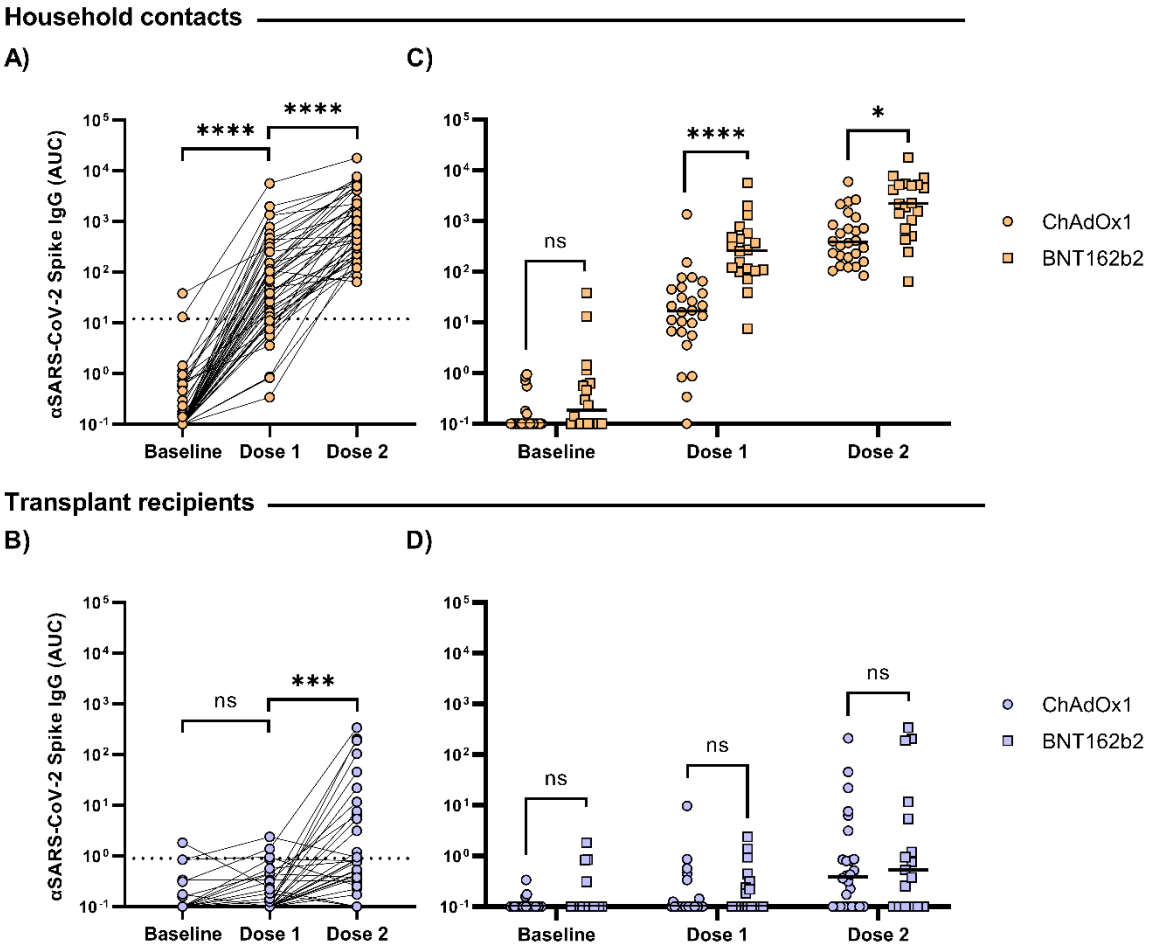


Figure S3.1.2. Anti-SARS-CoV-2 spike IgG. (A,B) Antibody titres in CHC and KTR at baseline, 3 weeks after dose 1, and 3 weeks after dose 2. (C,D) Antibody titres compared by vaccine received. Differences between groups tested by two-tailed Mann-Whitney.

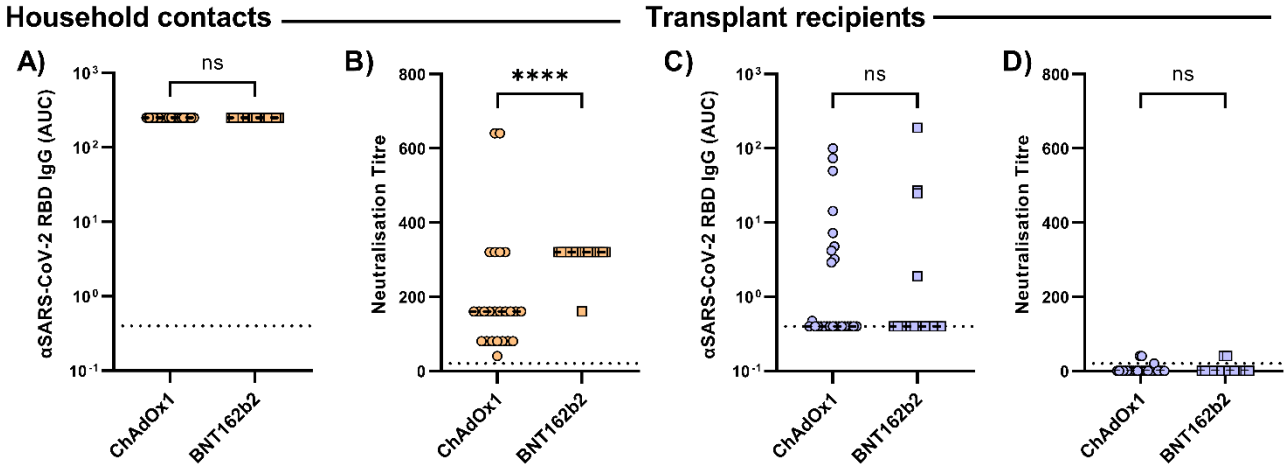


Figure S3.1.3. Anti-SARS-CoV-2 humoral immune response by vaccine. (A,C) Anti-SARS-CoV-2 RBD IgG titres in CHC and KTR, 3 weeks after second vaccine dose. (B,D) Serological live virus neutralisation in CHC and KTR 3 weeks after second vaccine dose. Differences between groups tested by two-tailed Mann-Whitney.

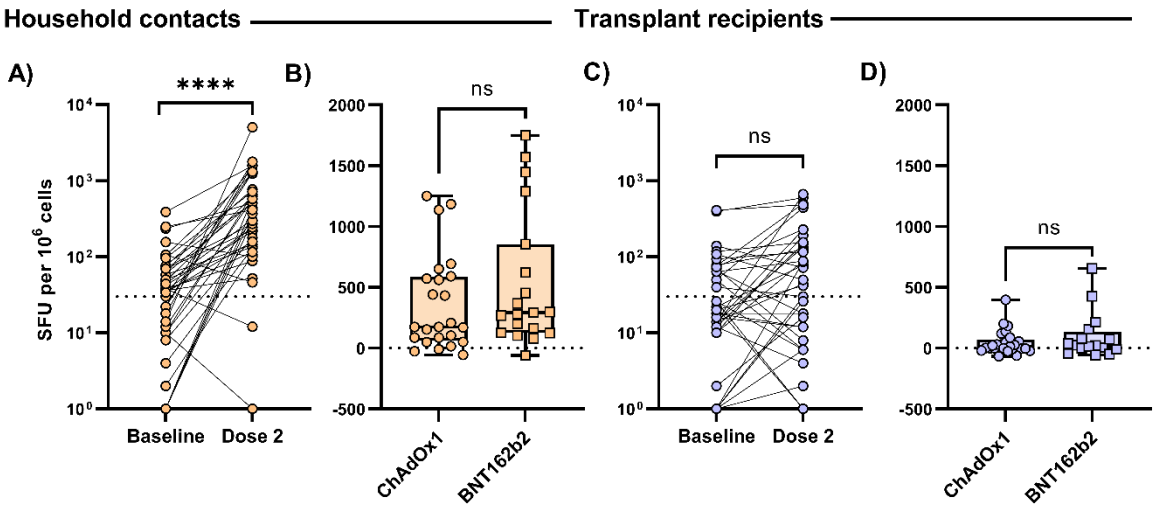


Figure S3.1.4. Vaccine-induced antiviral T cell response. (A,C) Spike-reactive IFNγ-secreting T cells in CHC and KTR at baseline and 3 weeks after dose 2. (C,D) Change in IFNγ spot-forming units between baseline and 3 weeks after second vaccine dose in CHC and KTR compared by vaccine received. Differences between groups tested by two-tailed Mann-Whitney.

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3.2 MANUSCRIPT 4-

mTOR inhibition boosts the formation of functional T cell memory following COVID-19 vaccination of kidney transplant recipients

Details-

Unpublished.

Preface-

The study presented in Manuscript 3 of this thesis revealed profound impairment of humoral (antibody) and cellular (T cell) immune responses to COVID-19 vaccination in kidney transplant recipients on standard-of-care immunosuppressive drug protocols. While ring vaccination represents an important approach in the immediate term, a strategy to directly improve vaccine immunogenicity in immunosuppressed individuals is needed. Such a strategy would likely centre on the temporary modification of patients' immunosuppression. In the present study, kidney transplant recipients receiving non-standard immunosuppressive drug protocols were actively recruited, with the aim of informing a drug modification strategy that could be tested in an interventional trial (Hypothesis V). Atypical drug protocols of interest included mycophenolate mofetil (MMF)-free protocols, as MMF is associated with strong suppression of humoral immunity, and mTOR inhibitor (mTORi)-inclusive protocols, as the mTORi rapamycin has been found to boost protective cellular immunity in preclinical models of vaccination. Manuscript 4 centres on immunogenicity of a two-dose COVID-19 vaccine schedule in patients on mTORi-inclusive protocols. Patients on MMF-free protocols were excluded from this analysis due to selection bias favouring patients with significant comorbidities.

Statement of Authorship

Title of Paper	Rapamycin boosts the formation of functional T cell memory following COVID-19 vaccination
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	N/A

Principal Author

Name of Principal Author (Candidate)	Griffith Perkins		
Contribution to the Paper	Project design Experimental design and optimisation Processing and storage of patients samples Data analysis Manuscript preparation		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01-07-2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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mTOR inhibition boosts the formation of functional T cell memory following COVID-19 vaccination of kidney transplant recipients

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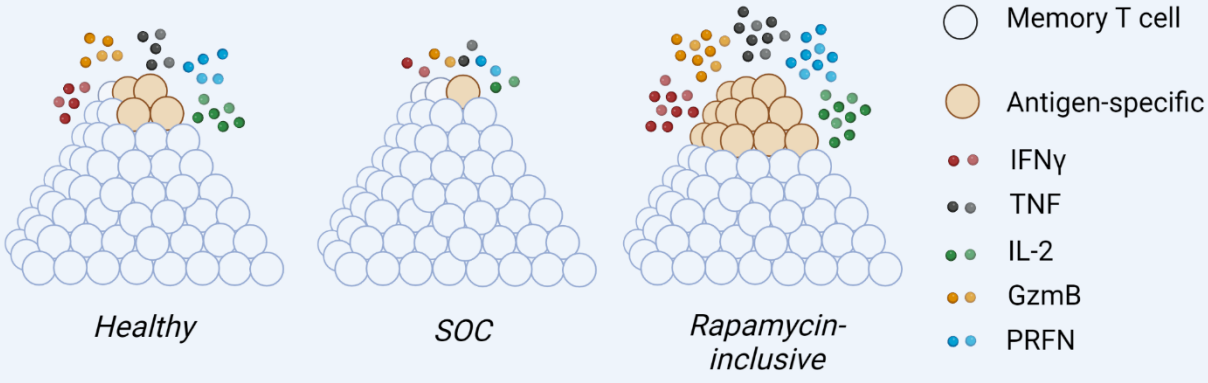
1. mTORi-based alternative to standard-of-care immunosuppression

Functional T cell mem.	*Increased*
Mem. T cell phenotype	*Normalised*
Specific IgM + IgG	*Increased*
Functional IgG	*Non-inferior*

	SOC	mTORi alt.
Calcineurin Inhibitor:	+	-
mTOR Inhibitor:	-	+
Antimetabolite:	+	+
Steroid:	+	+

2. Effect of rapamycin use on memory T cell response to vaccination in humans



Abstract

The COVID-19 pandemic has emphasised the importance of vaccine induced T cell immunity. Strong and effective T cell immunity is especially important when considering poor antibody cross-reactivity to circulating and emerging SARS-CoV-2 variants, and impaired neutralising antibody responses to vaccination in immunocompromised groups such as kidney transplant recipients. Previously, preclinical studies have demonstrated that peri-vaccination treatment with inhibitors of mechanistic target of rapamycin complex 1 (mTORC1) promotes the formation of memory $CD8^+$ T cells and improves viral control upon rechallenge, but concomitant suppression of humoral immune responses has discouraged further investigation in human subjects.

In this study, we comprehensively evaluated vaccine-induced T cell immunity in kidney transplant recipients (KTRs) treated with mTORC1 inhibitors everolimus and rapamycin (sirolimus) after vaccination with two doses of COVID-19 vaccines BNT162b2 or ChAdOx1. We demonstrate that KTRs receiving rapamycin develop enhanced T cell immunity with an increase in frequency of vaccine-specific memory $CD4^+$ and $CD8^+$ T cells and robust Spike-specific central and effector T cell memory. Memory T cells in KTRs receiving rapamycin are highly functional and display a 'healthy' effector profile and phenotype. In contrast, significant impairment in T cell immunity is observed for KTRs receiving standard-of-care immunosuppression with a calcineurin inhibitor, antimetabolite and steroid, characterised by a reduced frequency of Spike-specific memory T cells displaying a skewed phenotype.

Direct comparison between common three-drug immunosuppressive regimens revealed that use of mTOR inhibitors in place of calcineurin inhibitors is associated with a significant increase in vaccine-specific T cell immunity, without impairment of neutralising antibody responses against the ancestral SARS-CoV-2, as well as Delta and Omicron variants. Unexpectedly, patients on mTORi-based protocols had increased

titres of anti-Spike IgG and IgM, coupled with increased frequencies of Spike-specific circulating follicular helper $CD4^+$ T cells, when compared to patients on standard-of-care immunosuppression.

The high incidence of severe COVID-19 and mortality in KTRs, coupled with reduced real-world protection afforded them by current vaccination strategies, makes this population especially vulnerable in the ongoing pandemic. Our findings demonstrate, for the first time, a positive effect of rapamycin on vaccine-induced T cell immunity in humans, and provide evidence for immunosuppression modification with rapamycin as an adjuvanting therapy to boost vaccine response in KTRs.

Introduction

Kidney transplant recipients (KTRs) are at high risk of severe disease from SARS-CoV-2 infection (1, 2). Immunocompromised individuals have been prioritised for vaccination in numerous countries, however it is now clear that COVID-19 vaccines are poorly immunogenic in this group, and afford limited real-world protection from severe disease (3-12). Vaccine booster dosing has demonstrated efficacy in those with low antibody responses, however neutralising titres remain suboptimal in the majority of patients after a fourth dose, and a subgroup of patients remain seronegative (10, 11, 13). Indirect approaches to protect KTRs through ring vaccination may be beneficial in the immediate-term, however a strategy to directly boost immunity in immunosuppressed individuals is urgently needed (12, 14). Such a strategy would likely center on transient modification of immunosuppression, and would need to strike a balance between enhancing vaccine immunogenicity without increasing the risk of transplant rejection.

The combination of a calcineurin inhibitor (CNI; commonly tacrolimus), antimetabolite (commonly mycophenolate mofetil), and steroid (commonly prednisolone) is the standard-of-care maintenance immunosuppressive drug regimen employed for the prevention of transplant rejection in KTRs. Mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) inhibitors, rapamycin and everolimus, provide some advantages over CNIs, particularly in cases of cancer-risk, however are rarely employed for long-term maintenance. In preclinical models of vaccination, the mTOR inhibitor (mTORi) rapamycin (sirolimus) has been found to promote the expansion of antigen-specific T cell memory (15-17). Treatment of mice or non-human primates with rapamycin during the peri-vaccination period results in an increase in the frequency and (per-cell) function of the resultant antigen specific $CD8^+$ memory T cell pool (15, 16). This effect is mediated by direct inhibition mTORC1 in $CD8^+$ T cells, and provides enhanced viral control in mice upon secondary antigen exposure (15). Consistent with this, mTORi use by transplant recipients is associated with reduced viral reactivation and a lower incidence of some cancers, although a causative role for T cell memory is yet to be established (18-

22). Importantly, this augmentation of the T cell memory response in mice is selectively observed when T cell antigens are presented in the context of viral infection, and not in the context of transplantation (23). As such, rapamycin may be uniquely able to enhance antiviral immunity while suppressing the transplant allo-response.

While inhibiting mTOR enhances the T cell memory response in preclinical models, mTORC1 activity is required for germinal center formation and for the development of T follicular helper (T_{FH}) cells, components of effective humoral immunity (24, 25). Despite this, a recent study trialling selective mTORC1 inhibitors in the elderly found improved humoral responses to influenza vaccination with a six week course of treatment (26). This finding was attributed to the beneficial effects of mTOR inhibitors on immune ageing, and related studies observed reductions in markers of immune senescence and incidence of infection (27, 28). Of interest, while animal studies consistently show impairment in IgG titres as a result of mTOR inhibition, treatment of mice with rapamycin during primary influenza infection resulted in a broader IgM antibody repertoire that provided remarkable cross-protection against a lethal 'pandemic' influenza strain (29).

Here, we assess immunity to the SARS-CoV-2 Spike antigen in healthy individuals and in transplant recipients on standard-of-care and mTORi-based immunosuppressive protocols following two doses of BNT162b2 or ChAdOx1 COVID-19 vaccines. We first compare vaccine responses in patients receiving standard-of-care (CNI, antimetabolite, and steroid) and mTORi-based (mTORi, antimetabolite, and steroid) three-drug regimens, in order to evaluate which of these comparable protocols is preferable for effective vaccine response. We then compare the response of patients on mTOR inhibitors with that of healthy individuals and, in doing so, provide the first evidence in humans for a boosting effect of mTOR inhibition on the formation of functional, antigen-specific T cell memory. Based on these findings, we suggest that transient

immunosuppression modification with rapamycin be trialled as a strategy to improve vaccine responses in KTRs.

Results

mTOR inhibitors are preferable to calcineurin inhibitors in a three-drug regimen for the induction of humoral and cellular immunity following vaccination

Kidney transplant recipients on standard-of-care (SOC) triple therapy (calcineurin inhibitor, antimetabolite, and steroid) have profoundly impaired humoral and cellular immune responses to a two-dose COVID-19 vaccine schedule (4, 7, 8, 12). To assess the effects of alternative immunosuppressive drug protocols on vaccine response, healthy individuals and KTRs were prospectively recruited to receive two doses of BNT162b2 or ChAdOx1, as part of the REVAX trial (ACTRN12621000532808) and in accordance with health guidelines (Table 3.2.1; Table 3.2.2). Peripheral blood samples were collected three weeks after the second dose for analysis of SARS-CoV-2-specific immunity (Figure 3.2.1) and humoral and cellular correlates of immunity were evaluated and compared in KTRs who were receiving CNI-based (SOC) versus mTORi-based regimens at the time of vaccination (Figure 3.2.2).

Consistent with previous reports, measurement of total vaccine-induced anti-Spike IgG revealed a significantly lower titre in the SOC and mTORi groups (AUC 0.2848 and 6.766, respectively) compared to healthy controls (HC; AUC 1495) ($p < 0.0001$, Figure 3.2.2A). However, the median Spike-specific IgG titre in the mTORi group was significantly higher than that of the SOC group (AUC 0.2848 vs 6.766; $p < 0.05$, Figure 3.2.2A). This trend was independent of vaccine choice (Figure S3.2.1A, B), and was also observed for Spike-specific IgM and IgA titres (Figure S3.2.2A).

Anti-Spike receptor binding domain (RBD) IgG titres, and serological neutralisation of live SARS-CoV-2 virus, are correlates of effective humoral immunity against SARS-CoV-2 (30). Despite the greater anti-Spike IgG titre in the mTORi group (Figure 3.2.2A), the mTORi and SOC groups were equivalent in median titre and proportion of patients with detectable anti-RBD IgG (33.3% vs 33.3%; Figure 3.2.2B). This was reiterated in the

similar capacity of patients' sera to neutralise live SARS-CoV-2 virus (ancestral strain) between the mTORi and SOC groups (20.0% vs 11.9%; Figure 3.2.2C). Rates of neutralisation of the Omicron variant were greater in the mTORi group compared with SOC (13.3% vs 0.0%), although this difference was not statistically significant (Supplementary Figure 3.2.2B).

Vaccine-induced T cell responses were evaluated by interferon (IFN)-γ ELISpot, where PBMCs isolated from KTRs and healthy controls post-vaccination were stimulated for 18 hours with pooled peptides spanning the full-length SARS-CoV-2 (ancestral) Spike glycoprotein. KTRs on the standard-of-care protocol had a 10-fold lower median Spike-specific T cell response than healthy controls (43 vs 430 SFU/10^6 cells; $p<0.0001$, Figure 3.2.2D). Strikingly, however, this deficiency was not observed for KTRs receiving mTOR inhibitors. Rather, the median Spike-specific T cell response of the mTORi group was 520 SFU/10^6 cells, 12-fold higher than that of the SOC group ($p<0.0001$, Figure 3.2.2D). This was independent of vaccine choice (Figure S3.2.2C, D).

KTR on mTOR inhibitors have increased frequencies of Spike-specific memory T cells after COVID-19 vaccination

Based on the strong Spike-specific T cell response observed for the mTORi group, we next asked whether this was the result of a gain-of-function effect of mTORC1 inhibition on the formation of antigen-specific $CD8^+$ memory T cells, as has been described in animal models (15, 16). As an appropriate control cohort of KTRs (dual therapy with an antimetabolite and steroid) does not exist, we instead asked whether use of rapamycin or everolimus at the time of vaccination resulted in Spike-specific T cell responses significantly greater than that of healthy individuals. To perform this comparison, we extended our IFNγ ELISpot analysis from Figure 3.2.2D to include all patients receiving an mTORi as part of their immunosuppression, regardless of other immunosuppressive medications, and stratified these patients into those on rapamycin-inclusive and

everolimus-inclusive protocols. In doing so, we found that patients receiving rapamycin demonstrated a significantly greater vaccine-induced T cell response than healthy individuals (1050 vs 430 SFU/10^6 cells; $p<0.001$, Figure 3.2.3A), indicating a gain-of-function effect of mTORC1 inhibition, under the assumption that their status as KTRs and the other immunosuppressive medications used do not boost the T cell response. No significant difference in T cell response was observed between the healthy control and everolimus groups (Figure 3.2.3A).

To further characterise this boost in the Spike-specific T cell response imparted by rapamycin use, a subcohort of 15 healthy individuals were randomly sampled from the larger cohort for a more detailed comparison with patients receiving rapamycin or everolimus (Figure 3.2.1; Table 3.2.3; Table 3.2.4). Fifteen KTRs receiving SOC triple therapy were also included in the following analyses for comparison to the healthy group (Figure 3.2.1). The healthy and SOC sample groups were representative of the larger cohort in that the Spike-specific IFNγ ELISpot response of the full 'cohort' and 'sample' populations were not significantly different from one another (Figure S3.2.3).

IFNγ ELISpot gives a broad measure of the functional, antigen-specific cellular response, however does not provide information on particular cellular phenotypes and functions that are important to antiviral immunity. With respect to our analysis, it is also of interest whether the ELISpot data signify an increased frequency of Spike-specific T cells or an increased proportion of IFNγ competent T cells in the rapamycin group. These questions were addressed by activation-induced marker (AIM) assay. Importantly, the AIM assay provides information that is independent of cytokine secretion, which is particularly important as immunosuppressive drugs may selectively target features of cytokine production pathways. Circulating Spike-specific T cells were defined as $CD4^+CD134^+CD137^+$ or $CD8^+CD69^+CD137^+$ following a 24 hour stimulation of PBMCs with a Spike-derived peptide array (31, 32)(Supplementary Figure 3.2.4; Figure 3.2.3B). This analysis revealed that KTRs receiving rapamycin had increased frequencies

of Spike-specific T cells relative to healthy controls following two vaccine doses, irrespective of the type of vaccine administered (ChAdOx1 or BNT162b2)(Figure 3.2.3C).

Antigen-specific T effector memory (T_{EM}) and central memory (T_{CM}) populations play defined roles in antiviral immunity. T_{EM} cells maintain the capacity to produce cytokines rapidly in response to infection without further differentiation, and are likely to serve as a reservoir for the replenishment of resident memory T cell populations (33). T_{CM} cells are less differentiated, self-renew, and provide a long-term source of antigen-specific T cells capable of context-dependent polarisation upon reactivation (34). T_{CM} cells recirculate through secondary lymphoid organs and, in this capacity, can support the secondary B cell response (35). These populations are broadly defined by expression of the secondary lymphoid organ homing receptor CCR7 and marker of naivety CD45RA, where T_{CM} are defined as $CCR7^{+}CD45RA^{-}$ and T_{EM} as $CCR7^{-}CD45RA^{-}$. This gating strategy further identifies T effector memory cells re-expressing CD45RA (T_{EMRA}; $CCR7^{-}CD45RA^{+}$), which are associated with protection against severe disease from influenza infection in humans (36), and naïve T cells (T_{N}; $CCR7^{+}CD45RA^{+}$).

We assessed the frequency of Spike-specific T cell of each of these four phenotypes, in both the $CD4^{+}$ and $CD8^{+}$ compartments (Supplementary Figure 3.2.4; Figure 3.2.3B). The rapamycin group demonstrated increased frequencies of all three Spike-specific memory populations compared with healthy individuals, in both the $CD4^{+}$ and $CD8^{+}$ compartments (Figure 3.2.3D, E). While no difference in Spike-specific T_{N} cell frequency was observed in the $CD4^{+}$ compartment, rapamycin use was associated with a significantly greater frequency of Spike-specific $CD8^{+}CCR7^{+}CD45RA^{+}$ 'naïve' T cells relative to healthy controls (Figure 3.2.3D). While vaccination does not increase frequencies of antigen-specific naïve T cells, the $CCR7^{+}CD45RA^{+}$ phenotype includes T stem cell memory, a population which maintains the potential to differentiate into the full spectrum of T cell memory and the role for which we have an increasing appreciation in the context of vaccination and immune memory (37).

Elevated frequencies of these antigen-specific memory populations in the rapamycin group together produced a 3.3-fold higher frequency of total Spike-specific $CD4^+$ T cells, and a 3.1-fold higher frequency of $CD8^+$ T cells, than healthy individuals (Figure 3.2.3D, E). Interestingly, preclinical studies have focussed on $CD8^+$ T cells and have not reported an increase in antigen-specific $CD4^+$ T cell memory as a result of mTORC1 inhibition (15, 38-41).

These trends were consistent out to 3 months post-second vaccine dose in a subgroup of participants who had not received their third vaccine dose by this time point (Figure S3.2.5).

While KTRs in the everolimus group demonstrated elevated frequencies of Spike-specific $CD4^+$ and $CD8^+$ T_N cells, and $CD4^+$ T_{EMRA} cells, relative to the healthy group, the effect was not observed for the total $CD4^+$ and $CD8^+$ T cell analyses (Figure 3.2.3C, D, E). Therefore, while everolimus may have a similar effect to rapamycin of boosting vaccine-induced T cell memory formation, this cannot be confirmed by our analysis. Whether this represents an intrinsic difference between the two mTOR inhibitors, or a difference between the characteristics of the rapamycin-inclusive and everolimus-inclusive patient groups, is unclear and remains to be further investigated.

In contrast to the rapamycin and everolimus treatment groups, a significant decrease in the frequencies of Spike-specific $CD4^+$ and $CD8^+$ T cells was observed for patients receiving SOC triple therapy relative to healthy controls (Figure 3.2.3C, D, E). This deficit was evident in the $CD4^+$ T_N, T_{EM}, T_{EMRA} populations, and the $CD8^+$ T_{CM} population (Figure 3.2.3D, E).

These data indicate that the strong Spike-specific T cell response observed by ELISpot

for KTRs receiving rapamycin, and the poor response observed for those on SOC triple therapy, were, at least in part, the result of altered formation or contraction of the Spike-specific memory T cell pool.

Abnormal phenotypic distribution of Spike-specific T cells in KTRs on standard-of-care immunosuppression - but not in KTRs receiving rapamycin

It is of interest to understand whether the gain-of-function effect of rapamycin use, and the loss-of-function effect associated with the SOC immunosuppression regimen, preferentially influence certain T cell populations over others. While the rapamycin group displayed a similar distribution of phenotypes within the Spike-specific (AIM^+) $CD4^+$ and $CD8^+$ populations to that of the healthy group, Spike-specific $CD4^+$ T cells in patients on SOC therapy were preferentially of a T_{CM} rather than T_{EM} phenotype relative to healthy controls (Figure 3.2.4A). A similar bias was observed in the $CD8^+$ T cell compartment, with a lower proportion of Spike-specific memory cells forming of a T_{EM} phenotype (Figure 3.2.4B). Interestingly, Spike-specific $CD4^+$ and $CD8^+$ T cells in the everolimus group were preferentially of a 'T_N' phenotype relative to healthy individuals (Figure 3.2.4A, B).

To our knowledge, this bias in phenotype of antigen-specific T cells that form following vaccination is a previously unreported abnormality associated with SOC therapy. While it is tempting to suggest that rapamycin use normalises this abnormality, the heterogeneous immunosuppression within the rapamycin group precludes this conclusion. What is evident, however, is that the subset makeup of the Spike-specific T cell compartment in KTRs on an mTORi-based three drug regimen is closer to that of healthy individuals than is that of KTRs on a CNI-based SOC regimen (Figure S3.2.6).

Functional assessment of Spike-specific CD4$^+$ and CD8$^+$ T cells in vaccinated KTRs

As the direct indicator of T cell effector function is their ability to produce immunomodulatory effector molecules upon antigen stimulation, we next assessed the functionality of Spike-specific memory T cells in the vaccinated KTRs compared to healthy individuals. Production of antiviral effector molecules IL-2, IFNγ, TNF, granzyme B (GZMB) and perforin (PRFN) by CD4$^+$ and CD8$^+$ T cells in response to Spike peptides was assessed by an intracellular cytokine staining (ICS) assay (Figure 3.2.5A; Figure S3.2.4). Cells producing cytokines (IL-2, IFNγ, TNF) in response to stimulation are considered to be antigen-specific, and frequencies correlate closely with AIM positive cell frequencies (Figure S3.2.8). As intracellular levels of GRMB and PRFN may be indirectly upregulated in response to inflammatory cytokines released by antigen-specific cells in the culture, GZMB$^+$PRFN$^+$ T cells are assessed separately, as a readout of the cytotoxic response to the Spike antigen rather than as Spike-specific cells (Lin et al, 2014).

First, we assessed the frequency of cells producing one or more cytokines in response to antigen stimulation. KTRs on rapamycin-based protocols demonstrated a greater frequency of CD4$^+$ T cells producing one, two or three cytokines compared to healthy individuals, resulting in a marked increase in total frequency of cytokine-producing CD4$^+$ T cells (Figure 3.2.5B). This elevated frequency of functional CD4$^+$ cells was consistent for all cytokines assessed individually, as well as for GZMB/PRFN single and dual positive populations (Figure S3.2.9A; Figure 3.2.5D). Unlike we saw for the frequency of Spike-specific T cell by AIM expression, functional antigen-specific CD8$^+$ T cells were not found to be elevated relative to healthy individuals (Figure 3.2.5C). This was consistent for all effector molecules measured individually, and KTRs on rapamycin demonstrated similar frequencies GZMB/PRFN single and dual positive popualtions to healthy individuals (Figure S3.2.9B; Figure 3.2.5E). While this analysis is unable to demonstrate that rapamycin use provides a boost in the frequency of functional CD8$^+$ T cells because no increase relative to healthy individuals was observed, the mTORi-based

three-drug regimen was superioir to the SOC protocol for the generation of functional $CD8^+$ T cell immunity (Figure S3.2.10B).

By contrast, KTRs receiving SOC therapy demonstrated significant impairment in cytokine-producing, Spike-specific T cell frequency relative to healthy controls, in both the $CD4^+$ and $CD8^+$ compartments (Figure 3.2.5B, C). A similar impairment in frequency of GZMB and PRFN producing $CD4^+$ and $CD8^+$ T cells was also observed in comparing these groups (Figure 3.2.5D, E; Figure S3.2.9A, B).

KTRs receiving everolimus demonstrated similar functional Spike-specific $CD4^+$ and $CD8^+$ T cell frequencies to healthy individuals (Figure 3.2.5B, C, D, E).

The relative contribution of each effector molecules to the total effector response (what may be referred to as the 'effector profile') was very similar between groups. Relative to healthy individuals, a smaller proportion of $CD4^+$ T cells in the SOC and everolimus groups produced PRFN in response to stimulation, while no differences in effector profile were observed in the $CD8^+$ compartment (Figure S3.2.9C, D).

Simultaneous expression of multiple cytokines is associated with increased effector function and viral control (42-47). While KTRs receiving rapamycin demonstrated an increase in frequency of $CD4^+$ T cells producing two or three cytokines relative to healthy individuals (Figure 3.2.5B), preclinical models have suggested that treatment with rapamycin peri-vaccination may not only increase the frequency of functional antigen-specific memory T cells, but increase the 'per cell' functional response of these cells upon viral challenge, i.e. a given number of rapamycin-treated memory T cells transferred into a naïve host will confer greater viral control than the same number of untreated memory T cells (15). This is at odds with knockout studies which have found that genetic ablation of mTORC1 signalling has the opposite effect, strongly impairing the effector

capacity of memory cells (39). As a measure of 'per-cell' functionality, we compared the proportion of cytokine-producing cells that were polyfunctional between groups. Interestingly, none of the KTR groups demonstrated a significant difference from healthy individuals in the proportion of cytokine-producing cells that were polyfunctional (Figure 3.2.5G, H). This suggests that mTORC1 inhibition at the time of COVID-19 vaccination enhances the expansion of functional antigen-specific cells, but does not improve the average functional capacity of these cells. This does not rule out other measures of functionality, for example increased capacity for steady-state self-renewal and proliferation upon challenge. Thus, it is likely that the effect of immunosuppression on T cell immunity post-COVID19 vaccination in KTRs is mediated by modulation of antigen-specific memory cell frequency rather than function.

Combinations of cytokine and effector molecules were further explored (data not shown). IFNγ and TNF were the most commonly co-expressed molecules by $CD4^+$ T cells, and granzyme B and perforin were most commonly co-expressed by $CD8^+$ T cells (data not shown). Penta-functional $CD4^+$ T cells were detected in 6/15 healthy individuals and 8/15 KTRs on rapamycin, and in 1/15 KTRs on SOC therapy (data not shown). Thus, our data clearly demonstrate that the Spike-specific T cell response to COVID-19 vaccination in KTRs on rapamycin is highly polyfunctional.

Correlates of antibody response in patients on mTORi-based protocols

Circulating T follicular helper cell (cT_{FH}) frequency correlates with antibody titres following vaccination of healthy individuals and transplant recipients (5, 48). While mTOR inhibition has been found to impair the development of cT_{FH} cells (23), it is possible that mTORi-driven outgrowth of antigen-specific cells within the cT_{FH} compartment may compensate and promote the higher Spike-specific IgG response observed in the mTORi-based three drug regimen group compared with the SOC group (Figure 3.2.2A). To test this hypothesis, we measured CD154 expression on circulating

$CXCR5^+$ $CD4^+$ T cells following stimulation with Spike peptides, using CD154 expression to define functional, Spike-specific cT_{FH} cells. Our data showed that while circulating $CXCR5^+$ $CD4^+$ T cells were reduced as a proportion of $CD4^+$ T cells relative to the SOC group (Figure S3.2.11A), $CD154^+$ $CXCR5^+$ (antigen-specific cT_{FH}) cells were increased in the mTORi group, both as a proportion of $CXCR5^+$ $CD4^+$ T cells and as a proportion of total $CD4^+$ T cells (Figure S3.2.11B, C). Despite this, the frequency of $CD154^+$ $CXCR5^+$ $CD4^+$ T cells did not correlate with anti-Spike IgG titres in the mTORi three drug regimen cohort, and therefore is unlikely to account for the increased antibody titres in this group compared to the CNI-based SOC group (Figure S3.2.11D). Additional correlation analysis identified strong positive correlations of anti-Spike IgG with total AIM^+ $CD4^+$ T cells ($r = 0.4476$, $p = 0.0158$), and with the proportion of AIM^+ $CD8^+$ T cells that were of an effector memory phenotype ($r = 0.5613$, $p = 0.0035$) (Figure S3.2.11E, F). Reciprocally, AIM^+ T_{EMRA} bias was negatively correlated with anti-Spike IgG ($r = -0.5064$, $p = 0.0098$) (Figure S3.2.11G). This correlation between antibody response and T_{EM}:T_{EMRA} skewing in the $CD8^+$ T cell compartment was also observed for anti-RBD IgG titres, and hints at a possible association between antibody responses and the 'normalised' phenotype of antigen-reactivity $CD8^+$ memory T cells observed in patients on rapamycin (Figure S3.2.11H, I; Figure 3.2.4B).

Discussion

Improving the memory T cell response has been a long-term goal of viral vaccine development. The importance of this goal has come into sharp focus during the COVID-19 pandemic and global vaccination campaigns, and was highlighted recently in an open letter to the U.S. Food and Drug Administration (FDA) stressing the need for standardised research into T cell immunity (49). An effective T cell response confers protection against severe disease from viral infection, a function that is of particularly importance in the face of SARS-CoV-2 variants, which have reduced susceptibility to vaccine-elicited neutralising antibodies but which do not evade the T cell response (2, 50, 51). Many approaches have been explored to promote the formation of T cell memory

following vaccination, with mTORC1 inhibition using rapamycin identified as a potential method in 2009 (15). However, to-date, this effect of rapamycin on the T cell response to vaccination has not been investigated in human subjects, likely discouraged by the reciprocal suppression of the IgG response observed in mice treated with rapamycin (24). Despite this, recent trials of mTORC1 inhibitors in elderly patients have described improvements in humoral immunity to vaccination, attributed to reversal of age-related immune senescence (26, 27).

Effective cellular immunity is of particular importance in immunocompromised individuals who are unable to mount an effective neutralising antibody response (52). This is true of KTRs, who remain particularly vulnerable to COVID-19 (3). A strategy to directly boost T cell and antibody responses in this group is urgently needed. Such a strategy would likely involve the modulation of patients' immunosuppression at the time of vaccination, however any significant risk to patients' transplants would not be acceptable. Preclinical models suggest that rapamycin may be uniquely able to boost the cellular immune responses to vaccination without augmenting the transplant allo-response (23).

In this study, we comprehensively evaluated the most common mTORi-based protocol at our centre (mTORi, antimetabolite, and steroid) as an alternative to standard-of-care maintenance therapy (CNI, antimetabolite, and steroid) for the generation of humoral and cellular immunity to COVID-19 vaccination. Patients receiving the mTORi-based three-drug regimen at the time of vaccination produced significantly higher titres of Spike-specific IgM and IgG (Figure 3.2.2A; Figure S3.2.2A), however this was not reflected in the titre of, nor the proportion of patients that produced, anti-RBD IgG (Figure 3.2.2A, B). IgG antibodies directed against the Spike RBD block viral entry into host cells and, as such, no significant difference in capacity to neutralise live SARS-CoV-2 virus (ancestral, Delta, nor Omicron) was observed between the two protocols (Figure 3.2.2.C; Figure S3.2.2B), suggesting that any potential increase in humoral immune

response imparted by switching of immunosuppression to the mTORi-based protocol would not be expected to confer a significant functional advantage. Despite the lack of evidence of a functional increase in humoral immunity, it is worth noting that mice treated with rapamycin during primary influenza infection are afforded remarkable cross-protection against a lethal 'pandemic' influenza strain due to a broad IgM response, despite showing reduced virus neutralisation (29).

While the elevated titres of Spike-specific IgM observed in the mTORi group are consistent with observations in mice treated with rapamycin peri-vaccination (24, 29), the higher titres of Spike-specific IgG were unexpected, as mTORC1 signalling is known to be important for the germinal centre response and, by extension, for T_{FH} differentiation and class-switching (24). As similar effects of CNI on the humoral immune response have been described (53, 54), the relative increase in anti-Spike IgG in the mTORi group may simply reflect greater suppression of the Spike-specific IgG response by CNI use than mTORi use. However, we may alternatively speculate that, as the patients in this study are older, any negative impact of mTORC1 inhibition on the humoral immune response may be offset by reversal of immune senescence, as has been demonstrated in randomised controlled trials (26, 27).

A similar increase in IgG response to COVID-19 vaccination was recently reported for KTRs receiving CNI, mTORi, and steroid, compared with SOC (CNI, antimetabolite, and steroid) (55), however mycophenolate use is strongly associated with impairment of humoral immunity in KTRs and is therefore likely the major contributor to the observed effect (5, 56-58).

While use of the mTORi-based protocol was not associated with superior functional humoral immunity, the median T cell response, as measured by IFNγ ELISpot, was 12-fold higher than that of the SOC group (Figure 3.2.2D).

To further investigate this impressive boost in cellular immunity, we assessed the Spike-specific T cell response to vaccination in a cohort of KTRs receiving rapamycin-inclusive or everolimus-inclusive drug regimens, compared with that of a healthy cohort of similar age. Using three independent measures of T cell immunity, we demonstrated that, relative to healthy controls, patients receiving rapamycin had elevated frequencies of Spike-specific memory T cells following two doses of BNT162b2 or ChAdOx1, independent of vaccine received. While elevated in frequency, the Spike-specific T cells in this group were phenotypically and functionally equivalent to those in the healthy control group (Figure 3.2.4; Figure 3.2.5). As such, patients receiving rapamycin had elevated frequencies of Spike-specific $CD4^+$ and $CD8^+$ T cells of the three major memory subsets, T_{CM}, $T_{EM,}$ and T_{EMRA}, as well as elevated frequencies of polyfunctional antiviral effector cells (Figure 3.2.3; Figure 3.2.5).

Thus, we demonstrate for the first time a gain-of-function effect of rapamycin on the formation of functional T cell memory to vaccination in humans.

In contrast, KTRs on standard-of-care immunosuppression had impaired frequencies of Spike-specific $CD4^+$ and $CD8^+$ T cells following two doses of COVID-19 vaccine. Vaccine-induced memory T cells that formed in these patients were not impaired in their capacity to produce multiple effector molecules, however proportionally favoured a T_{CM} or T_{EMRA} phenotype at the expense of T_{EM} (Figure 3.2.4).

While this study succeeds in describing the gain-of-function effect of rapamycin use on T cell response to vaccination, it is limited by its reliance on comparison with healthy individuals. The conclusion that rapamycin use confers a boost in the formation of functional T cell memory is predicated on the assumption that the other immunosuppressants received by the patients in the rapamycin-inclusive group, and their status as kidney transplant recipients more broadly, has a neutral or negative

influence on the T cell response relative to the healthy control group. While we believe that this is a reasonable assumption, as the Spike-specific T cell response was widely impaired amongst KTRs that were not receiving an mTORi, this precludes accurate assessment of the magnitude of the observed effect. A randomised, controlled trial, in which transplant recipients or healthy individuals are started on rapamycin peri-vaccination, is needed to accurately gauge the magnitude of this effect.

Furthermore, while we described the effect of rapamycin in this cohort, we did not investigate the mechanism underlying the effect. In 2009, Araki *et al* described a similar effect in mice to what we have observed in humans (15). Based on the findings of this study, the authors proposed a mechanism by which inhibition of mTORC1 biases activated naïve T cells towards a memory-precursor effector cell (MPEC) phenotype, in preference to a short-lived effector cell (SLEC) phenotype (17). Following contraction of the SLEC compartment, rapamycin treated mice retained a higher frequency of antigen-specific memory T cells. This effect was dependent on rapamycin exposure during the expansion phase of the antigen-specific T cell response (~Day 0 to Day 8). In their study, Araki *et al* described an independent effect of rapamycin treatment during the contraction phase (~Day 8 to Day 35) of improving the 'per-cell' functionality of the resultant memory T cells, however we did not find evidence of such an effect in our human cohort. It should be noted that, while the effect of rapamycin on the T cell response was observed for both the $CD4^+$ and $CD8^+$ memory compartments, studies that have investigated the effect of mTORC1 inhibition on T cell memory response (15, 38, 39, 41), including that of Araki *et al*, have reported only an effect on the $CD8^+$ T cell response.

How mTORC1 inhibition biases activated naïve T cells towards an MPEC fate is unclear. One mechanism that has been proposed is direct modulation of the transcription factors T-BET and EOMES, which are linked to effector and memory identity, respectively (41, 59-61). However, as a key signalling node integrating nutrient and cytokine signals and

regulating cell cycle progression and cellular metabolism accordingly, inhibition of mTORC1 activity has the potential to modulate many additional aspects important to T cell fate, particularly given the intimate link between cell division and differentiation (15, 17, 38-41, 59, 62, 63). During the expansion phase, which follows productive antigen-encounter by naïve $CD8^+$ T cells, mTORC1 activity promotes cell cycle progression and aerobic glycolysis, which are critical aspects of the effector response (62, 64). The absence (or inhibition) of mTORC1 activity results in cells cycling more slowly and maintains the capacity to switch to oxidative phosphorylation utilising fatty acids as an energy source (fatty acid oxidation, FAO) (63, 65). As nutrients and growth factors (particularly IL-2) in the microenvironment are made scarce by the high demands of the effector expansion, the capacity to incorporate fatty acids as an alternative energy source is important for the persistence of memory precursor effector cells (MPECs) as the bulk of the effector population undergoes contraction due to nutrient deprivation (65). This fate bias has been identified as early as the first cell division of an activated naïve T cell, in which mTORC1 complexes are asymmetrically partitioned between daughter cells (40, 66). This partitioning is governed by proximity to the antigen presenting cell, wherein the proximal daughter cell receives a larger share of mTORC1 complexes in conjunction with growth factor and nutrient receptors required to drive mTORC1 activity and integrate signals that promote a SLEC fate (40, 67). By contrast, the distal daughter cell lacks the resources for high intensity cell division conducive of the SLEC fate, and is thought to preferentially give rise to MPECs (40, 67). The influence of mTORC1 activity on cellular identity of the daughter cells is so significant that $mTORC1^{high}$ and $mTORC1^{low}$ cells can be identified by size alone (68). mTORC1 is therefore intimately linked to aspects of the T cell fate decision, and inhibition of mTORC1 may bias memory formation from the very first cell division of an activated naïve T cell.

Given the importance of cellular immunity in protection against COVID-19 (69), particularly in those lacking an effective neutralising antibody response and given the emergence of antibody escape variants such as Omicron (52), this gain-of-function boost

in T cell immunity, and relative advantage of a mTORi-based three-drug immunosuppressive regimen over the SOC, is strong motivation to trial modification of immunosuppression with rapamycin as a strategy to help protect this vulnerable group against COVID-19 and other infectious diseases. To this end, systematic meta-analyses have demonstrated no difference in acute rejection risk in kidney transplant recipients receiving mTORi in place of CNI in a three-drug regimen, and reduced rates of acute rejection when used in place of an antimetabolite (70-71). Temporary modification of immunosuppression with rapamycin is common and safe given appropriate expertise (72).

Figures

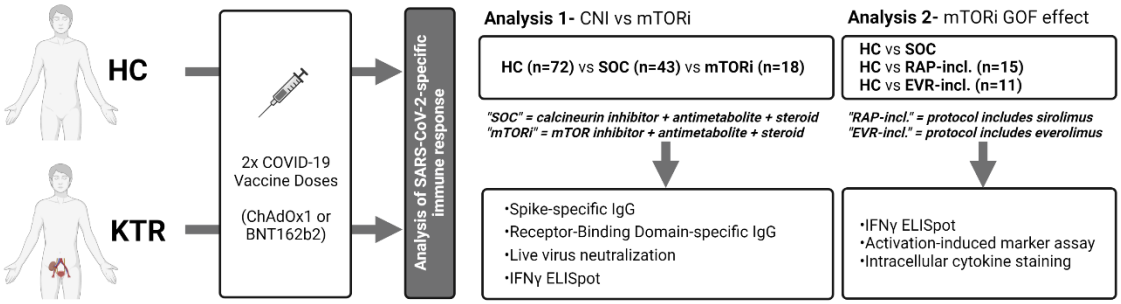


Figure 3.2.1. Study design. Unvaccinated, infection naïve kidney transplant recipients (KTR; n = 76) and healthy controls (HC; n = 72) were given two doses of ChAdOx1 or BNT162b2 vaccine as part of the REVAX trial (ACTRN12621000532808), and peripheral blood samples collected 21 days post-second dose. A comprehensive overview of the functional humoral and cellular SARS-CoV-2 Spike-specific immune response was performed. **Analysis 1:** Spike-specific immunity was compared between KTRs receiving standard-of-care (SOC) immunosuppression (n = 43) and an alternative mTOR inhibitor (mTORi)-based three-drug regimen (n = 18). Responses in both groups were compared with those of a healthy control cohort of comparable age (n = 72). **Analysis 2:** Based on a high IFNγ ELISpot response observed for the mTORi group in Analysis 1, a secondary analysis was performed comparing Spike-specific T cell response in KTRs receiving an mTORi (independent of immunosuppresion protocol) to that of healthy individuals, in order to test the hypothesis that mTORi use is associated with a gain-of-function (GOF) boost in the antigen-specific T cell memory response to vaccination. mTORi users were stratified into those receiving rapamycin (n = 15) and everolimus (n = 11), and the phenotype and function of Spike-specific T cells in this subcohort assessed by expression of activation-induced markers (AIM) and intracellular cytokine staining (ICS) in response to stimulation with Spike peptides by flow cytometry. A group of KTRs on SOC therapy (n = 15) were included in this additional analysis to better characterise the impairment in T cell response to vaccination of this group relative to healthy individuals. Created with BioRender.com.

Table 3.2.1. Cause of end-stage kidney disease- cohort for Analysis 1 (see Figure 3.2.1).

Cause of End-Stage Kidney Disease	CNI-based triple therapy regimen (n = 42)	mTOR-based triple therapy regimen (n = 18)
Glomerulonephritis	15 (36%)	8 (44%)
Other	11 (26%)	2 (11%)
Polycystic kidney disease	9 (21%)	5 (28%)
Diabetes mellitus	4 (9%)	0 (0%)
Hypertensive nephropathy / renovascular	2 (5%)	0 (0%)
Unknown	1 (3%)	3 (17%)

Table 3.2.2. Patient demographics- cohort for Analysis 1 (see Figure 3.2.1).

Characteristic	CNI-based triple therapy regimen (n = 42)	mTOR-based triple therapy regimen (n = 18)
Age	60.8 ± 12.5 years	67.3 ± 9.7 years
Sex	F: 18 (43%) M: 24 (57%)	F: 4 (22%) M: 14 (78%)
Vaccine	ChAdOx1-S: 24 (57%) BNT162b2: 18 (43%)	ChAdOx1-S: 12 (67%) BNT162b2: 6 (33%)
Graft number	First graft: 38 Second graft: 4	First graft: 12 Second graft: 6

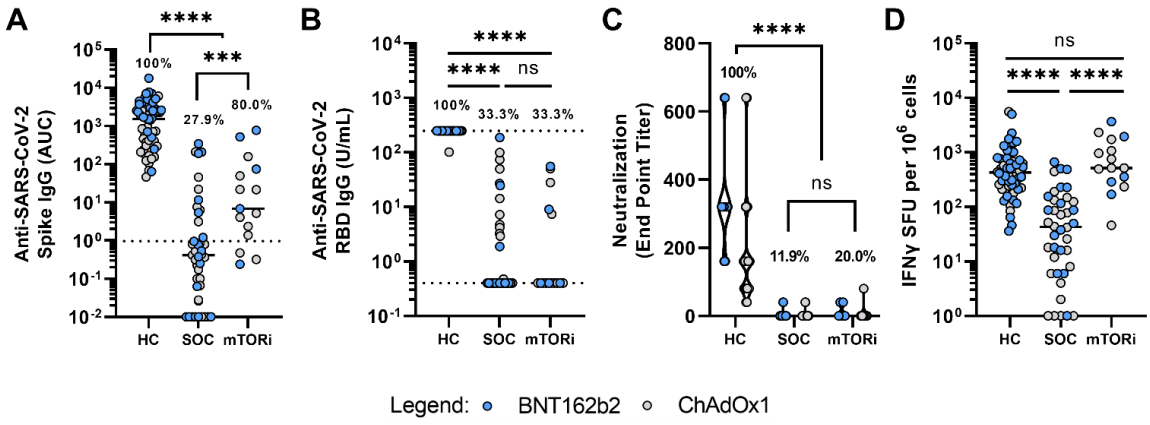


Figure 3.2.2. Immunity to SARS-CoV-2 in kidney transplant recipients- direct comparison of calcineurin inhibitor (CNI; n = 42) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 72) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. **(A)** Log-scale comparison of full-length Spike protein-specific IgG. Titres were assessed for eight serum dilutions and are presented as area under the curve (AUC). The detection threshold of AUC = 0.969 is delineated. **(B)** Log-scale comparison of SARS-CoV-2 Spike receptor-binding domain-specific IgG. The detection range for the assay is 0.4 – 250 U/mL, as demarcated by dotted lines. Percentage of seropositive individuals (above 0.4 U/mL) is shown for each group. **(C)** Minimum serum dilution for 50% neutralisation of live SARS-CoV-2 virus (ancestral strain) entry into target cells. **(D)** Log-scale comparison of vaccine-induced IFNγ-secreting T cell responses. Isolated peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with spike glycoprotein-derived peptides and the frequency of IFNγ-secreting cells measured as spot-forming units (SFU) by ELISpot. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

Table 3.2.3. Cause of end-stage kidney disease- cohort for Analysis 2 (see Figure 3.2.1).

Cause of End-Stage Kidney Disease	CNI-based triple therapy regimen subgroup (n = 15)	Rapamycin-containing regimen* (n = 15)	Everolimus-containing regimen (n = 11)
Glomerulonephritis	9 (60%)	6 (40%)	5 (45%)
Other	4 (26%)	1 (7%)	1 (9%)
Polycystic kidney disease	1 (7%)	2 (13%)	3 (27%)
Diabetes mellitus	1 (7%)	0 (0%)	0 (0%)
Hypertensive nephropathy / renovascular	0 (0%)	1 (7%)	1 (9%)
Unknown	0 (0%)	4 (26%)	1 (9%)

*Excluding 1 patient without end-stage kidney disease, recipient of islet-cell transplant for type 1 diabetes mellitus

Table 3.2.4. Patient demographics- cohort for Analysis 2 (see Figure 3.2.1).

Characteristic	CNI-based triple therapy regimen subgroup (n = 15)	Rapamycin-containing regimen (n = 15)	Everolimus-containing regimen (n = 11)
Age	62.3 ± 11.8 years	61.5 ± 10.1 years	66.9 ± 9.9 years
Sex	F: 4 (33%) M: 11 (67%)	F: 6 (40%) M: 9 (60%)	F: 2 (18%) M: 9 (82%)
Vaccine	ChAdOx1-S: 10 (67%) BNT162b2: 5 (33%)	ChAdOx1-S: 11 (73%) BNT162b2: 4 (27%)	ChAdOx1-S: 6 (55%) BNT162b2: 5 (45%)
Graft number	First graft: 15 Second graft: 0	First graft: 10 Second graft: 5	First graft: 9 Second graft: 2

Table 3.2.5. Transplant characteristics for patients receiving rapamycin

Characteristic	**Sirolimus regimens (n = 15)**	**Everolimus regimens (n = 11)**	**Triple therapy regimen (n = 15)**
Age of transplant	18.1 ± 4.1 (μ ± SD)	11.5 ± 8.3 (μ ± SD)	4.7 ± 4.3 (μ ± SD)
Type of transplant	Deceased donor 12 (80%) Live unrelated 2 (13%) Live related 1 (7%)	Deceased donor 4 (36%) Live unrelated 4 (36%) Live related 3 (27%)	Deceased donor 5 (33%) Live unrelated 5 (33%) Live related 5 (33%)
Graft number	1: 10 (67%) 2: 5 (33%)	1: 9 (82%) 2: 2 (18%)	1: 14 (93%) 2: 1 (7%)
Blood group	O+ 7 (47%) O- 1 (7%) A+ 4 (27%) B+ 2 (13%) AB+ 1 (7%)	O+ 3 (27%) A+ 4 (36%) A- 1 (9%) B+ 2 (18%) AB+ 1 (9%)	O+ 3 (20%) O- 3 (20%) A+ 6 (40%) A- 2 (13%) AB+ 1 (7%)
HLA mismatches	0: 0 (0%) 1: 3 (20%) 2: 3 (20%) 3: 2 (13%) 4: 2 (14%) 5: 4 (27%) 6: 1 (7%)	0: 0 (0%) 1: 1 (9%) 2: 1 (9%) 3: 3 (27%) 4: 1 (9%) 5: 4 (36%) 6: 1 (9%)	0: 1 (7%) 1: 1 (7%) 2: 2 (13%) 3: 2 (13%) 4: 5 (33%) 5: 3 (20%) 6: 1 (7%)
Induction therapy	Unknown: 6 (40%) Basiliximab: 5 (33%) ATG: 2 (13%) Daclizumab: 1 (7%) Steroids alone: 1 (7%)	Unknown: 2 (18%) Basiliximab: 6 (55%) ATG: 2 (18%) ATG + Rituximab: 1 (9%)	Basiliximab: 8 (53%) ATG: 6 (40%) Steroids alone: 1 (7%)
Initial immunosuppression therapy	Unknown: 6 (40%) TAC/MMF/PRED: 3 (20%) CYA/MMF/PRED: 4 (27%) TAC/MMF: 1 (7%) SIR/TAC/PRED: 1 (7%)	Unknown: 2 (9%) TAC/MMF/PRED: 8 (82%) Belatacept/MMF/PRED: 1 (9%)	TAC/MMF/PRED: 15 (100%)
Current immunosuppression therapy	SIR/MMF/PRED: 9 (60%) SIR/AZA/PRED: 1 (7%) SIR/MMF: 2 (13%) SIR/AZA: 1 (7%) SIR/PRED: 2 (13%)	EVR/MMF/PRED: 6 (55%) EVR/AZA/PRED: 1 (9%) EVR/TAC/MMF: 3 (27%) EVR/MMF: 1 (9%)	TAC/MMF/PRED: 15 (100%)

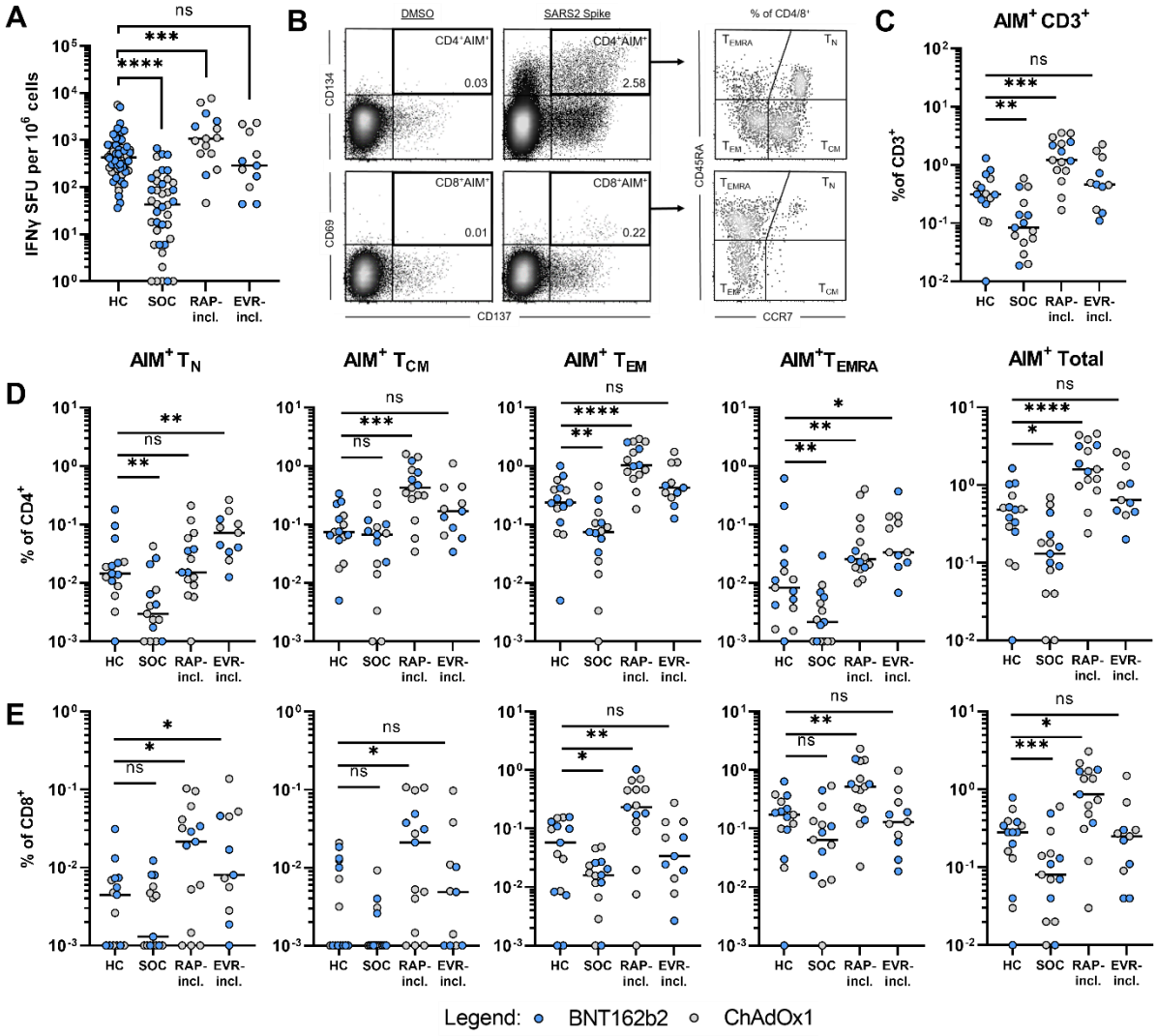


Figure 3.2.3. Frequency of Spike-specific T cell memory populations in kidney transplant recipients on standard-of-care (SOC; n = 15), rapamycin-inclusive (RAP-incl; n = 15) and everolimus-inclusive (EVR-incl; n = 11) immunosuppressive therapies, and healthy controls (HC; n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. **(A)** Log-scale comparison of vaccine-induced IFNγ-secreting T cell responses. Isolated peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with spike glycoprotein-derived peptides and the frequency of IFNγ-secreting cells measured as spot-forming units (SFU) by ELISpot. **(B, C, D)** Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (ancestral) Spike protein. T cells were gated as $CD3^{+}CD19^{-}CD14^{-}$, and

antigen-specific T cells defined as $CD4^+CD134^+CD137^+$ or $CD8^+CD69^+CD137^+$ by flow cytometry. **(B)** Gating strategy for AIM positive $CD4^+$ and $CD8^+$ T cells. Within the AIM^+ gates, four populations were defined by expression of CCR7 and CD45RA, and then back-gated to give the frequency of these AIM^+ subpopulations as a percentage of total $CD4^+$ or $CD8^+$. **(C)** Frequency of AIM^+CD4^+ and AIM^+CD8^+ T cells as a percentage of total $CD3^+$ T cells. **(D, E)** Frequency of each AIM^+ subpopulation as a percentage of the total $CD4^+$ (C) or $CD8^+$ (D) T cell population. Naïve: T_N, $CCR7^+CD45RA^+$; Central Memory: T_{CM}, $CCR7^+CD45RA^-$; Effector Memory: T_{EM}, $CCR7^-CD45RA^-$; Terminally Differentiated Effector Memory: T_{EMRA}, $CCR7^-CD45RA^+$. **Each KTR group compared to healthy controls by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

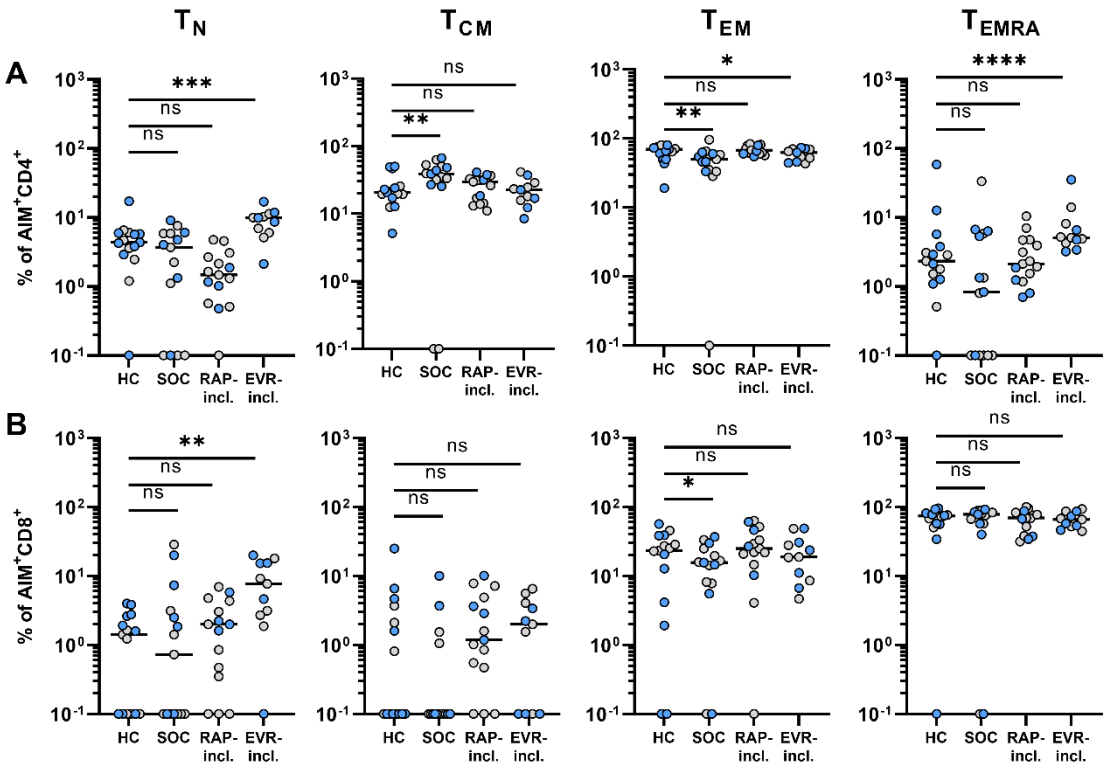


Figure 3.2.4. Phenotype of SARS-CoV-2 Spike-specific T cells in kidney transplant recipients on standard-of-care (SOC; n = 15), rapamycin-inclusive (RAP-incl; n = 15) and everolimus-inclusive (EVR-incl; n = 11) immunosuppressive therapies, and healthy controls (HC; n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and T cells defined as $CD3^{+}CD19^{-}CD14^{-}$. The extent to which each phenotype contributes to the total antigen-reactive $CD4^{+}$ **(A)** and $CD8^{+}$ **(B)** T cell pools was assessed as the percentage of naïve (T_N; $CCR7^{+}CD45RA^{+}$), central memory (T_{CM}; $CCR7^{+}CD45RA^{-}$), effector memory (T_{EM}; $CCR7^{-}CD45RA^{-}$), and terminally differentiated effector memory (T_{EMRA}; $CCR7^{-}CD45RA^{+}$) phenotype cells within the $CD4^{+}CD134^{+}CD137^{+}$ and $CD8^{+}CD69^{+}CD137^{+}$ activation-induced marker gates. **Each KTR group compared to healthy controls by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

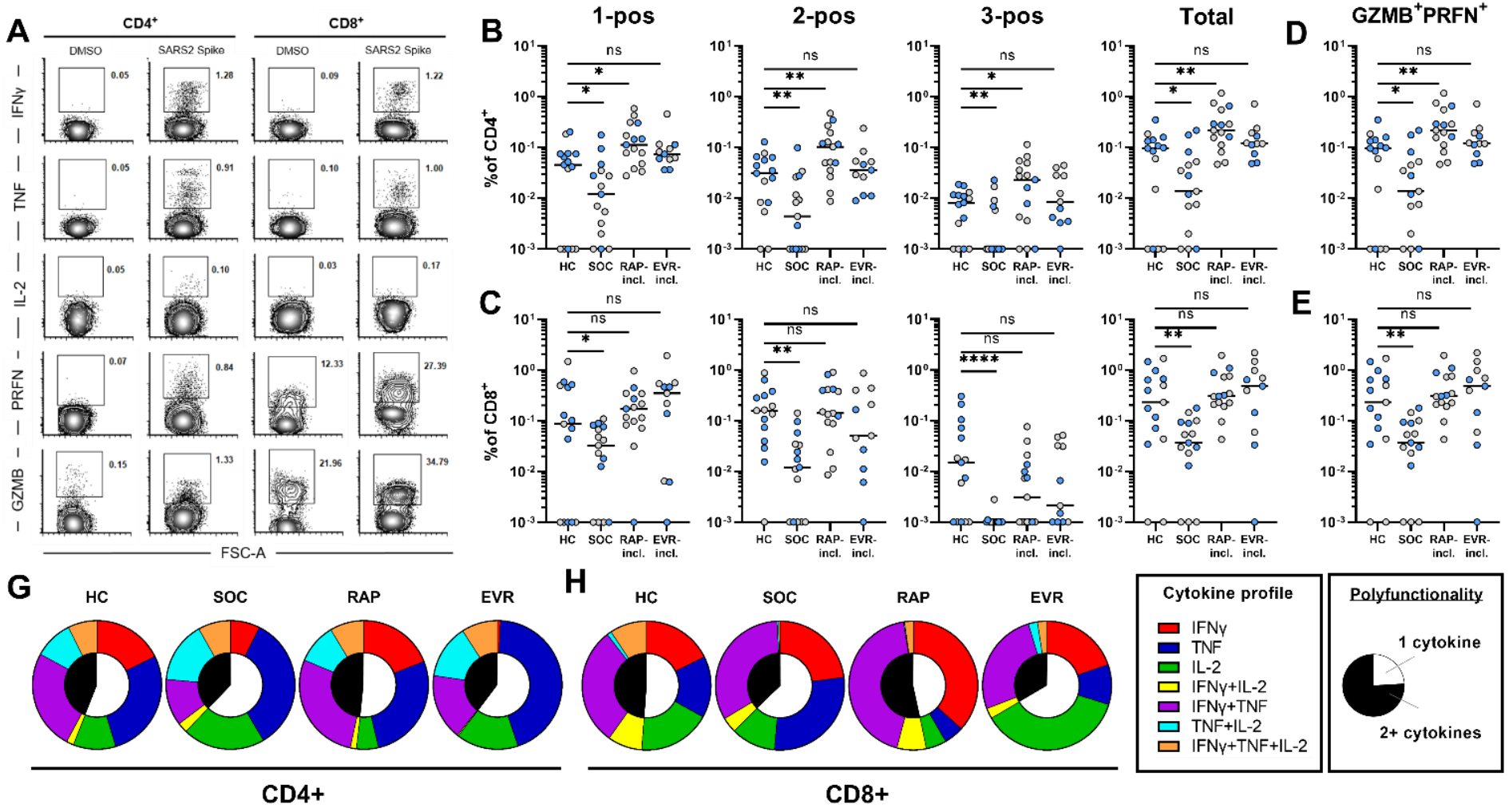


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Figure 3.2.5. Frequency and functional capacity of Spike-specific effector T cells in kidney transplant recipients on standard-of-care (SOC; n = 15), rapamycin-inclusive (RAP-incl; n = 15) and everolimus-inclusive (EVR-incl; n = 11) immunosuppressive therapies, and healthy controls (HC; n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and Golgi transport blocked for the final 4 hours prior to staining for flow cytometry. **(A)** Representative flow cytometry dot plots showing gating of effector-molecules positive $CD4^+$ and $CD8^+$ T cells. **(B, C)** Log frequency of $CD4^+$ (C) and $CD8^+$ (D) Spike-specific T cells co-producing one (1-pos), two (2-pos) or three (3-pos) cytokines. **(D, E)** Log frequency of granzyme B/perforin double positive $CD4^+$ (D) and $CD8^+$ (E) T cells following stimulation with Spike peptides. **(G, H)** Mean cytokine expression profile (outer pie chart) and proportion polyfunctionality (inner pie chart) of $CD4^+$ (G) and $CD8^+$ (H) Spike-specific T cells. **Each KTR group compared to healthy controls by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

Supplementary Figures

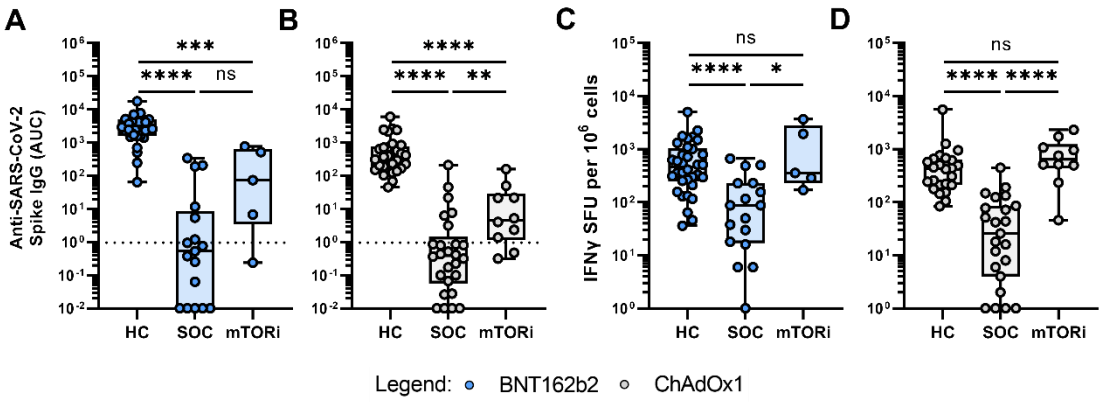

Figure S3.2.1 (relates to Figure 3.2.1). Spike-specific IgG and IFNγ ELISpot by vaccine in kidney transplant recipients receiving calcineurin inhibitor (BNT162b2, n = 17; ChAdOx1, n = 25) and mTOR inhibitor (BNT162b2, n = 8; ChAdOx1, n = 10) based three drug immunosuppressive regimens and healthy controls (BNT162b2, n = 31; ChAdOx1, n = 29) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. **(A, B)** Log-scale comparison of full-length Spike protein-specific IgG. Titres were assessed for eight serum dilutions and are presented as area under the curve (AUC). The detection threshold of AUC = 0.969 is delineated. **(C, D)** Log-scale comparison of vaccine-induced IFNγ-secreting T cell responses. Isolated peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with spike glycoprotein-derived peptides and the frequency of IFNγ-secreting cells measured as spot-forming units (SFU) by ELISpot. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

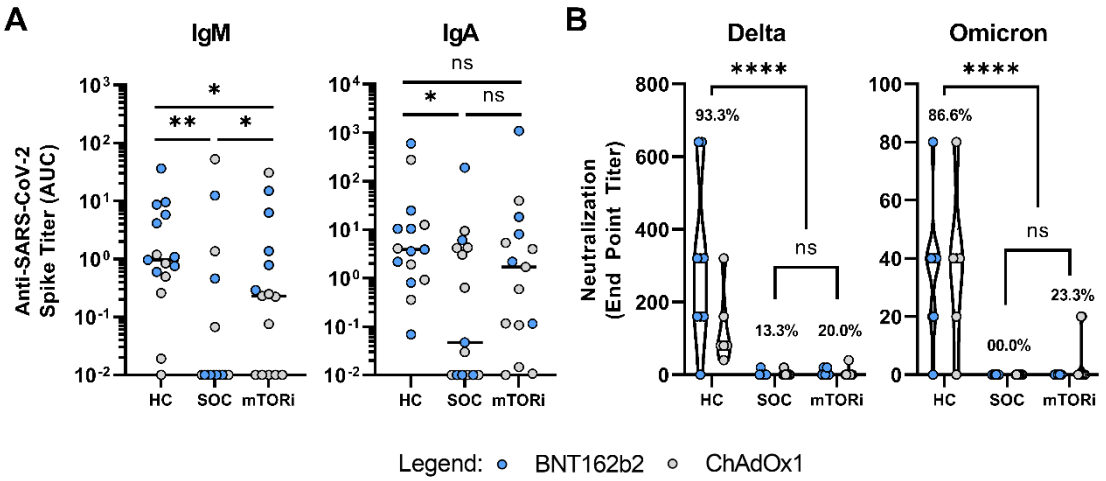

Figure S3.2.2. IgM, IgA and live virus neutralisation (Delta, Omicron) in kidney transplant recipients receiving calcineurin inhibitor (CNI; n = 15) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. **(A)** Log-scale comparison of full-length Spike protein-specific IgM and IgA. Titres were assessed for eight serum dilutions and are presented as area under the curve (AUC). **(B)** Log comparison of live virus neutralisation endpoint titre- minimum serum dilution for 50% neutralisation of live SARS-CoV-2 virus (Delta and Omicron) entry into target cells. Graphs are coloured by vaccine. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

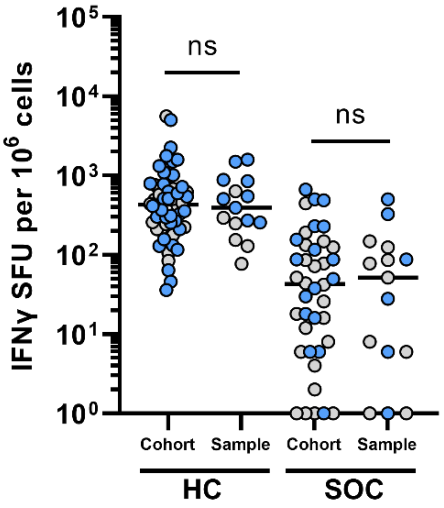

Figure S3.2.3. Comparison of IFNγ ELISpot response between the full cohort (Figure 3.2.1) and sample populations (Figures 3.2.2 – 3.2.5) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Isolated peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with spike glycoprotein-derived peptides and the frequency of IFNγ-secreting cells measured as spot-forming units (SFU) by ELISpot. **Comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

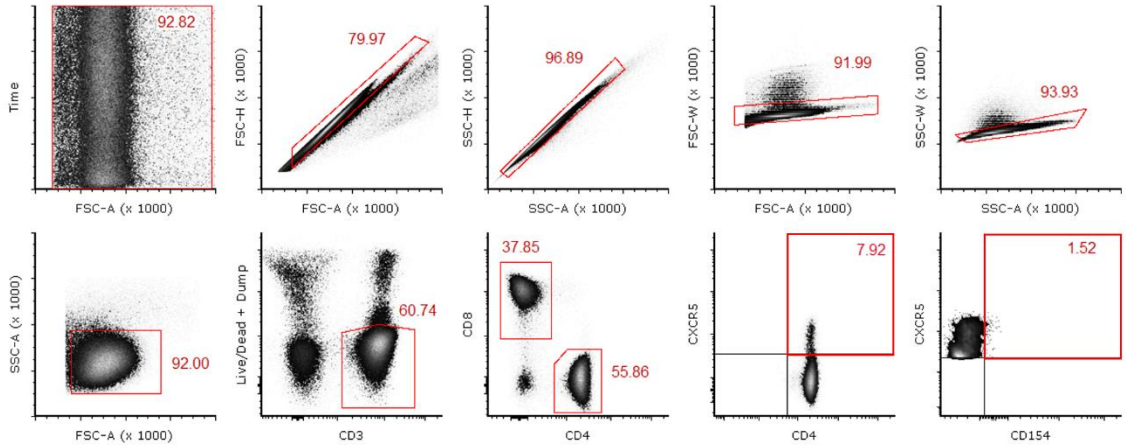

Figure S3.2.4 (relates to Figures 3.2.3, 3.2.4 and 3.2.5). Base gating strategy used for analysis of activation-induced marker (AIM) expression and intracellular cytokine staining (ICS). Singlets were identified (top row) and T cells defined as $CD3^+$ at the exclusion of $CD14^+CD19^+$ (dump gate) and dead cells. AIM and ICS markers of interest were quantified within the $CD4^+$ and $CD8^+$ gates, or cT_{FH} cells were defined as $CD4^+CXCR5^+$, and Spike-specific cT_{FH} cells quantified based on expression of CD154 (CD40L) following antigen stimulation.

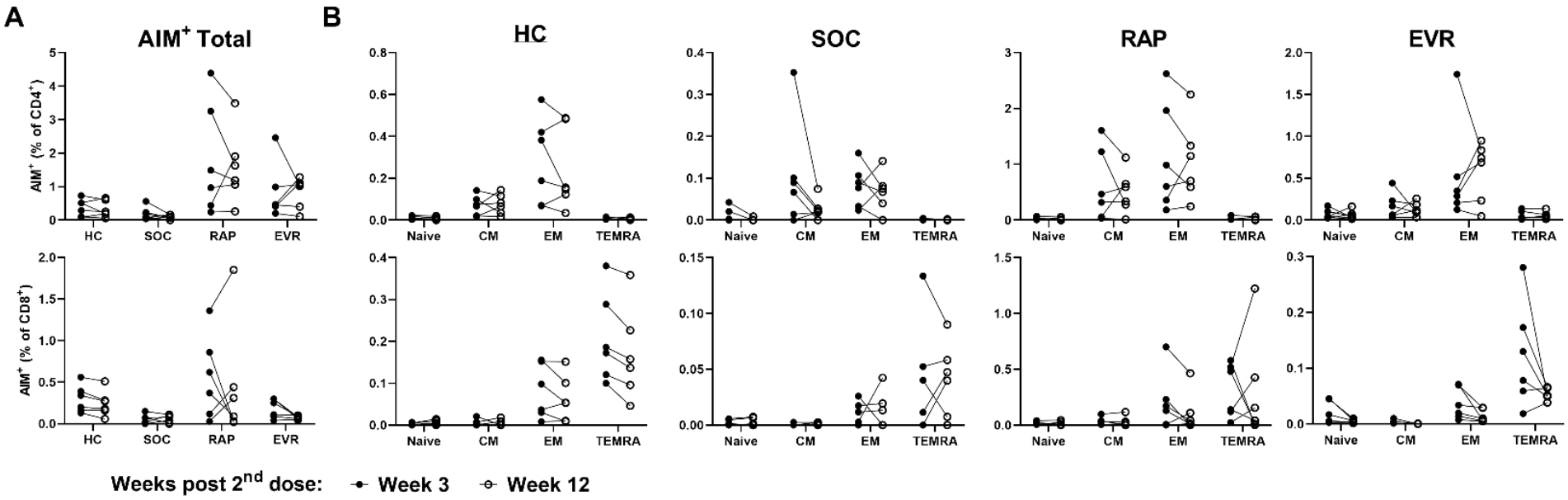

Figure S3.2.5 (relates to Figure 3.2.3). Follow up analysis of Spike-specific T cell responses by AIM assay at 3 weeks versus 12 weeks post second COVID-19 vaccine dose in KTRs on SOC (n = 6), rapamycin-inclusive (n = 6) or everolimus-inclusive (n = 6) therapies, and healthy controls (n = 6). Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (ancestral) Spike protein. T cells were gated as $CD3^{+}CD19^{-}CD14^{-}$, and antigen-specific T cells defined as $CD4^{+}CD134^{+}CD137^{+}$ or

$CD8^+CD69^+CD137^+$ by flow cytometry. **(A)** Frequency of AIM^+CD4^+ and AIM^+CD8^+ T cells. **(B)** Frequency of AIM^+ naïve (T_N, $CCR7^+CD45RA^+$), central memory (T_{CM}, $CCR7^+CD45RA^-$), effector memory (T_{EM}, $CCR7^-CD45RA^-$) and terminally differentiated effector memory (T_{EMRA}, $CCR7^-CD45RA^+$) cells as a percentage of total $CD4^+$ and $CD8^+$ T cell compartments. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

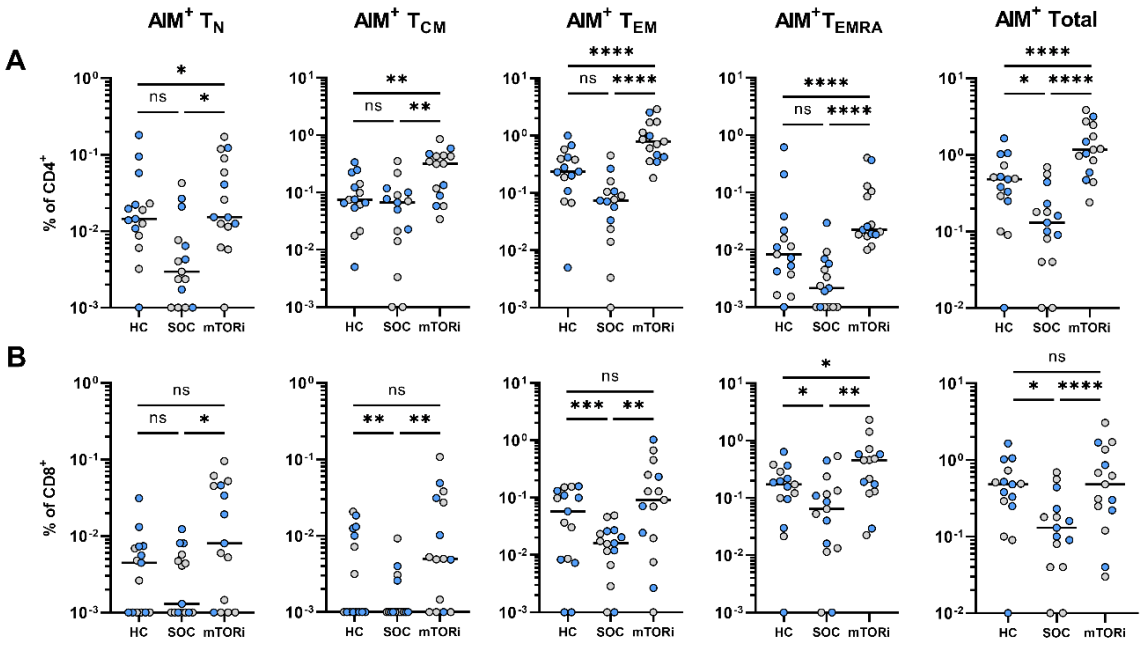

Figure S3.2.6 (relates to Figure 3.2.3). Direct comparison of Spike-specific T cell frequencies between kidney transplant recipients receiving calcineurin inhibitor (CNI; n = 15) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (ancestral) Spike protein. T cells were gated as $CD3^{+}CD19^{-}CD14^{-}$, and antigen-specific T cells defined as $CD4^{+}CD134^{+}CD137^{+}$ or $CD8^{+}CD69^{+}CD137^{+}$ by flow cytometry. **(A, B)** Frequency of each AIM^{+} subpopulation as a percentage of the total $CD4^{+}$ (C) or $CD8^{+}$ (D) T cell population. Naïve: T_N, $CCR7^{+}CD45RA^{+}$; Central Memory: T_{CM}, $CCR7^{+}CD45RA^{-}$; Effector Memory: T_{EM}, $CCR7^{-}CD45RA^{-}$; Terminally Differentiated Effector Memory: T_{EMRA}, $CCR7^{-}CD45RA^{+}$. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

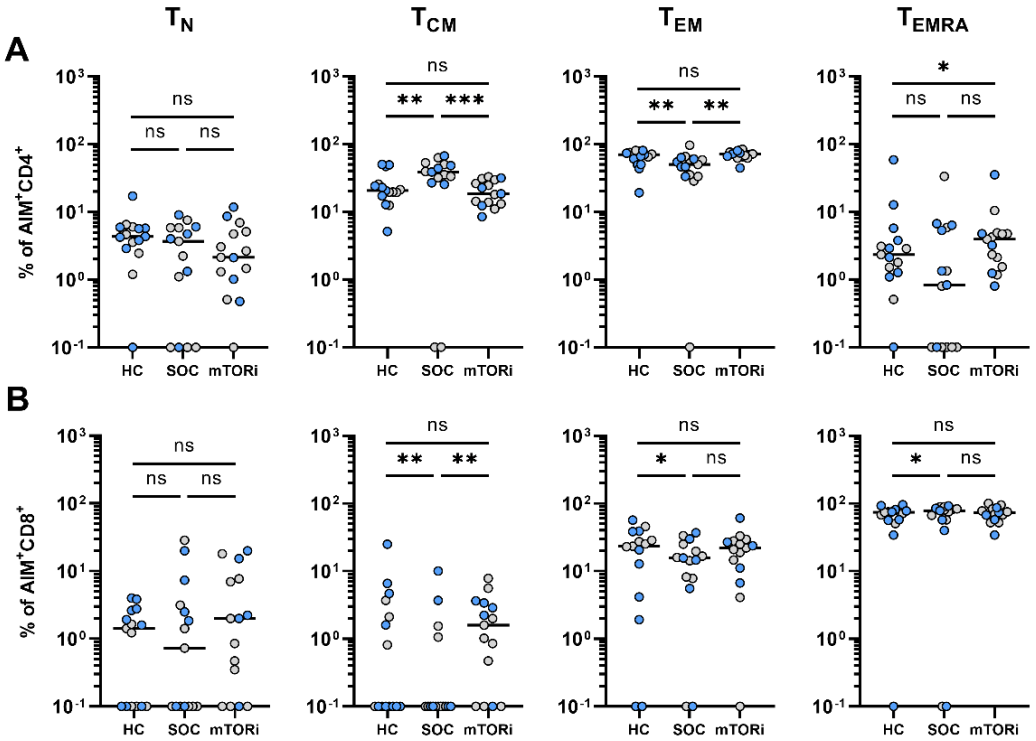

Figure S3.2.7 (relates to Figure 3.2.4). Direct comparison of Spike-specific T cell phenotype between kidney transplant recipients receiving calcineurin inhibitor (CNI; n = 15) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and T cells defined as $CD3^+CD19^-CD14^-$. The extent to which each phenotype contributes to the total antigen-reactive $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell pools was assessed as the percentage of naïve (T_N; $CCR7^+CD45RA^+$), central memory (T_{CM}; $CCR7^+CD45RA^-$), effector memory (T_{EM}; $CCR7^-CD45RA^-$), and terminally differentiated effector memory (T_{EMRA}; $CCR7^-CD45RA^+$) phenotype cells within the $CD4^+CD134^+CD137^+$ and $CD8^+CD69^+CD137^+$ activation-induced marker gates. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

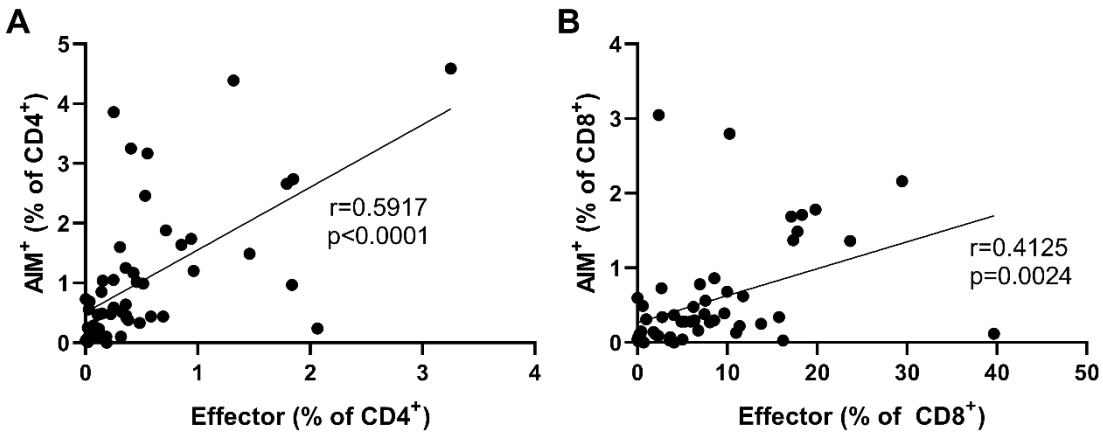

Figure S3.2.8. Correlation analysis of Spike-specific T cell frequencies as defined by activation-induced marker (AIM) expression (Figure 3.2.3) and intracellular cytokine staining (ICS; Figure 3.2.5) for kidney transplant recipients (n = 41) on varied immunosuppressive therapies and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine.

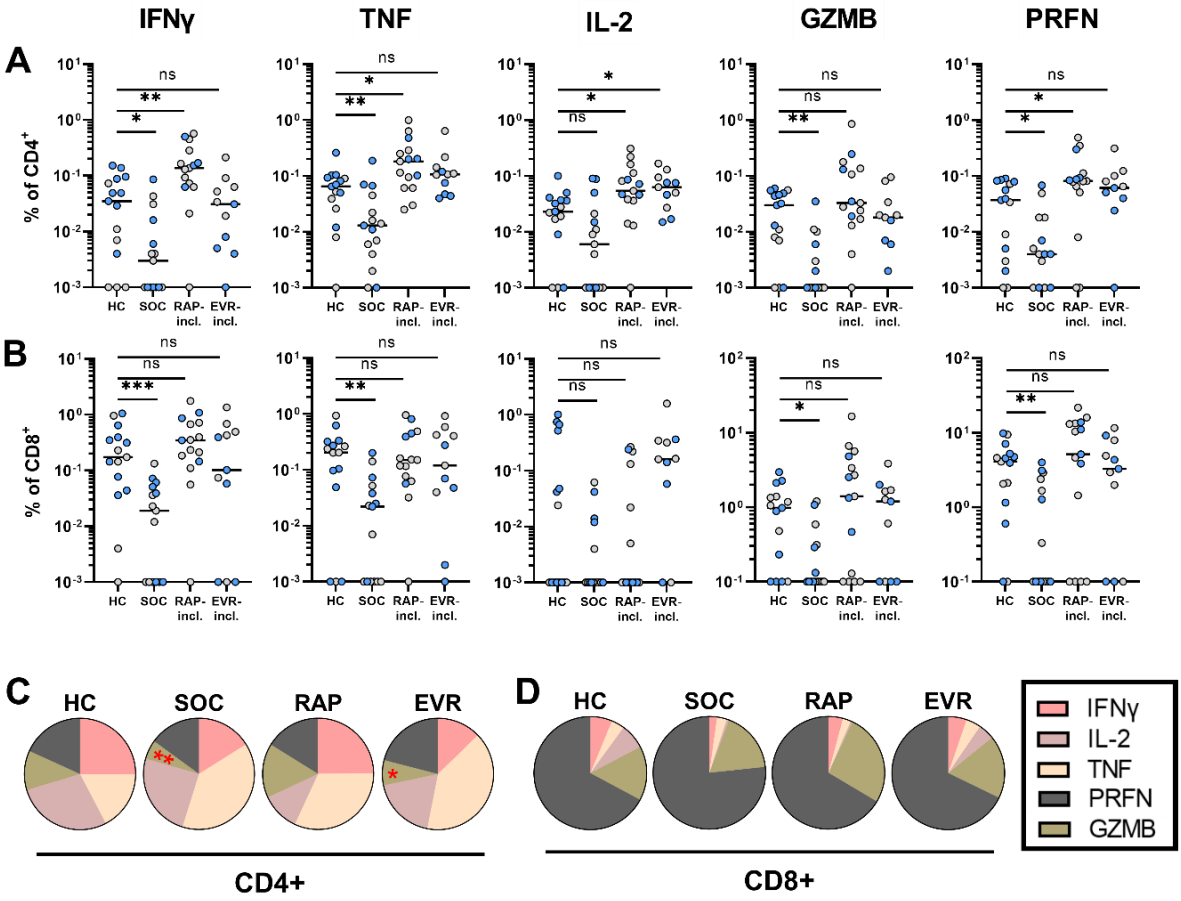

Figure S3.2.9 (relates to Figure 3.2.5). Effector profile of Spike-specific T cells in kidney transplant recipients on standard-of-care (SOC; n = 15), rapamycin-inclusive (RAP-incl; n = 15) and everolimus-inclusive (EVR-incl; n = 11) immunosuppressive therapies, and healthy controls (HC; n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and golgi transport blocked for the final 4 hours prior to staining for flow cytometry. **(A, B)** The total frequency of T cells (defined as $CD3^+CD19^-CD14^-$) producing each effector molecule was assessed within the $CD4^+$ (A) and $CD8^+$ (B) compartments. **(C, D)** Effector molecule production was then plotted as a proportion of total effector-molecule-producing cells within the $CD4^+$ (C) and $CD8^+$ (D) compartments. **Each KTR group compared to healthy controls by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

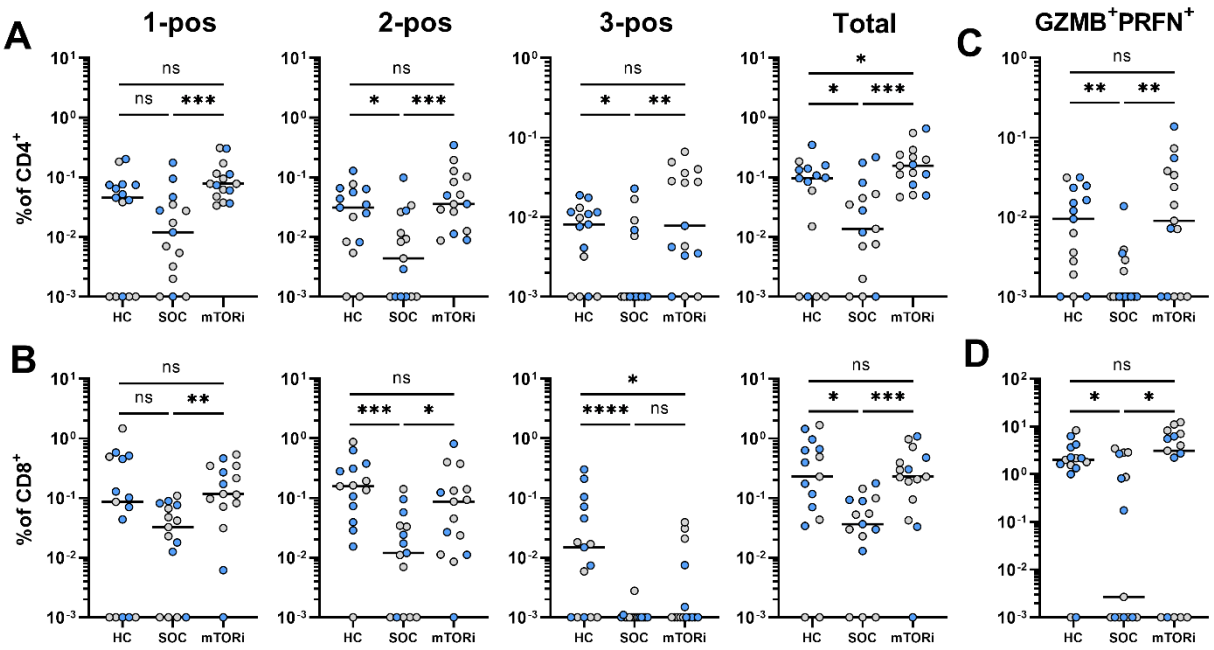

Figure S3.2.10 (relates to Figure 3.2.5). Frequency of Spike-specific effector T cells in kidney transplant recipients receiving calcineurin inhibitor (CNI; n = 15) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and Golgi transport blocked for the final 4 hours prior to staining for flow cytometry. **(A, B)** Log frequency of $CD4^+$ (A) and $CD8^+$ (B) Spike-specific T cells co-producing one (1-pos), two (2-pos) or three (3-pos) cytokines. **(D, E)** Log frequency of granzyme B/perforin double positive $CD4^+$ (D) and $CD8^+$ (E) T cells following stimulation with Spike peptides. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

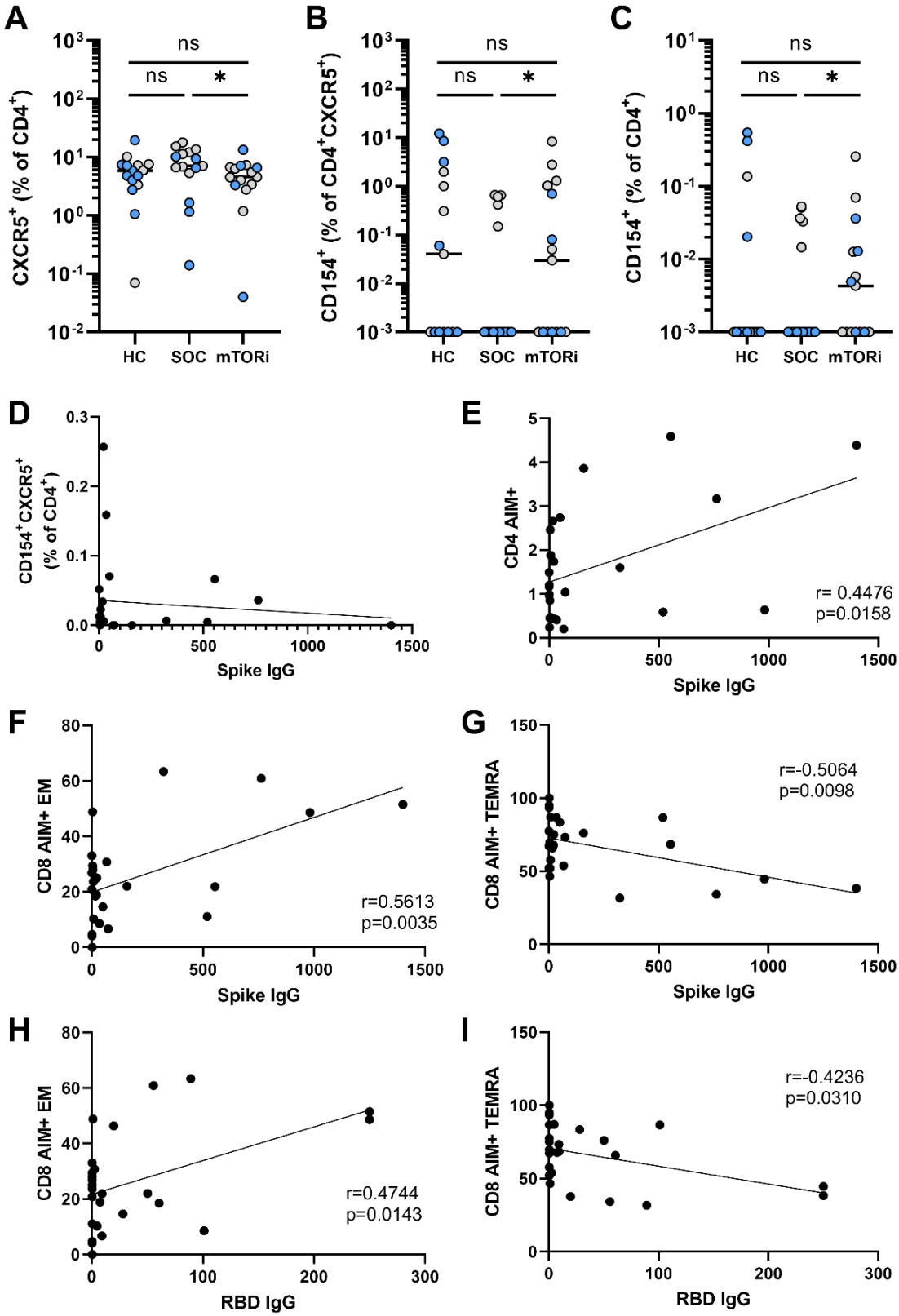

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Figure S3.2.11 (related to Figure 3.2.2). Investigation of IgG response. (A–C) Peripheral blood was collected from kidney transplant recipients receiving calcineurin inhibitor (CNI; n = 15) and mTOR inhibitor (mTORi; n=18) based three drug immunosuppressive regimens and healthy controls (n = 15) following 2 doses of coronavirus disease 2019 (COVID-19) vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated for 24 hours with a peptide pool derived from the full-length SARS-CoV-2 (Ancestral) Spike protein, and Golgi transport blocked for the final 4 hours prior to staining for flow cytometry. Frequencies of circulating T follicular helper cells (cT_{FH}) were assessed as the percentage of total $CD4^+$ T cells expressing CXCR5 (A). Spike-specific cT_{FH} were measured as $CD4^+CXCR5^+$ expressing CD154 following stimulation , and reported as a percentage $CD154^+$ within $CD4^+CXCR5^+$ population (B), and percentage $CD4^+CXCR5^+CD154^+$ of total $CD4^+$ population (C). **(D–I)** Correlation analysis of Spike-specific IgG responses in KTRs receiving mTORi-based therapy. **Analyses with significant variance identified by Kruskal-Wallis *H* test and comparisons between groups by Mann-Whitney *U* test: ns, non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.**

Methods

Study population.

This study is part of the REVAX trial (ACTRN12621000532808), for which a partial cohort was previously published (12). This study was conducted on a single center population of KTRs and their healthy cohabitants commencing in February 2021. Participants were identified by clinical nephrologists and screened. Inclusion criteria were kidney-only transplant over 18 years old with an available cohabitant without kidney disease. Exclusion criteria were patients with past COVID-19 infection, those who had already received a COVID-19 vaccine prior to enrolment, those who could not provide informed consent, or those who did not have a non-immunosuppressed and unvaccinated cohabitant. Demographics including gender, age, cause of kidney disease, and graft details were collected and presented as percentages for ordinal variables, and median ± range for continuous variables. See consort diagram below.

SARS-CoV-2 Spike protein production and ELISA.

Prefusion SARS-CoV-2 Spike ectodomain (isolate WHU1, residues 1-1208) with HexaPro mutations (kindly provided by Dr Adam Wheatley)(73) was used in ELISA. Recombinant protein was overexpressed in Expi293 cells (Thermo Fisher) and 72 h later purified by Ni-NTA affinity and size-exclusion chromatography. Purified proteins were quantified using the Bradford protein assay (Bio-Rad) and analysed by SDS-PAGE and Western blot before being stored at -80°C.

T cell ELISpot.

Millipore 96-well plates with nitrocellulose membranes (Merck, Branchburg, NJ) were activated with 35% ethanol for 30 s, before washing twice with PBS. Wells were coated with anti-IFNγ capture antibody (Clone 2G1, ThermoFisher, Cambridge, MA) overnight at 4°C, then washed twice with PBS. PBMCs were thawed by dropwise addition of

complete media complete media (RPMI + 20% FCS, glutamate, penicillin, streptomycin) and benzonase (Merck, Kenilworth, NJ) to prevent aggregation, and rested for 2 h before counting. PBMCs were treated with 4 pools of overlapping peptides spanning the length of the spike protein. PHA (7.5 μg/mL; Merck, Branchburg, NJ, USA) was used as a positive control. After 18 h at 37°C, wells were washed five times with PBS, then ten times with PBS + 0.05% tween-20. Captured IFNγ was detected with a biotinylated anti-IFNγ antibody (Clone B133.5; ThermoFisher, Cambridge, MA) at 4 °C overnight. Unbound detection antibody was removed by washing with PBS + 0.05% tween-20, and a streptavidin:HRP conjugate (BD Biosciences, NJ) was added for four hours at 4 °C. AEC substrate (BD Biosciences, New Jersey, USA) was added for 10 min at room temperature, before rinsing with deionised water and enumeration of spots using an ImmunoSpot analyser and software (Cellular Technology Ltd., Bonn, Germany). All washing steps were performed using an automated plate washer.

SARS-CoV-2 live-virus neutralisation assay.

HEK-ACE2/TMPRSS cells (Clone 24)(74) were seeded in 384-well plates at 5×10^3 cells/well in the presence of the live cell nuclear stain Hoechst-33342 dye (NucBlue, Invitrogen) at a concentration of 5% v/v. Two-fold dilutions of patient serum samples were mixed with an equal volume of SARS-CoV-2 virus solution (1.25×10^4 TCID50/ml) and incubated at 37°C for 1 h before adding 40 μl, in duplicate, to the cells (final MOI = 0.05). Viral variants used included the key variants of concern, Delta (B.1.617.2) and Omicron (B.1.1.529), as well as 'wild-type' control virus (A.2.2) from clade A and presenting no amino acid mutations in Spike (similar to Wuhan ancestral variant). Plates were incubated for 24 h post infection and entire wells were imaged by high-content fluorescence microscopy, cell counts obtained with automated image analysis software, and the percentage of virus neutralisation was calculated with the formula: $\%N = (D-(1-Q)) \times 100/D$, as previously described (74). An average %N > 50% was defined as having neutralising activity.

SARS-CoV-2 Spike peptide pools for AIM and ICS.

The SARS-CoV-2 peptide pool used for AIM and ICS analyses was kindly provided by Prof Alessandro Sette (La Jolla Institute of Immunology, CA, USA) (75). 15-mer peptides overlapping by 10 amino acids and covering the entire Spike protein sequence were used (total of 253 peptides). All peptides were synthesised and resuspended in DMSO at 1 mg/ml.

Activation-induced cell marker (AIM) T cell assay.

Thawed PBMCs were rested for 2 h at 37°C, 5% CO_2 in complete RPMI (cRPMI) medium (40 U/mL penicillin, 40 ug/mL streptomycin, 2 mM L-Glutamine) with 5% (v/v) heat-inactivated human AB serum. Cells were then plated at 10^6 PBMC/well in u-bottom 96-well plates and stimulated with 1 μg/mL of different SARS-CoV-2 Spike peptide pool. PHA 10 μg/mL (Sigma Aldrich) was included as a positive control. An equimolar amount of dimethyl sulfoxide (DMSO, vehicle) was used as a negative control. PBMCs were stimulated for 24 h at 37°C, 5% CO_2, washed and stained with Zombie Green Fixable Live/Dead Stain (L/D, Biolegend) for 20 min, RT, in the dark. Cells were then washed and stained with (CD3 BUV737, CD4 BUV496, CD8 BUV395, CD14 FITC, CD19 FITC, CD45RA BV650, CCR7 (CD197) APC, CD69 PE, CD134 (OX40) PE-Cy7, CD137 (41-BB) BV421) for 20 min, at room temperature in the dark. Fluorescence minus one (FMO) control for antigens: CD45RA, CCR7, CD134, CD69 and CD137 were added to PHA stimulated cells. PBMCs were washed and FACS Fix (0.4%PFA, 20 g/L Glucose, Sodium Azide 0.02% in PBS) was added for 20 min at room temperature, in the dark. Fixed cells were washed, resuspended in FACS wash buffer and data was acquired on BD FACS Symphony. Data analysis was performed using FCS Express ™ (DeNovo Software, Pasadena, CA, USA). All percentages of activated cells were calculated subtracting unspecific DMSO background for each cell phenotype and individual patient.

Intracellular cytokine staining and Spike-specific T follicular helper cell quantification.

As described previously (76), PBMCs were thawed and prepared for cell culture as described for the AIM assay. Cells were pre-treated with 0.555 μg/mL of anti-CD40 blocking antibody (HB14, Miltenyi Biotec) for 15 min. Spike peptide pool was then added to a final concentration of 1 μg/mL (making anti-CD40 concentration 0.5 μg/mL for the remainder of the stimulation period). After a 20 h incubation, 2 μM GolgiStop™ (containing monensin, BD, 554724) and 1 μg/mL GolgiPlug™ (containing Brefeldin A, BD, 555029) were added to the cells and incubated for an additional 4 h. Cells were then co-incubated with Fixable Viability stain 780 (BD) and Fc Block (BD) for 20 min, RT, in the dark, washed with FACS buffer solution and stained with surface stain mix (CD3 BUV737, CD4 BUV496, CD8 BUV395, CXCR5 BUV563, CD14 APC-Cy7, CD20 APC-Cy7) for 20 min, RT, in the dark. Cells were washed with PBS and subsequently fixed and permeabilised with Cytofix/Cytoperm™ (BD, 51-2090KZ) for 20 min, RT, in the dark. Cells were then washed with Perm/Wash™ (BD, 51-2091KZ) and stained with ICS stain Mix (CD154 PE, IFNγ PE-Cy7, TNFα APC, PRF1 FITC, IL-2 BV711, GZMB BV421) for 20 min, RT, in the dark. Cells were then washed twice with Perm/Wash™ and once with PBS. Finally, cells were resuspended in PBS and kept at 4°C until data was acquired on BD FACS Symphony. Data analysis was performed using FCS Express ™ (DeNovo Software, Pasadena, CA, USA).

Statistical analysis.

All statistical analyses were performed using GraphPad Prism 9.0.0 (San Diego, CA, US). No assumptions were made about the distribution of the data sets; non-parametric tests were used in all cases for comparisons. Accordingly, Krustal-Wallis tests were applied to group comparisons to identify significant variance, and two-tailed Mann-Whitney tests applied for pair-wise comparisons of antibody and T cell frequency data.

Spearman's correlation coefficient was used to calculate all correlation coefficients, and a significance level of 5% was used to assess whether the correlation coefficients were significant different from 0. All p-values were corrected using a false discovery rate of 5%.

For AIM and ICS analyses, 15 HC and 15 SOC KTRs were sampled from the larger cohort. This was performed by generating a randomly ordered number list using the random number generator on Random.org, which produces true randomness based on atmospheric noise, and selecting the first 15 entries in each group for further experiments. No experiments were blinded in this study.

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3.3 MANUSCRIPT 5-

Rapamycin and Inulin for booster VAccine response STIMulation (RIVASTIM) – Rapamycin: study protocol for a randomised, controlled trial of immunosuppression modification with rapamycin to improve SARS-CoV-2 vaccine response in kidney transplant recipients

Details-

Published in Trials.

Impact factor (2021): 2.728

Submitted March 18, 2022; Revised July 04, 2022; Accepted for publication July 20, 2022; Available online September 15, 2022.

Preface-

Based on the outcomes of Manuscript 3 and 4, we have designed and initiated a randomised, controlled trial of immunosuppression modification as a strategy to boost immunogenicity of a third COVID-19 vaccine dose for kidney transplant recipients. Manuscript 5 is a protocol paper outlining the rationale and design of this trial, and pre-specifying primary and secondary outcomes. Ethics approval and trial registration are presented in Appendix VIII and Appendix IX, respectively. A second, related trial investigating dietary modification with the pre-biotic inulin will be conducted under the umbrella of RIVASTIM, however is not discussed as part of this thesis. The protocol for RIVASTIM- Inulin can be found online:

Singer J, Tunbridge M, Perkins GB, Salehi T, Ying T, Coates PT, Chadban SJ. Rapamycin and inulin for third dose vaccine response stimulation (RIVASTIM) – Inulin: study protocol for a pilot, multicentre, randomized, double-blinded, controlled trial of dietary inulin to improve SARS-CoV-2 vaccine response in kidney transplant recipients. BMJ Open 2022; 12(12):e062747.

Statement of Authorship

Title of Paper	Rapamycin and inulin for booster vaccine response stimulation (RIVASTIM) – Rapamycin: study protocol for a randomized, controlled trial of immunosuppression modification with rapamycin to improve SARS-CoV-2 vaccine response in kidney transplant recipients
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in Trials (BioMed Central) Impact Factor 2.728 (2021) Accepted July 2022

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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Rapamycin and inulin for booster vaccine response stimulation (RIVASTIM) – Rapamycin: study protocol for a randomized, controlled trial of immunosuppression modification with rapamycin to improve SARS-CoV-2 vaccine response in kidney transplant recipients

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Abstract

Background: Kidney transplant recipients are at an increased risk of severe COVID-19-associated hospitalisation and death. Vaccination has been a key public health strategy to reduce disease severity and infectivity, but the effectiveness of COVID vaccines is markedly reduced in kidney transplant recipients. Urgent strategies to enhance vaccine efficacy are needed.

Methods: RIVASTIM-rapamycin is a multicentre, randomised, controlled trial examining the effect of immunosuppression modification prior to a third dose of COVID-19 vaccine in kidney transplant recipients who have failed to develop protective immunity to a 2-dose COVID-19 vaccine schedule. Participants will be randomised 1:1 to either remain on standard of care immunosuppression with tacrolimus, mycophenolate, and prednisolone (control); or cease mycophenolate and commence rapamycin (intervention) for 4 weeks prior to, and following, vaccination. The primary outcome is the proportion of participants in each trial arm who develop protective serological neutralisation of live SARS-CoV-2 virus at 4-6 weeks following a third COVID-19 vaccination. Secondary outcomes include SARS-CoV-2 receptor binding domain IgG, vaccine-specific immune cell populations and responses, and the safety and tolerability of rapamycin switch.

Discussion: Immunosuppression modification strategies may improve immunological vaccine response. We hypothesise that substituting the mTOR inhibitor rapamycin for mycophenolate in a triple drug regimen will enhance humoral and cell-mediated responses to COVID vaccination for kidney transplant recipients.

Introduction

Background and rationale

Kidney transplant recipients (KTRs) are at an increased risk of COVID-19 associated morbidity and mortality, with the combined effects of immunosuppression and prevalent comorbidities contributing to the high rates of adverse outcomes (1, 2). Meta-analyses have suggested a 28-day mortality approaching 25% for KTRs positive for SARS-CoV-2, and survivors have significant risk of morbidity including hospitalisation, acute kidney injury, and graft loss (2).

The development of effective vaccines which target the SARS-CoV-2 spike protein has been crucial to reducing disease burden and the development severe COVID-19 disease (3). However, immunocompromised populations such as KTRs were excluded from initial vaccine trials. KTRs exhibit suboptimal vaccine responses, and are inadequately protected by current standard two and three-dose vaccine regimes (4, 5).

To address the inadequate vaccine response observed in KTRs and other immunocompromised groups, additional doses of mRNA vaccine have been recommended. In KTRs, data from a randomised controlled trial suggest that a third mRNA vaccine dose increases the proportion of patients with protective neutralising antibodies to 60%, compared to 25% in the placebo group (5). Whilst a third vaccine dose improves vaccine immunogenicity, to what extent seropositive patients are protected is unclear, and a significant minority of KTRs fail to seroconvert and therefore require additional strategies (5-7).

Observational data suggests that vaccine responses are significantly affected by immunosuppression regimen. The use of mycophenolate has been identified as a key factor associated with vaccine hypo-responsiveness (4). Such observations are consistent with earlier reports identifying mycophenolate use as being strongly associated with

infection risk among maintenance-phase KTRs, and that use of mycophenolate was associated with inadequate responses to influenza vaccination (8-11). Conversely, mechanistic target of rapamycin inhibitors (mTORi) have been found to boost vaccine-elicited cytotoxic T cell memory responses in non-human primates, and to improve antibody responses to influenza vaccination in elderly individuals (12, 13). A recent, multicentre study of over 2,000 KTRs reported a 50% reduction in viral infections among KTRs randomised to an mTORi based regimen, as compared to those receiving mycophenolate, calcineurin inhibitor, and steroid (14, 15).

These observations led to recognition of mTOR complex 1 (mTORC1) activity as a key determinant of the effector versus memory fate decision of antigen-experienced T cells following vaccination (16, 17). Rapamycin is a potent inhibitor of mTORC1, and the use of mTOR inhibitor-based immunosuppressive protocols has been associated with superior rates of seroconversion, as well as greatly enhanced T cell mediated immunity against SARS-CoV-2 spike protein (18). Vaccine responses may also be modulated by commensal gastrointestinal microorganisms, collectively the microbiome (19-22). mTOR inhibitors such as rapamycin have been associated with immunosuppression-regimen-specific changes in the microbiome (23, 24), which may play a role in vaccine immune responses (25).

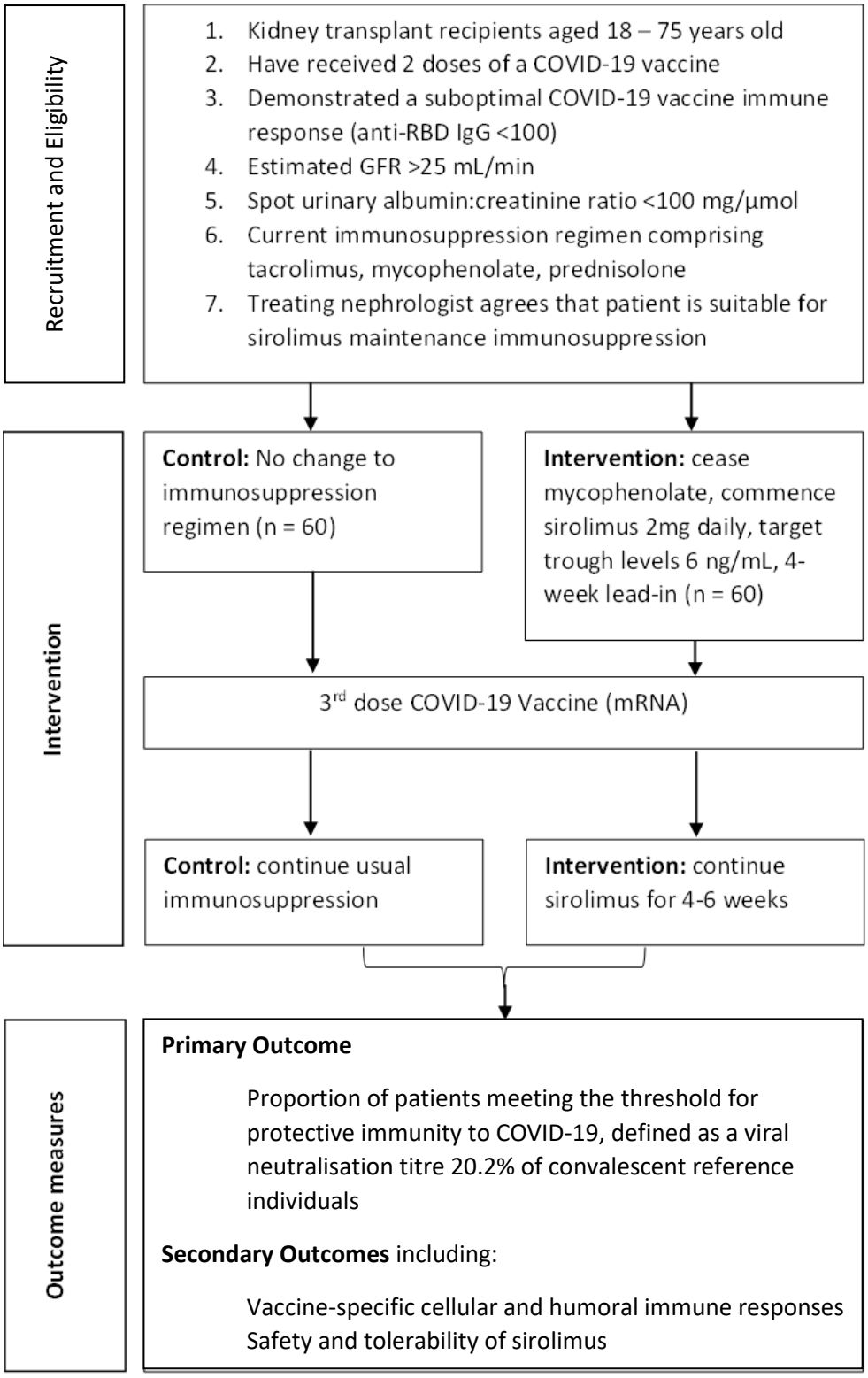
The RIVASTIM trials are designed to investigate potential strategies to enhance vaccine immunological responses in KTRs using supplementation of the pre-biotic inulin, or immunosuppression regimen alteration. RIVASTIM-Rapamycin is a multicentre, randomised, controlled trial of immunosuppression alteration in KTRs who have failed to develop vaccine-induced protective immunity to COVID-19, prior to a third vaccination. We hypothesise that cessation of mycophenolate and commencing rapamycin in a 3-drug regimen (tacrolimus, rapamycin, prednisolone) will improve the immune response to a third mRNA COVID-19 vaccine.

Objectives

The primary objective is to compare the proportion of participants in each trial arm who develop protective serological neutralisation of live SARS-CoV-2 virus (ancestral strain) at 4-6 weeks following a third COVID-19 vaccination. The secondary objectives are to determine whether the rapamycin switch [1] improves the proportion of patients that achieve protective anti-receptor binding domain (RBD) IgG antibody, [2] improves the magnitude of vaccine induced T cell response, and [3] is safe and well-tolerated.

Trial design

Figure 3.3.1. Study overview.



RIVASTIM-rapamycin is a multicentre, parallel-arm, randomised, controlled, superiority trial, seeking to examine the effect of ceasing mycophenolate and commencing rapamycin in a 3-drug regimen on the immune response to a third dose of mRNA COVID-19 vaccine in kidney transplant recipients who have failed to demonstrate protective immunity following a two-dose vaccine schedule. KTRs on triple therapy who have received 2-doses of a COVID-19 vaccine will be enrolled and their immune response to vaccination assessed by measurement of serological anti-RBD IgG titre. Those with a satisfactory immune response (anti-RBD IgG ≥ 100 U/mL) will exit the study and be advised to receive a third mRNA COVID-19 vaccination as per recommended guidelines. KTRs who fail to demonstrate protective immunity (anti-RBD IgG <100 U/mL) will proceed to randomisation.

Randomisation will occur at a participant level with an allocation ratio of 1:1, stratified by study site and the magnitude of immune response following 2 doses of vaccine (anti-RBD IgG titre; non-responder: < 0.4 U/mL; low responder: 0.4 – 99 U/mL). An outline of the trial is shown in Figure 1. Following randomisation patients will either continue their usual immunosuppression regimen of tacrolimus, mycophenolate, and prednisolone (control arm), or have mycophenolate ceased and rapamycin commenced (intervention arm). Rapamycin will be commenced at a standard dose of 2 mg daily, with a target trough level of 6 ng/mL and weekly dose titration. Tacrolimus dosage will be adjusted to achieve trough concentration within a range of 3-6 ng/mL. Following a 4-week lead in period, participants will receive a third dose of mRNA COVID-19 vaccine, with the subsequent antibody response measured at 4-6 weeks post vaccination. Participants will continue immunosuppression as per group at least until the time of antibody assessment, and thereafter as determined by their usual nephrologist. The first study participant was enrolled on the 8th November 2021 and recruitment continued through to 15th February 2022, with the final study visit of the last recruited patient in April 2022.

Methods: Participants, interventions and outcomes

Study setting

The trial will be conducted at the renal transplant units of two tertiary referral hospitals in Australia: [1] The Royal Adelaide, Hospital, Adelaide, South Australia, and [2] The Royal Prince Alfred Hospital, Sydney, New South Wales.

Eligibility criteria

The inclusion criteria are:

1. Kidney transplant recipients
2. Aged 18-75 years
3. estimated GFR >25 mL/min
4. Spot urinary albumin:creatinine ratio <100 mg/μmol
5. Current immunosuppression regimen comprising tacrolimus, mycophenolate, prednisolone
6. Treating nephrologist agrees that patient is suitable for rapamycin maintenance immunosuppression
7. Have received 2 doses of a COVID-19 vaccine regimen (either adenoviral vector or mRNA-based) and have demonstrably not responded (anti-spike RBD IgG antibody titre below 100 U/mL)

The exclusion criteria are:

1. Aged <18 years or >75 years
2. Significant kidney dysfunction, estimated GFR ≤25 mL/min or spot urinary albumin:creatinine ratio ≥100 mg/μmol
3. Unable or unwilling to provide informed consent to participate in the trial
4. Have received 2 doses of a COVID-19 vaccine regimen (either adenoviral vector or mRNA-based) and have mounted an adequate immune response (anti-spike RBD IgG antibody titre above 100U/mL)

5. Have had documented infection with COVID-19 and/or have detectable SARS-CoV-2 nucleocapsid-specific IgG
6. Known allergy to or intolerance of rapamycin or everolimus

Who will take informed consent?

The patient's treating nephrologist will determine participant eligibility and approach the patient regarding their interest in participating in the clinical trial. Trained research staff will then discuss the trial, provide written information, and seek informed consent during a scheduled clinic appointment. All clinical and research staff involved in consent and enrolment have received trial-specific training and adhere to Good Clinical Practice (GCP) requirements.

Additional consent provisions for collection and use of participant data and biological specimens

Participants will provide informed consent to the sampling of biological material (blood and stool), which will be deidentified and stored in the secure research facilities of the participating trial sites and may be used for future studies. Consent for the genomic sequencing of stool microbiota is addressed, with the understanding that no human genomic information will be collected.

Interventions

Explanation for the choice of comparators

Standard of care immunosuppression for transplant patients consists of a calcineurin inhibitor (CNI, most commonly tacrolimus), an antimetabolite (most commonly mycophenolate), and a corticosteroid (prednisolone / prednisone) (26). Mycophenolate has been associated with suppressed humoral vaccine response, while rapamycin is associated with improved humoral and cellular vaccine response (18). There is less risk

of acute rejection when replacing mycophenolate with rapamycin, as opposed to replacing CNI with rapamycin (27). When rapamycin is used in combination with tacrolimus, lower target concentrations of tacrolimus can be used to minimise treatment-related adverse events (27).

Intervention description

Participants will be randomly allocated to their inclusion in one of two groups:

1. Continuation of current immunosuppression regimen including mycophenolate, available in the form of mycophenolate sodium or mycophenolate mofetil
2. Cessation of mycophenolate, and commencement of rapamycin, available as sirolimus (Rapamune®, Pfizer Australia Pty Ltd)

The study products will be provided through the patients' elected pharmacy. Control arm participants will continue their usual immunosuppression regimen as directed by their treating nephrologist. Intervention arm participants will cease mycophenolate the day prior to commencing rapamycin 2 mg daily. Trough drug levels will be taken at Day 5-7 with dose titration targeting a trough level of 6 ng/mL and ongoing weekly levels to ensure stabilisation prior to vaccination.

All trial participants will receive a third "booster" dose of a COVID-19 mRNA vaccine, either Pfizer-BioNTech BNT162b2 (30 μg, IM) or Moderna mRNA-1273 (50 μg, IM) determined by local practice and vaccine availability. Study participants will receive written pre-vaccination information on the benefits and potential risks and harms of the COVID-19 vaccine and be screened for contraindications to immunisation such as serious adverse events attributable to a previous dose of a mRNA COVID-19 vaccine. All patients will be advised of the need to continue with additional public health measures (i.e. physical distancing, hand washing, wearing a face mask, and COVID-19 testing and isolation as required).

Criteria for discontinuing or modifying allocated interventions

Rapamycin has known dose-related adverse effects (AEs). Trough level targets of 6

ng/mL are generally well tolerated (28). Participants who experience severe adverse events will cease rapamycin and return to their usual immunosuppression regimen. Participants who experience mild AEs related to rapamycin trough levels above target will have their dose reduced. Participants who continue to experience mild AEs (mouth ulcers, peripheral oedema) at target trough level will be offered the option of discontinuing the study intervention and continuing with trial follow-up.

Strategies to improve adherence to interventions

RIVASTIM-rapamycin uses the combination of tacrolimus and rapamycin to facilitate lower target rapamycin and tacrolimus exposure without an increase in the risk of graft rejection (27). This reduces the likelihood of rapamycin-associated adverse events which are generally dose-related. Following randomisation, participants in the intervention arm will have weekly blood tests for dose titration and to ensure adequate adherence.

Relevant concomitant care permitted or prohibited during the trial

All participants will continue with usual transplant management as per local standard of care and at the discretion of their treating nephrologist. Any changes to medications will be recorded. Participants will be asked to continue with their usual diet and medications.

Provisions for post-trial care

Following completion of the trial intervention, patients will be contacted within one week to monitor for adverse events. In conjunction with their treating nephrologist, patients will be offered continuation of the trial drug regimen or return to their previous immunosuppression regimen. Patients converting back to mycophenolate will have this arranged by the trial team, with usual post-transplant care and follow-up then continuing per routine practice under the guidance of the treating nephrologist. The trial Sponsor has indemnity to compensate those who suffer from potential harm from as a result of their participation in the research study.

Management of COVID-19 positive participants during the trial

Study participants who returned a positive COVID-19 result during the trial will be managed in consultation with their treating transplant unit as per local best practice. Participants who contract COVID-19 following randomisation but prior to a third vaccination may have their third vaccine dose delayed. Where possible, participants will be asked to continue with their allocated treatment regimens and attend study visits and follow-up. Positive COVID cases will be excluded from the primary analysis as SARS-CoV-2 infection will confound assessment of the primary outcome measure.

Outcomes

Primary outcome measure:

1. The primary outcome is the proportion of participants in each trial arm who develop protective serological neutralisation of live SARS-CoV-2 virus (original Wuhan strain) at 4-6 weeks following a third COVID-19 vaccination. The protective level is defined as 20.2% of the mean neutralisation level of a standardised cohort of COVID-19 convalescent individuals. This threshold correlates with 50% protection from infection with SARS-CoV-2 (original Wuhan strain) in the general population (29).

Secondary outcome measures:

The secondary outcome measures include the following:

2. The proportion of participants in each trial that reach a threshold of serological anti-SARS-CoV-2 (Wuhan) RBD IgG antibody ≥ 100 units/mL (measured with an Elecsys Anti-SARS-CoV-2 immunoassay [Roche], and equivalent to 100 BAU/mL). This RBD IgG threshold was chosen on the basis of pre-clinical and

clinical studies, and is consistent with the reported outcomes in published COVID-19 clinical vaccine trials (5).

3. The development of COVID-19 following randomisation, determined by:
    a. Positive SARS-CoV-2 PCR test, or rapid antigen test in the setting of symptomatic disease
    b. Detection of SARS-CoV-2 anti-nucleocapsid antibodies at the time of primary outcome assessment.
4. Change in the median magnitude of the SARS-CoV-2 spike-specific, antiviral T cell response prior to and at 4-6 weeks following vaccination, determined as the frequency of cells that secrete IFNγ in response to stimulation with spike-protein (original Wuhan strain)-derived peptides.
5. Phenotypic and functional characterisation of T and B lymphocyte populations
6. Tolerance of rapamycin as determined by drug cessation, drug adherence, and drug-related adverse events including proteinuria, anaemia, leukopenia, rash, mouth ulcers, and pneumonitis.
7. Adverse events following immunisation (AEFI) including adverse events of special interest (AESI) will be assessed via phone consultation at 1 week, and again at 4-6 weeks post-vaccination during the final follow-up visit, and include:
    a. Changes in kidney allograft function, determined by serum creatinine, eGFR (CKD-EPI equation), and proteinuria.
    b. The occurrence of biopsy proven acute allograft rejection.
    c. The recurrence of primary kidney disease.
    d. Patient reported quality of life as recorded by the EQ-5D questionnaire.
8. Changes in the community structure, relative abundance, and functional characteristics of the gut microbiome following 4 weeks of rapamycin intervention, determined by 16S-rRNA metagenomic sequencing of participant stool samples.

Participant timeline

Participants are followed from the time of enrolment through until study close-out, 1-week following their final assessment visit. The schedule of enrolment, randomisation, interventions, and assessments is shown in Figure 2.

	STUDY PERIOD				
	Enrolment	**Randomisation**	**Follow-up**		**Close-out**
TIMEPOINT	***-2-7 days***	**0**	***Day 28***	***4-6 weeks after vaccination***	***+ 7 days***
Visit window			***-2 + 7 days***		
ENROLMENT:					
Eligibility screen	X				
Informed consent	X				
Baseline characteristics	X				
Randomisation		X			
INTERVENTIONS:					
Rapamycin arm (active)		←	—	→	
Mycophenolate arm (control)		←	—	→	
COVID-19 mRNA Vaccine			X		
ASSESSMENTS:					
Anti-RBD IgG titre	X			X	
Routine biochemistry and drug levels	X	x	X (weekly in active group)	X	
Blood draw for cellular and humoral assays		X		X	
Faecal microbiota assessment		X	X		
4-day food diary		X	X		
Medication Review	X		X	X	
Adherence Assessment		X	X	X	
EQ-5D		X	X	X	
AE/SAE			X	X	X

Figure legend over the page…

Figure 3.3.2. Participant timeline. Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) checklist. Enrolment, interventions, and assessments. GSRS, gastrointestinal symptom rating scale; EQ-5D, EuroQol five dimensions questionnaire; AE, adverse events; SAE, serious adverse events

Sample size

This study aims to enrol 120 patients across both sites, with 60 assigned to the rapamycin intervention group, and 60 assigned to control. This will provide 80% statistical power (alpha 0.05) to detect an absolute difference of 25% in the proportion of patients who achieve the serological neutralisation titre threshold necessary to provide clinical protection from COVID-19 disease, allowing for a 10% drop-out rate.

Recruitment

Prospective participants will be identified through the following means:

1. Review of local transplant recipient databases at each trial site. (Only local site clinical staff will have access to identifying information for the purpose of recruitment.)
2. During routine clinical review with their treating nephrologist or transplant centre.
3. Potential participants may have also indicated their interest in trial participation by responding to a QR code displayed during the Transplant Australia COVID Vaccination Update Webinar, broadcast in November 2021.

Potential participants will be approached by their treating nephrologist and offered the opportunity to participate in the trial. Prior to enrolling, patients will be provided with written information regarding the rationale behind the trial, the potential risk and benefits of participation, and the personal commitment involved. Recruitment will continue until target recruitment is fulfilled, or until recruitment of dual-vaccinated transplant recipients is no longer feasible, or if delaying a third vaccination becomes no

longer ethically permissible due to clinical urgency. Participants will not receive payment for participation.

Assignment of interventions: allocation

Sequence generation

Participants will be randomised 1:1 to either rapamycin (intervention) or mycophenolate (control). Randomisation will occur via computer-generated stratified block randomisation with randomly permuted block sizes of 2, 4 and 6. Stratification will occur by site and the response to a two dose COVID-19 vaccine schedule (low responder anti-RBD IgG 0.4 - 99 U/mL; or non-responder, anti-RBD IgG < 0.4 U/mL).

Concealment mechanism

The allocation sequence is contained and administered centrally through an external web-based randomisation module contained within a purpose-built Research Electronic Data Capture (REDCap) data management platform. The randomisation algorithm and treatment allocation are not accessible to study investigators or research staff.

Implementation

The allocation sequence will be generated by an independent and blinded statistician. Trained study investigators will enrol participants, at each study site, and will perform randomisation via the web-based platform and assign the intervention to each participant.

Assignment of interventions: Blinding

Who will be blinded?

Given the significant changes in prescribed immunosuppression, neither the study participants, nor clinical staff will be blinded to treatment allocation. Scientific staff

performing the laboratory assays for primary and secondary outcomes will be blinded to patient treatment allocation.

If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

The design is open label with only outcome assessors being blinded so unblinding will not occur.

Data collection and management

Plans for assessment and collection of outcomes

Trial data are collected prospectively by trial staff and entered on to web-based electronic case record forms (eCRF) maintained on a bespoke REDCap database.

Clinical and laboratory data are collected by study staff from the participant's electronic medical record. Safety laboratory tests (haematology, chemistry, urinalysis) and enrolment criteria (anti-RBD IgG) are performed at the hospital laboratory of each study site. Blood samples for immunological assessment will be collected by clinical research staff and processed in the on-site immunology laboratory at the Royal Adelaide Hospital. Estimation of participants habitual diet will be captured using a 4-day food diary, completed at the time stool samples are collected. Validated questionnaires are completed by participants to capture health-related quality of life (EQ-5D) information.

Plans to promote participant retention and complete follow-up

Participants who withdrawal from the study, are lost to follow-up, or permanently discontinue the study intervention will be asked to continue with scheduled study visits and follow-up. Outcome data relevant to the trial will be collected from clinical records unless the participants specifically withdraws consent. Reasons for withdrawal, discontinuation, or deviations from the study protocol will be captured in an eCRF.

Data management

All study data are collected by trained research staff and entered directly onto study-specific electronic data capture forms created and housed within a secure, web-based data management tool (REDCap). The data capture forms contain inbuilt protections to promote data quality, including range checks for numerical data values, restrictions on alphanumeric entries, and prevention of duplicate records. The RIVASTIM REDCap database is stored on secure servers in an on-site limited access data centre at the Royal Prince Alfred Hospital and operated behind the Sydney Local Health District (SLHD) firewall. All electronic information and transmissions are protected via Secure Sockets Layer (SSL) encryption. Access to the RIVASTIM REDCap database is limited to approved research staff, with individual user authentication and logging of all data entry and modification, and access to restricted modules (randomisation, scheduling, and data export) privileged. The database in maintained by the SHLD Information and Communication Technology (ICT) Services with regular back-up processes in place.

Confidentiality

Prior to study enrolment, participants will consent to research staff accessing their electronic medical record to obtain baseline and demographic information, and the results of laboratory assessments. The privacy and confidentiality of screened and enrolled participants will be preserved with all study data stored in the RIVASTIM REDCap database under a unique numerical study identifier. Access to personal identifying information for participant contact and safety will be limited to trial research staff with privileged access to the REDCap database. Privacy mechanisms within the RIVASTIM REDCap database will remove potential identifiers from data exported for downstream analysis.

No identifying information or individually identifiable participant data will be reported in publications, presentations, or in any report arising from this study.

Plans for collection, laboratory evaluation and storage of biological specimens for genetic or molecular analysis in this trial/future use

Blood samples will be taken from participants for immunological assessment at randomisation and at 4-6 weeks following vaccination. Blood will be drawn from participants by clinical research staff and collected in 7 x 9mL lithium heparin and 1 x 8mL CAT serum separator vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) will be isolated from whole blood by density gradient centrifugation in Ficoll-Paque and aliquoted and cryopreserved in liquid nitrogen for batch testing. Sera will be aliquoted and stored at -80°C. Phenotypic and functional assessments of vaccine specific T and B-cell responses will be determined using a variety of laboratory techniques including but not limited to: cytometric analysis with intracellular cytokine staining and activation-induced marker (AIM) assays, and IFNγ enzyme-linked immunosorbent spot (ELISpot) assays. Using participant serum, the titres of SARS-CoV-2 spike-protein-specific IgG and the capacity of participant serum to neutralise SARS-CoV-2 viral entry into $ACE2^{+}$ cells will be assessed. The capacity of pre- and post-immunisation serum to induce spike-protein-specific antibody-dependent innate immune responses will be measured.

Stool samples will be self-collected by participants using an at home collection kit (OMNIgene GUT OM-200, DNA Genotek, Canada) at baseline and at time of 3rd vaccine dose. Stool samples will be aliquoted and stored at -80°C until batch testing. Analysis of the faecal metagenome will be performed by comparative sequencing of the 16S-rRNA amplicons (V3-V4 region) to identify changes in community structure, relative abundance, and functional characteristics of the gut microbiome.

All biological specimens will be deidentified and labelled with the participants unique study identifier. Stool and blood samples will be stored and maintained in access-restricted laboratory freezers at their corresponding trial site (Adelaide Health and Medical Sciences building, Adelaide, or the Transplant Institute, RPA, Sydney).

Statistical methods

Statistical methods for primary and secondary outcomes

The primary analysis will be by intention-to-treat, with participants assessed according to their treatment allocation. Participants who develop a positive SARS-CoV-2 PCR result during the study will be excluded from the primary analysis to avoid confounding. A per-protocol analysis will also be reported, with participants who failed to adhere or tolerate rapamycin, and participants who withdrew or were lost to follow-up excluded from the analysis. A sensitivity analyses adjusting for potential confounding may be performed should significant imbalances in baseline characteristics between the treatment groups occur.

The primary endpoint is the proportion of patients who achieved a post-intervention serological neutralisation of live SARS-CoV-2 virus (20.2% of the mean neutralisation level of a standardised cohort of COVID-19 convalescent individuals) in both groups using the chi-square test. An unadjusted and adjusted relative risk (RR) will be calculated. For the adjusted RR estimate, the primary outcome of a threshold SARS-CoV-2 serological neutralisation titre will be analysed using a log-binomial regression model. The initial immune response to a two-dose vaccine schedule (anti-RBD IgG titre; low responder: 0.4 – 99 U/mL; or non-responder: <0.4 U/mL) will be included in the model as a fixed effect, with study site as a random effect.

Secondary outcomes will be analysed using univariate and multivariate methods dependant on the outcome type. Baseline characteristics and demographic data will be reported as mean ± SD for normally distributed data and median ± IQR for non-normally distributed data, with categorical variables reported as frequencies. All statistical analyses will be described in detail with arising publications. A two-sided significance level of 5% will be used for all analyses.

Interim analyses

No interim analyses are planned.

Methods for additional analyses (e.g. subgroup analyses)

Subgroup analyses will be performed to examine for statistical interaction between the treatment arm and [1] initial response to 2-dose vaccine schedule (non-responder or low-responder), [2] duration between previous vaccine dose (less than, or greater than 6 weeks) and randomisation. Patients who develop primary COVID-19 infection during the study period will have both primary and secondary outcomes analysed as a pre-specified subgroup analysis.

Methods in analysis to handle protocol non-adherence and any statistical methods to handle missing data

A per-protocol analysis will also be reported, with participants who failed to adhere or tolerate rapamycin, and participants who withdrew or were lost to follow-up excluded from the analysis. Multiple imputation will be used to handle data missing at random from baseline characteristics. Data missing at random for the primary and secondary outcome will not be imputed, with these cases excluded from ITT analysis. If > 10% of the primary outcome data is determined to be missing not at random, a best-worst and worst-best case sensitivity analyses will be performed.

Plans to give access to the full protocol, participant level-data and statistical code

The complete trial protocol and statistical code used for analyses will be made publicly available following publication of the primary results. Following publication of all study results, deidentified participant level data may be made available upon reasonable request to the principal investigator, or in the case of published works, through the corresponding author.

Oversight and monitoring

Composition of the coordinating centre and trial steering committee

The coordinating trial centre is located at the Royal Adelaide Hospital. The Trial Steering committee (TSC) is co-chaired by the Principal Investigator (PI) at each study site and includes the trial associate investigators. The TSC is responsible for the study conception, drafting and completion of the study protocol and associated documents, recruitment plan, data monitoring and integrity, end point adjudication, and approving publications arising from the study.

Composition of the data monitoring committee, its role and reporting structure

A data safety monitoring board has not been established and was not warranted in this study, given the short duration of the intervention and follow-up. The TSC is responsible for the scientific integrity of the trial and will monitor safety and operational data and will fulfil reporting obligations to the trial sponsor.

Adverse event reporting and harms

All protocol deviations and AEs will be documented, regardless of their potential relationship to the study intervention. AEs will be recorded using an adaptation of the National Institute of Health's Common Terminology Criteria for Adverse Events by a study team member on an eCRF. Recorded information on each AE will include: a descript of the AE, the onset date, duration, and resolution of the AE, the severity and seriousness, any action taken as a result of the AE, the outcome of the AE, and the likelihood of the relationship of the AE to a study intervention. Screening for adverse events will occur during each study visit and during scheduled clinical follow-up with their treating nephrologist, and will be captured up to 7 days following the final study visit. Adverse events following immunisation (AEFIs) with the exception of mild and/or short-lived symptoms, will be reported to the Therapeutic Goods Administration (TGA). Serious adverse events (SAEs) will be reported to the trial sponsor with 24-hours of the study team being made aware of the event.

Frequency and plans for auditing trial conduct

There are no plans for trial audit given the short duration of the trial intervention and follow-up.

Plans for communicating important protocol amendments to relevant parties (e.g. trial participants, ethical committees)

Amendments to the study protocol will be approved by the human research ethics committee (HREC) at the coordinating trial centre, followed by local site governance prior to implementation. The trial registration information contained with the Australia New Zealand Clinical Trial Registry (ANZCTR) will be updated with any protocol modifications.

Dissemination plans

The results of the RIVASTIM-Rapamycin trial will be published in peer-reviewed academic journals and presented at national and international scientific meetings. Additionally, a lay summary containing the study aim, salient findings, conclusions, and a take home message will be prepared and distributed to trial participants, research staff, and interested members of the transplant community. The lay summary will be distributed via direct approaches to trial participants and be made widely available through electronic media including newsletters, social media, and websites.

Discussion

Interventions to improve COVID-19 vaccine responses in transplants recipients are required. Preclinical studies, and observational findings in KTRs, suggest that the mTOR inhibitor rapamycin may enable enhanced immunological responses to COVID-19 vaccination, as compared to standard of care immunosuppression which includes mycophenolate.

This multi-centre, prospective, randomised, controlled trial has been designed to measure the effect of temporarily modifying maintenance immunosuppression with rapamycin, in conjunction with withdrawal of mycophenolate, on correlates of immune protection from COVID-19. The trial uses a pragmatic, established immunosuppression protocol to boost vaccine responses. Rapamycin is a commonly used immunosuppressant that is well tolerated at trough target levels of 3-6 nmol/L. As such, improved immunity in the treatment arm would provide strong justification for the use of rapamycin switch to enhance vaccine-induced protection against COVID-19 among KTRs.

There are limitations to a rapidly designed trial in the context of the COVID-19 pandemic. At the commencement of the trial, Australia had a largely SARVS-CoV2-naïve population with minimal community transmission. However, with the easing of social restrictions and border controls there is increasing community prevalence. Thus, trial recruitment may be limited by the prerogative to not delay booster vaccination in a vulnerable population, and in the loss of potential recruits to either having already had booster vaccination or having developed COVID-19. Additionally, while this study will provide a targeted strategy for immunosuppression modification, it will not be possible to discern the individual contributions of withdrawing mycophenolate versus addition of rapamycin on the immune response.

Abbreviations

AEFI: Adverse events following immunisation

AEs: Adverse events

ANZCTR: Australia and New Zealand Clinical Trials Registry

CALHN: Central Adelaide Local Health Network

CKD-EPI: Chronic Kidney Disease - Epidemiology Collaboration

eCRF: Electronic case report form

eGFR: Estimated glomerular filtration rate

EQ-5D: EuroQol five dimensions

GCP: Good Clinical Practice

GSRS: Gastrointestinal symptom rating scale

HREC: Human research ethics committee

KTRs: Kidney transplant recipients

mTOR: mechanistic target of rapamycin

mTORi: mechanistic target of rapamycin inhibitor

mTORC1: mechanistic target of rapamycin complex 1

RBD: receptor-binding domain

REDCap: Research Electronic Data Capture

RIVASTIM: Rapamycin and inulin for booster vaccine response stimulation

SAEs: Serious adverse events

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SCFA: Short-chain fatty acids

SLHD: Sydney Local Health District

TGA: Therapeutic Goods Administration

TSC: Trial steering committee

Declarations

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to acknowledge the contribution of the staff at the Royal Adelaide Hospital Specialist Immunisation Clinic.

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CHAPTER 4:

SUMMARY AND CONCLUDING REMARKS

4.1. Summary

This thesis addressed two key immunological challenges that have hampered global efforts to vaccinate against COVID-19: vaccine allergy, and reduced vaccine efficacy in kidney transplant recipients. Due to the novelty of the mRNA and DNA vaccines developed, and the pace at which these vaccines were, and continue to be, disseminated, strategies to provide protection to these groups have lagged behind the rollout. In the case of vaccine allergy, identification of individuals at risk of anaphylaxis to the vaccines has been a major challenge. Conversely, transplant recipients are a well-defined group, however are highly vulnerable to severe COVID-19 and are afforded variable protection from vaccination due to immunosuppression use.

Chapter 2 Summary. In South Australia, the Specialty Vaccination Clinic was established at the Royal Adelaide Hospital to evaluate patients considered potentially at-risk of an adverse reaction to vaccination, develop guidelines for the management of these individuals, and administer supervised vaccinations. The outcome of this process is summarised in the extended analysis and discussion in Chapter 2. Allergy testing protocols published by the American Academy of Allergy, Asthma and Immunology recommended evaluation of vaccine hypersensitivity based on skin testing with a combination of drugs containing polyethylene glycol (PEG) and polysorbate 80 (1), the likely allergenic components of the BNT162b2 (Pfizer) and ChAdOx1 (AstraZeneca) vaccines, respectively. This testing protocol was widely adopted, as most centres do not have access to the vaccines themselves for allergy testing purposes. We compared this approach of allergy testing with surrogate drugs to that of testing with the vaccines themselves in patients with a history suggestive of PEG allergy. Of the nine patients that returned positive skin test outcomes to the BNT162b2 vaccine, only two were positive to other PEG-containing drugs in the recommended testing protocol, indicating that testing with surrogate compounds is a poor alternative to testing with the BNT162b2 vaccine itself.

Exploring this discrepancy, we found that basophils from all nine individuals responded strongly to stimulation with BNT162b2 vaccine *ex vivo* but were not responsive to purified PEG molecules at a wide range of concentrations and molecular weights. One possible explanation for this was that PEG was not the inciting allergen in the BNT162b2 vaccine. However, all patients had a strong history of allergic reactions to PEG-containing substances, including one patient who was demonstrably allergic to PEG (developed symptoms of anaphylaxis upon oral challenge with PEG3350 [MOVICOL®]) despite showing no reactivity to MOVICOL® upon skin prick or intradermal testing. We confirmed that PEG was the inciting allergen in the vaccine by demonstrating that purified PEG2000 could competitively inhibit basophil activation in response to the vaccine. This was not a non-specific, inhibitory effect of PEG on degranulation as PEG2000 did not inhibit non-specific basophil activation with polyclonal αIgE. We therefore suggest that unbound PEG, as a non-protein antigen, can occupy binding sites (presumably IgE on the basophil surface) without necessarily inducing activation, and that this poor capacity to induce degranulation is overcome by presentation of PEG moieties on the surface of a nanoparticle. In support of this, basophils from all patients were reactive to another PEGylated liposomal drug, doxorubicin.

PEG allergy has found difficulty in achieving widespread recognition. This is because PEG is not a protein as are most allergens, and because skin test outcomes are variable and often change over time. Our data point to PEG being poorly immunogenic in its unbound state (purified or as a soluble excipient in PEG-containing drugs), and this could account for these observations. Despite this, two patients were skin test positive to PEG-containing compounds and all patients had prior reactions to ingestion or intramuscular administration, suggesting that the capacity of basophils and/or mast cells to react to PEG is dependent on quantity, route of administration, and/or *in vivo* modification. The practical application of these findings in that PEGylated lipid nanoparticles may be used in future to diagnose PEG allergy, and that basophil

activation testing with PEGylated liposomal doxorubicin may be employed immediately for PEG allergy testing in centres without access to BNT162b2 vaccine.

The mechanism of anaphylaxis to the vaccine also remains a matter of future enquiry. We have demonstrated that BNT162b2 can induce basophil activation in patients with a history of PEG allergy, and while this appears identical to IgE-mediated type I hypersensitivity, there is the possibility that it is IgG/complement-mediated. How representative the patients in our study are of those experiencing anaphylaxis upon administration of the vaccine also remains unclear.

Chapter 3 Summary. Ninety to one hundred kidney transplants are performed in South Australia each year, and the recipients remain on immunosuppressive medications for the life of their transplant. KTRs were prioritised in Phase 1B of the vaccine rollout in Australia. At this time, we initiated the REVAX trial to concurrently vaccinate close household cohabitants of 80 KTRs in order to help protect the patients from COVID-19 and to study their immune responses to vaccination compared with closely matched healthy controls. As patient and cohabitant were overwhelmingly spousal pairs, age and gender were well controlled in this study. Furthermore, patient and cohabitant were vaccinated and had their blood collected at the same time, resulting in a very high quality control group. We found that immunogenicity of the BNT162b2 and ChAdOx1 vaccines was profoundly impaired in KTRs. KTRs receiving the standard of care immunosuppressive therapy demonstrated a > 1,000-fold reduction in median total IgG response (area-under the curve), and a 10-fold reduction in functional T cell response to two vaccine doses compared with their cohabitants. Utilising anti-RBD IgG and live virus neutralisation as the gold-standard correlates of protection from SARS-CoV-2 infection and COVID-19 disease, we found that very few KTRs achieved levels estimated to provide a meaningful level of protection. By contrast, all close household cohabitants achieved high levels of protective humoral immunity. Based on these findings, we have recommended to the Australian Technical Advisory Group on Immunisation (ATAGI)

that ring vaccination be considered as the primary strategy to protect transplant recipients against COVID-19.

While ring vaccination represents an important approach in the immediate-term, a strategy to directly improve immunogenicity of COVID-19 and other vaccines in immunosuppressed individuals is needed. Such a strategy would likely centre on the temporary modification of patients' immunosuppression. To explore the influence of immunosuppressive protocol on vaccine efficacy, we enrolled an extended cohort of KTRs receiving non-standard immunosuppression. Due to low numbers of patients receiving most atypical immunosuppressants, and confounding variables relating to the use of mycophenolate-free protocols (namely, high levels of comorbidities), our analysis focussed on patients on mTORi-based protocols. We found that patients receiving rapamycin or everolimus (as part of mTORi + antimetabolite + steroid protocol) had increased spike-specific IgM and IgG compared with patients on the standard of care therapy (calcineurin inhibitor + antimetabolite + steroid). The most striking difference in patients on mTORi, however, was observed for the T cell response. While transplant recipients on standard of care therapy demonstrated a 10-fold lower median vaccine-induced T cell response than healthy controls, this deficit was not observed for those on mTOR inhibitors. On the contrary, patients on rapamycin demonstrated a ~2.5-fold *greater* T cell response than healthy controls. This is the first study to confirm that mTOR inhibition confers a gain-of-function effect on antigen-specific T cell immunity in humans following vaccination. The antigen-specific $CD4^+$ and $CD8^+$ T cells from patients on rapamycin were found to be highly polyfunctional, with cells producing up to 5 effector molecules identified in some individuals. Where a clear bias away from an effector memory phenotype was observed in the antigen-specific compartment of patients on standard of care therapy, the phenotype of antiviral T cells in patients on rapamycin was equivalent to that of healthy controls. In a sub-cohort of patients that were followed up 3 months after their second vaccination, robust $CD4^+$ central memory was maintained. These findings suggest that rapamycin may be used as an adjuvant

therapy to improve vaccine response in transplant recipients, and potentially to improve cross-protective T cell immunity in healthy individuals in light of antibody-evasive SARS-CoV-2 variants.

To directly address this hypothesis, we have initiated a randomised, controlled trial of immunosuppression modification with rapamycin to enhance immune responses to a third vaccine dose in KTRs. This trial will screen an estimated 126 KTRs on standard of care triple therapy across two sites, with the aim of enrolling 120 participants with anti-SARS-CoV-2 RBD IgG titres below 100 U/mL (equivalent to ~ 100 BAU/mL) following two vaccine doses. In this way, patients considered to already have reasonable seroprotection will not be subject to immunosuppression modification. Patients will be randomised 1:1 to either remain on their existing protocol, or to cease mycophenolate mofetil and begin a course of rapamycin targeting a trough level in serum of 4 - 6 ng/mL. Tacrolimus will also be reduced in switched patients, targeting a trough of 3 - 5 ng/mL. Following a four week lead time, participants will receive a third vaccine dose of BNT162b2, and have their immune response assessed four weeks later. Given the inherent risks in modifying immunosuppression, good renal function (eGFR > 25 mL/min and urinary ACR < 100 mg/mmol) is a key inclusion criteria for this trial.

As the best correlate that we have of real-world protection, live virus neutralisation will be measured as the primary outcome, specifically, the proportion of patients that achieve a neutralisation titre at or above 20.2% of the average titre achieved by a standardised convalescent cohort. Anti-RBD IgG titre will be measured as a secondary outcome, specifically, the proportion in each arm achieving an anti-RBD IgG titre > 100 U/mL (~100 BAU/mL) four weeks after booster vaccination. Anti-RBD IgG does not correlate as closely with real-world protection against SARS-CoV-2 as live virus neutralisation, however it is widely measured in diagnostic laboratories and it is important that the findings of this study can be directly applied to the clinical setting. While we hypothesise that the combination of switching to rapamycin and ceasing

mycophenolate will precipitate a stronger antibody response to vaccination, we expect that the most significant change will be observed in the T cell response, measured by IFNγ ELISpot.

At the time of submission of this thesis, 54 KTRs have been enrolled. 28 have been randomised to rapamycin switch; 71.4% male, mean age 57.4 ± 10.5 years, with mean graft age 6.2 ± 5.4 years. Mean serum trough concentrations of rapamycin and tacrolimus at present are 6.4 μg/L and 6.1μg/L, respectively, in the switch group. No serious adverse events or tolerability issues have been reported, and renal function in these patients has remained stable (mean baseline creatinine 117.8 μmol/L vs 119.3 μmol/L, p = 0.6), with a small increase observed in urinary ACR (mean baseline 5.4 vs 17.4, p = 0 .1).

4.2. Conclusion and Impact Statement

The rapid development and dissemination of COVID-19 vaccines has prevented in the region of half a million deaths in Europe, and over one million deaths in the United States (2-4). However, the vaccination strategy applied to the majority population overlooks particular groups. This thesis has addressed the immunological challenges to effective vaccination against COVID-19 presented by vaccine allergy and by immunosuppression of kidney transplant recipients. In addressing these issues, we have challenged current allergy testing recommendations, and have provided evidence for ring vaccination and immunosuppression modification with rapamycin as strategies to protect kidney transplant recipients against COVID-19.

Findings from this thesis have been presented at 5 international and 7 domestic scientific conferences, as well as at local symposia and invited talks. Manuscript 1 of this thesis, which relates to vaccine allergy, has been cited over 40 times in the year since it was published. Manuscript 3, which relates to vaccine immunogenicity in kidney transplant

recipients, was recently picked up by the American Society of Nephrology's newsletter *In The Loop* and highlighted to its readership of 14,000. Findings from Chapter 2 and Chapter 3 were communicated to the public via two news articles in South Australia's largest newspaper, *The Advertiser*, and, as the only prospective clinical trial examining vaccine response in transplant recipients in Australia mid-2021, data from Chapter 3 were presented to the Australian Technical Advisory Group on Immunisation (ATAGI) in support of a ring vaccination strategy and a third vaccine dose for immunocompromised groups in Australia. In South Australia, basophil activation testing with BNT12b2 vaccine is now routinely performed as part of the diagnostic workup of patients with suspected allergy to PEG, and, to-date, the immunosuppression protocol of 28 kidney transplant recipients has been modified with rapamycin prior to vaccination in our randomised, controlled trial.

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APPENDIX I:

BONUS CHAPTER

Regulation of interleukin-10 secretion by human B cells

Preface-

The following chapter contains research conducted prior to the COVID-19 pandemic. This work is relevant to the fields of allergy and transplant immunology, however is presented as an appendix so as not to divert from the central thesis of the main text.

Introduction

In 1974, two groups hypothesised the existence of an immunosuppressive population of B cells. Both groups, Katz *et al.* from London and Neta & Salvin from Pittsburgh, Pennsylvania, were trying to dissect the involvement of lymphocytes in a guinea pig model of delayed hypersensitivity (1, 2). Although this model had been around since the 1880s[a], it was not until the 1940s[b] that delayed-type hypersensitivity was recognised as being mediated by the cellular immune system (as distinct from humoral immunity). Neta & Salvin had previously found that guinea pigs sensitised to ovalbumin lost their hypersensitivity after 5 days. Adoptive transfer of splenocytes (but not peritoneal cells) from such a guinea pig that had lost its hypersensitivity could suppress hypersensitivity in a newly sensitised host (3). Katz *et al.* demonstrated that this effect was lost when γ-globulin positive cells were depleted prior to transfer, i.e. this transferable tolerance was mediated by IgG^+ B cells. The same year, Liew & Parish noted an apparent inverse relationship between effective cellular immunity (delayed-type hypersensitivity) and

[a] In developing Koch's postulates (which require that for a disease to be attributed to an infectious agent, the disease must be reproducible by isolation of the pathogen and infection of a new, susceptible host), Robert Koch managed to infected a guinea pig with Mycobacterium tuberculosis. Intradermal injection of tuberculin then precipitates a local reaction, as with the Mantoux test.

[b] Landsteiner & Chase were experimenting with passive transfer of immunity from immunised to naïve animals via antibodies in serum. They were weren't having success with tuberculosis until Chase inadvertently used 'unprepared' serum that still contained blood cells for the transfer and generated a response.

antibody production (4), which led to Mosmann *et al.* proposing the Th1/Th2 dichotomy for cellular versus antibody-mediated immunity in 1986 (5, 6).

Although several publications suggesting the presence of suppressive lymphocytes were published over the next few years, nobody was able to clone B or T cells with demonstrable suppressive activity and the concept of suppressor cells went out of fashion in favour of the Th1/Th2 balance determining reactivity to antigens.

Suppressor T cells remerged as 'regulatory' T (Treg) cells from research into oral tolerance and central tolerance/autoimmunity (7-10).[c] Similarly, Fillatreau *at al* demonstrated in 2002 that recovery from experimental autoimmune encephalomyelitis (EAE) was dependent on IL-10 production by auto-reactive B cells (12). Two other publications made similar findings relevant to rheumatoid arthritis and inflammatory bowel disease that same year (13, 14). The identification of the IL-2 receptor alpha chain, CD25, as a marker of Tregs, and FoxP3 as a master transcription factor regulating suppressive gene programs in Tregs, facilitated an explosion in Treg research. As no equivalents were identified for B cells with suppressive function, the concept of the regulatory B cell did not enjoy the same resurgence in popularity.

[c] Abandonment and rediscovery may even have been the easiest route to progress, as 'suppressor T cells' were thought by those in the field of 'suppressorology' to bind antigen directly, secrete their antigen receptors, and lack expression of the TCR beta chain (11).

In the last decade there has been renewed interest in B cells producing the regulatory cytokine IL-10, which has coincided with the growth of B cell depleting monoclonal antibody therapies. While a universal marker (or combination of markers) is yet to be found that identifies all cells with the capacity to produce IL-10, headway is being made in understanding the factors that regulate IL-10 competency in B cells.

Several populations of B cells have been defined with increased propensity for IL-10 production, and numerical and functional impairment of these populations have been linked to various disease states,including autoimmunity, allergy and transplant rejection (15-19). The most convincing link that has been drawn between immunosuppressive B cells and disease in humans is that of the transitional B cell cytokine response with kidney transplant outcome, discussed below.

IL-10 producing B cells. While IL-10 secretion by B cells is well-established as an important regulatory mechanism, the study of regulatory B cells has been hampered by an inability to identify a particular subset of dedicated IL-10 producers. Upon isolation, very few B cells are found to spontaneously secrete IL-10, with two potential populations of 'natural' Bregs described thus far: adipose Bregs in mice and IL-10^{+} plasmablasts (20, 21). Instead, various signals, and combinations of signals, that induce B cell activation are able to induce the production of IL-10. These include antigen receptor stimulation, CD40 engagement, and TLR activation (22).

CpGB, a synthetic agonist of TLR9, in particular induces significant IL-10 production, however this is confined to small percentage of cells (22). Early work by Thomas Tedder's group found that the optimal strategy for inducing IL-10 producing B cells was an initial stimulation with CD40 ligand followed by CpGB treatment (22). CD40 engagement appeared to have a licencing effect that induced an IL-10 permissive state in cells, and CpGB was able to induce the production and secretion of IL-10 in these IL-10 competent (or proB10) cells. While Tedder and colleagues determined in their system that these B10 cells were best captured by a $CD24^{hi}CD27^{+}$ phenotype, this phenotype did not capture all $IL\text{-}10^{+}$ cells and only a small percentage of B cells of this phenotype produced IL-10. Several phenotypes have since been proposed that capture as large of a portion of $IL\text{-}10^{+}$ cells as possible in various settings, however, these phenotypes will invariably be highly heterogeneous and capture < 30-40% of $IL\text{-}10^{+}$ cells (14-16, 22).

The signals that induce IL-10 production by B cells are notably generic and can similarly induce the production of the pro-inflammatory cytokines IL-6 and TNF. In fact, under most conditions, $IL\text{-}10^{+}$ cells will co-express one or more pro-inflammatory cytokine, and for this reason, more recent analyses have concluded that a dedicated IL-10 producing regulatory B cell subset is unlikely to exist (23, 24). Rather, IL-10 expression is likely to be induced in a variety of B cell subsets by environmental factors, including determinants of cellular metabolism, and is therefore a transient, context-dependent response rather than a feature of one or more regulatory B cell lineages (25-27). One key scenario that has been explored in mice is the induction of IL-10 in response to

recognition of apoptotic cells by surface TIM-1, in order to prevent the activation of autoreactive T cells (28). This system can be manipulated to promote transplant tolerance (29).

B cell cytokines and transplant rejection. Despite concomitant expression of IL-10 and pro-inflammatory cytokines, in 2017, Cherukuri *et al* described a strong bias towards IL-10 (and away from TNF) production by $CD24^{hi}CD38^{hi}$ B cells stimulated with CpGB (a synthetic microbial DNA mimic and TLR9 agonist) and CD40 ligand, relative to other B cell populations. Following 24 hours stimulation, clear $IL\text{-}10^{+}TNF^{-}$ and $IL\text{-}10^{-}TNF^{+}$ populations are observed, with few cells producing both IL-10 and TNF. Cherukuri *et al* found that the ratio of $IL\text{-}10^{+}$ to TNF^{+} transitional B cells not only corresponded with the capacity of an individual's B cells to suppress pro-inflammatory polarisation of $CD4^{+}$ T cells *in vitro*, but it could be used as a biomarker to predict both short-term (acute) rejection, as well as long-term (5 year) graft outcomes of kidney transplant recipients (18, 19).

Transitional B cells. Several groups have identified $CD19^{+}CD24^{hi}CD38^{hi}$ B cells as strong producers of IL-10 in response to microbial products and T cell help. $CD19^{+}CD24^{hi}CD38^{hi}$ B cells overlap almost entirely with transitional B cells (generally defined as $CD19^{+}CD24^{hi}CD38^{hi}CD27^{-}$). Transitional B cells have been best described in mice where B cell precursors can be tracked through their egress from the bone marrow into peripheral circulation. Transitional B cells transit from the bone marrow to the spleen where they undergo further selection to limit autoreactivity in the mature B cell pool.

As such, transitional B cells constitute a semi-mature, intermediate stage of development (30). While a similar semi-mature population of transitional B cells exists in humans, it is not as phenotypically distinct from mature B cells, and maturation of this population does not appear to be confined to the spleen (31). In humans, transitional B cells are defined as $CD27^-$ cells (CD27 is a standard marker of antigen-encounter although $CD27^-$ memory cells do exist) with high expression of CD24 and CD38. Although this population blurs into the mature naïve B cell pool, which expresses low levels of CD24 and CD38, stepwise reconstitution of $CD24^{+++}CD38^{+++}$ (transitional 1; T1) followed by $CD24^{++}CD38^{++}$ (transitional 2; T2) and finally $CD24^{+}CD38^{+}$ B cells can be observed following B cell depletion with anti-CD20 monoclonal antibodies (32). While phenotypically indistinguishable from mature naïve B cells, a third maturation stage of transitional B cells (transitional 3; T3) can be discriminated based on its capacity to extrude rhodamine-123 dye, a function that is peculiar to cycling transitional and memory B cells but is lost in quiescent naïve cells (32, 33).

Chapter Aims

1. To identify the optimal conditions to induce the production and secretion of IL-10 by human B cells
2. To identify culture conditions for the expansion of IL-10 competent human B cells

Results/Discussion

A culture system for the expansion of human B cells

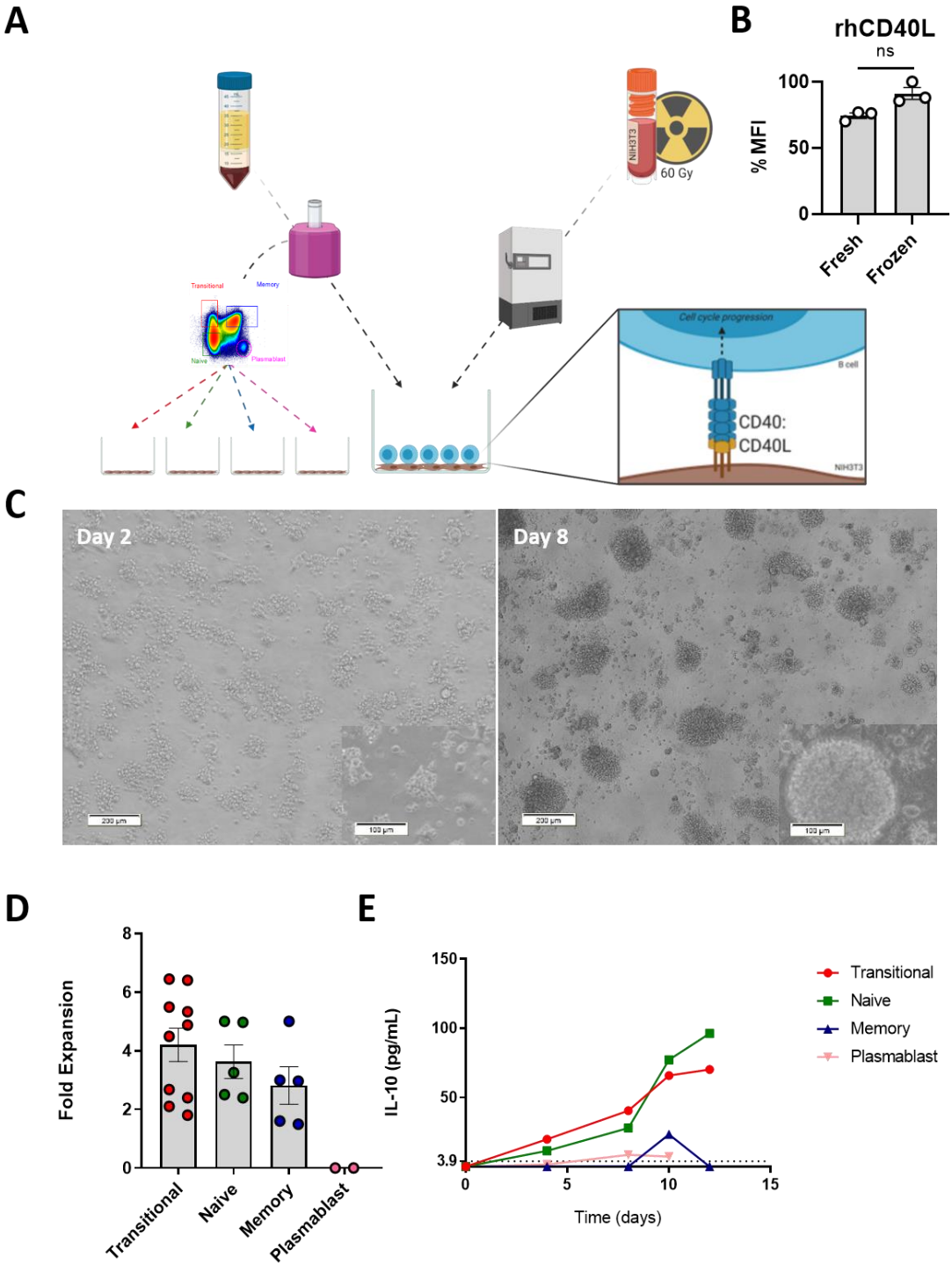
The aim of this chapter was to identify factors that control the capacity of cells to produce IL-10 upon activation, with the ultimate goal of deriving a set of culture conditions that would enable us to expand IL-10 competent cells that could be exploited for research an immunotherapy. Although IL-10 competence does not appear to be restricted to a particular B cell subset, some populations (notably transitional B cells) are more enriched for IL-10 competent cells and there are fundamental differences in how the major developmental B cell subpopulations respond to stimulation via TLRs, antigen-receptor, and CD40. Therefore, in order to best evaluate regulation of IL-10 competence and secretion, we examined transitional, naïve, and memory B cells and plasmablasts, individually. Separation of these populations was based on Gating Strategy 1, Appendix Figure S1.

In Appendix Figure 1, we optimised conditions for the expansion of human B cells and assessed the natural capacity of each of the major B cell subsets to expand and secrete IL-10 in this system. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood by density gradient centrifugation, and total B cells enriched by magnetic separation (negative selection, STEM CELL Technologies). Untouched B cells were stained for CD19, CD24 and CD27, and sorted as per Gating Strategy 1 (Appendix Figure S1). Whole B cells or subpopulations were cultured on a monolayer of CD40L-expressing NIH3T3 fibroblastic cells in complete IMDM culture medium supplemented

with insulin, transferrin and selenium. The 3T3:CD40L cell line was initially expanded, then irradiated and stored at -80°C. As frozen feeder cells had not been previously used by our group for this purpose, expression of CD40L on the surface of cells was assessed in fresh and frozen specimens to ensure that the freezing process did not alter CD40L expression (Appendix Figure 1B). One millilitre of 3T3:CD40L cells was added per well of a 48-well plate at a concentration of 2.5 x 10^5 cells/mL in complete DMEM media, and left to attached overnight at 37°C. Non-adhered cells were washed off prior to addition of B cells.

Appendix Figure 1C depicts whole $CD19^+$ B cells after 2 days of co-culture beginning to form aggregates on top of adherent 3T3:CD40L cells. Large spherical structures of dividing cells formed later in the culture (Appendix Figure 1D), the size and density of which was increased by the addition of IL-4 (10 ng/mL) to the culture (Appendix Figure S2). Surprisingly, transitional and naïve B cells expanded more readily than memory cells over a 12 day culture. This suggests that, while the threshold for activation of memory cells is classically lower than that of naïve cells due to appropriate licencing accompanying initial antigen encounter, the capacity for long-term expansion in response to T cell help (CD40 engagement) was greater in naïve cells. The high proliferative capacity of transitional B cells is surprising as the antigen-receptor repertoire of this population has a high frequency of auto-reactive specificities. It may be that T cell help is sufficient to permit the expansion of TrB cells, however assessment of the purity of this population found that <30% could be considered to be 'true' transitional cells (Appendix Figure S1), which may account for the observed behaviour.

Gating Strategy 2 was therefore used for future experiments (Appendix Figure S1). Measurement of IL-10 in culture supernatants over 12 days found a peak in cumulative IL-10 at Day 8 of culture (n=2, Appendix Figure 1E). Interestingly, IL-10 produced by memory cells at this time appeared to be consumed from the culture, hinting at an auto/paracrine role for B cell-derived IL-10 (Appendix Figure 1E).



Appendix Figure 1. A system for expanding human B cells for assessment of IL-10 competency. (A) Schematic of expansion system. B cells are isolated from human buffy coats by density gradient centrifugation, followed by magnetic negative selection to

enrich for B cells from peripheral blood mononuclear cells. Isolated B cells are seeded onto a monlayer of irradiated NIH3T3:CD40L cells to promote B cell proliferation. Created with BioRender.com. (B) Comparison of CD40L expression on the surface of fresh versus frozen NIH3T3:CD40L cells. (C) Light field microscopy images of proliferating human B cells at Day 2 and Day 8 of expansion. (D) Fold expansion of transitional, naïve, memory B cells, and plasmablasts, after 8 days of expansion. (E) Cumulative IL-10 secreted into the supernatant of expansion culutres. Concentrations do not account for cell numbers as they change during the expansion. Note: details of flow cytometry gating strategies can be found in Appendix Figure S1.

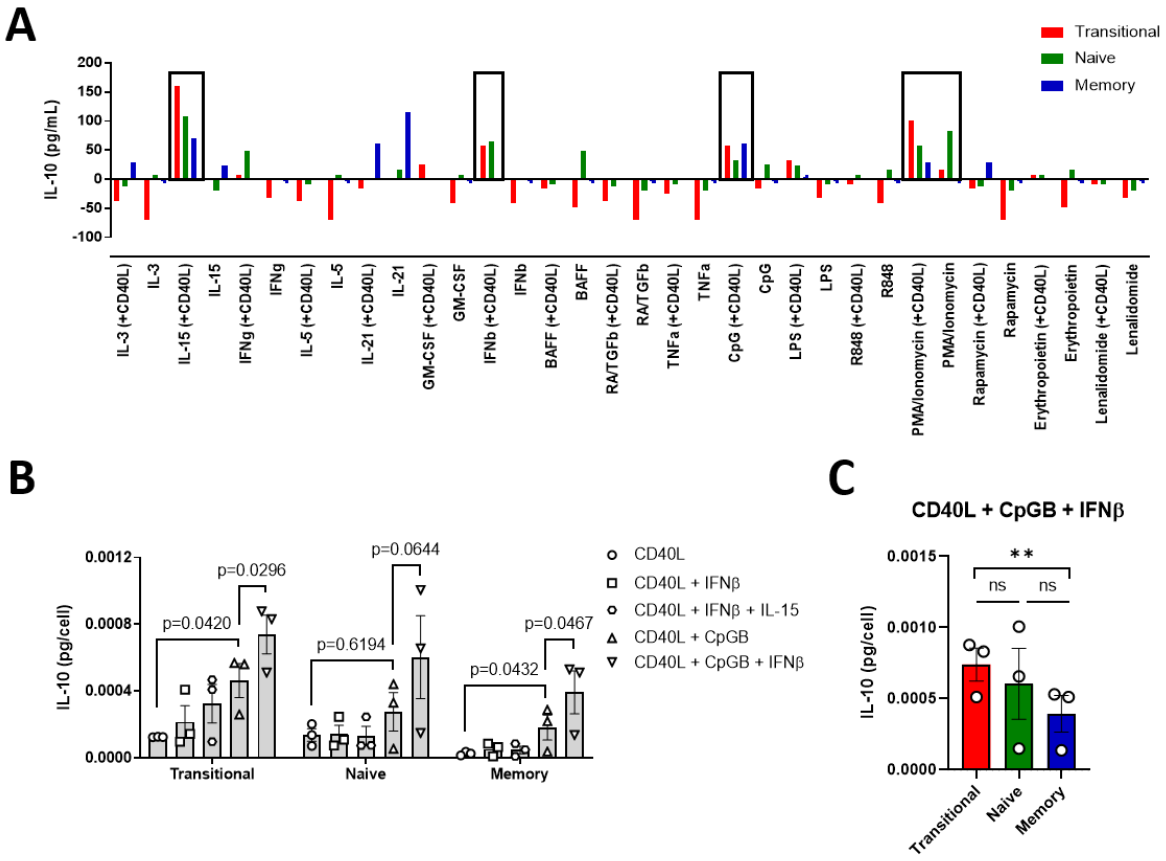
An immunostimulatory role for B cell derived IL-10

The possibility that B cells produce IL-10 for auto/paracrine use is attractive. While IL-10 is classically a potent immunosuppressive cytokine, its effects on B cells are more stimulatory than suppressive. Prior to the discovery of IL-21 as the key driver of antibody-forming cell development, this role was primarily attributed to IL-10 due to the effects of IL-10 on B cell cytokine production *in vitro* (34). Memory B cells and marginal zone (MZ) B cells (and human equivalent) are classically capable of differentiating into antibody-forming cells independent of T cell help. Rather, these cells express high levels of TLRs and rapidly differentiate into short-lived plasmablasts in response to TLR ligands or upon encounter of T-independent antigens capable of cross-linking multiple B cell receptors; paracrine IL-10 would potentially assist in this process. T follicular helper cells produce IL-10 to support B cell differentiation and extrafollicular $CD4^+$ T cells secrete IL-10 early in parasitic infection to promote rapid antibody responses (35). Furthermore, IL-10 secretion by T cells likely supports autoantibody production in lupus erythematosus in humans, a disease classically driven by inappropriate activation of TLR9 (36). Although tangential to the aim of this chapter, we took the opportunity to assess the contribution of B cell-derived IL-10 to CpGB-induced activation marker expression and antibody isotype switching. The results of this experiment, presented in Appendix Figure S3, support a role for auto/paracrine IL-10 in B cell activation. Blockade of IL-10 in purified B cell cultures resulted in reduced expression of the early activation marker CD69, and increased expression of the co-stimulatory molecule CD86 (Appendix Figure S3). As CD86 is crucially important for productive B cell:T cell interaction, a down-regulatory effect of autocrine IL-10 on CD86

expression supports preference for T cell-independent extrafollicular B cell development. At Day 3, inhibition of IL-10 resulted in reduced IgG1 and IgG3 in supernatant (Appendix Figure S3). As an effect on class-switch recombination is unlikely to be observable at such an early time point, this finding likely reflects impaired antibody secretion by IgG^+ memory B cells. While this effect was lost by Day 9, a reduction in IgG4 secretion was observed at this time point, recapitulating a role for IL-10 in IgG4 class-switching that has been proposed by others (37). IgG4 plays a role in tolerance to protein allergens by sequestering allergen epitopes to prevent binding by pathogenic IgE (38), and IL-10 blockade in our system resulted in a switch in the ratio of IgE:IgG4 at Day 9 (Appendix Figure S3). Interestingly, this finding may explain the association of IgG4 and IL-10 positivity observed of B cells in allergic patients undergoing desensitising immunotherapy (15). These findings are consistent with literature assessing the effect of IL-10 on B cell responses, and extend such considerations to B cell-derived IL-10 consumed in an auto/paracrine manner following TLR stimulation. However, while this experiment incorporated n=3 biological replicates, these replicates were run in a single experiment, and should be repeated in independent experiments in order to have a high degree of confidence in the results.

Culture conditions for the optimal induction of IL-10 secretion by human B cells

Returning to our aim of defining culture conditions for the expansion of IL-10 competent B cells, we assessed a variety of immunomodulatory factors that have been reported to induce IL-10 production (or target intracellular pathways that have been linked to IL-10 production in B cells or other immune cell populations) to identify a set of conditions that would induce maximum IL-10 secretion by IL-10 competent B cells within each major B cell subpopulation. Appendix Figure 2A shows the outcome of n=2 independent experiments. Sorted B cell populations were cultured with or without 3T3:CD40L feeder cells for 24 hours prior to the addition of immunomodulatory factors. IL-10 in supernatants was measured 48 hours later by ELISA (Biolegend). Results were standardised to the average IL-10 concentration for unstimulated or CD40L-alone stimulated cells. CD40L alone induced minimal IL-10 secretion (data not shown), however was required for IL-10 production in response to activating factors, consistent with CD40 engagement as a licencing signal that increases IL-10 permissiveness but not production/secretion. IL-15, CpGB and IFNβ were identified as significant drivers of IL-10 secretion in CD40L primed cells (Appendix Figure 2A). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were included for comparison; PMA and ionomycin are non-physiological activators of protein kinase C and calcium flux, respectively, which together drive cytokine production and secretion in permissive cells. Combinations of these factors were tested in an identical experiment (n=3), and the combination of CD40L, CpGB and IFNβ determined to be the optimal combination of factor for the secretion of IL-10 by all subpopulations (Appendix Figure 2B,C).

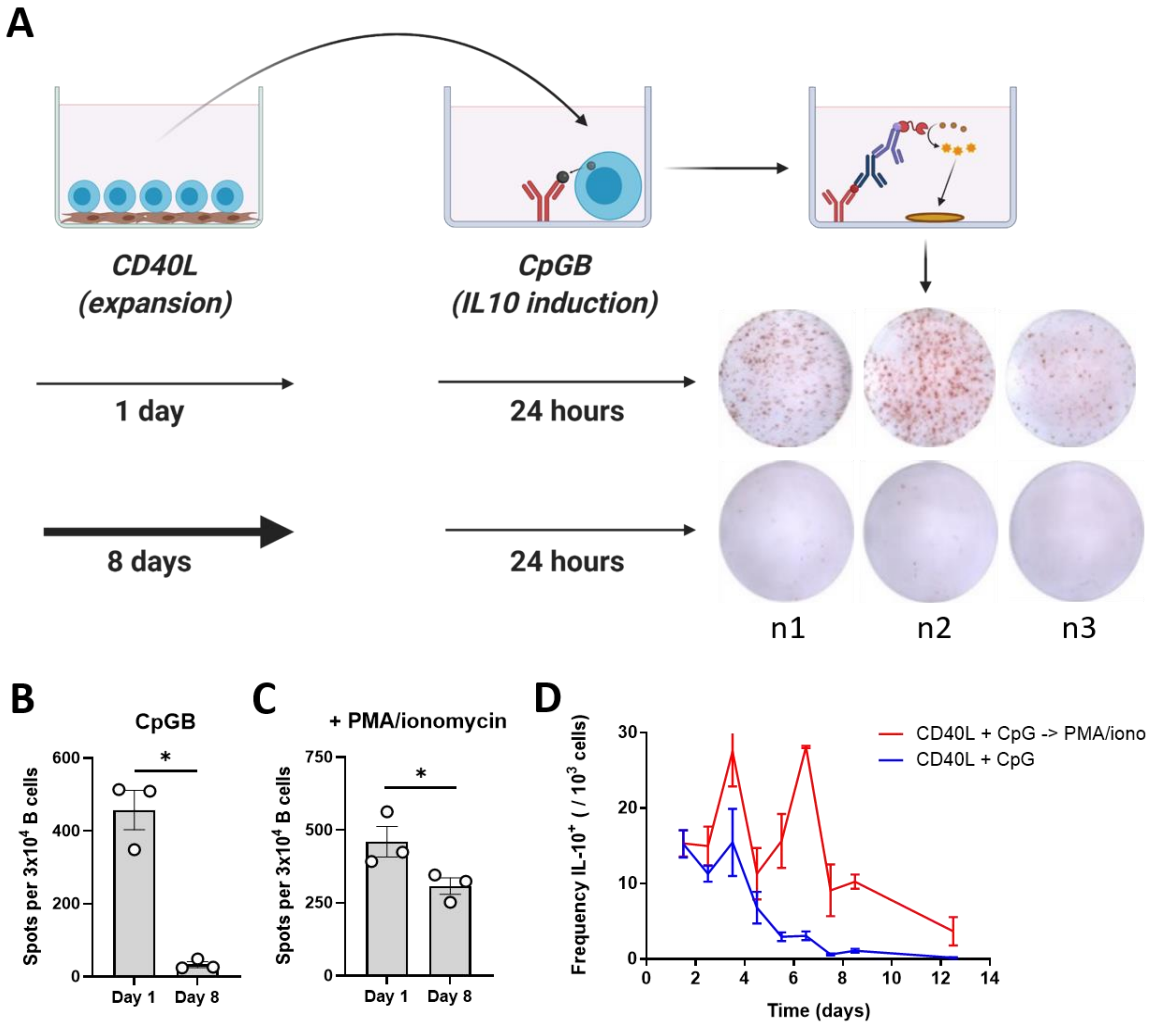


Appendix Figure 2. Optimal conditions for induction of IL-10 secretion by human B cells. (A) Average of N=2 independent experiments measuring IL-10 in the culture supernatants of purified transitional, naïve and memory B cells stimulated for 48 hours with indicated immunomodulatory factors +/- CD40L. Concentrations are normalised to unstimulated or CD40L alone to isolate the effect of each factor on IL-10 secretion. (B) Further investigation of potential immunomodulatory factors identified in A, presented as concentration per cell count (n=3 independent experiments). (C) Direct comparison of IL-10 secretion per cell between transitional, naïve and memory B cells stimulated with the optimal culture condition identified, using data from B. Statistics by paired, two-tailed Student's T test.

IL-10 competence is lost with extended culture

Having established the best conditions for inducing IL-10 secretion by IL-10 competent cells, we assessed IL-10 competence in the expanded population of $CD19^+$ B cells. As it is unknown whether IL-10 competence in hereditary or solely regulated by environmental conditions, this experiment could be interpreted as either measuring the capacity of IL-10 competent cells to expand in our system, or as the likelihood of expanded B cells to assume an IL-10 competent state. For this experiment we optimised an in-house IL-10 ELISpot assay that enabled quantification of the number of IL-10 secreting cells rather than total secreted IL-10 (capture and detection antibodies were derived from the Biolegend human/viral IL-10 ELISA). In this assay, whole $CD19^+$ B cells were seeded to a 24-well plate coated with 3T3:CD40L cells (at 50% confluence), for 1 or 8 days. Cells were then detached, washed, and viable cells counted and transferred at 10^5 cells per well to an ELISpot plate with CpGB or PMA/ionomycin to induce IL-10 secretion. After 24 hours, plates were developed and IL-10 spot-forming units counted (CTL Immunospot counter). Culture conditions were as established in Appendix Figure 1: complete IMDM medium supplemented with insulin, selenium, transferrin and rhIL-4 (10 ng/mL). The frequency of B cells competent to secrete IL-10, as assessed by stimulation with CpGB or PMA/ionomycin, was significantly reduced after 7 days of expansion in our system (Appendix Figure 3). This difference was much more pronounced in the CpGB condition than the PMA/ionomycin condition, suggesting that, while IL-10 competence is significantly reduced following expansion, this may be partially due to reduced TLR9 responsiveness. To better understand this loss in IL-10 competence, we assessed IL-10 competence every 24 hours. We found that IL-10

competence peaked early in expansion, and then again at Day 6, before dropping off significantly by Day 8 (Appendix Figure 3D). By Day 12, non-physiologic stimulation with PMA and ionomycin was able to induce IL-10 secretion by very few cells (Appendix Figure 3D).



Appendix Figure 3. IL-10 competence is lost with extended culture of human B cells.

(A) Schematic representation of the experimental design. Purified human B cells were expanded on a monolayer of CD40L-expressing NIH3T3 cells for 1 day or 8 days. B cells

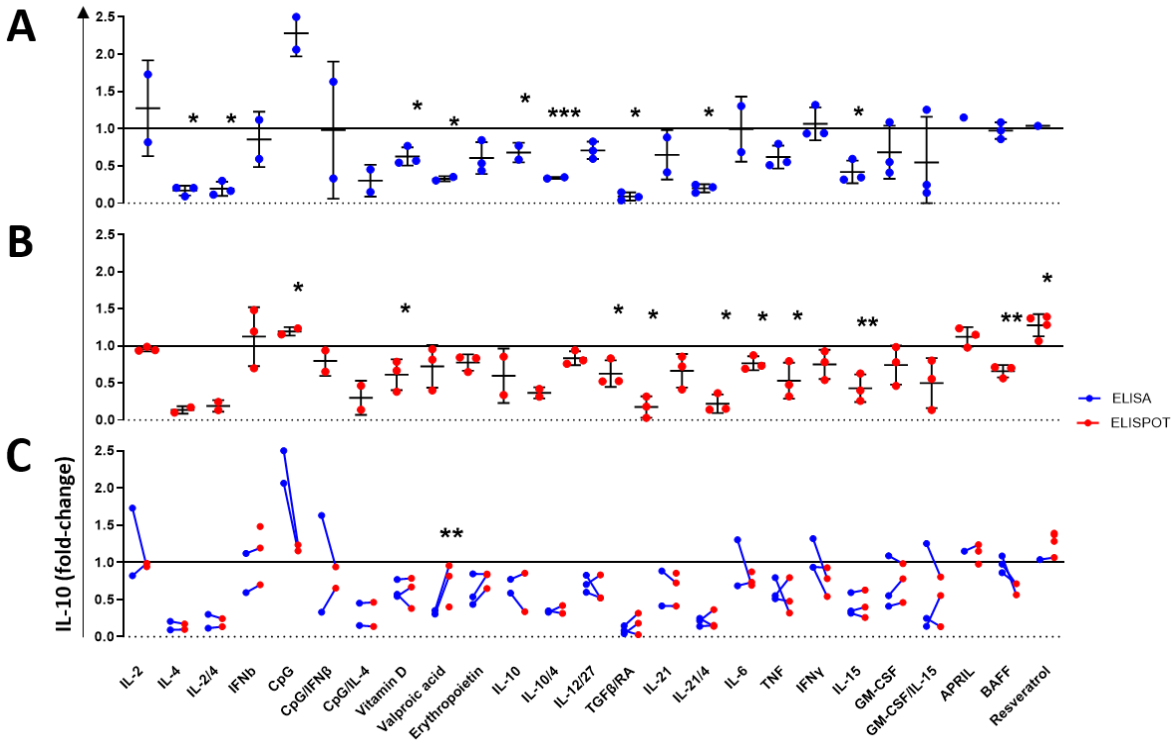
were detached, counted, and seeded to an ELISpot plate in CpGB containing medium for 24 hours. IL-10 secreting cells were counted as spot-forming units. **(B)** Frequency of B cells secreting IL-10 in response to CpGB stimulation following 1 day versus 8 days of expansion culture. **(C)** Frequency of B cells secreting IL-10 in response to CpGB when PMA + ionomycin was added to the ELISpot culture for the final 6 hours. This may be interpreted as the 'competency' of the cells to secrete IL-10. **(D)** Longitudinal assessment of IL-10 secretion and IL-10 competency in the expanding B cell population out to Day 12. Statistics by two-tailed paired Student's T test: *$p<0.05$.

IL-10 competence is altered by expansion conditions

In an attempt to restore IL-10 competence in the expanded population, we expanded whole $CD19^+$ B cells for 8 days, as previously, under 25 different culture conditions. Culture conditions were as previous, but without IL-4 to allow for the effect of IL-4 on IL-10 competence to be assessed. Cells were then detached from 3T3:CD40L monolayers, viable cells counted, and equal numbers transferred to an ELISpot plate or 96-well tissue culture plate for assessment of total IL-10 secretion by ELISA. Cells were stimulated for 24 hours with CpGB, and IL-10 spot-forming units and IL-10 concentrations in supernatants were standardised to that of cells expanded with CD40L alone then stimulated with CpGB. Several factors were found to modulate IL-10 competence in the expanded population. While inclusion of CpGB or resveratrol in the expansion medium resulted in a slight increase in IL-10 competence, multiple factors further impaired IL-10 competence (Appendix Figure 4A,B). Most notable amongst these was IL-4 (independent of co-factor) and the combination of TGFβ and retinoic acid (which together are potent inducers of regulatory T cell differentiation). While the ELISpot and ELISA results were generally in agreement, valproic acid reduced the amount of IL-10 secreted into the supernatant without significantly impairing the frequency of $IL\text{-}10^+$ cells (Appendix Figure 4C).

The capacity of IL-4 to impair IL-10 competence is of particular note as IL-4 has been included in all expansion cultures thus far. Removal of IL-4 from the expansion culture significantly restored IL-10 secretion following 8 days of expansion when CpGB was used to quantify $IL\text{-}10^+$ cells (data not shown). However, removal of IL-4 did not

significantly affect outcomes when PMA and ionomycin were used to stimulate IL-10 secretion. Therefore, while IL-4 impairs CpGB-induced IL-10 secretion, it was not responsible for the loss of IL-10 competence in the expanded population (Appendix Figure 3C,D).

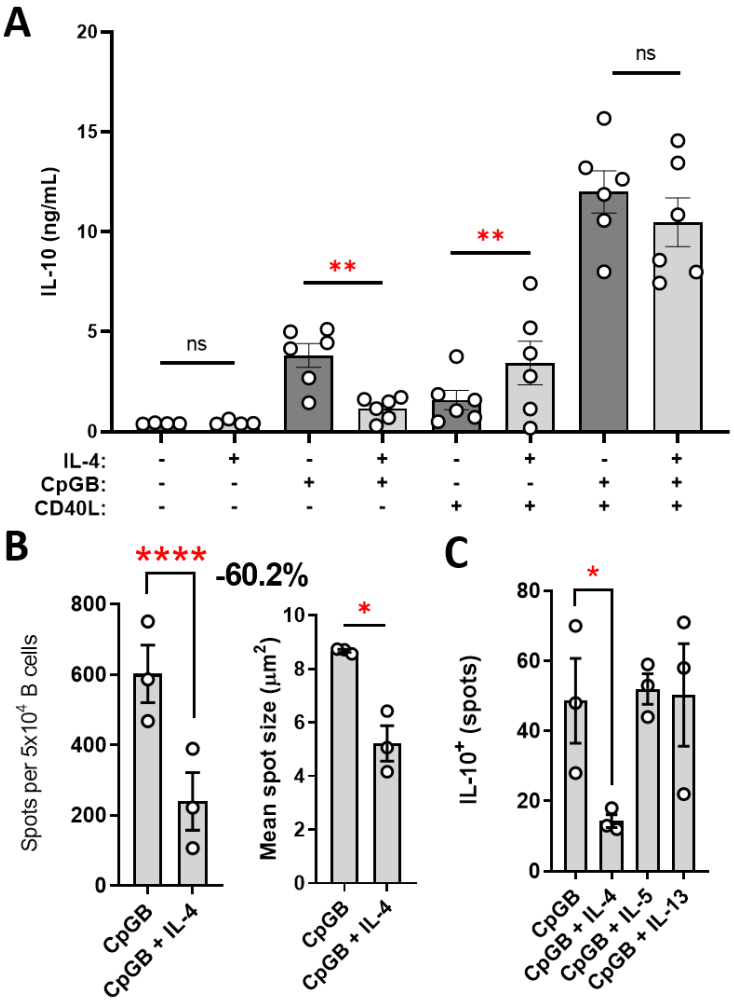


Appendix Figure 4. Effect of expansion conditions on IL-10 competency in the expanded B cell population. Purified B cells were expanded for 8 days in the presence of 25 different immunomodulatory factors or combinations of factors. Cells were then detached from 3T3:CD40L monolayers, washed, viable cells counted, and equal numbers transferred to an ELISpot plate, or 96-well tissue culture plate for assessment

of total IL-10 secretion by ELISA. Cells were stimulated for 24 hours with CpGB, and IL-10 spot-forming units, and IL-10 concentrations in supernatants, were standardised to that of cells expanded with CD40L alone then stimulated with CpGB. **(A)** Normalised IL-10 concentration in supernatant following 24 hour stimulation with CpGB. **(B)** Normalised IL-10 secreting B cell frequency following 24 hour stimulation with CpGB. **(C)** Comparison of the effect of culture condition on IL-10$^+$ cell frequency (by ELISpot; red) and concentration of secreted IL-10 (by ELISA; blue). Statistics by two-tailed paired Student's T test: *$p<0.05$, **$<p<0.01$, ***$p<0.001$.

IL-4 suppresses CpGB-induced IL-10 secretion

Having failed to identify conditions that promote IL-10 competence in the expanded B cell population, we focussed on the observed effect of IL-4 on IL-10 secretion. IL-4 acts as a growth and differentiation factor for B cells, and is involved in almost all aspects of B cell biology. To begin to understand this effect in our system, we assessed the effect of IL-4 on IL-10 production in response to CpGB and CD40L treatment for 48 hours. IL-4 significantly impaired CpGB-induced IL-10 secretion but did not impair CD40L-induced IL-10 production (Appendix Figure 5A). Rather, IL-4 enhanced IL-10 secretion in combination with CD40L, and did not make a significant net impact on IL-10 secretion in response to CpGB + CD40L. Thus, IL-4 selectively impairs IL-10 secretion in response to CpGB, but not in the context of T cell help. This effect corresponded to a 60.2% reduction in the frequency of IL-10 secreting cells detectable by ELISpot (Appendix Figure 5B). The spots observed in the IL-4 treated condition were on average significantly smaller than that of the CpGB alone condition, suggesting that IL-4 impairs the amount of IL-10 secreted rather than acting as an on/off switch for IL-10 secretion (Appendix Figure 5B). This effect was not observed in response to the related Th2 cytokines, IL-5 and IL-13, which is of particular interest given that IL-4 and IL-13 receptor complexes share a common alpha chain (Appendix Figure 5C).



Appendix Figure 5. IL-4 suppresses CpGB-induced IL-10 secretion by human B cells. **(A)** Purified human B cells were culture for 48 hours with combinations of IL-4, CpGB and CD40L (NIH3T3:CD40L monolayer), and IL-10 in supernatants measured by ELISA. Conditions containing IL-4 are coloured light grey for comparison with conditions without IL-4 (dark grey). **(B)** Purified B cells were stimulated with CpGB +/- IL-4 for 24 hours in an IL-10 ELISpot assay, and spot frequency and size measured. **(C)** Purified B cells were stimulated with CpGB +/- IL-4, IL-5 or IL-13 for 24 hours and IL-10 spot forming units counted. Statistics by two-tailed paired Student's T test: *p<0.05, **<p<0.01, ***p<0.001, ****p<0.0001.

IL-4 does not impair TLR9 signalling nor B cell activation in response to CpGB

The major source of IL-4 for B cells is T cells in follicles/germinal centres, and NKT cells in the interfollicular areas within lymph nodes (39). As such, the provision of IL-4 to B cells is strongly associated with T cell driven responses, and drives B cell behaviours congruent with T cell help. This is at odds with the innate and T cell-independent responses of B cells to TLR ligands. Furthermore, microbial DNA analogues are used as adjuvants to drive Th1-type cellular immune responses, which is at odds with the role of IL-4 as a driver of antibody production and Th2 immunity (40). This broad interpretation of the roles of TLR ligands and IL-4 in B cell development and activation supports an antagonistic relationship between IL-4R and TLR signalling, and such a relationship has been described in mouse dendritic cells (41). As the effect of IL-4 was specific to CpGB-induced IL-10 secretion, we questioned whether IL-4 exerts its effect by antagonising TLR9 signalling.

To test whether IL-4 treatment impairs TLR9 signalling, we pre-treated purified B cells with IL-4 (10 ng/mL) for 24 hours, then added CpGB (3.2 μg/mL) to the culture. After 24 hours of TLR9 stimulation with CpGB, cells were harvested and stained for expression of surface activation markers known to be upregulated in response to TLR9 stimulation. CpGB treatment alone upregulated the expression of CD40, CD69, CD80 and CD86 to varying extents (Appendix Figure 6A). Treatment with IL-4 alone did not significantly impact expression of any of these TLR9 activation-induced markers, although a non-significant increase in CD69 was observed (Appendix Figure 6A). Surprisingly, the

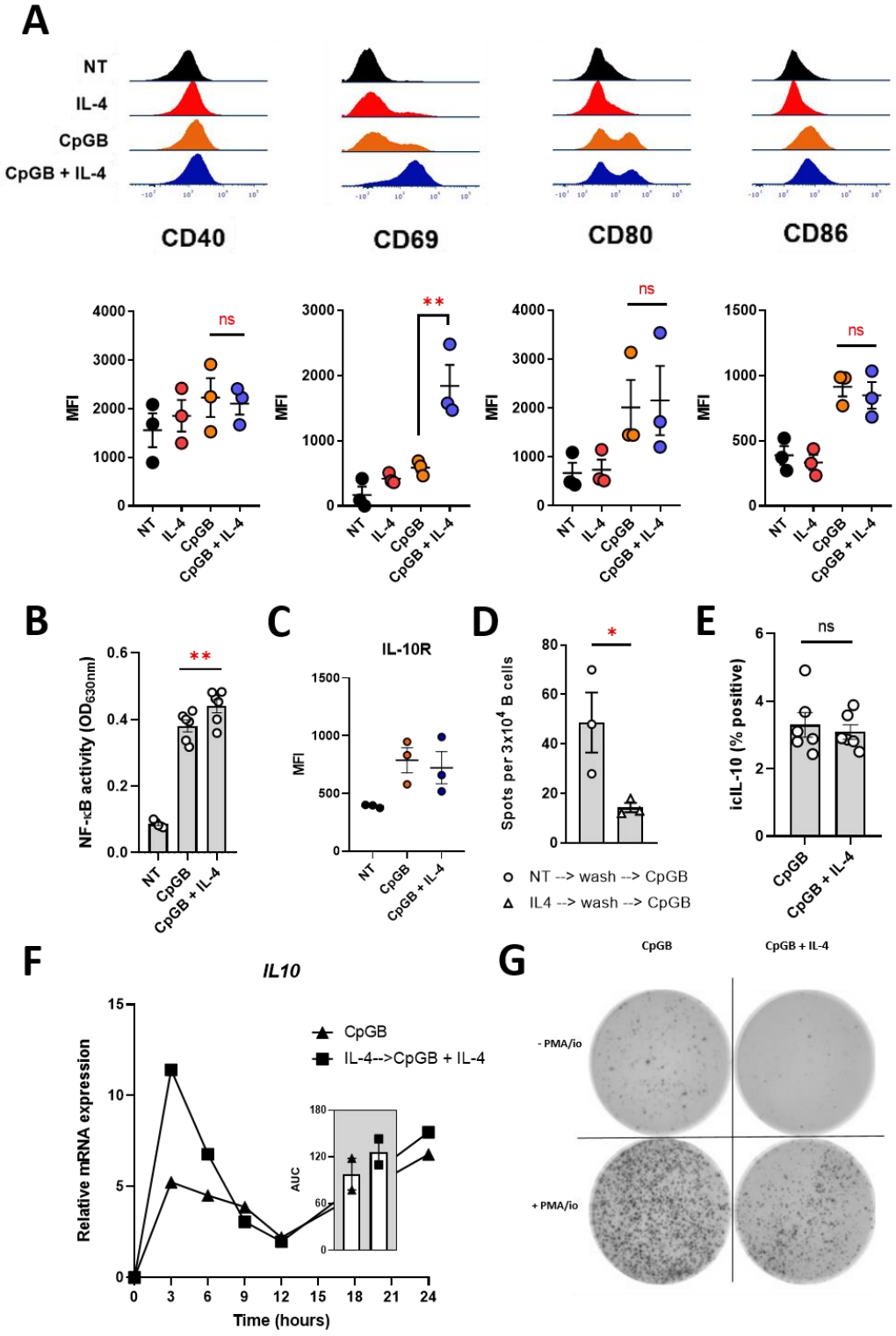
combination of CpGB and IL-4 synergistically increased CD69 expression (Appendix Figure 6A).

The major transcription factor downstream of TLR9 signalling is NF-κB. In order to confirm that IL-4 does not impact TLR9 signalling, we measured NF-κB activity in response to CpGB and IL-4 using a B cell reporter line, Ramos Blue. Ramos Blue is a Burkitt's lymphoma-derived cell line that expresses an alkaline phosphatase (ALP) gene under an NF-κB controlled promoter. Activation of NF-κB therefore drives expression of ALP, which is secreted into the culture supernatant and can be quantified using a QantiBlue substrate and colourimetric plate reader. Using this system we found that, rather than suppressing TLR9 signalling, IL-4 treatment enhanced TLR9-driven NF-κB activity (Appendix Figure 6B). This may partially account for the synergistic effect on CD69 upregulation (Appendix Figure 6A).

An alternative hypothesis for the effect of IL-4 on secreted IL-10 is that IL-4 upregulates IL-10R expression on B cells, thereby increasing consumption of IL-10 and resulting in less IL-10 detectable in the supernatant. We therefore measured IL-10R expression on purified B cells treated with CpGB and IL-4, or CpGB alone. We found no significant increase in IL-10R expression with IL-4 treatment (Appendix Figure 6C). This hypothesis of increased IL-10 consumption is further discredited by ELISpot results, as the speed at which secreted IL-10 would need to be consumed from the culture would need to be sufficiently rapid to prevent binding by capture antibodies on the well membrane.

The data presented thus far indicate that IL-4R signalling produces a state in the cell that impairs transcription, translation, or secretion of IL-10, or increases intracellular turnover of the IL-10 mRNA or protein. To investigate this further we asked whether this state in the cell was dependent on constant IL-4R signalling, or whether transient IL-4 treatment would induce a lasting state that was refractory to CpGB-induced IL-10 secretion. We found that transient priming of purified B cells with IL-4 for 24 hours was sufficient to reduce the IL-10 response to CpGB treatment (Appendix Figure 6D), although the effect was reduced compared with constant IL-4 exposure (data not shown). To better understand which aspect of the IL-10 secretion process is affected by IL-4 priming, we measured IL-10 protein and mRNA levels following CpGB treatment. In cells primed with IL-4, we observed no reduction in il10 mRNA levels over 24 hours following CpGB stimulation (Appendix Figure 6F, n=2). To determine the effect of IL-4 on the production of IL-10 protein in response to CpGB treatment, purified human B cells were stimulated for 48 hours with CpGB and then cytokine secretion blocked for the last 5 hours allowing the accumulation of IL-10 protein in the cytoplasm. In cells primed with IL-4, we observed no change in the percentage of B cells expressing intracellular IL-10 by flow cytometry (Appendix Figure 6E), suggesting that IL-4 does not impair the amount of IL-10 protein produced. As inhibiting golgi transport may interfere with cellular machinery involved in protein recycling, this does not necessarily rule out a role for enhanced IL-10 degradation. Despite our findings suggesting that intracellular levels of il10 mRNA and IL-10 protein are unaffected by IL-4 treatment, impairment of secretion remains clearly observable (Appendix Figure 6G).

It is thus possible that IL-4R signalling induces a cellular state that impairs the secretion of cytoplasmic IL-10 protein. While selective regulation at the stage of secretion is uncommon as it is energetically wasteful (the protein has already been produced), IL-10 is tightly controlled as a potent immunosuppressive cytokine, and regulation of IL-10 at the level of secretion has been observed in other cell types (42).

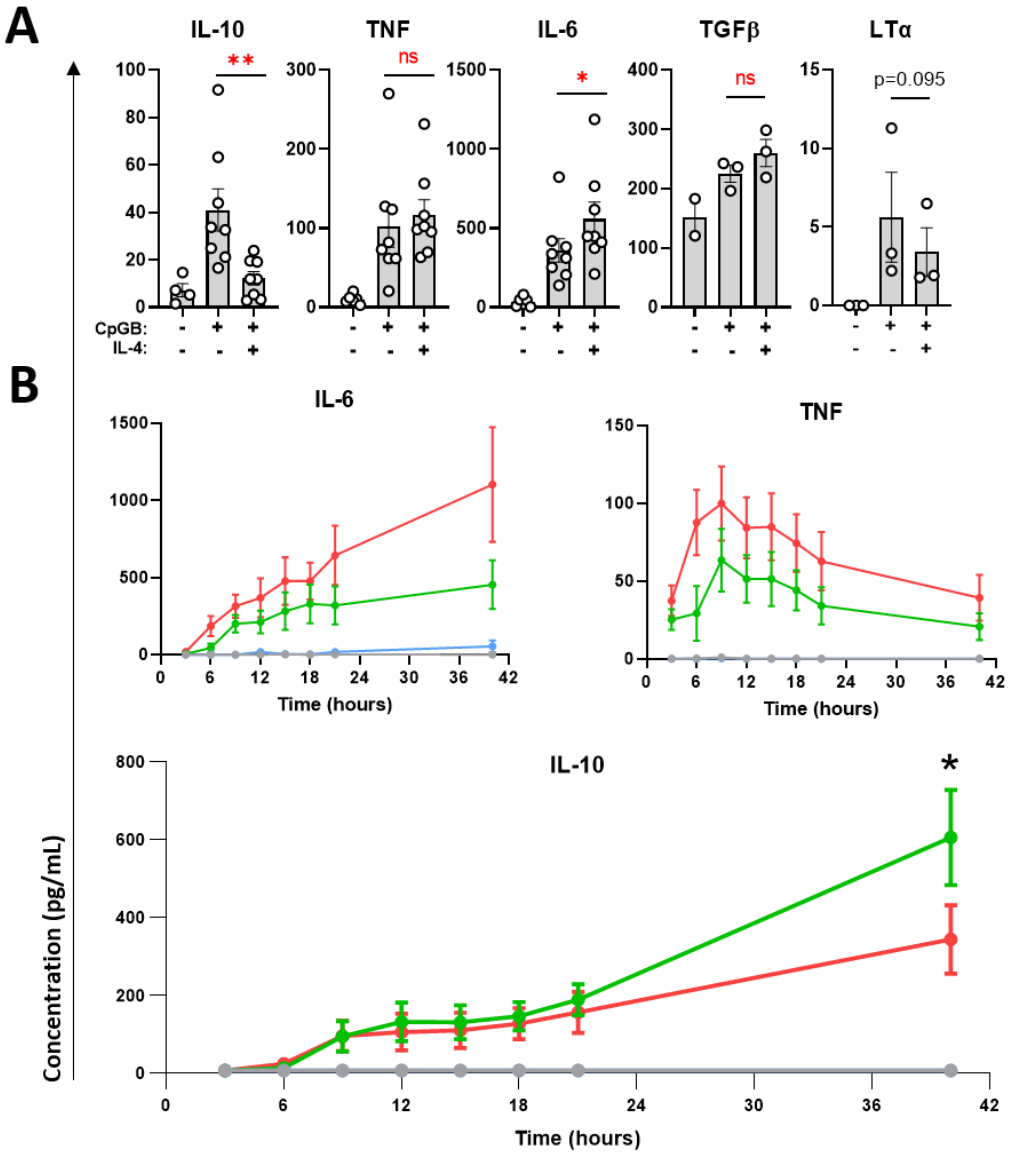


Appendix Figure 6. IL-4 does not impair TRL9 signalling. (A) Purified human B cells were cultured with IL-4 and CpGB, together and separately, for 24 hours, and activation

marker expression measured by flow cytometry. **(B)** Ramos Blue cells were cultured with CpGB +/- IL-4 for 24 hours and NF-κB activity measured by enzymatic activity of alkaline phosphatase in the supernatant. **(C)** Purified human B cells were stimulated with CpGB +/- IL-4 for 48 hours and surface IL-10Ra expression measured by flow cytometry. **(D)** Purified human B cells were cultured for 24 hours with or without IL-4, then washed and stimulated with CpGB for 24 hours in an IL-10 ELISpot assay. **(E)** Purified human B cells were stimulated with CpGB +/- IL-4 for 48 hours. Cytokine secretion was blocked for the final 6 hours of culture with brefeldin and monensin. Cells were fixed and permeabilised, stained for intracellular IL-10, and analysed by flow cytometry. **(F)** Purified human B cells were cultured for 24 hours with or without IL-4, then washed and stimulated with CpGB of CpGB + IL-4 for 24 hours. Cells were harvested every 3 hours and il10 mRNA expression measured by RT-PCR using the housekeeper RPL13 as a reference gene. **(G)** Purified human B cells were cultured for 48 hours with CpGB +/- IL-4 in an IL-10 ELISpot assay. In the conditions displayed by the lower well images, PMA/ionomycin was added to the culture for the final 6 hours. This image is representative of n=6 biological replicated performed across n=3 independent experiments. Statistics by two-tailed paired Student's T test: *$p<0.05$, **$<p<0.01$.

IL-4 selectively suppresses IL-10 secretion

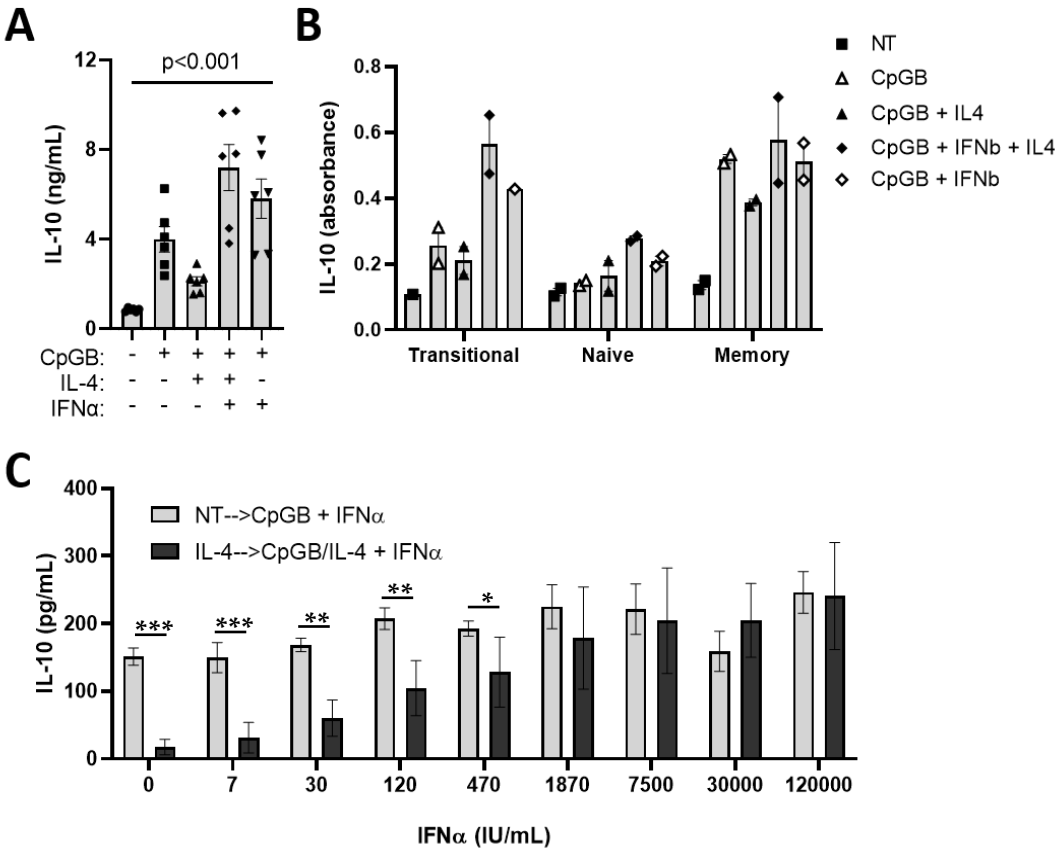
As multiple cytokines may be targeted by common regulators of secretion, we assessed the effect of IL-4 on the secretion of 13 cytokines. Purified B cells were stimulated with CpGB +/- IL-4 for 48 hours and cytokine levels in supernatant assessed by cytometric bead array (Legendplex, Biolegend). We found that while IL-4 impaired CpGB-induced IL-10 secretion, similar impairment was not observed for other cytokines (cytokines of interest are presented in Appendix Figure 7A). While most cytokines were unaffected by IL-4, IL-6 was enhanced, which may reflect the capacity of IL-4 to boost CpGB-driven NF-κB signalling. TGFβ and lymphotoxin-α levels were assessed by ELISA and, although we observed a trend towards increased and decreased secretion, respectively, this was not significant for an n value of 3 (Appendix Figure 7A). In the hope of better understanding the effect of IL-4 on IL-10 secretion, levels of IL-10, TNF and IL-6 were measured over a period of 40 hours (Appendix Figure 7B). Interestingly, a pattern of early production followed by auto/paracrine consumption of TNF was observed for CpGB stimulated B cells (Appendix Figure 7B).



Appendix Figure 7. IL-4 alters the cytokine profile of human B cells in response to CpGB. (A) Purified human B cells were stimulated with CpGB +/- IL-4 for 48 hours and cytokines in supernatant measured by cytometric bead array (n=9) or ELISA (TGFβ; n=3). **(B)** Purified human B cells were cultured in complete media alone (grey), or with IL-4 (blue), CpGB (green), or CpGB + IL-4 (red) for 42 hours, and concentrations of IL-10, TNF and IL-6 measured in supernatants at Hours 3, 6, 9, 12, 15, 18, 21, 24 and 39. Statistics by two-tailed paired Student's T test: *p<0.05, **<p<0.01.

Interferon-α alleviates IL-4 mediated suppression of IL-10 secretion

While the physiological relevance of the effect of IL-4 on TLR9-mediated IL-10 secretion is unclear, forcing IL-10 secretion by B cells has resulted in functional tolerance in other systems (29). We therefore designed an experiment to screen factors for their capacity to relieve suppression of IL-10 secretion. We screened 11 factors (data not shown) and identified type I interferons as having the potential to override suppression of IL-10 secretion. In fact, the combination of CpGB, IL-4, and IFNα resulted in significantly more secretion of IL-10 by purified B cells than CpGB and IFNα alone (Appendix Figure 8A). Similar patterns of IL-10 regulation were observed for isolated transitional, naïve and memory B cells, although the absorbance data for this single experiment could not be fitted to a standard curve in order to derive absolute concentrations (Appendix Figure 8B). The capacity of IFNα to restore IL-10 secretion in IL-4 treated cells was dose-dependent, and most effective at very high concentrations (Appendix Figure 8C). While increasing concentrations of IFNα enhanced CpGB induced IL-10 secretion to a small extent (as observed in Appendix Figure 2), this relationship was significantly enhanced in the presence of IL-4 (Appendix Figure 8C).



Appendix Figure 8. IFNα alleviates IL-4 mediated suppression of IL-10 secretion. Bulk **(A)** or subpopulations of **(B)** purified human B cells were cultured for 48 hours with combinations of CpGB, IL-4 and IFNα, and IL-10 measured in the supernatant by ELISA. **(C)** Purified human B cells were cultured for 24 hours in complete media alone or containing IL-4, and then CpGB added to cultures in combination with increasing concentrations of IFNα for an additional 24 hours. IL-10 in supernatants was measured by ELISA. Statistics by two-tailed paired Student's T test: *p<0.05, **<p<0.01, ***p<0.001.

Discussion

In this chapter we identify IL-4 as a potent regulator of TLR-induced IL-10 secretion by B cells. In attempting to expand IL-10 competent B cells, we found that a CD40L-based expansion system resulted in the loss of IL-10 competence in the expanded population. This was partially due to inclusion of IL-4 in the culture conditions, however IL-10 competence was still lost in the absence of IL-4. Treatment with IL-4 induced a persistent state in purified B cells that was refractory to IL-10 secretion. This was not due impairment of TLR signalling, as IL-4 did not affect NF-kB activation nor B cell activation in response to CpGB treatment. Furthermore, secretion of pro-inflammatory cytokines, notably IL-6 and TNF, was not impaired by IL-4. While we were not able to elucidate the mechanism by which IL-4 impaired IL-10 secretion, IL-4 did not appear to affect the production of IL-10 mRNA nor protein. This effect was only observed with TLR9 activation, as IL-4 did not affect IL-10 secretion in response to combined activation with CpGB + CD40L.

The physiological relevance of this finding is unclear. IL-4 is produced at relevant concentrations in multiple physiological niches, including in the serum of atopic individuals (43). Early during viral infection, IL-4 is secreted at high concentrations by NKT cells in interfollicular regions of lymph nodes with the outcome of promoting B cells to seed germinal centres (39). IL-4 is similarly produced in significant quantities by T follicular helper cells to promote cell cycling and class-switch recombination (44). When considering IL-4 in this context of driving humoral immunity, we might speculate

that the effect that we have observed is related to the quality of humoral immune response. Where IL-4 is a hallmark of a T cell driven antibody response, TLR signalling promotes extrafollicular antibody production and antagonises T cell help (45). As IL-10 has suppressive effects on $CD4^+$ T cells, IL-4 might play a role in promoting T cell help by limiting IL-10 secretion. IL-10 production by B cells may be further detrimental in the germinal centre. Where IL-4 is a quintessential switch factor, additional factors drive differential isotype switching outcomes. Where IL-4 in combination with IL-13 promotes IgE class-switching, IL-10 preferential promotes IgG4 switching in humans (37), which we demonstrate in a TLR-driven system (Appendix Figure S3). Therefore, while autocrine/paracrine IL-10 may promote extrafollicular plasmablast differentiation in response to microbial products, it might be of evolutionary importance that production of IL-10 and similar switch factors in germinal centres be regulated exclusively by T cells. Given the potent immunosuppressive effects of IL-10 on T cells, tight control of IL-10 secretion in germinal centres and at the T:B border may be another explanation for the effect that we have described.

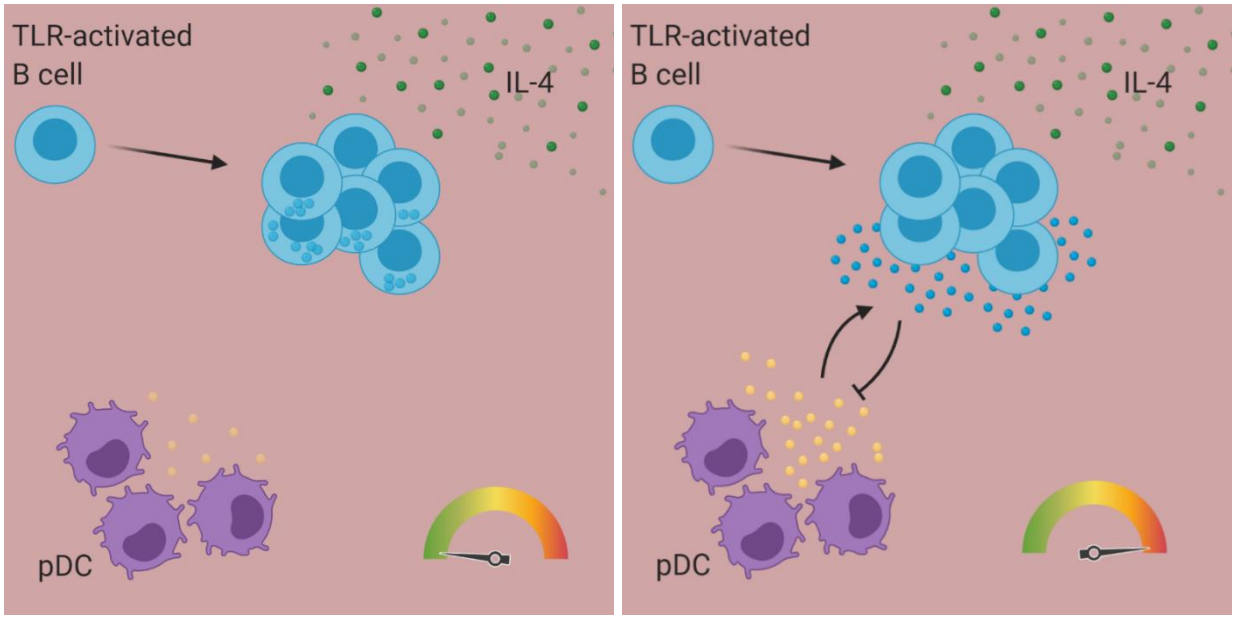
IFNα and IFNβ were able to reverse the effect of IL-4 on IL-10 blockade. Type I interferons are critically important in innate immune defence against intracellular pathogens. Expression of these proteins occurs downstream of a variety of conserved intracellular pathogen-sensing receptors, and results in the rapid expression of a host of antiviral genes by neighbouring cells to prevent viral dissemination (46). Within the specialised immune system, plasmacytoid dendritic cells (pDCs) are considered professional IFN producing cells as they secrete large amounts of IFNα in response to

TLR7 and TLR9 activation. A signature of elevated IFN responsive genes has been associated with disease severity in patients with systemic lupus erythematosus (SLE), and pDCs have been proposed as the source of this IFN. This makes sense as pDCs produce large quantities of IFNα in response to TLR7/9 stimulation by extracellular nucleic acids, which are abundant during active SLE, however their role is debated (47-49). Recently, Menon *et al* reported that IFNα is able to induce IL-10 production by human B cells, and proposed a feedback loop between B cells and pDCs wherein excessive IFNα production by pDCs would result in the development of IL-10 competent B cells that would reciprocally limit the pDC IFNα response (17). Correlations were observed between pDCs activation and frequency of this regulatory B cell population in the peripheral blood of patients with SLE, adding physiological significance to this relationship. The model described by Menon *et al* shares significant similarities with what we have observed, and we therefore propose our observations as an extension to this relationship. Where Menon's model does not account for TLR signalling, we would suggest that negative feedback between pDCs and B cells would be most relevant in an environment where both the B cells and pDCs are exposed to microbial signals; bacterial DNA would induce both IFNα production by pDCs and IL-10 secretion by B cells (Appendix Figure 9). However, this scenario is complicated by the requirement for an IL-4 rich environment.

Where the Th1/Th2 dichotomy (typified by mutual antagonism of IL-4 and IFNγ-driven outcomes in cells) is widely appreciated, a reciprocal relationship between interferons and Th2 cytokines has also been described in allergy (50, 51). This relationship

predominates at barrier tissues where history of allergic inflammation and viral infection determine regulatory circuits and remodelling of the epithelium (51). In this setting, it makes sense that IFN-I signalling might antagonise effects of IL-4, and promote outcomes of intracellular pathogen-sensing pathways like TLR9. The implications of our findings in mucosal immunity is a matter of future research.

This research was cut short by the COVID-19 pandemic. In continuing this work, we would aim to develop functional assays to assess how modulating IL-10 secretion by B cells affects suppression of $CD4^+$ T cell proliferation in the context of the allergic response and the mixed lymphocyte reaction. The relevance of these findings in disease might be explored in the context of high serum IL-4 in patients with hay fever during pollen season, and in patients with SLE with elevated serum IFN-I during disease flares.



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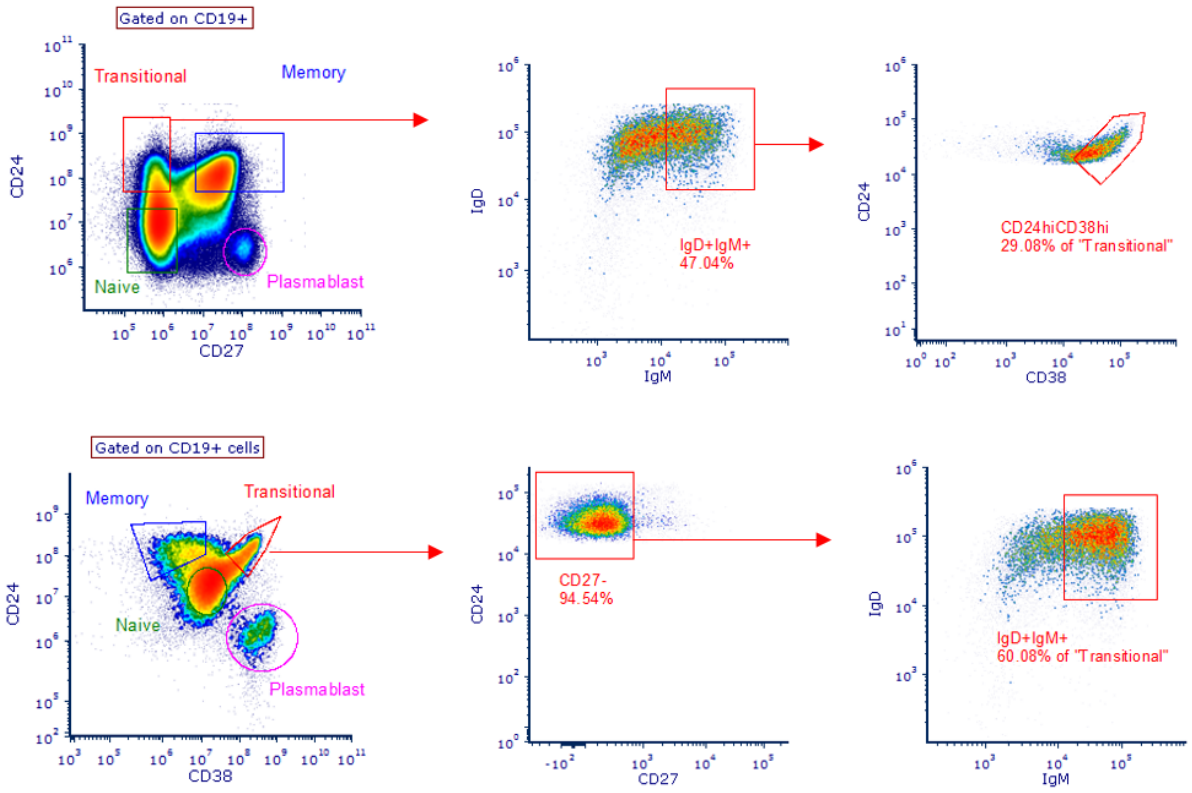
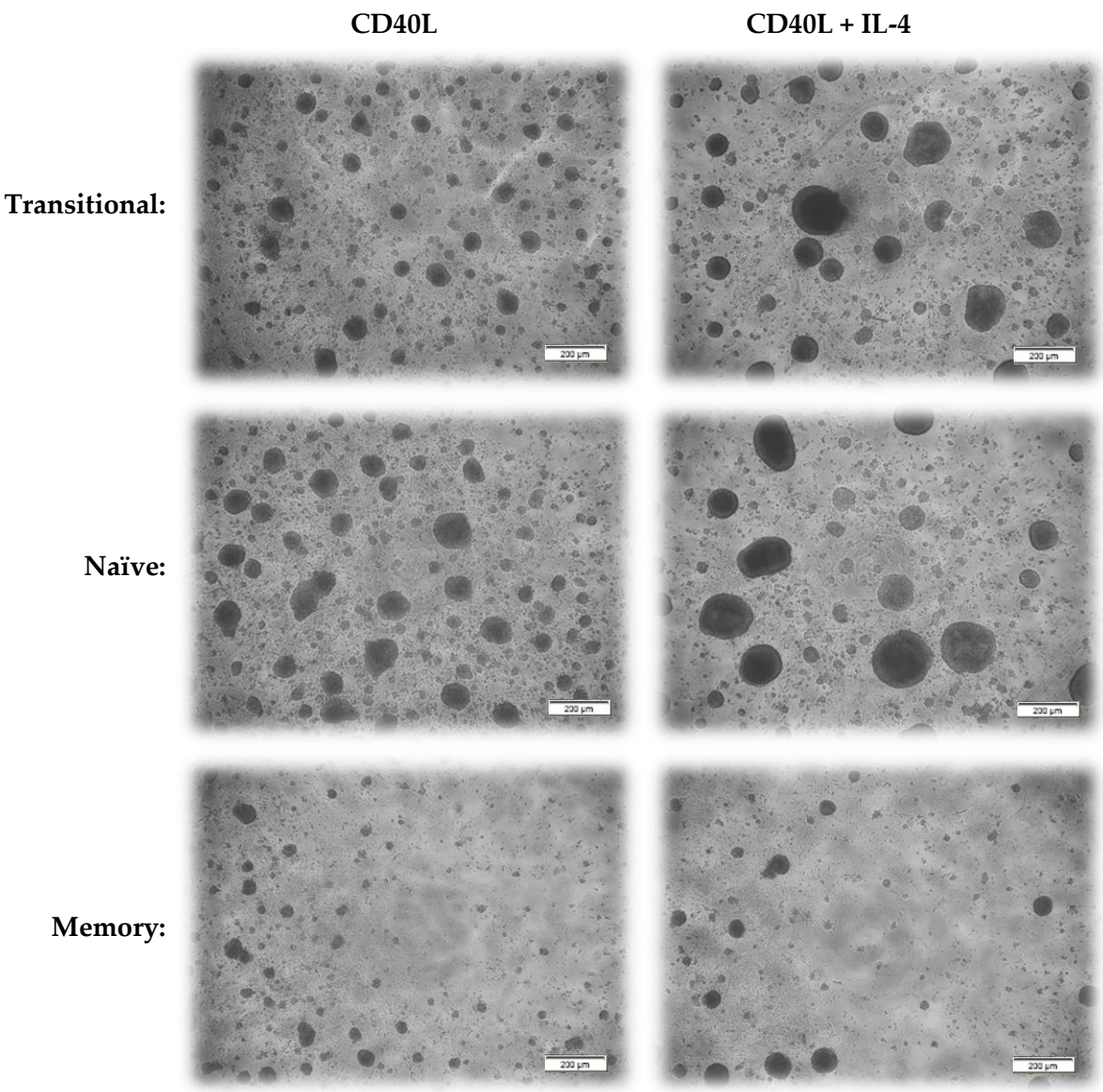
Appendix Figure 9. Cartoon representation of hypothesised regulatory feedback loop between IL-10 producing B cells and plasmacytoid dendritic cells in response to CpGB in the presence of IL-4. Created with BioRender.com.

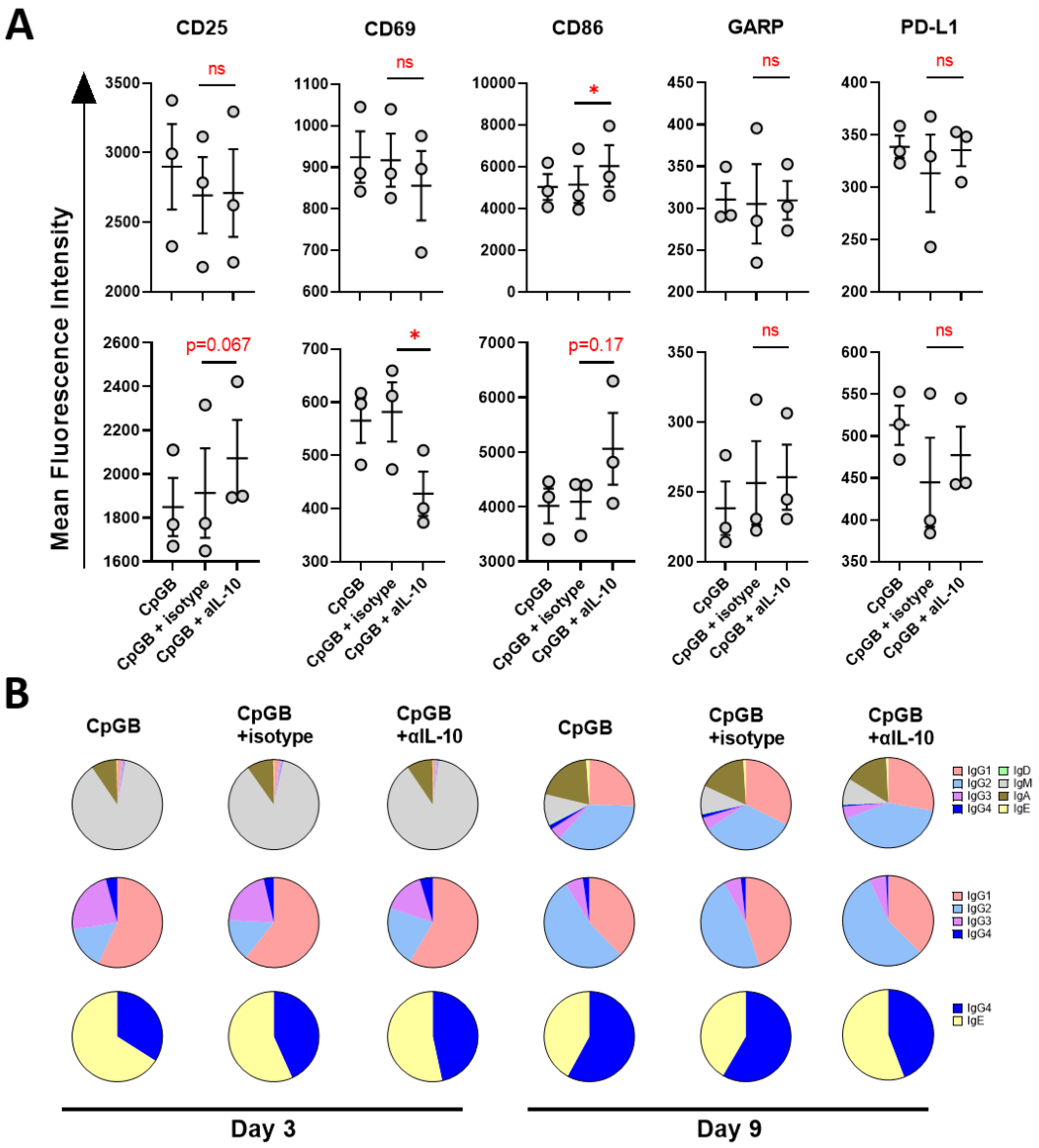
Supplementary Figures



Appendix Figure S1. Alternative gating strategies for sorting transitional B cells. We compare two simple gating strategies on the basis of the purity of the transitional B cell gate. We find that a sorting strategy based on CD19, CD24 and CD38 best enriches for what might be considered to be true transitional B cells ($CD19^{+}CD27^{-}CD24^{hi}CD38^{hi}IgD^{+}IgM^{+}$).



Appendix Figure S2. Effect of IL-4 on the morphology of expanding human B cells. Purified human B cells were sorted into transitional, naïve and memory cells, and cultured on a monolayer of CD40L-expressing NIH3T3 feeder cells for 8 days, with or without IL-4 (10 ng/mL).



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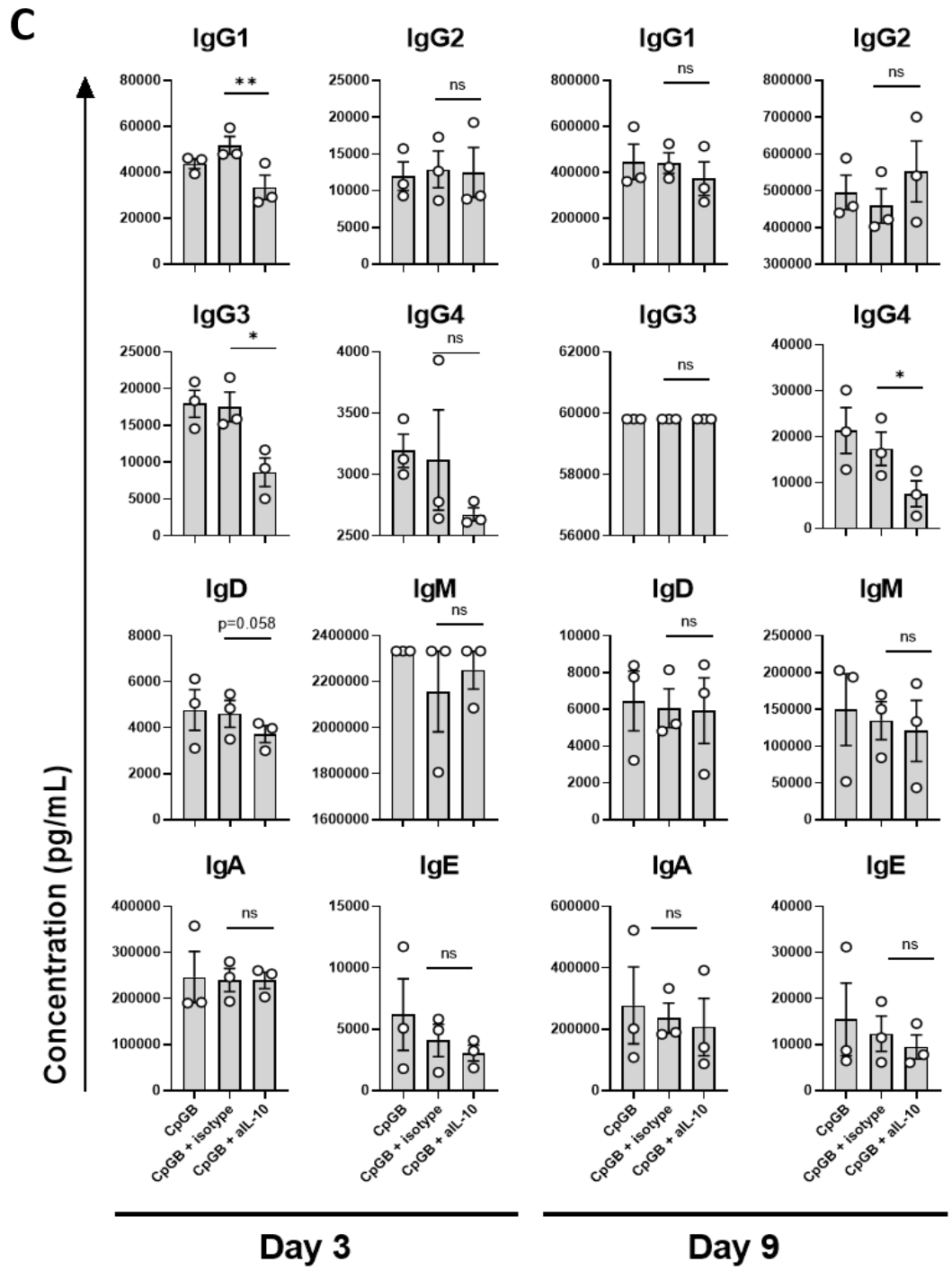

Appendix Figure S3- Contribution of auto/paracrine IL-10 to activation marker expression and isotype of secreted antibodies upon TLR9 stimulation. Purified human B cells were cultured with CpGB alone, or with polyclonal IL-10 neutralising antibody

(PeproTech) or appropriate isotype control (rabbit IgG) as per manufacturer's instructions. Cells were collected at Day 3 and Day 9 for assessment of activation markers, and supernatant was collected for analysis of antibody isotypes produced. Statistics by two-tailed, paired Student's T test; *p<0.05, **p<0.01/

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APPENDIX III:

MANUSCRIPT 2 IN PRINT

APPENDIX IV:

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It is also available online to authorised users at:
https://doi.org/10.1016/j.kint.2022.02.015

APPENDIX V:

MANUSCRIPT 5 IN PRINT

APPENDIX VI:

ETHICS GOVERNANCE FOR THE REVAX STUDY

Government of South Australia
Health
Central Adelaide Local Health Network

Central Adelaide Local Health Network
Research Services

Tel: 08 7117 2224

Postal Address:
Royal Adelaide Hospital
Clinical Trial Centre
Level 3, Wayfinder 3D460.02
Port Road
ADELAIDE SA 5000

Health.CALHNResearchLNR@sa.gov.au
www.health.sa.gov.au

ABN: 96 269 526 412

Authorisation Date: 11 March 2021

Prof Toby Coates
CNARTS Department
The Royal Adelaide Hospital

Dear Prof Coates

CALHN Reference Number: 14541

Project Title: Immunogenicity of the ChAdOx1 nCoV-19 (Oxford-AstraZeneca) and the BNT162b2 (Pfizer) Covid-19 Vaccines in a South Australian Cohort of Immunocompromised Patients and their Close Household Contacts

Thank you for submitting the above proposal for review. This project has undergone ethics and governance review via the expedited processes of the Central Adelaide Local Health Network (CALHN) Human Research Ethics Committee (HREC) and CALHN Research Services.

I am pleased to advise that your project has been granted full ethics approval and meets the requirements of the National Health and Medical Research Council (NHMRC) *National Statement on Ethical Conduct in Human Research 2007* incorporating all updates. The project is **authorised** by CALHN Research Services for conduct at the Royal Adelaide Hospital and SA Pathology.

The CALHN HREC is constituted in accordance with the NHMRC *National Statement on the Ethical Conduct of Human Research* (2007).

Documents reviewed and approved:

Document	Version	Date
Ethics and Governance Application	-	1 March 2021
Study Team Declaration Emails	-	1 March 2021
Randall Faull Head of Department support Email	-	1 March 2021
Protocol	2	2 March 2021
Participant Information Sheet Group 1&2	2	2 March 2021
Participant Information Sheet Group 3	2	2 March 2021
Participant Information Sheet Group 4	2	2 March 2021
Consent Form Group 1&2	2	2 March 2021
Consent Form Group 3	2	2 March 2021
Consent Form Group 4	2	2 March 2021

Sites covered by CALHN HREC approval:

Site	State	Principal Investigator
The Royal Adelaide Hospital	SA	Prof Toby Coates
SA Pathology	SA	Prof Toby Coates

Project authorisation is valid for **one (1) year** from **11 March 2021 to 11 March 2022**. An annual progress report requesting an extension must be submitted if the duration of the project continues beyond this period.

Government of South Australia | Health Central Adelaide Local Health Network

GENERAL TERMS AND CONDITIONS OF PROJECT AUTHORISATION:

1. The CALHN HREC is the South Australian (SA) 'lead HREC' for the purpose of this ethics approval. Any study sites that are not listed on this letter are not covered by the CALHN HREC approval.
2. The study must be conducted in accordance with the standards outlined in the National Statement on Ethical Conduct in Human Research (2007), the Australian Code for the Responsible Conduct of Research (2018), and SA Health policies.
3. Adequate record keeping must be maintained in accordance with Good Clinical Practice, and the NHMRC, state, and national guidelines. The duration of record retention for all low risk research data is five years from the date of publication.
4. Proposed amendments to the research protocol or conduct of the research which may affect the ongoing ethical acceptability of the project and/or the site acceptability of the project must to be submitted to CALHN Research Services. Researchers are required to immediately report anything which might warrant review of ethical approval of the study, including:
   a) Adverse events which warrant protocol change or notification to research participants;
   b) Changes to the protocol;
   c) Changes to the safety or efficacy of the investigational product, device or method;
   d) Matters that may affect the conduct of the project;
   e) Premature termination of the study.
   5. Confidentiality of the research participants must be maintained at all times as required by law.
6. A report of the progress of the project at least annually, and related to the degree of risk to participants. The report is due on the anniversary of project authorisation. Continuation of approval is contingent on submission of this report, due within 14 days of the approval anniversary. Failure to comply may result in suspension of the project
7. A final report if the outcome of the project must be submitted on completion of the project. A copy of any published material must also be provided with the report, or following when available.
8. A copy of this letter should also be maintained on file by the Coordinating Principal Investigator as evidence of project authorisation.
9. If University personnel are involved in this project, the Principal Investigator should notify the University before commencing their research to ensure compliance with University requirements including any insurance and indemnification requirements. **A copy of compliance confirmation must be forwarded to CALHN Research Services upon receipt.**

You are reminded that this letter constitutes ethical approval only and governance authorisation for CALHN sites.

Should you have any queries about the consideration of your project, please contact Health.CALHNResearchLNR@sa.gov.au.

All future correspondence regarding this study must include the CALHN reference number in the subject header.

We wish you every success in your research.

Yours sincerely,

Ian Tindall
Chair, CALHN Human Research Ethics Committee

Bernadette Swart
Manager, CALHN Research Services

11 March 2021

APPENDIX VII:

REVAX TRIAL REGISTRATION

Trial registered on ANZCTR

Registration number
ACTRN12621000532808
Ethics application status
Approved
Date submitted
15/04/2021
Date registered
6/05/2021
Date last updated
6/05/2021
Date data sharing statement initially provided
6/05/2021
Type of registration
Retrospectively registered

Titles & IDs

Public title
Vaccination of kidney transplant and dialysis patients and their close household contacts against COVID-19
Scientific title

Immunogenicity of the ChAdOx1 nCoV-19 (Oxford-AstraZeneca) and the BNT162b2 (Pfizer) COVID-19 Vaccines in a South Australian Cohort of Immunocompromised Patients and their Close Household Contacts

Secondary ID [1]

None

Universal Trial Number (UTN)

Trial acronym

REVAX

Linked study record

Health condition

Health condition(s) or problem(s) studied:

Kidney Transplant

Dialysis

Condition category

Condition code

Renal and Urogenital

Other renal and urogenital disorders

Intervention/exposure

Study type

Interventional

Description of intervention(s) / exposure

Immune responses in kidney transplant and dialysis patients are compared in each case with that of a close household contact (usually a spouse) over the age of 18.

This study is interventional in so much as close household contacts of transplant and dialysis patients will, in some instances, receive their vaccinations earlier than they otherwise would.

Both patients and cohabitants will be asked to provide up to four blood samples starting prior to-, and then up to 6 months post-, receipt of the initial ChAdOx1 nCoV-19 (Oxford-AstraZeneca) or BNT162b2 (Pfizer-BioNTech) COVID-19 vaccine. Blood samples will be collected at the Royal Adelaide Hospital by trained staff. Samples for some participants will be collected at the Queen Elizabeth Hospital or at SA Pathology collection centres as dictated by convenience.

Number and timing of vaccine doses received will not differ from that of the rest of the population i.e. an initial and booster dose, 21 days apart in the case of Pfizer-BioNTech and 12 weeks apart in the case of Oxford-AstraZeneca.

Participants will also be asked to provide a one-off stool sample prior to vaccination using a provided at-home collection kit. In conjunction with this, participants will be asked to complete a four-day food diary.

Participation in this study will amount to four appointments for blood collection, and two visits to vaccination clinics.

Intervention code [1]

Early detection / Screening

Comparator / control treatment

In this study, cohabitants will act as reference comparators for the vaccine response of the transplant and dialysis patients.

Both patients and cohabitants will be asked to provide up to four blood samples starting prior to-, and then up to 6 months post-, receipt of the ChAdOx1 nCoV-19 (Oxford-AstraZeneca) or BNT162b2 (Pfizer-BioNTech) COVID-19 vaccine. Blood samples will be collected at the Royal Adelaide Hospital by trained staff. Samples for some participants will be collected at the Queen Elizabeth Hospital or at SA Pathology collection centres as dictated by convenience.

Number and timing of vaccine doses received will not differ from that of the rest of the population i.e. an initial and booster dose, 21 days apart in the case of Pfizer-BioNTech and

12 weeks apart in the case of Oxford-AstraZeneca.

Participants will also be asked to provide a one-off stool sample prior to vaccination using a provided at-home collection kit. In conjunction with this, participants will be asked to complete a four-day food diary.

Participation in this study will amount to four appointments for blood collection, and two visits to vaccination clinics.

Control group

Active

Outcomes

Primary outcome [1]

Capacity of serum to neutralise SARS-CoV-2 spike protein-expressing pseudovirus will be assessed by a pseudovirus neutralisation assay.

Timepoint [1]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose (primary timepoint), 26 weeks post vaccine booster dose.

Primary outcome [2]

Detectable seroconversion of IgG against SARS-CoV-2 spike protein and receptor-binding domain will be assessed by enzyme-linked immunosorbent assay.

Timepoint [2]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose (primary timepoint), 26 weeks post vaccine booster dose.

Primary outcome [3]

SARS-CoV-2 spike protein-specific T cell response will be assessed by IFN-gamma ELISpot.

Timepoint [3]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose (primary timepoint), 26 weeks post vaccine booster dose.

Secondary outcome [1]

SARS-CoV-2 spike protein-specific memory T cell quality and frequency will be assessed by activation-induced marker analysis by flow cytometry.

Timepoint [1]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [2]

SARS-CoV-2 spike protein and RBD-specific memory B cell quality and frequency will be assessed by B cell tetramer staining for flow cytometric analysis,

Timepoint [2]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [3]

Quality of the antibody response to SARS-CoV-2 spike protein receptor-binding domain will be assessed by enzyme-linked immunosorbent assay measuring specific IgM in participant serum.

Timepoint [3]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [4]

Quality of the antibody response to SARS-CoV-2 spike protein receptor-binding domain will be assessed by enzyme-linked immunosorbent assay measuring specific IgG1 in participant serum.

Timepoint [4]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [5]

Quality of the antibody response to SARS-CoV-2 spike protein receptor-binding domain will be assessed by enzyme-linked immunosorbent assay measuring specific IgG3 in participant serum.

Timepoint [5]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [6]

Quality of the antibody response to SARS-CoV-2 spike protein receptor-binding domain will be assessed by enzyme-linked immunosorbent assay measuring specific IgA in participant serum.

Timepoint [6]

Prior to initial vaccine dose, 20 days post initial vaccine dose, 20 days post vaccine booster dose, 26 weeks post vaccine booster dose.

Secondary outcome [7]

Gut microbiome species diversity will be compared between participants who do and do not seroconvert in response to vaccination based on an Inverse Simpson diversity score derived from 16s ribosomal sequencing of genetic material extracted from stool samples.

Timepoint [7]

The comparison will be made between baseline stool sample, sero-status measured at 20 days post initial vaccine dose, 20 days post vaccine booster dose, and 26 weeks post vaccine booster dose.

Eligibility

Key inclusion criteria

For patient group:

Have a current kidney transplant >3 months or currently on dialysis.

Have a close household contact able to participate.

Minimum age

18 Years

Maximum age

No limit

Gender

Both males and females

Can healthy volunteers participate?

Yes

Key exclusion criteria

For patient group:

Prior exposure to SARS-CoV-2 (positive serology).

Have previously received a COVID-19 vaccination.

Due to receive a transplant within the ensuing 6 months.

Control/comparison group:

Prior exposure to SARS-CoV-2 (positive serology).

Have previously received a COVID-19 vaccination.

Study design

Purpose of the study

Prevention

Allocation to intervention

Non-randomised trial

Procedure for enrolling a subject and allocating the treatment (allocation concealment procedures)

Methods used to generate the sequence in which subjects will be randomised (sequence generation)

Masking / blinding

Who is / are masked / blinded?

Intervention assignment

Other design features

Phase

Not Applicable

Type of endpoint(s)

Statistical methods / analysis

A priori power calculations were performed based on data from previously published vaccination studies.

Recruitment

Recruitment status

Recruiting

Date of first participant enrolment

Anticipated

Actual

1/04/2021

Date of last participant enrolment

Anticipated

31/05/2021

Actual

Date of last data collection

Anticipated

1/12/2021

Actual

Sample size

Target

200

Accrual to date

84

Final

Recruitment in Australia

Recruitment state(s)

SA

Recruitment hospital [1]

The Royal Adelaide Hospital - Adelaide

Recruitment hospital [2]

The Queen Elizabeth Hospital - Woodville

Recruitment postcode(s) [1]

5000 - Adelaide

Recruitment postcode(s) [2]

5011 - Woodville

Funding & Sponsors

Funding source category [1]

Charities/Societies/Foundations

Name [1]

The Hospital Research Foundation

Address [1]

1 Port Road, Adelaide, South Australia 5000

Country [1]

Australia

Funding source category [2]

University

Name [2]

The University of Adelaide

Address [2]

North Terrace, Adelaide, South Australia 5005

Country [2]

Australia

Funding source category [3]

Charities/Societies/Foundations

Name [3]

Kidney, Transplant & Diabetes Research Australia

Address [3]

1 Port Road, Adelaide, South Australia 5000

Country [3]

Australia

Primary sponsor type
Government body
Name
Central Adelaide Local Health Network
Address
1 Port Road, Adelaide, South Australia 5000
Country
Australia
Secondary sponsor category [1]
University
Name [1]
University of Adelaide
Address [1]
North Terrace, Adelaide, South Australia 5005
Country [1]
Australia
Secondary sponsor category [2]
Hospital
Name [2]
Royal Adelaide Hospital
Address [2]
1 Port Road, Adelaide, South Australia 5000
Country [2]
Australia
Other collaborator category [1]
Hospital
Name [1]
Royal Prince Alfred Hospital (Transplantation Institute)
Address [1]
50 Missenden Rd, Camperdown NSW 2050
Country [1]

Australia
Other collaborator category [2]
Other
Name [2]
Basil Hetzel Institute
Address [2]
37 Woodville Rd, Woodville South SA 5011
Country [2]
Australia
Other collaborator category [3]
Government body
Name [3]
SA Pathology
Address [3]
Frome Road, Adelaide, South Australia, 5000
Country [3]
Australia

Ethics approval

Ethics application status
Approved
Ethics committee name [1]
Central Adelaide Local Health Network HREC
Ethics committee address [1]
136 North Terrace, Adelaide, South Australia 5000
Ethics committee country [1]
Australia
Date submitted for ethics approval [1]
01/03/2021
Approval date [1]
11/03/2021

Ethics approval number [1]

14541

Summary

Brief summary

This study aims to measure the efficacy of the ChAdOx1 nCoV-19 (Oxford-AstraZeneca) and BNT162b2 mRNA (Pfizer-BioNTech) COVID-19 vaccines in transplant recipients and patients on dialysis, and in their close household contacts. Early reports indicate that kidney transplant recipients receiving immunosuppressive medications have a reduced protective immune response to COVID-19 vaccines. This is of concern as these are the individuals most at risk. These patient groups are adept at avoiding exposure to pathogens out in the world, and are therefore most likely to be exposed to the SARS-CoV-2 virus via a close household contact e.g. a spouse.

In this study, transplant recipients and dialysis patients will receive their vaccination at the same time as a close household contact. By comparing the immune response of patient and cohabitant, we will develop an understanding of whether priority vaccination of household contacts is a worthwhile strategy for protecting transplant and dialysis patients from COVID-19 in the future.

Trial website

Trial related presentations / publications

Public notes

Contacts

Principal investigator

Name

Prof Toby Coates

Address

Royal Adelaide Hospital, 1 Port Road, Adelaide, SA 5000

Country

Australia

Phone

+61 439901856

Fax

Email

toby.coates@sa.gov.au

Contact person for public queries

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Prof Toby Coates

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+61 8 70740000

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toby.coates@sa.gov.au

Contact person for scientific queries

Name

Prof Toby Coates

Address

Royal Adelaide Hospital, 1 Port Road, Adelaide, SA 5000

Country

Australia

Phone

+61 439901856

Fax

Email

toby.coates@sa.gov.au

Data sharing statement

Will individual participant data (IPD) for this trial be available (including data dictionaries)?
No
No/undecided IPD sharing reason/comment
What supporting documents are/will be available?
No other documents available

APPENDIX VIII:

ETHICS GOVERNANCE FOR THE RIVASTIM TRIAL

Government of South Australia
Health
Central Adelaide Local Health Network

Central Adelaide Local Health Network
Research Office
Level 3, Roma Mitchell House
North Terrace, Adelaide SA
Australia 5000

T : 08 7117 2209
T : 08 8222 6841
E : Health.CALHNResearchGovernance@sa.gov.au

14 October 2021

Professor Toby Coates
Royal Adelaide Hospital
Port Road, Adelaide SA 5000

Dear Professor Coates,

HREC Reference Number: 2021/HRE00354

Governance Reference Number: 15528

Project Title: Rapamycin and Inulin for booster VAccine response STIMulation (RIVASTIM)

RE: Governance Review

Thank you for submitting an application for authorisation of this project. I am pleased to inform you that authorisation has been granted for this study to commence at the Royal Adelaide Hospital, SA.

Authorisation is valid from **14 October 2021 to 13 October 2024**. Proposed extensions beyond this term must be submitted as an amendment to the CALHN Research Office.

The following conditions apply to the authorisation of this research project. These are additional to those conditions imposed by the Human Research Ethics Committee (HREC) that granted ethical approval to this project:

1. Authorisation is limited to the site/s identified in this letter only.
2. Project authorisation is granted for the term specified above.
3. The study must be conducted in accordance with the conditions of ethical approval provided by the lead HREC, SA Health policies, and in conjunction with the standards outlined in the *National Statement on Ethical Conduct in Human Research* (2007) and the *Australian Code for the Responsible Conduct of Research* (*2007).*
4. Proposed amendments to the research protocol or conduct of the research which may affect the ethical acceptability of the project, and which are submitted to the HREC for review, are copied via email to the CALHN Research Office;
5. Proposed amendments to the research protocol or conduct of the research which only affects the ongoing site acceptability of the project, are to be submitted via email to the CALHN Research Office;
6. For all clinical trials, the study must be registered in a publicly accessible trials registry prior to enrolment of the first participant.
7. Proposed amendments to the research protocol or conduct of the research which may affect both the ongoing ethical acceptability of the project and the site acceptability of the project are to be submitted to the CALHN Research Office after a HREC decision is made.
8. A copy of this letter should also be maintained on file by the Coordinating Principal Investigator as evidence of project authorisation.
9. Notification of completion of the study at this site is to be provided to the CALHN Research Office.

You are reminded that continuation of governance approval is contingent on submission of the Annual Progress Report to the reviewing HREC, a copy of which must be submitted to the CALHN Research Office along with acknowledgement of the report by the reviewing HREC. Failure to comply may result in suspension of the project.

If University personnel are involved in this project, the Principal Investigator should notify the University before commencing their research to ensure compliance with University requirements including any insurance and indemnification requirements.

We wish you every success in your research project.

Yours sincerely

Bernadette Swart
Manager, CALHN Research Office
Ph: 7117 2209
Email: Health.CALHNResearchGovernance@sa.gov.au

APPENDIX IX:

RIVASTIM TRIAL REGISTRATION

Trial registered on ANZCTR

Registration number
ACTRN12621001412820
Ethics application status
Approved
Date submitted
11/10/2021
Date registered
20/10/2021
Date last updated
20/10/2021
Date data sharing statement initially provided
20/10/2021
Type of registration
Prospectively registered

Titles & IDs

Public title
The effect of sirolimus-based immunosuppression and dietary fibre supplementation on booster COVID-19 vaccine responses in kidney transplant recipients - Part 1: sirolimus-based immunosuppression

Scientific title
Rapamycin and Inulin for booster VAccine response STIMulation (RIVASTIM) - Part 1: The effect of rapamycin on booster COVID-19 vaccine responses in kidney transplant recipients
Secondary ID [1]
Nil known
Universal Trial Number (UTN)
U1111-1270-1439
Trial acronym
RIVASTIM
Linked study record
N/A

Health condition

Health condition(s) or problem(s) studied:
COVID-19
Drug-induced immunosuppression
Vaccine non-response
Condition category
Condition code
Infection
Other infectious diseases
Inflammatory and Immune System
Normal development and function of the immune system
Inflammatory and Immune System
Other inflammatory or immune system disorders
Respiratory
Other respiratory disorders / diseases

Intervention/exposure

Study type

Interventional

Description of intervention(s) / exposure

Kidney transplant recipients receiving standard of care immunosuppression with tacrolimus, mycophenolate, and steroids, who are deemed to be non-responders or low-responders to conventional 2-dose COVID-19 vaccination, will be randomised to switch from mycophenolate to sirolimus. Participants will initially have mycophenolate ceased, and sirolimus tablets commenced at 2mg daily. The dose of sirolimus will be adjusted weekly to target trough levels of 3-6 ng/mL. Once sirolimus levels are therapeutic, tacrolimus dose will be adjusted to target trough levels of 3-5 ng/mL. Adherence will be monitored through weekly drug level monitoring. Four weeks after randomisation, participants will receive a 3rd COVID-19 mRNA vaccine dose (Pfizer Comirnaty). All participants will receive COVID vaccination during a clinical trial visit to ensure adherence. Participants will continue the altered immunosuppression regimen until measurement of vaccine responses at 4-6 weeks post vaccination. At that stage patients will be able to either continue the intervention immunosuppression or switch back to usual immunosuppression in conjunction with their treating nephrologist.

Intervention code [1]

Treatment: Drugs

Comparator / control treatment

Kidney transplant recipients receiving standard of care immunosuppression with tacrolimus, mycophenolate, and steroids, who are deemed to be non-responders or low-responders to conventional 2-dose COVID-19 vaccination, will be randomised to stay on the same immunosuppression regimen. Participants will receive a 3rd COVID-19 mRNA vaccine dose (Pfizer Comirnaty) 4 weeks after randomisation. All participants will receive COVID vaccination during a clinical trial visit to ensure adherence.

Control group

Active

Outcomes

Primary outcome [1]

Absolute proportion of patients in each arm meeting the threshold of 20.2% neutralising antibody required for clinical protection from SARS-CoV2 infection. Neutralising antibody

titres will be assessed by dilution at which paCoV-2 live virus entry into angiotensin converting enzyme (ACE) 2 receptor positive cells by 50%.

Timepoint [1]

4-6 weeks post-vaccination. During the intervening post-vaccination period patients will be clinically assessed once by phone 1 week post vaccination. Patients in the intervention group will also continue fortnightly immunosuppression level checks with phone call follow up for 6 weeks post-vaccination.

Secondary outcome [1]

Quantification of T cell responses as measured by interferon gamma release assay (ELISPOT) to SARS-CoV2 viral peptides in each arm

Timepoint [1]

4-6 weeks post-vaccination. During the intervening post-vaccination period patients will be clinically assessed once by phone 1 week post vaccination. Patients in the intervention group will also continue fortnightly immunosuppression level checks with phone call follow up for 6 weeks post-vaccination.

Secondary outcome [2]

Capturing adverse events following immunisation (AEFI) including adverse events of special interest (AESI):
- Changes in kidney function (as measured by eGFR)
- 50% increase in proteinuria (as measured by spot urine albumin:creatinine ratio)
- Biopsy-proven acute rejection
- Recurrence of primary kidney disease. This will be assessed based on known primary kidney disease (prospectively collected information at enrolment using both patient history and electronic medical records) and either typical re-presentation of a known pathology (e.g. typical flare of IgA vasculitis) or transplant kidney biopsy.
- Patient reported experiences using the EQ-5D questionnaire

Timepoint [2]

Incidence of AEFI will be captured during the 4 week period post-vaccination. This information will be collected using clinical assessment by phone 1 week following vaccination, and then again at the physical follow-up visit 4-6 weeks post-vaccination.

Secondary outcome [3]

Safety and tolerability of sirolimus switch as measured by proportion of patients ceasing study drug, patient-reported adherence, and incidence of drug-related adverse events including:
- new proteinuria (as measured by spot albumin:creatinine ratio)
- new anaemia
- new leukopaenia
- rash
- mouth ulcers
- pneumonitis

This information will be assessed at each clinical visit (phone or physical) by active questioning from the trial investigator. Patients will complete an EQ-5D questionnaire at each clinical visit. Investigations to monitor for the adverse events (blood tests, urine tests) will be conducted at baseline, at vaccination 4 weeks later, and at the concluding study visit 4-6 weeks post vaccination.

Timepoint [3]

During the 8-10 week clinical follow-up period following randomisation. Clinical visits for all patients will be at randomisation, vaccination 4 weeks later, follow up phone call 1 week post vaccination, and vaccination response assessment 4-6 weeks post vaccination.

Patients in the intervention arm will additionally have regular monitoring of immunosuppression levels, these will be assessed weekly during the first 4 weeks, and then fortnightly during the subsequent 4-6 weeks (or more frequently if indicated).

Secondary outcome [4]

Measurement of sirolimus effect on faecal microbiome as measured by DNA sequencing on faecal samples collected at randomisation and at vaccination

Timepoint [4]

Comparison between randomisation visit sample and vaccination visit sample at week 4

Eligibility

Key inclusion criteria

- Kidney transplant recipients
- Aged 18-70 years
- estimated GFR >25 mL/min
- Spot urinary albumin:creatinine ratio <100 mg/µmol
- Current immunosuppression regimen comprising tacrolimus, mycophenolate, prednisolone
- Treating nephrologist agrees that patient is suitable for sirolimus maintenance immunosuppression
- Have received 2 doses of a COVID-19 vaccine regimen (either adenoviral vector or mRNA-based) and have demonstrably not responded (anti-Receptor Binding Domain antibody titre < 100 U/mL)

Minimum age

18 Years

Maximum age

70 Years

Gender

Both males and females

Can healthy volunteers participate?

No

Key exclusion criteria

- Multi-organ transplant recipients (e.g. kidney-pancreas)
- Aged <18 years or >70 years
- Significant kidney dysfunction, estimated GFR =25 mL/min or spot urinary albumin:creatinine ratio =100 mg/µmol
- Unable or unwilling to provide informed consent to participate in the trial
- Have received 2 doses of a COVID-19 vaccine regimen (either adenoviral vector or mRNA-based) and have mounted an adequate immune response (anti-Receptor Binding Domain antibodies >100 U/mL)
- Have had documented infection with COVID-19
- Known allergy to or intolerance of sirolimus or everolimus
- Patients who are currently pregnant

Study design

Purpose of the study
Treatment
Allocation to intervention
Randomised controlled trial
Procedure for enrolling a subject and allocating the treatment (allocation concealment procedures)
Allocation will be concealed. Central computerised randomisation will occur following enrolment using a centralised REDCaps database.
Methods used to generate the sequence in which subjects will be randomised (sequence generation)
Randomisation will occur via permuted blocked randomisation, stratified by site (RAH and RPA), time post vaccination, original vaccine type (AstraZeneca Vaxzevria or Pfizer Comirnaty) and antibody titre.
Masking / blinding
Blinded (masking used)
Who is / are masked / blinded?
The people analysing the results/data
Intervention assignment
Parallel
Other design features
Phase
Not Applicable
Type of endpoint(s)
Safety/efficacy
Statistical methods / analysis

This study will enrol 120 patients, with 60 assigned to the sirolimus intervention group, and 60 assigned to control. This will provide 80% statistical power (alpha 0.05) to detect an absolute difference of 25% in the proportion of patients who achieve the 22% neutralising antibody threshold necessary to provide clinical protection from COVID-19 disease, allowing for a 10% drop-out rate.

The primary outcome will be analysed as a dichotomous variable, considering the absolute proportion of patients achieving the target 22% neutralising antibody threshold in each group using the Wald statistic. A two-sample unpaired T test will also be conducted to compare total neutralising antibody levels between groups.

Secondary outcomes will be similarly analysed depending on whether they are categorical variables (comparison of proportions) or continuous variables (comparison of means).

Recruitment

Recruitment status

Not yet recruiting

Date of first participant enrolment

Anticipated

1/11/2021

Actual

Date of last participant enrolment

Anticipated

1/12/2021

Actual

Date of last data collection

Anticipated

1/03/2022

Actual

Sample size

Target

120

Accrual to date

Final

Recruitment in Australia

Recruitment state(s)

NSW,SA

Recruitment hospital [1]

Royal Prince Alfred Hospital - Camperdown

Recruitment hospital [2]

The Royal Adelaide Hospital - Adelaide

Recruitment postcode(s) [1]

2050 - Camperdown

Recruitment postcode(s) [2]

5000 - Adelaide

Funding & Sponsors

Funding source category [1]

Charities/Societies/Foundations

Name [1]

Kidney, Transplant, Diabetes Research Australia

Address [1]

Kidney, Transplant, Diabetes Research Australia

The Hospital Research Foundation Group Head Office

Level 1, 62 Woodville Road

Woodville, SA 5011

Country [1]

Australia

Primary sponsor type

Individual

Name

Professor P. Toby H Coates (MBBS FRACP PhD)

Address

Director of Kidney and Islet Transplantation

Central and Northern Adelaide Renal and Transplantation Service

Royal Adelaide Hospital

Port Road ADELAIDE SA 5000

Country

Australia

Secondary sponsor category [1]

None

Name [1]

Address [1]

Country [1]

Ethics approval

Ethics application status

Approved

Ethics committee name [1]

Central Adelaide Local Health Network Human Research Ethics Committee (CALHN HREC)

Ethics committee address [1]

CALHN HREC Executive Officer

The Queen Elizabeth Hospital

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Ethics committee country [1]

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Date submitted for ethics approval [1]

08/10/2021

Approval date [1]

13/10/2021

Ethics approval number [1]

2021/HRE00354

Summary

Brief summary

The RIVASTIM trials aim to identify strategies to improve immunological responses to a 3rd booster dose of the mRNA Pfizer Comirnaty COVID-19 vaccine in a cohort of kidney transplant patients who have failed to achieve an adequate immune response to a standard two-dose COVID-19 vaccine course. Kidney transplant patients are a highly vulnerable group of immunosuppressed patients who suffer from disproportionately high COVID-19-related morbidity and mortality. The transplant medication sirolimus shows promise in enhancing immune responses to COVID-19 vaccination.

In this study kidney transplant patients receiving standard of care immunosuppression with tacrolimus, mycophenolate, and steroids will be randomised to either switch from mycophenolate to sirolimus, or remain on standard of care immunosuppression. Four weeks after randomisation, participants will receive a 3rd COVID-19 vaccine dose, and immunological responses will be assessed 4-6 weeks later.

Trial website

Trial related presentations / publications

Public notes

Contacts

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Data sharing statement

Will individual participant data (IPD) for this trial be available (including data dictionaries)?

Yes

What data in particular will be shared?

Identifiable data will not be publicly released, however deidentified individual participant data including relevant demographic and health information, primary outcome (neutralising antibody), and secondary outcomes (other measures of immunological response, measures of vaccine safety/tolerability, measures of study medication safety/tolerability) will be made available as per below criteria.

When will data be available (start and end dates)?

3 months following publication of all study results. Data will no longer be available after 10 years.

Available to whom?

Applications from experienced investigators for trial data can be made through correspondence with the Principal Investigator (or the corresponding author in the case of published works)

Available for what types of analyses?

All reasonable requests from experienced investigators will be considered, including re-analysis of trial results, secondary outcomes analysis, and sub-group analysis

How or where can data be obtained?

After approval, deidentified data will be provided through access on a secure electronic platform. Approval can be sought through contacting the Principal Investigator by email (toby.coates@sa.gov.au), or the Corresponding Author for published data.

What supporting documents are/will be available?

Informed consent form

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