MONASH University

Immune biomarkers and infections in kidney transplant recipients

A thesis submitted to Monash University in total fulfilment of the requirements for the degree of Doctor of Philosophy

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I would like to thank the 168 kidney transplant patients who volunteered to participate in this study. The path to transplantation is not an easy one but the patients in this study have been overwhelmingly generous with their time and genuinely put themselves out to contribute to research that may help others in their situation.

My main supervisor Professor Stephen Holdsworth has taught me the secret of research supervision; if the candidate enjoys the process, then the failed experiments, journal rejections and ideas that lead to nothing are seen as challenges to be worked around rather than insurmountable roadblocks. Steve has shown me that good supervision allows autonomy of research ideas, promotion of internal drivers for success rather than micromanagement and the ability to recognise when specific academic support is needed. Steve has helped me use this PhD to create a career pathway in immunocompromised patient care, rather than a stand-alone piece of work, which seems to be key to foster longevity in researchers. I would like to thank Steve and feel I was very lucky to have him as my supervisor.

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Abbreviations

AUROC	area under receiver operating curve
BKV	BK virus
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CI	confidence interval
CMV	cytomegalovirus
CNI	calcineurin inhibitors
CT	computerised tomography
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
eGFR	estimated glomerular filtration rate
HHV	human herpes virus
HLA	human leukocyte antigen
HR	hazard ratio
HSV	herpes simplex virus
iATP	intracellular adenosine triphosphate
Ig	immunoglobulin
IPD	invasive pneumococcal disease
IQR	interquartile range
IV	intravenous
JC	John Cunningham
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MMF	mycophenolate
NK	natural killer
PCR	polymerase chain reaction
PCP	*Pneumocystis jirovecii*
PCV13	13-valent pneumococcal conjugated vaccine
PPV23	23-valent pneumococcal polysaccharide vaccine
OI	opportunistic infection

OPA	opsonophagocytic assay
OR	odds ratio
sCD30	soluble CD30
SOT	solid organ transplant
spp.	species
ROC	receiver operating curve
RR	relative risk

Declaration

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Research Masters regulations the following declarations are made: I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Signature: 

Abstract

Infection is an important cause of morbidity and mortality in kidney transplant recipients. The risk of infection in this population is largely driven by the level of immunosuppression, which is in place to prevent graft rejection. Currently there are no reliable ways in which to identify immunosuppressed patients' risk of infection before the infection itself becomes clinically apparent. The primary aim of this research was to find immune biomarkers or a scoring system that predicts the risk of severe infection in kidney transplant recipients in order to better develop strategies that reduce that risk, including the reduction of immunosuppression, use of primary and secondary antimicrobial prophylaxis and more intensive clinical and laboratory monitoring.

One hundred and sixty-eight clinically stable kidney transplant recipients were enrolled in a prospective cohort study at an academic health network in Melbourne, Australia. Patients underwent baseline testing for a selection of immune biomarkers and then were followed prospectively for the development of a severe infection, defined as infection that required admission to hospital. A range of biomarkers were chosen to broadly represent each of the innate, humoral and cellular immune systems and included natural killer cell number and cytotoxic function, lymphocyte subsets, immunoglobulin concentrations and vaccine seroresponses.

After two years follow-up, 35% of patients had developed a severe infection and 21% recurrent severe infections.

In the innate immune system, natural killer cell cytotoxic function and natural killer cell number measured at study entry were predictive for the development of severe infection.

In the humoral immune system, reduced B cell number, particularly when combined with reduced IgG, was associated with sino-pulmonary infection. A series of studies were performed evaluating vaccine efficacy, safety and whether vaccine seroresponses could be used as a biomarker to predict subsequent infections. Seroresponses to trivalent influenza vaccination were suboptimal and as such could not be used as a biomarker of humoral immune competence to stratify patients into those at high risk of infection. Vaccination with 13-valent conjugate pneumococcal vaccine resulted in measurable seroresponses and no increased rates of rejection or formation of de novo HLA antibodies. A meta-analysis of vaccination in solid organ transplant recipients found that vaccination did not result in adverse immunological sequalae.

In the cellular immune system, reduced CD4+ cell number was a strong predictor of severe infection and superior to more complicated T cell proliferative assays.

A composite score, containing clinical and immune biomarkers (CD4+ cell count and NK cell count, eGFR and mycophenolate use) predicted admission with severe infection in the subsequent two years and was a better predictor than the biomarkers alone.

Findings from this body of work have advanced our understanding of biomarkers that could successfully discriminate between patients at very high risk of developing infection in kidney transplantation and those at a lower risk. These findings can inform future studies aimed at personalising antimicrobial prevention and finding safe ways to reduce immunosuppression in kidney transplant recipients.

Manuscript status

This section gives a summary of the manuscript status and author contributions for this thesis. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

The main body of the thesis incorporates two published original papers (the papers themselves are included in the body of the thesis, in Section 2.2 and 3.5 respectively) and four papers submitted to peer-reviewed journals. Section 3.3 discusses the manuscript "Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis". I was the second author and contributed 30% to the analysis of data and preparation of the manuscript. This article was published in the *Journal of Heart Lung Transplant* in 2018 and is included as Appendix 1. Section 4.2 discusses a manuscript still in preparation.

In addition to Appendix 1 (discussed in Section 3.3), there are a further five manuscripts included as appendices. Two are case reports describing opportunistic infections in kidney transplant patients, to which I contributed 20% each. The other three are my first author publications, published during my candidature, in the topic of immunocompromised patients.

The core theme of the thesis is measuring immune biomarkers to predict infection in kidney transplant recipients. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Centre for Inflammatory Disease in the Department of Medicine at Monash Medical Centre under the supervision of Professor Stephen Holdsworth, Associate Professor Rhonda Stuart and Professor Karin Thursky.

Thesis section	Manuscript	Status	Contribution	Co-author name and % contribution	Co-author a Monash student
1.2	Can immune biomarkers predict infections in solid organ transplant recipients? A review of current evidence	Submitted to Transplantation Reviews August 2018	1st (80%) Analysis of data, and preparation of manuscript	Mulley WR (5%) Holdsworth S (15%)	No
2.2	Natural killer cell function predicts severe infection in kidney transplant recipients	Published in the American Journal of Trans-plantation April 2018	1st (80%) Design, experimental work, analysis of data and preparation of manuscript	Gan PY (2%) Polkinghorne KR (2%) Ngui J (2%) Stuart RL (2%) Kanellis J (2%) Thursky K (2%) Mulley WR (2%) Holdsworth S (6%)	No
3.2	Measurement of humoral immune competence and the risk of sino-pulmonary infection in a cohort of kidney transplant recipients	In press Transplant Proceedings December 2018	1st (75%) Design, experimental work, analysis of data and preparation of manuscript	Stuart RL (2%) Mulley WR (2%) Polkinghorne KR (2%) Gan PY (2%) Kanellis J (2%) Ngui J (2%) Laurie K (2%) Thursky K, (2%) Leung V (2%) Holdsworth SR (7%)	No
3.4	Pneumococcal vaccination in solid organ transplant recipients: A review of current evidence	Submitted to Vaccine August 2018	1st (80%) Analysis of data and preparation of manuscript	Stuart RL (6%) Mulley WR (6%) Holdsworth SR (8%)	No
3.5	Seroresponses and safety of 13-valent pneumococcal conjugate vaccination in kidney transplant recipients	Published in Transplant Infectious Diseases April 2018	1st (80%) Design, experimental work, analysis of data and preparation of manuscript	Stuart RL (2%) Polkinghorne KR (2%) Balloch A (2%) Kanellis J (1%) Ling J (1%) Kummrow M (1%) Moore C (1%) Thursky K (1%) Buttery J (1%) Mulholland K (2%) Gan PY (2%) Holdsworth S (2%) Mulley WR (2%)	No
5.2	A simple score can identify kidney transplant recipients at high risk of severe infections over the following two years	Submitted to Clinical Transplantation June 2018	1st (80%) Design, experimental work, analysis of data and preparation of manuscript	Holdsworth S (4%) Gan PY (4%) Stuart RL (2%) Thursky K (2%) Mulley WR (2%) Kanellis J (2%) Polkinghorne KR (4%)	No

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Student's signature: [redacted] **Date:** 15 January 2019

Main supervisor's signature: [redacted] **Date:** 15 January 2019

Publications and submitted manuscripts arising from this thesis

The following publications constitute the body of this thesis and are included in the work, in the sections listed below. I have renumbered or slightly reformatted sections of submitted or published papers in order to generate a consistent presentation within the thesis. The presentation of citation and reference entries may vary, to conform with publication requirements.

[1] **Dendle C**, Mulley WR, Holdsworth S. Can immune biomarkers predict infections in solid organ transplant recipients? A review of current evidence.
Revisions submitted August 2018 – *Transplantation Reviews*
See Chapter 1, Section 1.2

[2] **Dendle C**, Gan PY, Polkinghorne KR, Ngui J, Stuart RL, Kanellis J, Thursky K, Mulley WR, Holdsworth S. Natural killer cell function predicts severe infection in kidney transplant recipients.
Published April 2018 – *American Journal of Transplantation*
See Chapter 2, Section 2.2 and the full published article following

[3] **Dendle C**, Stuart RL, Mulley WR, Polkinghorne KR, Gan PY, Kanellis J, Ngui J, Laurie K, Thursky K, Leung V, Holdsworth SR. Measurement of humoral immune competence and the risk of sino-pulmonary infection in a cohort of kidney transplant recipients.
In press December 2018 – *Transplant Proceedings*
See Chapter 3, Section 3.2

[4] **Dendle C**, Stuart RL, Mulley WR, Holdsworth SR. Pneumococcal vaccination in solid organ transplant recipients: A review of current evidence.
Revisions submitted August 2018 – *Vaccine*
See Chapter 3, Section 3.4

[5] **Dendle C**, Stuart RL, Polkinghorne KR, Balloch A, Kanellis J, Ling J, Kummrow M, Moore C, Thursky K, Buttery J, Mulholland K, Gan PY, Holdsworth S, Mulley WR. Seroresponses and safety of 13-valent pneumococcal conjugate vaccination in kidney transplant recipients.
Published April 2018 – *Transplant Infectious Diseases*
See Chapter 3, Section 3.5 and the full published article following

[6] **Dendle C**, Polkinghorne KR, Mulley WR, Gan PY, Kanellis J, Stuart RL, Thursky K, Holdsworth SR. A simple score can identify kidney transplant recipients at high risk of severe infections over the following two years.
Submitted June 2018 – *Transplant International*
See Chapter 5, Section 5.2

Other publications candidate contributed to during candidature not included in thesis

These further publications represent other projects or collaborations that were undertaken during the PhD candidature. All of these published manuscripts are consistent with the theme of infections in immunocompromised hosts. Some of the papers are included as appendices in this thesis, as noted.

[1] Mulley WR, **Dendle C**, Ling JEH, Knight SR. Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis. J Heart Lung Transplant. 2018 Jul;37(7):844–852.
See Chapter 3, Section 3.3 and Appendix 1

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See Appendix 3

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[6] **Dendle C**, Gilbertson M, Spelman T, Stuart RL, Korman TM, Thursky K, Opat S, McQuilten Z. Infection is an independent predictor of death in diffuse large B cell lymphoma. Sci Rep. 2017 Jun 30;7(1):4395.
See Appendix 4

[7] Gurry GA, Campion V, Premawardena C, Woolley I, Shortt J, Bowden DK, Kaplan Z, **Dendle C**. High rates of potentially infectious exposures between immunocompromised patients and their companion animals: an unmet need for education. Intern Med J. 2017 Mar;47(3):333–335.

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See Appendix 5

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See Appendix 6

[13] Azzam R, Badenoch PR, Francis MJ, Fernández C, Adamson PJ, **Dendle C**, Woolley I, Robson J, Korman TM, Graham M. Acanthamoeba encephalitis: isolation of genotype T1 in mycobacterial liquid culture medium. J Clin Microbiol. 2015 Feb;53(2):735–9.

[14] Egerton-Warburton D, Craig S, Stuart R, **Dendle C**. Improving patient safety by doing less rather than more: many peripheral intravenous catheters are unnecessary. GMS Hyg Infect Control. 2014 Mar 7;9(1).

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Chapter 1:
Immune biomarkers and infections in kidney transplant recipients

1.1 Introduction

Generation of the research question

Solid organ transplantation has changed the face of medicine and given new hope to patients who previously would have died or been dialysis dependent. For kidney transplant recipients, improvements in immunosuppression over recent decades has meant that one-year graft survival and five-year patient survival is very high. This is true success, but we can now focus on the adverse outcomes of transplantation and how to minimise them. One of the most important adverse outcomes of prolonged immunosuppression are infections. There are three research areas examining ways in which to reduce infections in kidney transplant recipients. The first is a search for alternatives to immunosuppression, such as the induction of tolerance or the use of non-living organs. The second is to personalise immunosuppression by finding the minimum effective dose for each patient. The third is to identify patients at the highest risk of infection, and enhance surveillance and preventative strategies in these patients.

There are currently no reliable biomarkers to identify patients at the highest risk of infection and it is this topic that formed the basis of this thesis.

There are two steps required to address this question from a research perspective:

1. Identify biomarkers to measure the net state of immunocompromise and use these biomarkers to identify a subgroup of patients who are at high risk of infection.
2. Test interventions to reduce infection in the group of patients identified as high risk by these biomarkers.

The studies in this thesis aim to address the first step. Importantly, we did not attempt to test any clinical interventions (such as reduction of immunosuppression) based on these biomarkers that may reduce the risk of infections. In addition, we also did not attempt to simultaneously identify biomarkers to predict the risk of allograft rejection.

Aim

The primary aim of this thesis was to identify immune biomarkers that were associated with severe infection in a cohort of kidney transplant recipients.

Selection of patient group

Monash Health is five-centre, 2100-bed tertiary referral health network in Victoria, Australia. Monash Health has a large kidney transplant program, with 1000 current active transplant recipients. It is also the state centre for kidney/pancreas transplantation. The kidney transplant cohort was selected as the study population because these patients have similar characteristics in terms of their immunosuppression. For example, the majority of the kidney transplant cohort were receiving mycophenolate, tacrolimus and prednisolone as their immunosuppressive combination. In addition, these patients received similar protocolised management. This patient cohort had differing clinical characteristics but shared the common vulnerability in that they must be immunosuppressed in order to maintain their renal allograft. One hundred and sixty-eight stable kidney transplant recipients were recruited for the study, from the renal outpatient clinic where they were attending for their regular review. The renal transplant physicians kindly provided assistance with the recruitment process. The author personally reviewed in detail all the transplant patients at study entry and then each time the patient was admitted to hospital. The cohort was analysed for different clinical and laboratory factors associated with severe infection. As such, there were several subgroups and not all of the cohort had complete data for all immune biomarkers at study entry. Most chapters in this thesis examine subgroups comprising more than 100 patients and the demographic characteristics of these are defined. As expected, the subgroups for different analyses have very similar characteristics.

Selection of biomarkers

The immune biomarkers for this study were specifically selected to represent the humoral, cellular and innate immune system. The design of this thesis was in part modelled on two studies performed at Monash Health over 10 years ago, which demonstrated that a composite score of immune biomarkers could predict infection in kidney transplant recipients. It was unclear whether these studies would have similar findings today, as there is an increased use of mycophenolate mofetil as a key immunosuppressive agent.[1,2] The same biomarkers used in these studies were selected for this study, with the addition of new biomarkers including influenza vaccine seroresponses, natural killer cell functional assay and a T cell proliferative assay. Immune biomarkers were performed cross-sectionally at study entry and study participants were followed prospectively for two years for the development of infection.

Selection of infectious outcomes

Studies that examine infectious outcomes in solid organ transplantation are difficult to compare because the definition of infection is heterogonous. Some studies examine all infections, including those that did not require admission to hospital, while others examine only certain infections. Rates of cytomegalovirus infection are also difficult to compare depending on whether the transplant centre has a prophylactic or pre-emptive approach to diagnosis and management. For this study, it was decided that hospital admission for infection was the most clinically relevant outcome. Therefore, the definition of severe infection was infection requiring admission to hospital.

Subgroup analysis of certain infections was performed aligned with the immune biomarkers being tested. These subgroup analyses were determined a priori.

Structure of the thesis

This thesis contains six chapters, which are divided according to each arm of the immune system. The basis for each chapter is reflected in actual publications or submitted works and these are included in their published or submitted format, with occasional renumbering or reformatting in order to generate consistent presentation within the thesis. The presentation of citations and reference entries may vary, to conform with publication requirements.

References

[1] Blazik M, Hutchinson P, Jose MD, Polkinghorne KR, Holdsworth SR, Atkins RC, et al. Leukocyte phenotype and function predicts infection risk in renal transplant recipients. Nephrol Dial Transplant. Oxford University Press; 2005 Oct;20(10):2226–30.

[2] Hutchinson P, Chadban SJ, Atkins RC, Holdsworth SR. Laboratory assessment of immune function in renal transplant patients. Nephrol Dial Transplant. 2003 May;18(5):983–9.

1.2 Can immune biomarkers predict infections in solid organ transplant recipients? A review of current evidence

HIGHLIGHTS

- Infection induced morbidity and mortality remains a problem for solid organ transplant recipients despite improved graft survival.
- Biomarkers are required to identify patients who are over-immunosuppressed and at high risk of severe infection.
- Adaptive immune system biomarkers associated with infections include immunoglobulins, lymphocyte numbers, lymphocyte subsets, intracellular concentrations of adenosine triphosphate in stimulated CD4+ cells and soluble CD30.
- Innate immune system biomarkers associated with infections include natural killer cell numbers, complement and mannose-binding protein.
- Quantification of viral nucleic acid can predict all-cause infections.
- Composite panels of immune biomarkers show the most promise.
- There is no current robust biomarker or panel thereof to guide clinical immunosuppression, but data is sparse.

SUBMITTED MANUSCRIPT

Dendle C, Mulley WR, Holdsworth S. Can immune biomarkers predict infections in solid organ transplant recipients? A review of current evidence.

Revisions submitted August 2018 – *Transplantation Reviews*

ABSTRACT

Despite improvements in graft survival, solid organ transplantation is still associated with considerable infection induced morbidity and mortality. If we were able to show that serious infection risk was associated with excessive suppression of immune capacity, we would be justified in "personalising" the extent of immunosuppression by carefully monitored reduction to see if we can improve immune compromise without increasing the risk of rejection. Reliable biomarkers are needed to identify patients at an increased risk of infection. This review focuses on the currently available evidence in solid organ transplant recipients for immune non-pathogen–specific biomarkers to predict severe infections with the susceptibility to particular pathogens according to the component of the immune system that is suppressed. This review is categorised into immune biomarkers representative of the humoral, cellular, phagocytic, natural killer cell and complement system. Biomarkers of the humoral and cellular systems that have demonstrated an association with infections include immunoglobulins, lymphocyte number, lymphocyte subsets, intracellular concentrations of adenosine triphosphate in stimulated CD4+ cells and soluble CD30. Biomarkers of the innate immune system that have demonstrated an association with infections include natural killer cell numbers, complement and mannose-binding lectin. Emerging evidence shows that quantification of viral nucleic acid (such as Epstein-Barr virus) can act as a biomarker to predict all-cause infections. Studies that show the most promise are those in which several immune biomarkers are assessed in combination. Ongoing research is required to validate non-pathogen–specific immune biomarkers in multi-centre studies using standardised study designs.

MANUSCRIPT

Introduction

An episode of infection for a solid organ transplant (SOT) recipient can result in increased mortality and serious morbidity, including an increased risk of allograft rejection.[1-4] While the underlying risk of admission to hospital for infection differs according to transplant type,[5] SOT recipients overall do have a substantially higher requirement for admission and higher case fatality rates related to infection than the general population.[6] Consequently, prevention, diagnosis and treatment of infection have become key goals in the care of SOT recipients.

One component of the risk of infection in individuals transplanted is the immunosuppressive regimen, often standardised at individual sites, but sometimes leading to quite markedly different immunological effects in patients. Despite the clear clinical benefit to accurately predicting in which patients infection or rejection will develop, or be likely to develop, there are no good clinical tools available.[7] Patients that develop both infection and rejection are a particularly important group because their optimal window of immunosuppression is narrowly set between that which protects them from rejection and that which leads inevitably to serious infection.

While immunosuppression may be seen as a balance between the risk of infection and rejection, there are a number of reasons why a SOT recipient may develop both syndromes. Firstly, the clinical risk factors for infection and rejection may overlap. Secondly, the risk of infection and rejection may be increased as a consequence of reactive changes to the immunosuppressive regimen. For example, if a patient developed an infection, immunosuppression may be reduced, thereby increasing the risk of rejection. Conversely, a patient with rejection may have their immunosuppression increased with a subsequent increased risk of infection. Thirdly, an

infectious episode itself has been reported to trigger allograft rejection through the development of heterologous immunity.[4]

Cippa et al. attempted to disentangle the individual risks for infection and rejection in the first year after kidney transplant.[7] They performed a post-hoc analysis of a large cohort of kidney transplant recipients, defined clinical risk factors for infection and rejection from the multivariable analysis and applied a risk weighting for both infection and rejection to each patient. Their model was able to discriminate between the groups and consequent external validation confirmed the applicability of the model in an independent cohort. This is one of the few studies that have demonstrated that clinical data can be used to stratify patients to predict infectious risk. Reliable biomarkers for the risk of infection would therefore be of great value as an adjunct to guide the clinician in adjusting the level of immunosuppression. Biomarkers could also be used in clinical trial design to personalise prophylaxis against infection and more precisely design an individual, risk-based immunosuppressive regimen.[8]

Biomarkers to predict infection can be pathogen specific or non-pathogen specific. A non-pathogen–specific biomarker is a biological assay with no specificity for a particular antigen or organism.[8] Pathogen-specific biomarkers have been examined in several studies, whereby viral-specific CD4+ and CD8+ lymphocyte responses have identified patients at high risk for cytomegalovirus (CMV),[9] Epstein-Barr virus (EBV)[10] and BK virus (BKV).[11]

This narrative review will focus on the currently available evidence for SOT recipients using non-pathogen–specific immune biomarkers to predict severe infections. While in clinical practice it is important that the risks for infection and rejection are not taken in isolation, risk factors for allograft rejection are beyond the scope of this review, which will focus on infection risk alone.

We searched MEDLINE and EMBASE from their inception until 1 July 2018. Pertinent articles were selected from the citations returned from the search. Literature searches included keywords and free text terms for solid organ transplantation, biomarkers, risk factors and the infectious outcomes of interest. Results were limited to human subjects and language of publication was restricted to English.

This review is categorised into immune biomarkers representative of the humoral, cellular, phagocytic, natural killer cell and complement system. This categorisation is arbitrary, as host defence against infection is achieved through the combined effect of all arms of the immune system. However, this categorisation could be of practical use for clinicians when selecting antimicrobial agents for empirical treatment of infection and infection prophylaxis. Table 1 presents the risk of susceptibility to particular pathogens according to the component of the immune system that is impaired.[12-47] Much of this data is derived from observational reports regarding patients with primary immune deficiencies. The risk of infection with a particular pathogen can be difficult to predict in transplant recipients because multiple components of the immune system are affected to varying degrees. There is little evidence directly comparing the relative contribution of each component of the immune system in mounting an effective response against different pathogens in SOT recipients. Figure 1 illustrates the role of different components of the innate and adaptive immune system in the control of infectious pathogens and their relative contribution.

Immunosuppression following SOT is predominately aimed at suppressing cellular immune function in order to prevent allograft rejection. With suppression of cellular immune function, innate and humoral immunity play an enhanced role in defence against infection. Calcineurin inhibitors (CNIs) and mycophenolate mofetil are the cornerstone immunosuppressants for SOT recipients. While they directly target T cell function they also suppress humoral and innate function, which might otherwise compensate for the reduction in cellular function.[48,49] In some patients, additional

treatment targeted towards specific elements of the immune system are used; for example, anti-thymocyte globulin and rituximab which respectively deplete T cells and B cells. Monitoring CNI drug levels guides dosing. However, this is only a surrogate for the degree of effect on T cell function from treatment and rarely assessed directly. It is notable that other aspects of the immune system effectively go unmonitored.

Humoral immune biomarkers

B cells

B cell numbers are frequently reduced following SOT.[49-51] While there is evidence that enhanced humoral immunity before transplantation, such as increased memory class-switched B cells, can identify heart transplant recipients at low risk of infection,[52] studies specifically linking B cell numbers and their kinetics to infectious outcomes post-transplantation are lacking.

Immunoglobulins

Hypogammaglobinemia is common in SOT recipients and a number of prospective studies have demonstrated that pre-transplant or early post-transplant hypogammaglobulinemia is associated with an increased infectious risk.[49,50,53-58] Florescu et al. performed a meta-analysis examining the risk of infection in SOT recipients with hypogammaglobulinemia in the first year post-transplant.[53] Pooled data from 18 studies revealed an increased risk of infection and death in those with severe hypogammaglobulinemia (< 400 mg/dL). However, this risk was not identified in patients with mild hypogammaglobulinemia. Immunoglobulin subclass deficiencies (IgG1) may be also be associated with infectious outcomes.[58]

Seroresponses to vaccination

There are limited data assessing other measures in humoral competence (such as vaccine responses) that link in vitro measurements with infectious outcomes. Recent

studies by Sarmiento et al. have reported that kinetics of IgA or IgM anti-pneumococcal polysaccharide antigens may have a role in predicting post-transplant infections in heart and lung transplant infection.[59,60] An Australian study demonstrated that seroresponses to annual influenza vaccination were very poor and were not associated with the development of all-cause sinopulmonary infection (in press, *Transplant Proceedings*).

Cellular immune biomarkers

Studies examining the association between biomarkers of the cellular immune system and infection are presented in Table 3.

Lymphocyte subsets (total lymphocyte count, CD4+ and CD8+ cells)

Monitoring of the absolute numbers and kinetics of lymphocyte subsets (such as total lymphocyte number, CD4+ cell number, CD8+ cell number, CD4+ cell nadir and CD4:CD8 ratio) to predict infections in SOT recipients has been to predict infection post-transplant.[61-66] CD4+ and CD8+ lymphopenia are associated with the development of opportunistic infections such as *Pneumocystis jirovecii* (PCP), herpes viral and fungal infections. The majority of studies have used CD4+ cell number monitoring before or early post-transplant and there are very few studies that have examined the association between CD4+ cell number and infection beyond the first post-transplant year.

Soluble CD30 (sCD30)

CD30 is a cell surface maker that expressed by activated T cells.[67,68] CD30 is a member of the tumour necrosis factor receptor superfamily and its soluble form (sCD30) is released by CD4+ and CD8+ T cell clones. It appears to have a role in the regulation between T helper 1 (Th1) and T helper 2 (Th2) responses and may be a biomarker for Th2 polarised T cell responses. As such, it has been studied as a biomarker of cellular immunity (Table 3).[69,70] Membrane bound CD30 can be proteolytically cleaved to

generate the soluble form of CD30 (sCD30), which can be measured in serum or plasma by enzyme linked immunosorbent assay.[8,71-75] Trials that have examined the relationship between sCD30 and infections have found discordant results, hence the utility of this biomarker remains to be defined.[76-79] Fernández-Ruiz et al. recently reported that high levels of sCD30 pre-transplant are associated with an increased risk of post-transplant bacterial infections but not other types of infections.[68] The authors propose that this relates to the immunomodulatory role of sCD30 which, by deviating immune response towards Th2, reduces antibacterial immunity by inhibiting production of cytokines and reducing macrophage killing.[68]

Intracellular concentrations of adenosine triphosphate in stimulated CD4+ cells (iATP)

The ImmuKnow immune cell function assay (Cyclex Inc., Columbia, MD, US) is a commercial test developed to measure T cell activation, as a surrogate marker of T cell function. This assay detects iATP production from activated CD4+ cells. After CD4+ cells are incubated with the mitogen phytohaemagglutinin, iATP production is measured by chemiluminescense. iATP production is categorised as weak, moderate or strong. Weak responses are indicative of excessive immunosuppression and an increased risk of infection.[80,81]

Since the test's introduction, there have been numerous studies correlating iATP with rejection and infection. Two meta-analyses and one systematic review have been performed to examine the value of iATP in predicting infection, with discordant findings.[82] The largest study, performed by Ling et al., found the test lacked sensitivity and specificity and concluded that the current evidence suggested that iATP is not able to identify individuals at risk of infection or rejection.[80,82,83] Vittoraki et al. demonstrated the test was not reproducible for a single patient at different time points.[84] They found that of 128 kidney transplant recipients, 43% exhibited fluctuations in their iATP levels among the three T cell function zones (weak, moderate

and strong). In this same study, because the majority of kidney transplant recipients and controls tested in the moderate range, the authors determined that they were not able to support this assay as an immune monitoring test in clinically stable renal transplant recipients. Suviolahti et al. demonstrated that there were differences in iATP results depending on the timing of testing in relation to when blood was drawn.[85] They studied 152 transplant patients and 18 healthy controls and found that iATP levels were lower in one-day-old blood compared with fresh blood, concluding that fresh blood should be used for assessing iATP to obtain the most accurate results.[85] Recently, iATP has been identified as a potential biomarker for the prediction of CMV disease.[86] One of the only studies to change immunosuppressive regimens based on an immune biomarker was a randomised, parallel, blinded, interventional trial comparing the outcomes of adult liver transplant recipients whose immunosuppressive therapy was managed by standard practice compared to adjusting therapy based on iATP responses (interventional group).[87] iATP testing was measured at several time points post-transplant with tacrolimus doses reduced by 25% when iATP values were < 130 ng/mL iATP (low immune cell response) and increased by 25% when values were > 450 ng/mL iATP (strong immune cell response). The one-year patient survival was higher (95% vs 82%; $p < 0.01$) and the incidence of infections was lower (42.0% vs 54.9%, $p < 0.05$) in the intervention arm relative to the standard care group. The difference in infections was due to a reduced incidence of bacterial (32% vs 46%; $p < 0.05$) and fungal infections (2% vs 11%; $p < 0.05$). iATP levels did not correlate with rejection in this study.

T cell proliferation

T cell proliferation can be measured in vitro through the use of mitogen or antigen stimulation.[88] A small study in heart transplant recipients demonstrated lower proliferative responses to mitogen in those that developed infection compared to those without infections.[88]

Innate immune biomarkers

Phagocytic biomarkers (neutrophils, phagocytes, macrophages)

Absolute neutrophil count and duration of neutropenia are powerful predictors of infection in haematopoietic stem cell transplants,[89] but there are less data regarding the risk of infection in SOT recipients. Egger et al. examined the use of polymorphonuclear leukocyte functional tests as predictive makers for infection shortly after surgery.[90] They found that levels after transplant surgery of the neutrophil derived enzyme elastase over 100mg/L, followed by a drop in polymorphonuclear leukocyte migration, were a marker for impending infection. Measurement of neutrophil phagocytic capacity and reactive oxygen species generation have also been performed in kidney transplant patients and shown to be predictive of infection when included in a composite immune score.[49,50]

Natural killer (NK) cell biomarkers

NK cells are innate immune cells that are capable of immediate defence against pathogens and cancer. NK cells do not require antigen-specific recognition of their targets, but rather are activated by generic stress signals.[91,92] NK cells are important in the control of viral infections and NK cell deficiency predisposes to viral infection, in particular herpes virus.[93,94] Calcineurin inhibitors used in SOT can reduce NK cell function.[48,95-97] In vitro studies have demonstrated a decrease in NK cell degranulation and interferon gamma release with increasing doses of tacrolimus and cyclosporine. Several studies have examined the association between NK cell number and infections in SOT recipients. A recent publication by Fernández-Ruiz et al. described an association between low NK cell number one month post-liver transplant and opportunistic infections, such as CMV disease.[44] The same authors demonstrated that low NK cell numbers are predictive of both invasive fungal and herpes zoster infections in SOT recipients.[44,98] Blazik et al.,[50] Hutchinson et al.,[49] Sarmiento et al.[99] and Fernández-

Ruiz et al.[100] included NK cell number as part of a composite score to predict infections in SOT recipients. Recent data suggests that certain NK cell subsets (e.g. cd94/NKG2C^{bright} activating lectin-like receptors) have a role in the control of CMV infection in kidney transplant recipients.[101] Additionally, Dendle et al. reported that NK cytotoxic function was a significant predictor of infection in stable kidney transplant recipients.[102]

Complement biomarkers

The complement system has an important role in opsonisation of infective pathogens and activation of the adaptive immune system. Table 4 summarises studies examining the association between biomarkers of the complement system and infectious outcomes in SOT.[54,99,103-106] Reduced levels of complement, measured within one month of kidney, heart and liver transplant, are associated with an increased risk of infections in the first post-transplant year.[99,103,104] When foreign antigen is presented, complement can be activated by the classical, the lectin and the alternate pathways. Functional assessment of the lectin pathway can be performed by measurement of serum mannose-binding lectin (MBL), which activates the pathway through binding to a broad range of microorganisms.[107] Genetic polymorphisms that lead to decreased MBL production have been identified[105] and recent data demonstrated that liver transplant recipients of MBL-deficient liver transplants have a higher risk of bacterial infections, pneumonia and bacterial-infection related mortality.[108] Three studies have identified an association between reduced MBL pre-transplant and an increased risk of infection post-transplant.[54,105,106]

Combinations of immune biomarkers

Several studies have assessed the correlation of a composite immune score with post-transplant infections (Table 5).[49,50,99,100,109,110] Five of the six studies have demonstrated a significant association. It is difficult, however, to compare these studies

directly due to differences in study designs and immune components included in the scores. The study performed by Blazik et al. in kidney transplant recipients was the only study to examine a composite score beyond the first post-transplant year.[50] This study and that of Hutchinson et al. also differed from others in that they were the only studies to include neutrophil and T cell functional assays.[49,50] Importantly, both were performed prior to the widespread usage of tacrolimus and mycophenolate mofetil and need to be validated in the modern era of immunosuppression. In two separate cohorts of heart transplant recipients it was demonstrated that decreased levels of serum complement and natural killer cells add to the predictive value of total IgG levels for severe infection.[99,109] Crepin et al. and Fernández-Ruiz et al. each performed a prospective study in kidney transplant recipients using CMV serostatus, CD4+:CD8+ ratio and CD8+ absolute number in a composite score. Crepin et al. found an association with the score and infection but Fernández-Ruiz et al. did not.[100,110]

Mian et al. performed a prospective cohort study of 137 SOT recipients using an immune monitoring assay to predict infections during the first year post-transplant.[111] The assay tested interferon gamma responses to stimulation of the innate (toll-like receptor 7 ligand) and adaptive (anti-CD3+ antibody) immune system. The assay predicted an increased risk of infections, with patients with low IFN-γ values being at the highest risk of subsequent infection.

Biomarkers using quantification of viral nucleic acid

Measurement of viruses through quantification of their DNA in plasma or serum can be used to predict the risk of other infections in SOT recipients.[112,113] Viral replication in SOT recipients depends on a number of factors that should be taken into account when using viremia as a non-pathogen–specific biomarker. Since some of the viruses proposed for use as non-pathogen–specific biomarkers can also cause infection in transplant recipients, it can become difficult to determine whether increasing viral

replication represents a biomarker for the level of immunosuppression or early infection with the virus itself. In addition, donor-recipient serostatus is predictive of viral infection and disease for CMV, EBV and BKV. The type of transplant can influence viral reactivation, and this may be virus and transplant specific. For example, BKV establishes latency in the reno-urinary tract and has a markedly higher risk of reactivation in kidney transplant recipients compared with other SOT recipients.[114] Certain immunosuppressive medications can influence viral replication.[115]

Epstein-Barr virus

EBV DNAemia is common among SOT recipients, ranging from 17% to 70%.[112,113,116-121] Morton et al. studied 499 kidney transplant recipients recruited between one month and 33 years post-transplant and followed them with serial measurements of EBV DNAemia.[121] EBV DNAemia prevalence and persistence appeared to increase, rather than fall, with time from transplant. The majority of studies linking clinical outcomes with EBV DNAemia use post-transplant lymphoproliferative disorder as the outcome measure. However, more recent studies have used serial measurements of EBV DNAemia to assess infection risk in SOT recipients.[112,113,120] These studies show that a high EBV viral load or persistent EBV infection is associated with an increased risk of severe infection.[112,113,120] A study of 62 lung transplant recipients showed that detectable EBV DNAemia within six months post-transplant was associated with an increased rate of overall and opportunistic infections and that mean peak EBV DNAemia was higher in those with later overall infection and opportunistic infection.[120] Another study of 383 kidney transplant recipients showed EBV DNAemia was associated with opportunistic infection but not bacterial infection or CMV.[113] San Juan et al. found that in liver, heart and lung transplant recipients, high level and persistent EBV DNAemia was associated with tumours, and severe and opportunistic infections.[112] Current guidelines recommend screening for EBV DNAaemia in high-risk recipients for one year

after transplantation for the purpose of early detection of EBV-related post-transplant lymphoproliferative disorder.[122]

Cytomegalovirus

CMV infection and disease is arguably the most important infection in SOT recipients. Established CMV disease is immunomodulatory and places patients at risk of subsequent infections and rejection. There are a number of CMV-specific biomarkers in use in research settings that can be used to predict CMV disease.[123] The majority of these strategies rely on measurement of CMV-specific CD8+ cells. The use of CMV DNAemia as a non-specific biomarker for all-cause infection is problematic. Firstly, donor-recipient serostatus is a key determinant of CMV DNAemia and will affect the likelihood of infection independent of other factors. Secondly, measurement of CMV DNAemia is influenced by the use CMV antiviral prophylaxis. Thirdly, the appearance of high level CMV DNAemia generally warrants antiviral treatment, making it difficult to measure longitudinally.

Human herpes virus 6 and 7

One hundred and twenty-nine liver transplant recipients were randomised to real-time monitoring of HHV6 and HHV7 viremia by PCR at regular intervals or to undergo usual care, with the primary outcome being a composite of adverse events indirectly attributable to viral reactivation (including opportunistic infection, graft rejection and severe hepatitis C virus recurrence). There were no differences in the cumulative incidence of the primary outcome between the “monitoring” and “no monitoring” groups at one year or five years.[124]

Torque teno virus

Torque teno viruses (TTV) are small non-enveloped viruses that are non-pathogenic in humans.[125] There is emerging interest in the use of TTV as a non-specific biomarker of immunosuppression as they have a prevalence of up to 90% in healthy and

immunocompromised individuals.[126] In SOT recipients, there have been studies that have demonstrated a correlation between the intensity of immunosuppression and TTV DNAemia.[126-129] A recent study prospectively quantified TTV viremia in the peripheral blood of 169 kidney transplant recipients. Patients who developed infections in the 14 months of follow-up had higher TTV levels compared to patients without infection. Logistic regression demonstrated independent association between TTV levels and infection.[130]

BK virus

BKV is an important pathogen, especially in kidney transplant recipients. Although BKV DNAemia is widely considered a marker of over-immunosuppression there are no specific studies linking BKV DNAemia to other infections. Further research is required into the association between BKV and all-cause infectious outcomes.

Conclusions

Better biomarkers useful to identify SOT recipients at an increased risk of infections are required so that strategies aimed at reducing the risk of infections can be well tested. The majority of studies that have used clinical factors to guide reductions in immuno-suppression have resulted in unacceptably high rates of allograft rejection.[1,131-133]

This review has summarised currently available evidence from studies that have used immune biomarkers to predict infection in SOT recipients. The current available evidence is insufficient to support the use of any one single or composite panel of diagnostic tests or algorithms to guide the clinician in tailoring the immunosuppressive regimen optimally for a given transplant recipient.[134] There is high quality evidence that severe hypogammaglobulinemia predisposes to infection, so monitoring of immunoglobulin concentrations does appear worthwhile. There is moderate quality evidence that monitoring of lymphocyte subsets can predict infection, making this simple test another feasible method of identifying patients at high risk of infection. The

quality of evidence for the remaining biomarkers is low and mostly derived from single-centre studies. The studies have differed in terms of study design, immunosuppressive regimens, follow-up, infectious outcomes and clinical parameters. Furthermore, the relative contribution of CMV to total infections has differed markedly. There are very few studies that have validated the use of biomarkers in different transplant cohorts and those that did found inconsistent results.[100,110] Studies that have utilised composite immune scores have been the most promising, however the presence of co-linearity, functional overlap and redundancy of immune biomarkers needs to be considered.[49,50,99,100,109,110,135,136] Emerging evidence for the use of monitoring of viruses for non-pathogen–specific infections is interesting but is yet to be tested in combination with immune biomarkers.

SOT recipients represent a unique group of patients in which to study immune biomarkers because although they are prescribed similar medications, there are widely variable degrees of immunosuppression and susceptibility to infection between individuals. Due to the changing epidemiology of SOT recipients, with less early graft loss and prolonged survival, increased research is required to develop robust tests to reflect the individual's level of immunosuppression or immune function. Ideally the tests should use standardised definitions and be validated in multiple transplant centres. The demographic trend towards older SOT recipients with increasing immune senescence may mean that CNI monitoring may be assaying a pathway (activation of naïve donor-specific T cells) that is of less importance than it was in previous eras. It might be that quantification (direct or indirect) of other aspects of immunity may be a better guide to functional immunity in such populations. Clearly, biomarkers that can predict both infection and rejection would be the most useful for clinicians. Furthermore, immune biomarkers found to be predictive of infection in SOT recipients may be relevant to other immunocompromised patients and this requires further research.

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Table 1. Defects in host immunity and associations with organisms

Bacteria	Viruses	Fungi	Parasites
Humoral system (B cells and immunoglobulins)[11-18]			
Streptococcus pneumoniae	Enterovirus spp.		*Giardia* spp.
Haemophilus influenzae	Echovirus spp.		
Campylobacter spp.	Norovirus (chronic)		
Mycoplasma spp.			
Ureaplasma spp.			
Cellular system (T cells)[19-33]			
Mycobacteria tuberculosis	Herpes viruses (HSV, CMV, EBV, VZV, HHV6, HHV8)	Invasive filamentous fungal	*Toxoplasmosis*
Non-tuberculous mycobacteria	Polyoma virus (BK and JC)	*Pneumocystis jirovecii*	*Cryptosporidium* spp.
Nocardia spp.		*Cryptococcus* spp.	*Microsporidium* spp.
Streptococcus pneumoniae		*Candida* spp. (mucocutaneous or invasive)	
Listeria spp. and			
Salmonella spp.			
Phagocytic system (neutrophils, phagocytes, macrophages)[33-37]			
Staphylococcus aureus		Candida (mucocutaneous or invasive)	
Klebsiella spp.		Invasive filamentous fungi	
Serratia spp.			

Bacteria	Viruses	Fungi	Parasites
Natural killer cell system[34,38-44]			
Non-tuberculous mycobacteria	Herpes viruses (HSV, CMV, EBV, VZV, HHV6, HHV8)	Invasive filamentous fungal	
Complement system[45-47]			
Neisseria meningiditis			
Streptococcus pneumoniae			
Haemophilius influenzae			

spp.: species, HSV: herpes simplex virus, CMV: cytomegalovrius, EBV: Epstein-Barr virus
VZV: varicella-zoster virus, HHV6: human herpes virus 6, HHV8: human herpes virus 8

Table 2. Associations between humoral biomarkers and infections in solid organ transplant recipients

Year	Biomarker	Recipient organ type (number)	Study design Follow-up (years)	Timing of biomarker measurement	Results
2006[56]	IgG, IgA, IgM	Heart (38)	Prospective 1.4	Pre-transplant and post-transplant day 7 and month 1	After adjustment for CMV serostatus, IgG pre-transplant and post-transplant day 7 were associated with infection (RR 3.69 and RR 11.21, respectively)
2008[57]	IgG, IgA, IgM	Liver (46)	Prospective 0.5	Pre-transplant and post-transplant day 7 and month 3	Pre-transplant IgG and IgA hypergammaglobulinemia were associated with infection (RR 2.78 and 2.77, respectively)
2012[55]	IgG, IgM, IgA	Kidney (290)	Prospective 1	Pre-transplant and post-transplant month 1 and 6	Hypogammaglobulinaemia at month 1 was a risk factor for overall infection and bacterial infection month 1–6 Hypogammaglobulinaemia at month 6 was a risk factor for overall infection and bacterial infection month 6–12
2008[54]	IgG	Kidney (152)	Prospective 1	Pre-transplant and post-transplant month 3 and 12	Hypogammaglobulinaemia pre-transplant and month 3 was associated with increased risk of infection
2013[53]	IgG	Lung, liver, kidney, heart (1756)	Meta-analysis 0.2–6.5	Various time points over 1 year post-transplant	Severe hypogammaglobulinaemia (IgG < 400 mg/dL) during the first year post-transplant was associated with overall infections, CMV (OR 2.4), *Aspergillus* spp. (OR 8.19), other fungal (OR 3.7) and respiratory infections (OR 4.8) and all-cause mortality (OR 21.9)
1996[58]	IgG, IgM, IgA IgG subclasses	Kidney (36)	Prospective Early post-transplant period	Post-transplant week 5	Lower IgG1 subclass in patients with infection compared with non-infected patients

Year	Biomarker	Recipient organ type (number)	Study design Follow-up (years)	Timing of biomarker measurement	Results
2018[59]	IgG, IgA, IgM, C3,C4, titres of antibodies to pneumococcal polysaccharide antigens (IgG, IgA, IgM), CMV antibodies, serum B cell activating factor	Lung (82)	Prospective 0.5	Pre-transplant and post-transplant day 7 and month 1	IgG post-transplant day 7 was associated with CMV disease and fungal infection (OR 8.15 and RR 8.03, respectively) Lower IgM antibody against pneumococcal polysaccharide antigens post-transplant day 7 was associated with bacterial (OR 3.96)
2018[60]	IgG, IgA, IgM, C3,C4, titres of antibodies to pneumococcal polysaccharide antigens (IgG, IgA, IgM), CMV antibodies, serum B cell activating factor	Heart (170)	Prospective 0.5	Pre-transplant and post-transplant day 7 and month 1	Low IgG and low C3 post-transplant day 7 were associated with severe infection and bacterial infection (OR 7.40 and 12.37, respectively) Low IgG antibody against pneumococcal polysaccharide antigens post-transplant day 7 and day 30 were associated with bacterial infection (OR 14.82)

IV: intravenous, CMV: cytomegalovirus, RR: relative risk, OR: odds ratio

Table 3. Association between cellular immune biomarkers and infections in solid organ transplant

Year	Biomarker	Recipient organ type (number)	Study design Follow-up (years)	Timing of biomarker measurement	Results
2012[64]	T lymphocyte subsets kinetics	Heart (48) Kidney (42)	Retrospective 1	Various time points in the first 8 months post-transplant	Heart transplant recipients with OIs had lower CD4+ and CD8+ cell numbers than those without infections Kidney transplant recipients with OIs had lower CD8+ cell numbers than those without infections
2015[65]	CD4+ cell number and CD8+ cell number	Lung (83)	Retrospective 1	Various time points in the first 12 months post-transplant	A nadir CD4+ cell number < 200 cells/μL in the first 3 months post-transplant predicted a higher frequency of viral OI in the subsequent 6-month period
2009[62]	Total lymphocyte count	Liver (63)	Prospective 2	Pre-transplant	Pre-transplant total lymphocyte count was associated with infection (OR 10.1)
2014[66]	Total lymphocyte count	Liver (276)	Retrospective 5	Pre-transplant	Pre-transplant lymphopenia < 500 cells/mm^3 was associated with CMV (HR 5.5) and non-CMV invasive infection (HR 1.6)
2014[62]	Peripheral blood lymphocyte subsets	Kidney (304)	Prospective 1	Pre-transplant and post-transplant months 1 and 6	Recipients who did not receive anti-thymocyte globulin, CD8+ cell number < 0.100 × 10^3 mm/μL was an independent risk factor for OI (HR 3.55) Recipients who received anti-thymocyte globulin, a CD4+ cell number < 0.050 × 10^3 cell/μL showed negative predictive values of 0.92 for the subsequent occurrence of overall OI and CMV disease
2006[63]	CD4+ cell number	Kidney (20) All HIV positive	Prospective 3	Various time points over 3 years post-transplant	Annualised risk of infection while CD4+ cell number < 200 cells/μL was over 10 times that when CD4+ > 200 cells/μL
2012[88]	T cell proliferation to antigen and mitogen stimulation	Heart (12) Controls (8)	Prospective 1	Pre-transplant and post-transplant month 3	Month 3 post-transplant CD3+ cell and CD8+ cell proliferative responses to mitogen were lower in infected patients than those without infection

Year	Biomarker	Recipient organ type (number)	Study design Follow-up (years)	Timing of biomarker measurement	Results
2010[77]	sCD30	Kidney (586)	Prospective 5	Pre-transplant	Low level sCD30 (< 120 U/mL) was associated with an increased risk of pneumonia
2008[79]	sCD30	Kidney or simultaneous kidney/pancreas (92)	Prospective 2	Pre-transplant	Low level sCD30 (< 90 U/mL) was associated with a decreased risk of infection
2007[78]	sCD30	Heart (100)	Prospective 2	Pre-transplant	Low level sCD30 (< 90 U/mL) was significantly associated with an increased risk of infection
2006[76]	sCD30	Heart (100)	Prospective 1	Pre-transplant	Low level sCD30 (< 90 U/mL) was associated with an increased risk of infection
2017[85]	sCD30	Kidney (100)	Prospective	Pre-transplant and post-transplant month 1, 3 and 6	Higher sCD30 levels pre-transplant were associated with increased risk of bacterial infection (HR 4.65)
2012[82]	iATP	Liver, lung, heart, kidney (2013)	Meta-analysis	Various time points	The pooled estimates for iATP in identification of infection risk were poor (sensitivity 0.58, specificity 0.69, positive likelihood ratio 2, negative likelihood ratio 0.39, diagnostic odds ratio 7.41)
2012[83]	iATP	Liver (651)	Systematic review < 0.1 to 4.2	Various time points	The pooled estimates for iATP in identification of infection risk were good (sensitivity 0.84, specificity 0.75, positive likelihood ratio 3.3 and an area under the receiver operator curve of 0.82)
2006[80]	iATP	Kidney, heart, simultaneous kidney/pancreas, liver, small bowel (504)	Meta-analysis	Various time points	Patients with an iATP < 25 ng/mL were 12 times more likely to develop an infection than a recipient with a stronger immune response

OI: opportunistic infection, CMV: cytomegalovirus, EBV: Epstein-Barr virus, HSV: herpes simplex virus, VZV: varicella-zoster virus, HHV6: human herpes virus 6, PCP: *Pneumocystis jirovecii*, CT scan: computerised tomography scan, sCD30: soluble CD30, IV: intravenous, spp.: species, iATP: intracellular adenosine triphosphate, HR: hazard ratio, OR: odds ratio

Table 4. Associations between biomarkers of the complement system and infections in solid organ transplant recipients

Year	Biomarker	Recipient organ type (number)	Study design Follow-up (years)	Timing of biomarker measurement	Results
2006[56]	C3 and C4	Heart (38)	Prospective 1.4	Pre-transplant and post-transplant day 7 and month 1	At month 1, C3 was lowest in patients who developed infections
2008[58]	C3 and C4	Liver (46)	Prospective 0.5	Pre-transplant and post-transplant day 7 and month 3	C3 hypocomplementemia as associated with infection (RR 3.0)
2008[55]	MBL	Kidney (152)	Prospective 1	Pre-transplant and post-transplant month 3 and 12	Low MBL at month 3 was associated with sepsis and viral infections
2013[104]	C3 and C4	Kidney (270)	Prospective 1	Pre-transplant and post-transplant month 1 and 6	Low C3 at month 1 was associated with overall infection and bacterial infection month 1–6 Low C3 at 6 months was associated with bacterial infection month 6–12
2008[106]	MBL	Simultaneous kidney/pancreas (152)	Retrospective 1	Pre-transplant	Urosepsis was more common in patients with low baseline MBL (< 400 ng/mL) compared with those with greater MBL levels No influence of MBL on the occurrence of wound infections and CMV disease
2005[105]	MBL MBL genotype polymorphisms were determined in liver donors and recipients	Liver (49)	Prospective	Pre-transplant and post-transplant month 12	The presence of MBL variant alleles in the MBL gene of the donor liver, but not in the recipient, was associated with an incidence of clinically significant infections

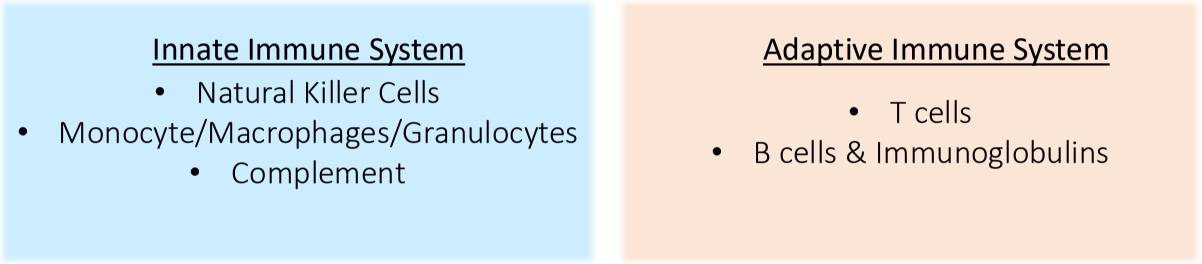
IV: intravenous, CMV: cytomegalovirus, MBL: mannose-binding lectin, RR: relative risk

Table 5. Associations between composite immune biomarker scores and infections in solid organ transplant recipients

Year	Immune biomarkers	Organ type	Study design Follow-up (years)	Timing	Results
2003[49]	Lymphocyte subsets Mitogen-induced T cell proliferative responses Neutrophil phagocytic capacity and reactive oxygen species generation Score based on 1 point allocated if test less than 10th centile of healthy controls	Kidney (152) Simultaneous kidney/pancreas (14)	Retrospective 5	Score performed cross-sectionally > 12 months post-transplant	The patients with a higher immune biomarker score had a higher infection score
2005[50]	Lymphocyte subsets NK cell number T cell proliferation IgG, IgM, IgA Neutrophil phagocytic capacity and reactive oxygen species generation Score based on 1 point allocated if test less than 10th centile of healthy controls	Kidney (70)	Prospective 5	Score performed cross-sectionally > 6 months post-transplant	The score was the only risk factor that was associated with infection The score predicted major and opportunistic infections but not minor infections
2012[99]	NK cell number IgG, IgA, IgM Immunoglobulin subclasses, C3, C4 Score based on hazard ratios for each single immune test	Heart (133)	Prospective 1	Pre-transplant and day 7 and 30 and month 12.	Day 7 post-transplant low IgG2, NK cell number and C3 were predictors of infection after adjustment for total IgG levels
2014[109]	IgG C3 and C4 NK cell number CD4+ cell number	Heart	Prospective 100	Post-transplant	Score was predictive of severe infection
2016[110]	Positive CMV serology plus at least one of CD4+:CD8+ ratio < 1 and/or CD8+ count number > 700 cells/mm^3	Kidney (486)	Prospective 1	Pre-transplant	Score was predictive of both opportunistic infection and severe bacterial infection (HR 2.9 and 2.3, respectively)

Year	Immune biomarkers	Organ type	Study design Follow-up (years)	Timing	Results
2016[100]	Positive CMV serology plus at least one of CD4+:CD8+ < 1 and/or CD8+ cell number < 0.850 × 10^3 cells/ul	Kidney (435)	Prospective 1	Pre-transplant and various time points post-transplant	Score was not predictive of either OI or severe bacterial infection

NK: natural killer, CMV: cytomegalovirus, VZV: varicella-zoster virus, IV: intravenous, OI: opportunistic infection, PCP: *Pneumocystis jirovecii*, HR: hazard ratio, spp.: species



	Bacteria	Mycobacteria	Fungi	Viruses	Parasites
NK cells	+	++	++	+++	+
Monocytes/Mϕ /Granulocytes	+++	++	+++	++	+++
Complement	+++	+	+	+	+
T cells	++	+++	+++	+++	+++
B cells & Ig	+++	+	+	++	+

Figure 1. An overview of the relative contribution of components of the innate and adaptive immune system in control of infectious pathogens

This figure is based on data from case reports and cohort studies.

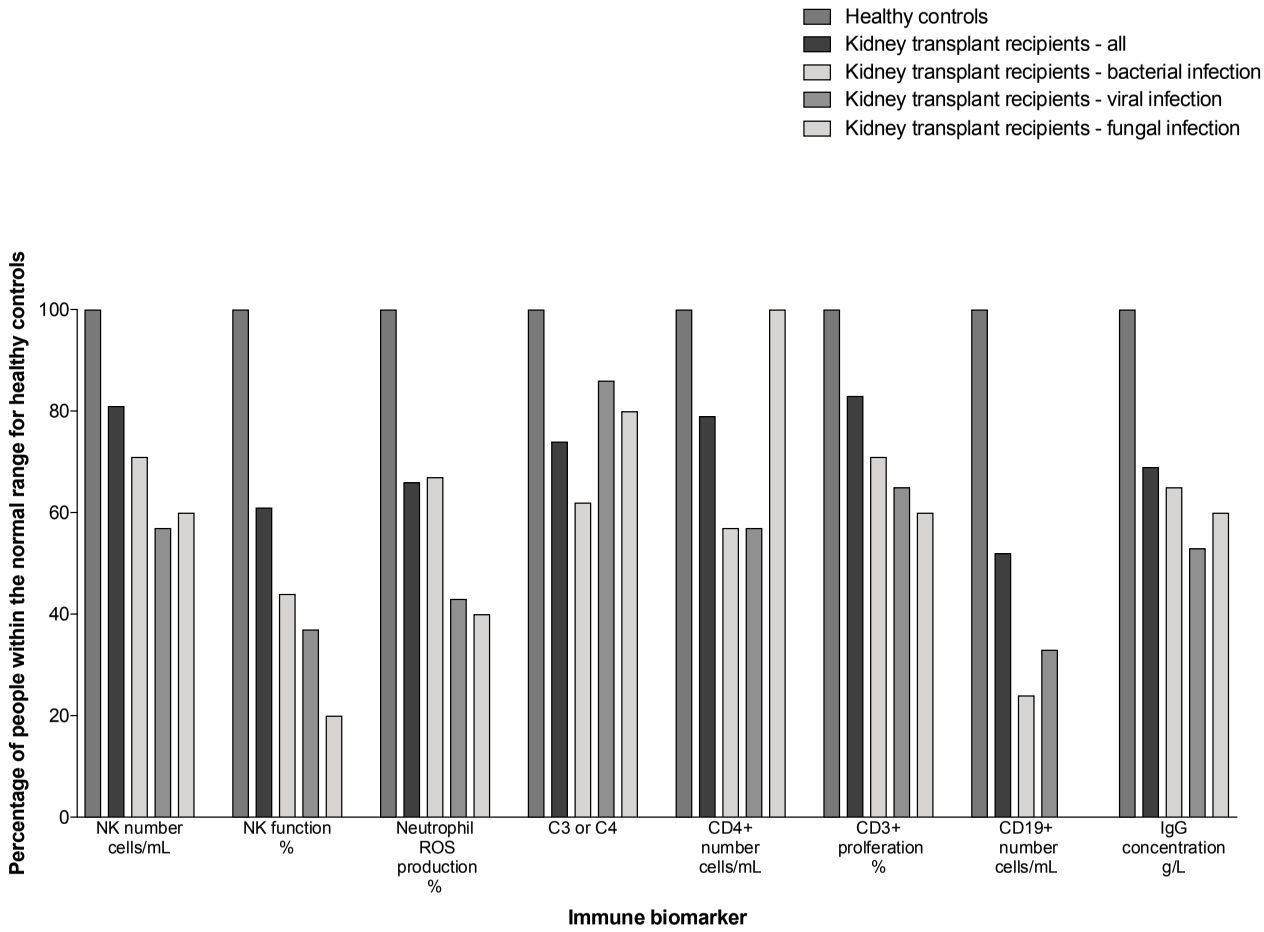


Figure 2. The relative impairment of immune biomarkers according to type of infection in a cohort of kidney transplant recipients

Biomarkers were tested at baseline in 168 stable kidney transplant recipients (median 4.1 years post-transplant) and patients were followed for two years for the development of infection. Any infection resulting in hospital admission whereby a causative microorganism was isolated were included. Infections were classified into bacterial, viral and fungal. Results for immune biomarkers were compared with a cohort of laboratory controls, who were healthy with no known infectious or inflammatory conditions. The immune biomarker was classified as reduced if the result was below the 5th percentile of healthy controls.

Chapter 2:
Natural killer cells and infections

2.1 Introduction

This section explores the association between natural killer (NK) cells and infectious outcomes in kidney transplant recipients.

NK cells are important effector cells of the innate immune system. They respond non-specifically to pathogens and kill with various methods including secretion of granzyme and perforin.

NK cells were selected to study because of their interesting and relevant association with infections. In 1978, Biron et al. published an observation in the *New England Journal of Medicine* that an adolescent with NK cell deficiency died of overwhelming herpes viral infection.[1] Primary deficiencies of natural killer cells are very rare but it is almost universally found that the patients suffer from severe and recurrent herpes viral and other viral infections.[2] They frequently succumb to virally driven malignancies such as lymphoma. Interestingly, kidney transplant patients are susceptible to the same spectrum of opportunistic infections and malignancies. Despite quite different underlying diseases, there is a striking similarity to the infectious phenotypes. For this reason, natural killer cell cytotoxic function was selected as a biomarker to study in relation to infections in transplant recipients.

In recent years there has been increasing interest in NK cell biology, in particular in the field of allograft rejection.[3] There have only been a few reports in solid organ transplant recipients about the spectrum of infections associated with NK cell deficiency.[4] There is emerging data regarding the role of peripheral blood NK subsets and the control of cytomegalovirus.[5] Future research could also focus on the relationship between polyoma virus (such as BK virus) and NK numbers and cytotoxic function.

The degree to which the iatrogenic immunosuppression used in transplantation affects NK number and function has been examined but the studies are small and the data inconclusive.[6,7] There are very few reports of the use of NK functional analysis and there are no studies to our knowledge about the association of NK cytotoxic function and infection.

The investigators wanted to determine whether measuring NK cell numbers, subsets and NK cytotoxic function could stratify patients at high risk of infection.

The hypothesis was that patients with reduced NK numbers and cytotoxic function would demonstrate an increased susceptibility to infection due to the importance of NK functional response in the control of viral pathogens commonly encountered in transplantation.

Section 2.2 in this chapter is represented by manuscript "Natural killer cell function predicts severe infection in kidney transplant recipients", published in the *American Journal of Transplantation* in 2018.

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2.2 Natural killer cell function predicts severe infection in kidney transplant recipients

HIGHLIGHTS

- 24% of stable kidney transplant recipients had NK cell cytotoxic function reduced below healthy controls.
- NK cytotoxic function predicted development of severe infection over two years of follow-up.
- The NK cytotoxic function area under the receiver operating curve for severe infection was 0.84 (95% CI 0.77–0.91).

PUBLICATION

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ORIGINAL ARTICLE

Natural killer cell function predicts severe infection in kidney transplant recipients

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The aim of this study was to determine if natural killer cell number ($CD3^-/CD16^{\pm}/CD56^{\pm}$) and cytotoxic killing function predicts severity and frequency of infection in kidney transplant recipients. A cohort of 168 kidney transplant recipients with stable graft function underwent assessment of natural killer cell number and functional killing capacity immediately prior to entry into this prospective study. Participants were followed for 2 years for development of severe infection, defined as hospitalization for infection. Area under receiver operating characteristic (AUROC) curves were used to evaluate the accuracy of natural killer cell number and function for predicting severe infection. Adjusted odds ratios were determined by logistic regression. Fifty-nine kidney transplant recipients (35%) developed severe infection and 7 (4%) died. Natural killer cell function was a better predictor of severe infection than natural killer cell number: AUROC 0.84 and 0.75, respectively ($P = .018$). Logistic regression demonstrated that after adjustment for age, transplant function, transplant duration, mycophenolate use, and increasing natural killer function (odds ratio [OR] 0.82, 95% confidence interval [CI] 0.74-0.90; $P < .0001$) but not natural killer number (OR 0.96, 95% CI 0.93-1.00; $P = .051$) remained significantly associated with a reduced likelihood of severe infection. Natural killer cell function predicts severe infection in kidney transplant recipients.

KEYWORDS

clinical research/practice, Cytomegalovirus (CMV), immunosuppression/immune modulation, infection and infectious agents, infection and infectious agents – bacterial, infection and infectious agents – viral: , infectious disease, kidney (allograft) function/dysfunction, kidney transplantation/nephrology, natural killer (NK) cells/NK receptors, translational research/science

1 | INTRODUCTION

Improvements in graft survival following solid organ transplantation have meant that the prevention of infection and malignancy have become a key focus in the care of transplant recipients.[1] Predicting the subset of transplant recipients at risk of infection is difficult using clinical factors alone and therefore requires biomarkers.[2] There is emerging evidence that natural killer (NK) cell quantification may be a predictor of infection in transplant recipients.[3-5] However, there are few studies in this area and none that examines the relationship of NK cell function with infection.

NK cells are innate immune cells that are capable of immediate defense against infective pathogens and cancer.[6] In transplantation,

Abbreviations: AUROC, area under the receiver operating characteristic; CMV, cytomegalovirus; EBV, Epstein-Barr virus; eGFR, estimated glomerular filtration rate; HPV, human papilloma virus; HSV, herpes simplex virus; IQR, interquartile range; LOIS, level of immunosuppression study; NK, natural killer; PBMC, peripheral blood mononuclear cell; TLF4, fluorescent target cell marker; VZV, varicella-zoster virus.

most current immunosuppressive medications target the adaptive immune system, allowing the innate immune system to have increasing importance in the protection against infections. NK cell subsets in the peripheral blood can be differentiated according to expression of surface molecules.[7-9] The 2 main subsets include $CD56^{bright}$NK cells, which represents an early stage of NK cell differentiation and lacks CD16 and $CD56^{dim}$NK cells, which represent a later stage differentiation and the majority are positive for CD16.[8] The $CD56^{bright}$ $CD16^{-}$ NK subset has unique receptor characteristics such as negativity of killer-cell immunoglobulin-like receptors and higher levels of CD94/NKG2A.[8] Functionally, the subsets have different roles. NK bright cells predominantly secrete cytokines such as interferon gamma and/or tumor necrosis factor and NK dim cells have a more prominent role in cytotoxicity.[9] These subsets also express different activating and inhibitory receptors.[7,8] Evidence suggests that NK subsets expressing $CD94/NKG2C^{bright}$ activating lectin-like receptor play a role in control of cytomegalovirus (CMV) infection in kidney transplant recipients.[10]

One of the most important immune functions of NK cells, learnt from observing patients with primary NK cell deficiency, is defense against herpesvirus infections.[11] NK cells recognize and respond to cells without MHC class 1.[6,11,12] The particular susceptibility to herpesviruses may relate to the fact that these viruses can downregulate MHC class 1 in order to evade host cytotoxic T cell response.[13] Other infections that are associated with NK cell deficiency include human papilloma virus, fungal, and mycobacterial infections.[3,7,13,14] Patients with primary NK cell deficiency are susceptible to virally driven malignancies.[11,15,16] Twenty-one percent of a cohort of patients with classical NK cell deficiency developed cancer, including an Epstein-Barr virus (EBV)–driven smooth muscle tumor, human papilloma virus (HPV)–associated cancers, and leukemia.[11,15-19]

The aim of this study is to determine whether NK cell function is a predictor of severe infection risk in kidney transplant recipients. Our hypothesis was that NK cell function predicts severe infection in kidney transplant recipients.

2 | MATERIALS AND METHODS

2.1 | Setting

In April 2015, the Level of Immunosuppression Study (LOIS) was commenced. This prospective cohort study was performed at Monash Health, a 1500-bed academic health service in Melbourne, Australia.

All kidney transplant recipients were offered the opportunity to have their immune parameters measured at study entry and were then followed for 2 years for episodes of infection and malignancy. Consenting participants underwent outpatient blood collection for NK cell number and functional testing.

To determine the incidence of infection, the investigators received notification from the treating physicians whenever a subject was admitted to the hospital, and they underwent clinical review by a specialist infectious diseases physician to determine the site of infection and to review the microbiological investigations.

2.2 | Inclusion and exclusion criteria

Kidney transplant recipients were eligible to participate if they were ≥18 years and had stable graft function for a minimum of 3 months (median 4 years). Kidney transplant recipients were excluded if they recently augmented immunosuppression to treat rejection or had an infectious illness just prior to or at the time of the study.

The study was approved by the human research ethics committee of Monash Health (Number 13085). Written informed consent was obtained from all participants.

2.3 | Data collection

Patient clinical and demographic details were assessed at enrollment.

2.4 | Laboratory methods

2.4.1 | NK cell number and percentage

Lymphocyte subset testing was performed on freshly collected peripheral blood. One hundred microliter aliquots of heparinized blood were labeled with appropriately titered monoclonal antibodies. Fluorescently labeled antibodies used in this study were obtained from Beckman Coulter and included combinations of CD3-APC, CD14-PE, CD16-FITC, CD56-PE, and CD45-PC7. Following incubation, red blood cells were lysed using the Beckman-Coulter Q-Prep system and acquired on a FC500 flow cytometer (Beckman Coulter, Brea, CA). NK cells were identified by the immunophenotype $CD3^{-}$/$CD16^{\pm}$/$CD56^{\pm}$ and percentages determined after gating on lymphocytes by forward and side-scatter characteristics. The following NK subsets were reported: $CD56^{+bright}CD16^{-}$, $CD56^{+dim}CD16^{+}$, $CD56^{+dim}CD16^{-}$, and $CD56^{-}CD16^{+}$. A representation of the gating strategy for NK cells is presented in Figure 1. Absolute numbers were calculated using the lymphocyte count provided by full blood examination.

2.4.2 | NK cell function

NK-cell function was calculated as the percent of target cells capable of cleaving Pantoxilux substrate. Peripheral blood mononuclear cells (PBMCs) were used as a source of NK cells and it was anticipated that cytotoxic activity would be lower than if isolated NK cells were used. PBMCs were isolated by density gradient centrifugation using Leucosep tubes pre-filled with Ficoll-Paque Plus (Greiner Bio-One, Austria). PBMCs were cryopreserved in liquid nitrogen. To measure NK cytotoxicity, PBMCs (effector cells) were exposed to a known quantity of target cells (K562 cells) and cytotoxicity was determined using a commercially available kit, Pantoxilux (OncoImmunin, Inc, Gaithersburg, MD). Target cell were K562 cells, a human immortalized myelogenous leukemia line

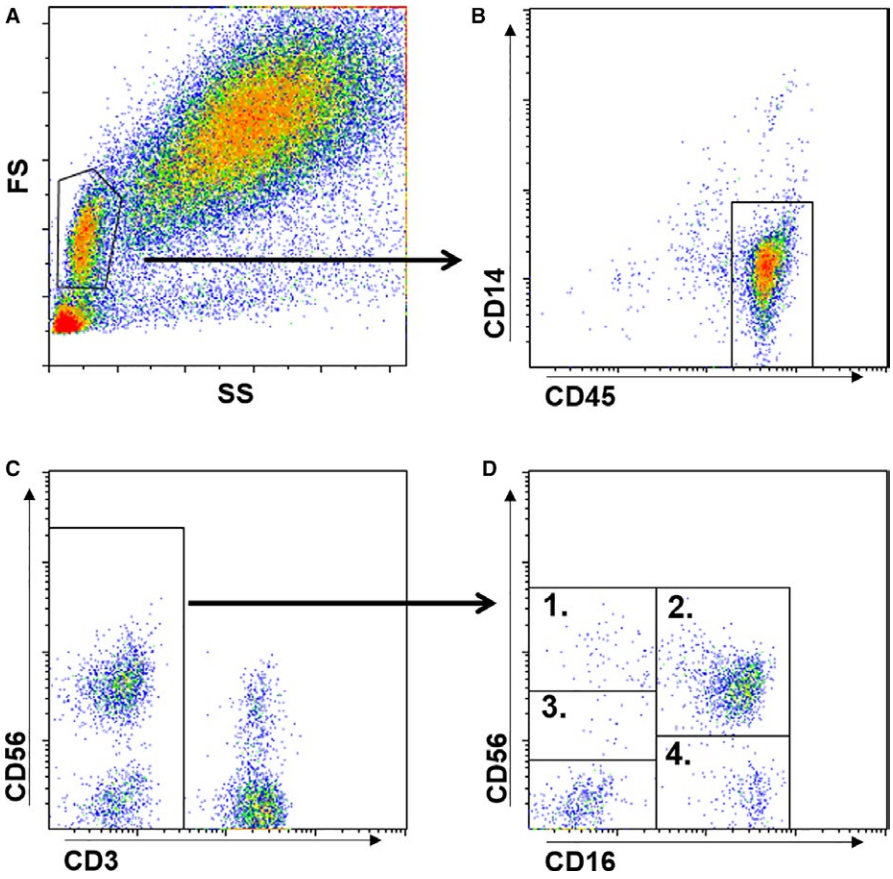

FIGURE 1 Representative flow cytometry plots from human peripheral blood mononuclear cells to demonstrate natural kill cell gating strategy. Natural killer cell subsets were identified by sequentially gating on lymphocytes (A), then excluding monocytes by gating on $CD14^{-}$ $CD45^{+}$ population (B), then CD3- expressing CD56 (C). NK cells were identified as $CD3^{-}$ lymphocytes that were (D) 1. $CD56^{bright}CD16^{-}$, 2. $CD56^{dim}CD16^{+}$, 3. $CD56^{dim}CD16^{-}$ and 4. $CD56^{-}CD16^{+}$

derived from a patient with chronic myelogenous leukemia patient in blast crisis. The cells can be killed by NK cells as they lack the MHC required to inhibit NK activity.[20] The Pantoxilux assay is based on the hydrolysis of a cell-permeable fluorogenic peptide substrate containing a sequence recognized by the serum protease Granzyme B and upstream caspase activity.[21] Following kit instructions, target cells were counted and resuspended in RPMI 1640 medium containing the fluorescent target cell marker (TLF4) and incubated at 37°C for 30 minutes and then washed twice with RPMI media. 2×10^4 TLF4 labelled target cells per well were co-cultured with effector PBMCs at different concentrations: 0, 5×10^5, 1×10^6, and 2×10^6 cells per well (effector-to-target ratios; 25:1, 50:1, and 100:1, respectively) together with Pantoxilux substrate (75 μL). Cells were incubated in 5% CO_2 at 37°C for 60 minutes. Co-cultured cells were spun down and washed in Pantoxilux wash buffer and resuspended in 250 μL of wash buffer for acquisition. Samples were acquired on a Navios flow cytometer (Beckman Coulter, Brea, CA) and data analyzed using Kaluza software (Beckman Coulter, Brea, CA).

NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells (NK cells) at a ratio of effector-to-target of 100:1.

Normal ranges for NK number and function were derived from cuts-offs using 10th to 90th percentile measurements from a random cohort of staff controls who were well, with no known medical, inflammatory, or infectious conditions.

2.4.3 | Clinical outcomes

Severe infection was defined as any infection requiring admission to hospital after the date of study enrollment. Details of all severe infections were collected. All infections reported in this study (including viral and urinary tract infections) were associated with hospitalization.

Infectious episodes were classified as microbiologically defined (whereby a microorganism related to the clinical presentation was isolated) or clinically defined (whereby no microorganism related to the clinical presentation was isolated but a clinical diagnosis of the site of infection could be determined by the study investigators).

CMV infection was defined as virus isolation, or detection of viral nucleic acid in any body fluid or tissue specimen. CMV disease was defined as the presence of appropriate clinical symptoms and/or signs required together with documentation of CMV in tissue from the relevant organ by histopathology, virus isolation, rapid culture, immunohistochemistry, or DNA hybridization.[22] Polyoma virus replication was defined by increasing polyoma viral loads. Probable polyoma virus disease was defined as viral replication $>10^4$ copies per mL or together with compatible symptoms and signs of viral syndrome or organ disease, but without histological confirmation. Proven polyoma virus disease was defined as evidence of virus replication plus corresponding specific histopathology.[23,24] Fungal infections was categorized as possible, probable, and proven, and bloodstream

infections and sepsis were defined by internationally recognized criteria.[25-27]

Each episode of infection was classified according to source. NK-associated infections or malignancies was a definition created by the authors. It is based on literature that suggests the types of organisms and malignancies to which patients with NK cell deficiency are susceptible. NK-associated infections were defined as any viral or fungal infection causing admission to hospital or probable or proven polyoma virus disease. NK-associated malignancies included any malignancies with a documented association with viral infections.[18] Details of the inclusions of the definition of NK-associated infections or malignancies are described in Table 1.

2.5 | Statistical analysis

We assumed an expected rate of severe infection of 20% based on previous data from our centre[28] and other data in the literature.[4,29] The minimum required sample size for an area under the receiver operating characteristic (AUROC) curve of 0.80 was 64 patients assuming a 90% power and an alpha at 0.05.

Categorical variables were summarized using frequency and percentage. Continuous variables were summarized using mean ± standard deviation or median and interquartile range (IQR), as appropriate. Categorical variables were compared using the chi-square test and continuous variables were compared using the Mann-Whitney *U* test.

The extent to which NK cell number influenced NK cell function was analyzed by calculating the ratio of the number of K562 cells per well to the number of NK cells/per well at an effector to target ratio of 100:1. This was defined as the absolute NK cell function.

The ability of baseline NK cell number and NK cell function to predict the first episode of severe infection within the study period was assessed using logistic regression. ROC curves were calculated from the logistic regression models for both baseline NK cell number and NK cell function and compared using the method from Hanley and McNeil.[30] Youden's index was determined to find the cut-off to maximize sensitivity and specificity. Variables were input into the multivariable logistic regression model if they had a *P*-value > 0.2 in the univariable logistic regression model. Age, time from transplant, mycophenolate use, and eGFR were included regardless of their *P*-value on the univariable analysis given the known influence of each factor on overall infection risk.[31,32] Adjustment for other factors was not performed due to the sample size. Statistical significance was set at a *P*-value of <.05. Analyses were conducted on STATA (Version 15, College Station, TX) and Graph Pad Prism (Version 7, La Jolla, CA).

TABLE 1 Definition of NK associated infections or malignancies

NK associated infections[7,11,20,45-48]
Viral infection
Proven or probable polyoma viral infection[a]
Mycobacterial infection
Fungal infection
NK-associated malignancies[11-15]
EBV-associated malignancies (Burkitt's lymphoma, immunoblastic lymphoma, posttransplant lymphoproliferative disease, smooth muscle tumor)
Polyoma virus–associated malignancies (Merkel cell carcinoma)
Human papilloma virus–associated malignancies (cervical and anogenital malignancy)

NK, Natural killer; EBV, Epstein-Barr virus.
[a]Polyoma virus may not require hospital admission.

3 | RESULTS

3.1 | Characteristics of kidney transplant recipients

One hundred sixty-eight of a possible 850 (20%) of kidney transplant recipients accepted the offer to participate in this study (Figure 2). All participants were followed for 24 months. The demographics details are presented in Table 2. The participants in this study were similar in age and other demographic details to the cohort of kidney transplant recipients at our institution.[33]

3.2 | NK cell number and function of healthy controls

Nineteen healthy controls underwent testing for NK cell number and function. The normal range for NK cell number among healthy controls was 61-776 cells/μL and NK cell percent was 7%-28%, based on the normal range for laboratory controls. The median percentage

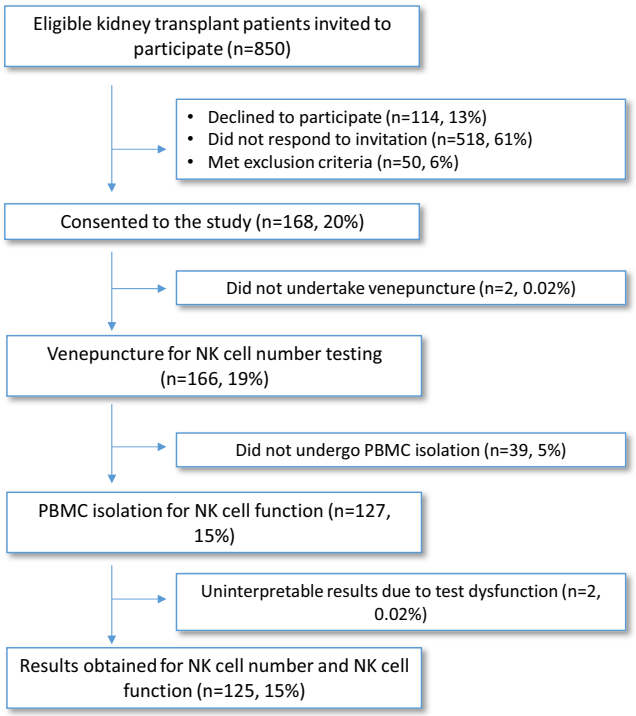

FIGURE 2 Flow chart of kidney transplant recipients

TABLE 2 Demographic, clinical, and immunological details of kidney transplant recipients

Characteristic		n (%) median (IQR)
Age (years)		54.6 (47.1-63.9)
Sex	Male	106 (63)
Ethnicity	Caucasian	106 (63)
	Asian	20 (12)
	Other	42 (25)
Cause of ESRF	Diabetes	37 (22)
	IgA	35 (21)
	Glomerulonephritis	31 (18)
	PCKD	18 (11)
	Reflux	15 (9)
	Other	32 (19)
No. of previous grafts	0	150 (89)
	≥1	18 (11)
Transplant duration (years)		4.1 (1.6-7.8)
Medications	Tacrolimus	137 (81)
	Mycophenolate	140 (83)
	Azathioprine	22 (13)
	Prednisolone	144 (86)
	mTORi	11 (6)
Tacrolimus level (μg/L)		4.6 (3.5-5.5)
Mycophenolate dose (mg/day)		1375 (1000-1500)
Serum creatinine μmol/L		113.0 (91.0-153.1)
eGFR mLs/min/m^3		54.9 (41.0-73.2)
CMV donor/recipient serostatus	D−R−	21 (12)
	D−R+	60 (36)
	D+R+	68 (40)
	D+R−	19 (11)
NK numbers and percent of total lymphocytes in the peripheral blood	$CD3^-56±16±$ number	140 (77-211)
	$CD3^-56±16±$ %	9 (6-15)
	$CD3^-16^-56^{+bright}$ number	11 (6.35-20)
	$CD3^-16^-56^{+bright}$ %	1 (1-1)
	$CD3^-56^{+dim}16^+$ number	106 (47-167)
	$CD3^-56^{+dim}16^+$ %	7 (4-12)
	$CD3^-56^{+dim}16^-$ number	1 (1-2)
	$CD3^-56^{+dim}16^-$ %	0 (0-0)
	$CD3^-16^-56^+$ number	17 (11-26)
	$CD3^-16^+56^-$ %	1 (1-1)
NK cytotoxic function (% cytotoxicity)	100:1	
Trimethoprim/sulfamethoxazole		62 (37)

IQR, interquartile range; ESRF, end-stage renal failure; IgA, IgA nephropathy; PCKD, polycystic kidney disease; mTORi, mammalian target of rapamycin inhibitor; eGFR, estimated glomerular filtration rate; CMV, cytomegalovirus; D, donor; R, recipient.

cytotoxicity with an effector to target ratio of 100:1 was 21.6% (IQR 13.7-30.4). The 10th percentile was 11.6% and the 90th percentile was 39.2%.

3.3 | NK number and function of kidney transplant recipients

The median NK cell number was 140 cells/μL (IQR 77-211.0) and the median NK cell percentage was 9.0% (IQR 6.0-15.0). There were 31 participants (19%) who had an NK cell number below the normal range for healthy controls.

Percentage NK cytotoxicity activity increased dose dependently and was maximal at an effector-to-target ratio of 100:1. For participants, the median percentage cytotoxicity for an effector-to-target ratio of 100:1 was 13.4 (IQR 9.4-18.8); this was significantly lower than that for healthy controls ($P = .0009$) (Figure 3).

One hundred twenty-six participants had both NK number and function tested. Using the cut-offs for healthy controls, 62 participants (47%) had NK number and function within the normal range; 16 (13%) had normal NK number but reduced function and 37 (29%) had reduced NK number but normal NK function. Eleven participants (9%) had both reduced NK number and function.

3.4 | Description of severe infections

Fifty-nine of the 168 participants (35%) had at least one severe infective episode, with a total of 141 episodes during the 24 months follow-up. Overall, 23 (39%) experienced 3 or more infections in the 24-month follow-up period.

Of the 141 episodes of severe infection, 68 (48.2%) were microbiologically proven, 72 (51.1%) were clinically defined, and 1 (0.7%) was fever without focus.

The microbiology of the severe infections is summarized in Table 3. Of the 141 severe infections, 33 (48%) were bacterial, 29 (43%) were viral, 5 (7%) were fungal, and 1 (2%) was parasitic. There were 4 proven fungal infections (3 cases of invasive pulmonary aspergillosis and one case of disseminated cryptococcosis), one probable fungal infection (*Pneumocystis jiroveci* pneumonia [PJP]).

The most common source of infection was respiratory, accounting for 53 (38%) of severe infections. There were 8 episodes (6%) of bloodstream infection and 10 episodes (7%) of sepsis.

Forty-eight participants (29%) developed NK-associated infection or malignancy. There were 9 episodes of herpesvirus infections (6 CMV, 1 EBV, 1 VZV, and 1 HSV), 40 probable or proven polyoma virus infections, 16 respiratory virus infections, and 5 invasive fungal infections. Three participants developed virally driven malignancy. One participant developed posttransplantation lymphoproliferative disorder, which is associated with latent EBV infection in the setting of immunosuppression. One patient developed Merkel cell carcinoma, which is associated with polyoma virus infection and another participant developed cervical intraepithelial neoplasia, which is associated with HPV infection.

Seven participants (9%) died during the follow-up period, 4 from malignancy and 3 from infection.

Table 4 compares the clinical characteristics and other immune cells in infected and uninfected patients.

3.5 | NK cell number, NK cell function, and severe infection

Figure 4 illustrates NK cell number and function between participants who developed infection and those who did not. Median NK cell number at entry into the study was lower in participants who developed severe infection compared to those who did not (P = .026, Figure 4A). Likewise, the proportion of NK targeted killing of K562 cells at entry into the study was lower in those who developed severe infection (P ≤.0001, Figure 4B).

When considering the influence of NK cell number on NK cell function by assessing the absolute NK cell function (ratio of killed K562 cells per well to the absolute number of NK cells per well), participants with severe infection had significantly lower absolute NK cell cytotoxic activity compared to those without infection (P = .044, Figure 4C).

3.6 | Prediction of a severe infection

Results of the logistic regression models are presented in Table 5. On univariate analysis both increasing NK cell function (odds ratio [OR] 0.83 per 1% increase 95% confidence interval [CI] 0.76-0.91, P < .0001) and number (OR 0.96, 95% CI 0.93-0.99, P = .049) were associated with a lower likelihood of severe infection over the 24-month follow-up. After adjustment for age, renal function (eGFR), mycophenolate use and transplant duration, increasing NK function (OR 0.82, 95% CI 0.74-0.90, P < .0001), but not NK number (OR 0.96, 95% CI 0.93-1.00, P = .051) remained significantly associated with a reduced likelihood of severe infection.

Figure 5A presents the ROC curves for NK cell number and NK function derived from the adjusted models described above. The model incorporating NK cell number demonstrated moderate predictive ability with an AUROC curve of 0.75 (95% CI 0.67-0.84).

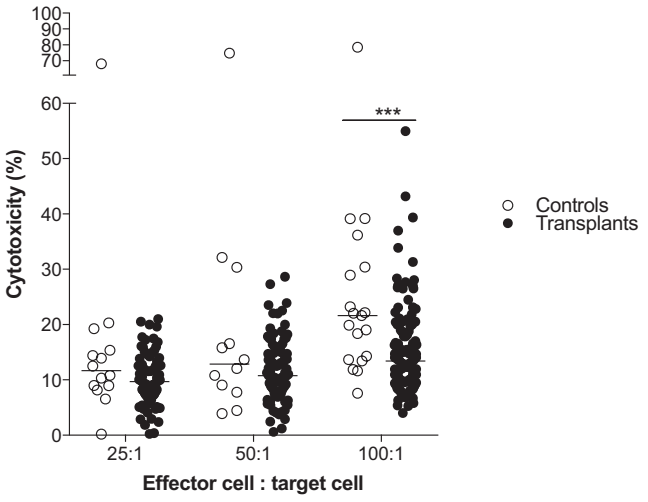

FIGURE 3 Percentage cytotoxicity according to effector cell-to-target cell ratios in healthy controls and kidney transplant recipients. Bars represent medians. Mann-Whitney test was used to compare healthy controls and kidney transplant recipients at each of the 3 target-to-effector ratios

TABLE 3 Microbiology of severe infections

Organism	Number	Site
Bacterial infection		
Staphylococcus aureus	3	Wound (3)
Staphylococcus epidermidis	2	BSI (2)
Streptococcus pneumoniae	1	BSI (1)
Streptococcus agalactiae	1	MSU (1)
Enterococcus faecium	2	MSU (2)
Escherichia coli	14	BSI (4), sputum (2), urine (14)
Klebsiella spp.	1	Urine (1)
Serratia spp.	1	BSI (1)
Morganella spp.	1	Urine (1)
Pseudomonas spp.	2	Urine (2), sputum (1)
Campylobacter spp.	2	Faeces (2)
Shigella spp.	1	Faeces (1)
Nocardia spp.	1	BAL (1)
Viral infection		
Adenovirus	1	Blood (1), urine (1)
Cytomegalovirus	7	Blood (7), BAL (1)
Influenza	9	NPA (9)
Picornavirus	4	NPA (4)
Respiratory syncytial virus	3	NPA (3)
Human metapneumovirus	1	NPA (1)
Herpes simplex virus 1	1	CSF (1)
Varicella-zoster virus	1	Wound (1)
Epstein-Barr virus	3	Blood (3), CSF (1)
Fungal infection		
Aspergillus spp.	3	BAL culture (3)
Cryptococcus spp.	1	Skin biopsy (1), Blood (1),CSF (1)
Pneumocystis jiroveci	1	BAL PCR (1)
Parasitic infection		
Microsporidia spp.	1	Kidney biopsy (1)

BSI, bloodstream infection; MSU, mid-stream urine; BAL, bronchoalveolar lavage; NPA, nasopharyngeal aspirate; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; spp, species.

TABLE 4 Comparison of the clinical characteristics and immune tests in patients with and without severe infection

Characteristic		No severe infection n (%) median (IQR)	Severe infection	*P*-value
Age (years)		55.7 (46.9-63.1)	57.9 (48.6-65.1)	.262
Sex	Male	67 (61)	39 (66)	.553
Ethnicity	Caucasian	73 (67)	33 (56)	
	Asian	14 (13)	3 (5)	
	Other	22 (20)	23 (39)	
Cause of ESRF	Diabetes	22 (20)	15 (25)	.233
	Glomerulonephritis	33 (30)	20 (34)	
	PCKD	11 (11)	7 (12)	
	Reflux	10 (9)	5 (8)	
	Other	33 (30)	12 (20)	
No. of previous grafts	0	96 (88)	54 (92)	.537
	≥1	13 (12)	5 (8)	
Transplant duration (years)		4.0 (1.7-8.3)	3.7 (1.1-7.5)	.261
Immunosuppressive regimen of mycophenolate plus tacrolimus plus prednisolone		69 (63)	44 (74)	.137
Medications	Tacrolimus	88 (81)	49 (83)	
	Mycophenolate	86 (79)	54 (91)	.249
	Azathioprine			
	Prednisolone	92 (84)	52 (88)	
	mTORi	8 (7)	3 (5)	
Tacrolimus level (μg/L)		4.6 (3.9-5.5)	5.1 (3.9-5.7)	.373
Mycophenolate dose (mg/day)		1500 (1000-1500)	1000 (1000-1500)	.249
eGFR mLs/min/m^3		58.6 (45.5-76.2)	44.5 (27.3-65.0)	**.002**
CMV donor/recipient serostatus	D−R−	16 (15)	5 (8)	.466
	D−R+	35 (35)	25 (42)	
	D+R+	41 (38)	27 (46)	
	D+R−	17 (16)	2 (3)	
NK numbers and percent of total lymphocytes in the peripheral blood	$CD3^-56±16±$ number	143 (97-226)	112 (54-194)	**.045**
	$CD3^-56±16±$ percent of lymphocytes in the peripheral blood	9 (6-14)	10 (6-15)	.962
	$CD3^-56^{+bright}16^-$ number	13 (7-21)	10 (6-14)	.373
	$CD3^-56^{+bright}16^-$ %	1 (1-1)	1 (0-1)	.065
	$CD3^-56^{+dim}16^+$ number	114 (52-169)	101 (30-162)	.505
	$CD3^-56^{+dim}16^+$ %	7 (4-12)	7 (3-13)	.840
	$CD3^-56^{+dim}$ 16^- number	1 (0-1)	1 (1-2)	.438
	$CD3^-56^{+dim}$ 16^- %	0 (0-0)	0 (0-0)	.942
	$CD3^-56^+16^-$ number	18 (11-28)	16 (10-23)	.429
	$CD3^-56^-16^+$ %	1 (1-2)	1 (1-2)	.663
NK cytotoxic function (% cytotoxicity)	K562 alone	15.6 (12.1-20.1)	9.8 (8.2-13.1)	<.0001

IQR, interquartile range; ESRF, end-stage renal failure; IgA, IgA nephropathy; PCKD, polycystic kidney disease; mTORi, mammalian target of rapamycin inhibitor; eGFR, estimated glomerular filtration rate; CMV, cytomegalovirus; D, donor; R, recipient.

The model incorporating NK cell function as opposed to NK number demonstrated improved model discrimination (AUROC curve 0.84 [95% CI 0.77-0.91, *P* = .0183] for the difference between the 2 AUCs).

For NK cell function, using the unadjusted model, the most appropriate cut-off to maximize sensitivity and specificity was 13.5%. The sensitivity and specificity of this cut-off value for predicting the occurrence of severe infection were 80% and 67%, respectively.

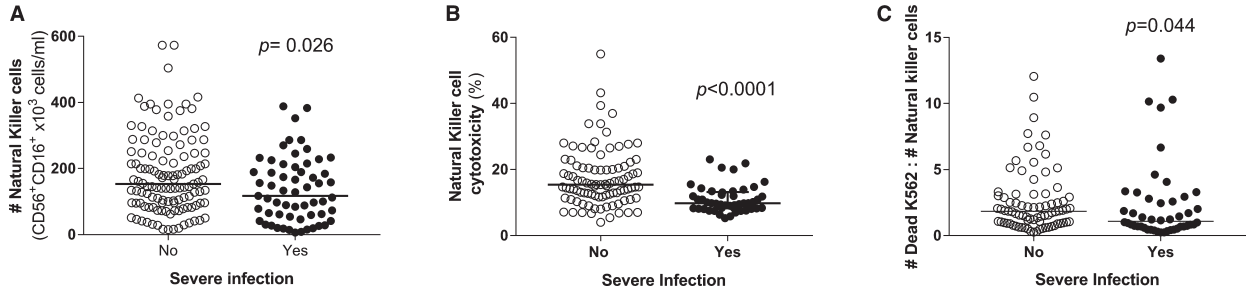

FIGURE 4 Study entry (A) NK cell number, (B) NK cell function, (C) absolute NK cell function (ratio of the absolute number of killed K562 cells per well to the absolute number of NK cells per well). Mann-Whitney test was used to compare kidney transplant recipients with and without infection for NK number, NK function and absolute NK function

TABLE 5 Regression analysis for predictors of a first severe infectious episode

	Univariable			Multivariable		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
NK cell number model n = 126						
Age (per 10 years)	1.10	0.83-1.45	.510	0.99	0.69-1.40	.968
Sex	0.81	0.37-1.73	.581	—	—	—
Transplant duration (per year)	0.94	0.86-1.02	.173	0.94	0.85-1.03	.226
Number of previous grafts	0.71	0.34-1.48	.368	—	—	—
Mycophenolate	3.53	0.97-12.84	.056	3.88	0.98-15.33	.540
eGFR (per mL/min/m^3)	0.71	0.60-0.86	<.0001	0.69	0.56-0.86	.001
NK cell number (per 10 cells/μL)	0.96	0.93-0.99	.049	0.96	0.91-1.00	.051
NK cell function[a] model n = 126						
Age (per 10 years)	1.10	0.83-1.45	.510	0.99	0.95-1.02	.579
Sex	0.81	0.37-1.73	.581	—	—	—
Transplant duration (per year)	0.94	0.86-1.02	.173	0.95	0.86-1.05	.354
Number of previous grafts	0.71	0.34-1.48	.368	—	—	—
Mycophenolate	3.53	0.97-12.84	.056	4.12	0.86-19.72	.076
eGFR (per mL/min/m^3)	0.71	0.60-0.86	<.0001	0.67	0.54-0.83	<.0001
NK cell function (per 1% increase)	0.83	0.76-0.91	<.0001	0.82	0.74-0.90	<.0001

OR, odds ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate; NK, natural killer cell.
[a]NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells at a ratio of effector-to-target of 100:1.

Figure 5B illustrates the probability of developing severe infection relative to NK cell function.

Of the 46 participants (36%) with NK cell function ≤13.5% of K562 target cells that were dead following 60-minute incubation with effector cells (NK cells) at a ratio of effector-to-target of 100:1, 37 (58%) developed severe infections compared with 9 (14%) with NK cell function >13.6% ($P < .0001$, Figure 5C).

4 | DISCUSSION

The key finding of this study was that reduced NK cell function was an independent predictor of severe infection in kidney transplant recipients. We identified that participants with NK cell function below 13.5% were at a higher risk of severe infection compared to those with NK cell function above this cut-off. This is the first study, to our knowledge, in

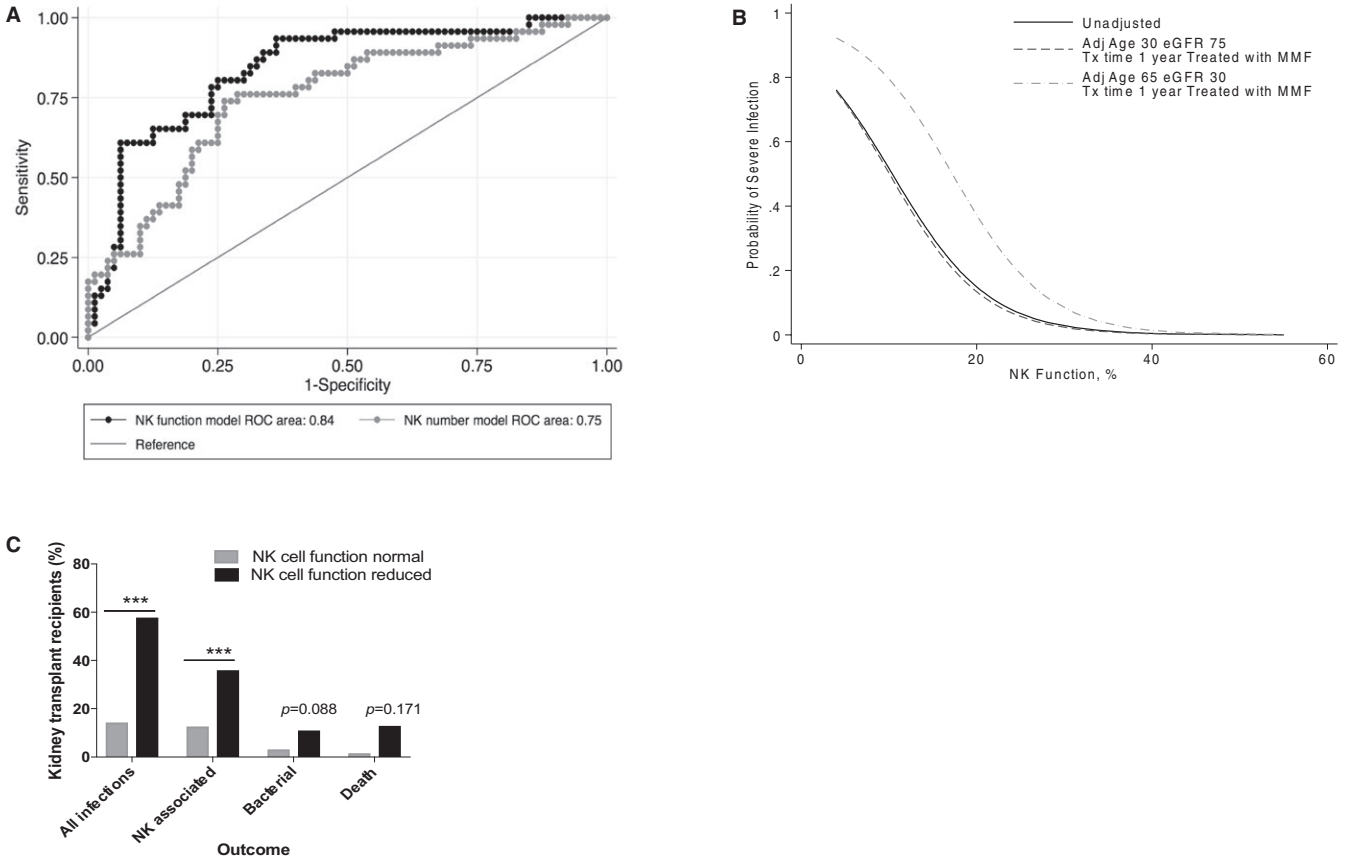

FIGURE 5 NK cell number and cytotoxic function and the association with infection. (A) Comparison of the ROC curve of the NK cell function model (NK cell function, age, time from transplant, mycophenolate used, and eGFR) and the NK cell number model (NK cell number, age, time from transplant, mycophenolate use, and eGFR) as a test to predict the development of severe infection in the following 24 months (P = .0183). (B) The probability of developing severe infection relative to NK cell function. Overall, a steep increase in the probability of severe infection occurs once NK cell function falls below approximately 20%. Two theoretical patients are illustrated on this graph. For an older patient with poor renal function, the curve is shifted to the right, demonstrating that the probability of severe infection occurs at higher levels of NK function. The opposite is true in younger patients with better renal function (probability curve shifts to the left). (C) Outcomes of severe infections and death over the 2-year period according to whether NK cell function as normal or reduced. NK cytotoxic function was reported as the percentage of K562 target cells that were dead following 60-minute incubation with effector cells at a ratio of effector: target of 100:1. Patients with an NK cytotoxic function below 13.5% were defined as having reduced NK function. NK-associated infections were defined as any viral or fungal infection causing admission to hospital or probable or proven polyoma virus disease. NK-associated malignancies included any malignancies with a documented association with viral infections. ***P < .002

solid organ transplant recipients that have specifically examined the use of NK cell function to predict infection. Impairment of NK cell function by iatrogenic immunosuppression has been examined in animal and in vitro models with conflicting results. Some studies have suggested minimal effects of calcineurin inhibitors on NK cell function,[34-36] whereas others have suggested dose dependent inhibition.[35,37,38]

NK cells are capable of several effector functions, the most important of which is the ability to mediate contact-dependent killing of target cells. This process is mediated by lytic granules contained within the NK cells that contain the pore-forming molecule perforin and death-inducing enzymes, such as granzymes.[39] The lytic granules are deposited at the interface of the NK cell with target cells resulting in cytotoxic killing. NK cells also release inflammatory cytokines (such as interferon gamma) to amplify the immune response.[40] The Pantoxilux assay measures cytotoxicity by Granzyme B and caspase activity. NK cells express Fas ligand, which when cross-linked by Fas on K562 cells results in granzyme B release by degranulation, which in turn activates caspase to induce apoptosis.[41,42] NK-induced cytotoxicity is dependent on both phenotypic and functional characteristics of NK cells.[42] The amount of granzyme and caspase produced by NK cells is dependent on the interaction of activating and inhibitory receptors on the NK cell surface.[6] In this study, we did not phenotype the NK cells; therefore the degree to which phenotype has influenced cytotoxicity is unknown.

We demonstrated that NK cell function was superior to NK cell number in the ability to predict infection. Although there is no literature on NK cell function as a predictor of infection, several studies have found NK cell number to be predictive, either independently or as part of a composite immune score.[3,4,28,29,43-45]A recent study examined NK cell number 1-month posttransplant to predict CMV disease and opportunistic infections in 92 liver transplant recipients. NK cell number at 1 month was a better predictor of opportunistic

infection compared with $CD3^+$, $CD4^+$, and $CD8^+$ cell number. In the multivariate models, an NK cell count of 0.05×10^3 cells/μL at 1-month posttransplant was an independent risk factor for CMV disease and opportunistic infection.[46] Another study found that NK cell number 7 days posttransplant was an independent predictor of infection in heart transplant recipients, after adjustment of total IgG.[4] Our findings cannot be directly compared with these studies, which were performed in the first posttransplant year (compared with a median duration of 4 years posttransplant in our study), in different organ transplant recipients and using different definitions of infection.

Reduced NK function was associated with an increased risk of all severe infections, NK-associated infections and malignancies, but not bacterial infections. The lack of association of NK cell function with bacterial infection is consistent with the observation that bacteria are infrequent pathogens in those with primary NK cell deficiency.[11] In solid organ transplant recipients, Fernandez-Ruiz et al also found that although reduced NK cell number predicted viral and fungal infections, it did not predict bacterial infections.[3] In our study there were too few invasive fungal infections to perform a subgroup analysis; however, NK cell number has been demonstrated to predict fungal infection in hematopoietic stem cell transplant[5] and solid organ transplant recipients.[3] Of interest, in our study, the NK bright subsets ($CD56^{bright}CD16^-$) were lower in patients who developed infection compared to those who did not, and this was approaching statistical significance ($P = .065$). This is consistent with emerging evidence that certain NK subsets are involved in control of infections such as CMV, as well as signatures of alloreactive humoral responses in kidney transplant recipients.[8,10,47]

In this study, 24% of participants developed the composite endpoint of NK-associated infections or malignancies. This definition was designed by the authors due to the paucity of literature describing the spectrum of infections and malignancies in patients with acquired NK deficiency.

In this study, eGFR was a predictor of severe infection. We found that older participants with poor renal function had reduced NK cell function and an increased probability of infection than younger participants with good renal function. This reinforces previous literature showing that advancing age and reduced graft function increases the risk of infection,[31,32] and suggests that poor NK function may be a factor associated with this increased risk.

This study was performed at a single transplant center, which may limit its generalizability. Although less than half of the infections were microbiologically proven, all patients admitted with infection were reviewed by an infectious diseases physician to confirm the diagnosis of severe infection. All patients with a severe infection received inpatient antimicrobial therapy, so it is unlikely that the number of clinically relevant infections were overestimated. It is possible, however, that patients developed infections that did not require admission such as labial herpes or shingles, and therefore the burden of infection may have been underestimated. PBMCs were used as a source of NK cells, rather than purified NK cells. This may influence results in that the proportions of NK cells in each assay. To accurately measure NK cytotoxic activity, we considered the fact that comparison of cytotoxicity among participants' PBMCs must consider both the number of NK cells and the functional capacity. To do this, we report cytotoxic capacity on target cells on a per cell basis as well as total circulating numbers of NK cells. PBMC killing of K562 target cells predominately reflects NK cell cytotoxicity, as other PBMCs have little or no cytotoxicity for K562 cells.[42] This is because K562 cells do not express MHC, and the observation that monocytes require activation and/or prolonged co-incubation (18 hours) rather than the 1 hour required by NK cells as used in this study.[48] This study did not measure cytokine secretion, only cytotoxicity.[7] In this study, we did not analyze the relationship between NK number and function and other immune cells (such as T cell number and function). Future research could examine the interaction between various measured components of the immune system, NK cell function, and the relationship with infection. The authors acknowledge that although the definition of NK-associated infection or malignancies contains conditions associated with NK cell deficiency, there are also conditions associated with excess immunosuppression. This definition was applied in a priori study and we believe this categorization is useful in trial design of targeted prophylactic strategies in patient with isolated NK cell deficiency.

NK cell function may predict severe infection in patients, allowing its potential use as a biomarker to identify the subset of transplant recipients at risk for infection. These findings should be validated in different transplant settings and other transplant organ groups and to correlate NK cells with other cell types; however, it would be important to develop standardized protocols for NK cytotoxic testing. The potential implications of finding reduced NK function may lead to enhanced antiviral prophylaxis, targeted vaccinations, intensified monitoring for viral infections, and tailoring of immunosuppressive regimens; however, further research is required. NK functional assays are relatively simple to perform and interpret and may represent an important strategy to identify and manage those transplant patients at risk of infection.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Chapter 3:
Humoral immune biomarkers, vaccination and infections

3.1 Introduction

This section explores the association between biomarkers of the humoral immune system and infectious outcomes in kidney transplant recipients.

High quality evidence data describes an association between severe hypogammaglobinaemia and infection is SOT recipients[1]; however, other than this, there are very limited studies examining other measures of humoral competence and their association with infection. For example, there are no studies in SOT recipients that correlate measurement of B cell subsets with infection or studies that examine vaccine responses and infection.

There are multiple reasons to study vaccination responses in SOT recipients: firstly, to determine the efficacy of vaccination for protection against clinical infection; secondly, to determine the risk of side effects, in particular rejection; and thirdly, to determine whether vaccine responses can quantify the patient's degree of immunosuppression. Vaccine responses are a well-described diagnostic test for humoral competence and are utilised clinically in the diagnosis of primary immune deficiency.[2] Their use in secondary immune deficiency is less well studied. The predominant vaccine for this purpose is the pneumococcal polysaccharide vaccine, which is a pure B cell vaccine.[2]

The investigators wanted to quantify seroresponses to vaccines that were recommended as standard of care for transplant recipients and determine whether responses to these vaccines offered any ability to predict infections in the future. The hypothesis was that if a patient is unable to mount an adequate seroresponse to vaccine antigen then it is possible they would be at higher risk of infection compared with those that were able to respond. Therefore, this section focuses on two clinically relevant vaccines: seasonal influenza vaccination and pneumococcal vaccination. The conjugate pneumococcal vaccine was of particular interest as this vaccine is specifically designed to have increased immunogenicity, which is relevant to immunocompromised SOT

recipients.[2] There is no data that examines the efficacy and safety of this vaccine in kidney transplant recipients.

The sections in this chapter are represented by manuscripts that have been published or submitted for publication, as outlined below.

Section 3.2 describes the association between three humoral biomarkers (seroresponses to influenza vaccination, CD19+ cell number and immunoglobulin concentrations) and sino-pulmonary infections. The outcome of hospital admission with sino-pulmonary infection was selected as the primary outcome due to the known association with deficiencies of humoral immunity[3] and the that fact it is a highly relevant clinical event.

Section 3.3 is a systematic review and meta-analysis of influenza vaccination and the association with adverse immunologic sequelae in SOT recipients.

Section 3.4 is a literature review of pneumococcal vaccination in SOT recipients.

Section 3.5 is a sub-study whereby 45 patients in the kidney transplant recipient cohort were vaccinated with 13-valent pneumococcal conjugate vaccine and followed up for seroresponses. A subset of patients underwent testing for the development de novo of HLA antibodies. This study was published in 2018 in *Transplant Infectious Diseases*. We did not analyse whether seroresponses to 13-valent pneumococcal vaccine were associated with all-cause infection due to the small numbers vaccinated.

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3.2 Measurement of humoral immune competence and the risk of sino-pulmonary infections in a cohort of kidney transplant recipients

HIGHLIGHTS

- Over two years, 31 (18%) kidney transplant recipients developed sino-pulmonary infections.
- Reduced B cell numbers and reduced immunoglobulin concentrations were common.
- Reduced B cells were associated with sino-pulmonary infections over two years of follow-up.
- Seroresponses to influenza vaccination were poor for all influenza vaccine strains.
- Seroresponses to influenza vaccination were not associated with sino-pulmonary infections.

SUBMITTED MANUSCRIPT

Dendle C, Stuart RL, Mulley WR, Polkinghorne KR, Gan PY, Kanellis J, Ngui J, Laurie K, Thursky K, Leung V, Holdsworth SR. Measurement of humoral immune competence and the risk of sino-pulmonary infections in a cohort of kidney transplant recipients.

In press December 2018 – *Transplant Proceedings*

ABSTRACT

Introduction: The aim of this study was to determine if measurement of B cell protective immunity was associated with susceptibility to sino-pulmonary infection in kidney transplant recipients.

Methods: A prospective cohort of 168 patients with stable graft function (median 4.1 years) underwent assessment of CD19+ cell number, IgG concentration and seroresponses to influenza vaccination upon study entry. Patients received a single dose of trivalent, seasonal influenza vaccine.

Results: After two years follow-up, 31 (18%) patients developed sino-pulmonary infections. CD19+ cell number was strongly associated with future sino-pulmonary infections. A higher proportion of patients with CD19+ cell counts below the 5th percentile for healthy controls developed sino-pulmonary infections than those above the 5th percentile: 30% (23/77) compared with 9% (7/79), p = 0.001. There was a trend towards a higher proportion of patients with reduced IgG concentrations developing infections than in the normal range for healthy controls: 29%(14/48) compared with 15% (16/108), p = 0.060. Influenza vaccination seroresponses were poor in patients and controls, such that they could not be used to identify a subgroup of patients at high risk for the development of severe pulmonary infection.

Conclusions: Monitoring B cell numbers represents a simple, inexpensive means of stratifying kidney transplant recipients' risk of sino-pulmonary infection.

MANUSCRIPT

Introduction

Sino-pulmonary infections are a frequent cause of morbidity and mortality among kidney transplant recipients. Deficiencies of humoral immunity are a recognised risk factor for sino-pulmonary infection.[1,2] Vaccine responses can be used as a diagnostic test for humoral immune competence,[3] and although many transplant recipients receive annual influenza vaccination, it has rarely been utilised for this purpose. The aim of this study was to determine if three components of humoral protective immunity (CD19+ cell numbers, concentrations of IgG and seroresponses to influenza vaccination) were associated with susceptibility to sino-pulmonary infection in kidney transplant recipients.

Materials and methods

A prospective cohort study was performed in Melbourne, Australia, in which 168 kidney transplant recipients underwent peripheral blood testing at study entry for CD19+ cell number, immunoglobulin concentration and influenza serology. Patients received a single intramuscular dose of the 2015 southern hemisphere seasonal trivalent influenza vaccine containing 15 μg hemagglutinin of each component: A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2) and B/Phuket/3073/2013 (B Yamagata lineage) (Vaxigrip, Sanofi Pasteur). Seroresponses to vaccination were assessed one-month post-influenza vaccination. Patients were followed from baseline testing for two years for the development of severe sino-pulmonary infections (defined as infection of the upper or lower respiratory tract requiring admission to hospital).

Antibody titres were measured using hemagglutination inhibition (HI) assays for antibodies against the vaccine influenza strains.[4-6] The normal ranges for influenza seroresponses were based on those of 149 healthy controls who received the same vaccine during the same time period as the study patients. The normal range for total

white cell number and B cell numbers were based on the 5th to 95th percentile of healthy controls.

Seroprotection for influenza vaccine was defined as an HI titre ≥ 40 for a specific strain. Seroconversion post-vaccination was defined as a ≥ four-fold rise in HI titre and a post-vaccination titre ≥ 40.[4,5]

The study was approved by the human research ethics committee of Monash Health (Number 13085). Written informed consent was obtained from all participants.

Results

Characteristics of kidney transplant recipients

The median age was 56.2 years, 106 (63%) were male and median time from transplant was 4.1 years. One hundred and-thirty-seven (81%) recipients were receiving tacrolimus, mycophenolate and prednisolone maintenance immunosuppression.

The median CD19+ cell number was 103 cells/μL (IQR 61–212), with 88 (48%) patients below the 5th percentile for healthy controls. The median IgG was 8.79 g/L (IQR 7.15–15.6) with 48 (31%) patients below the 5th percentile for healthy controls.

Seroresponses to influenza vaccination are presented in Table 1. Overall, the seroconversion rates for patients were poor. Only five (4%) patients seroconverted to all three influenza strains, while 56 (48%) failed to seroconvert to any strains.

Sino-pulmonary infection

There were 53 severe sino-pulmonary infections in 31 (18%) patients. Fourteen patients (8%) experienced two or more severe sino-pulmonary infections. The median length of stay was seven days (range 1–84). Nine (17%) patients were admitted to intensive care, nine (17%) required life support and one patient (2%) died from their infection.

Association between humoral biomarkers and sino-pulmonary infection

Patients who developed sino-pulmonary infection had a lower CD19+ cell number at study entry (67 [40–109] cells/mL vs 117 [IQR 67–235] cells/mL, $p = 0.001$) and a greater proportion with a CD19+ count below the 5th percentile for healthy controls developed sino-pulmonary infection compared with those above (30% [23 of 77] vs 9% [7 of 79], $p = 0.001$). A greater proportion of patients with IgG concentrations below the 5th percentile for healthy controls developed sino-pulmonary infections relative to those above, but this was not significant (29% [14 of 48] vs 15% [16 of 108], $p = 0.060$). There was no difference in influenza vaccine seroresponses in those that did and did not develop sino-pulmonary infection, however there was a trend towards seroconversion to H3N2 being associated with a reduced likelihood of sino-pulmonary infection ($p = 0.062$) (Table 2).

Relationship between CD19+, IgG and sino-pulmonary infection

The combination of CD19+ cell numbers and IgG concentrations below the 5th percentile for healthy controls was associated with twice the incidence of sino-pulmonary infection relative to those with normal IgG levels (43% [12 of 28] vs 22% [11 of 49] $p = 0.003$) (Figure 1).

Discussion

The key finding of this study was that the subgroup of kidney transplant recipients who developed severe and recurrent sino-pulmonary infections could be prospectively identified by reduced CD19+ cell counts. This is one of the first studies to demonstrate an association between reduced B cell numbers and infection in kidney transplant recipients.[7] It highlights for clinicians that a simple test can help stratify patients at high risk of pulmonary infections.

The three humoral biomarkers tested were markedly reduced compared with healthy controls. CD19+ cell number was the biomarker most strongly associated with sino-pulmonary infections and when it dropped below 98 cells/mL, more than a third of patients developed sino-pulmonary infections. There was an additive risk when IgG was also reduced below the 5th percentile of healthy controls, with over 40% developing sino-pulmonary infection, consistent with previous data demonstrating an association between hypogammaglobinemia and infections.[8,9]

This is the first report to our knowledge of seroresponses to influenza vaccination being used as a diagnostic test for humoral immunity in solid organ transplant recipients. The suboptimal seroresponses to the influenza vaccine used in this study among healthy controls and transplant recipients limited its utility as a biomarker of humoral immune competence. It is possible that a more immunogenic vaccine may have better discriminatory power.

Our study has highlighted the high frequency and substantial morbidity from sino-pulmonary infections (18% of patients over two years follow-up). This is substantially higher than the reported annual incidence of pneumonia-related hospitalisation for the general population (25 per 10,000 adults).[10]

This is a single-centre study with a small sample size. The study demonstrates an association between humoral biomarkers and infections but for more robust predictive models, other factors that influence infectious risk (such as such as time from transplantation) need to be analysed. Future studies could validate our findings in a larger transplantation cohort.

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Table 1. Seroresponses to influenza vaccination compared with healthy controls (n = 118 transplant patients and 149 healthy controls)

	H1N1		H3N2		B	
	Transplant recipients	Healthy controls	Transplant recipients	Healthy controls	Transplant recipients	Healthy controls
HI antibody titre (median/IQR)						
Baseline	40 (10–80)	40 (10–80)	10 (10–20)	10 (10–20)	20 (10–80)	40 (20–80)
Post-vaccination HI titre	40 (20–160)	80 (40–160)	40 (20–80)	80 (40–160)	80 (20–160)	80 (40–160)
Post-vaccination fold rise	1 (1–2)	2 (1–4)	2 (1–4)	8 (4–16)	2 (1–4)	2 (1–4)
Geometric mean (95% confidence intervals)						
Baseline	34.1 (27.8–41.9)	31.5 (26.9–36.9)	16.1(14.3–18.1)	13.9 (12.6–15.1)	32.4 (26.1–40.2)	33.8 (28.6–39.9)
Post-vaccination	50.9 (40.6–63.7)	59.9 (50.8–70.7.3)	39.5 (32.2–48.6)	68.9 (56.9–83.4)	62.9 (49.5–79.9)	76.4 (64.1–91.0)
Seroconversion n (%)						
	14 (11.9%)	36 (24.2%)	40 (33.9%)	90 (68.4%)	32 (27.1%)	50 (33.6%)
Seroresponses to influenza vaccination among patients without baseline seroprotection						
	H1N1		**H3N2**		**B**	
	Transplant recipients n = 58		Transplant recipients n = 97		Transplant recipients n = 60	
Post-vaccination HI titre	20 (10–40)		20 (10–60)		20 (10–80)	

Table 2. Humoral biomarkers in patients with and without severe sino-pulmonary infection

Characteristics at study entry[a]	No severe sino-pulmonary infection	Severe sino-pulmonary infection	*p* value[b]
CD19+ number (cells/mL) n = 156	117 (67–235)	67 (40–109)	**0.001**
≤ 5th centile healthy controls (≤ 98)	54 (70)	23 (30)	
6th–95th centile healthy controls	70 (92)	6 (8)	**0.001**
> 95th centile healthy controls (> 597)	2 (67)	1 (33)	
IgG concentration (g/L) n = 156	8.9 (7.2–11.0)	8.0 (6.5–10.1)	0.129
≤ 5th centile healthy controls (≤ 7.5)	34 (71)	14 (29)	
6th–95th centile healthy controls	87 (86)	14 (14)	0.060
> 95th centile healthy controls (> 11)	5 (71)	2 (29)	
H1N1 seroconversion n = 118	11 (79)	3 (21)	0.845
H1N1 no seroconversion	84 (81)	20 (19)	
H3N2 seroconversion n = 118	36 (90)	4 (10)	0.062
H3N2 no seroconversion	59 (76)	19 (24)	
B seroconversion n = 118	26 (81)	6 (19)	0.901
B no seroconversion	69 (80)	17 (20)	

a. Data are presented as median (interquartile range) or number (percentage) if categorical

b. Data are presented as median (interquartile range) or number (percentage) if categorical

Bold indicates *p* value < 0.05

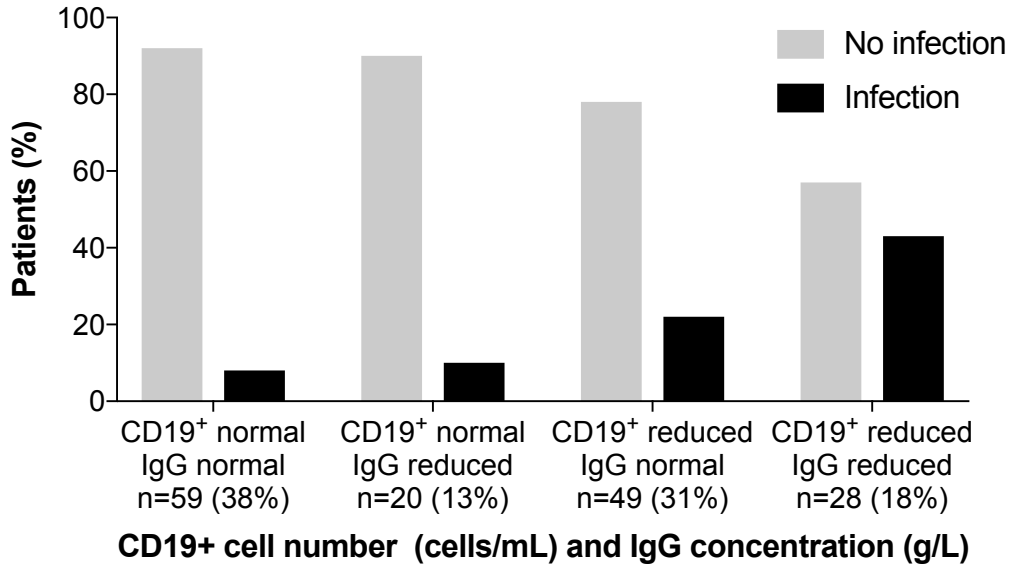

Figure 1. The distribution of patients with and without sino-pulmonary infection according to CD19+ cell number and IgG concentration at study entry

CD19+ cell concentration was defined as reduced if the CD19+ cell number was below the 5th percentile of healthy controls (≤ 98 cell/mL). IgG concentration was defined as reduced if the IgG concentration was below the 5th percentile of healthy controls (≤ 7.5 g/L) (p = 0.003 n = 156).

3.3 Does vaccination in solid organ transplant recipients result in adverse immunologic sequalae?

HIGHLIGHTS

- A systematic review and meta-analysis of 90 studies examining vaccination in SOT was conducted.
- The rate of de novo anti-HLA antibody formation post-vaccination was low.
- There was no difference in rejection rates between vaccinated and unvaccinated SOT recipients.
- Vaccination of SOT recipients does not result in adverse immunological sequalae.

PUBLICATION

Mulley WR, **Dendle C**, Ling JEH, Knight SR. Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis. J Heart Lung Transplant. 2018 Jul;37(7):844–852.

The published article is included as Appendix 1.

3.4 Pneumococcal vaccination in solid organ transplant recipients: A review of the literature

HIGHLIGHTS

- Data examining clinical protection against invasive pneumococcal diseases in SOT recipients is lacking.
- Seroresponse data suggests SOT recipients can mount a measurable antibody response following vaccination but it is suboptimal.
- There is no evidence that conjugate vaccine is superior to polysaccharide vaccine in SOT recipients.
- There is no evidence that pneumococcal vaccination increases rejection rates.

SUBMITTED MANUSCRIPT

Dendle C, Stuart RL, Mulley WR, Holdsworth SR. Pneumococcal vaccination in solid organ transplant recipients: A review of current evidence.

Revisions submitted August 2018 – *Vaccine*

ABSTRACT

This narrative review summarises the current literature relating to pneumococcal vaccination in adult solid organ transplant (SOT) recipients, who are at risk of invasive pneumococcal disease (IPD) with its attendant high morbidity and mortality.

The effect of the pneumococcal polysaccharide vaccine has been examined in several small cohort studies in SOT recipients, the majority of which were kidney transplant recipients. The outcomes for these studies have been laboratory seroresponses or functional antibody titres. Overall, in most of these studies the transplant recipients were capable of generating measurable serological responses to pneumococcal vaccination but these responses were less than those of healthy controls. A mathematical model estimated the effectiveness of polysaccharide vaccination in SOT recipients to be one-third less than that of patients with HIV.

The evidence for the efficacy of the pneumococcal conjugate vaccine in SOT is based on a small number of randomised controlled trials in liver and kidney transplant recipients. These trials demonstrated that SOT recipients mounted a serological response following vaccination; however, there was no benefit to the use of prime boosting (conjugate vaccine followed by polysaccharide vaccine). Currently there are no randomised studies investigating the clinical protection rate against IPD after pneumococcal vaccination by either vaccine type or linked to vaccine titres or other responses against pneumococcus. Concerns that vaccination may increase the risk of adverse alloresponses such as rejection and generation of donor-specific antibodies are not supported by studies examining this aspect of vaccine safety. Pneumococcal vaccination is a potentially important strategy to reduce IPD in SOT recipients and is associated with excellent safety. Current international recommendations are based on expert opinion from conflicting data, hence there is a clear need for further quality

studies in this high-risk population examining optimal vaccination regimens. Such studies should focus on strategies to optimise functional immune responses.

MANUSCRIPT

Introduction

International guidelines recommend pneumococcal vaccination for solid organ transplant (SOT) recipients to prevent sino-pulmonary infection and invasive pneumococcal disease (IPD).[1,2] Despite these recommendations, coverage with pneumococcal vaccination is suboptimal.[3,4] Pneumococcal polysaccharide vaccination rates have been reported to be 60% in liver transplant recipients,[5] and 62% in potential lung transplant recipients.[3] The evidence on which these recommendations are based is limited, with few randomised controlled studies in SOT recipients.[6] This review will summarise the literature regarding the seroresponse data, efficacy, effectiveness and safety of both the polysaccharide and conjugate pneumococcal vaccines in adult SOT recipients.

We searched Cochrane CENTRAL, MEDLINE, EMBASE and The Transplant Library from inception until 1 July 2017. We also reviewed article reference lists for additional studies. Literature searches included keywords and free text terms for solid organ transplantation, pneumococcal vaccination and the outcomes of interest. We only examined studies that included adult SOT recipients.

This literature review will summarise the current evidence for adult pneumococcal vaccination in SOT recipients.

Epidemiology of pneumococcal disease in SOT recipients

The incidence and mortality rate of IPD is higher in SOT recipients than the general population.[4,7-11] The incidence of invasive IPD differs according to transplanted organ but is estimated to be 13 to 41 times higher than the general population.[4,7-11] Table 1

summarises the data estimating these risks. The mortality rate from IPD is reported to be three times higher in an immunosuppressed population (24%) compared with the general population (9%).[4,7,8] IPD can occur any time after transplant, however is most common in the first three years post-transplant.[7] Infection with particular pneumococcal serotypes have been associated with different frequency, severity and types of clinical presentations.[12] Serotype 1 has a high invasive disease potential[13] while serotype 3 is associated with an increased case fatality rate compared with other serotypes.[14] Of concern, there is emerging evidence that serotypes not included in currently licensed pneumococcal vaccines are occurring with increased frequency in immunocompromised compared with immunocompetent patients. These include serotypes 6A, 23F, 11A and 33F.[12] This may relate to clones with capsular types that have a lower relative risk of causing IPD. These serotypes are more opportunistic and primarily affect immunocompromised patients.[10,13]

Types of pneumococcal vaccinations

When a SOT recipient is exposed to *Streptococcus pneumoniae* though colonisation or infection, antibodies are generated against the capsular polysaccharides.[15,16] Pneumococcal vaccination either induces or boosts serotype-specific antibody concentrations against these polysaccharides.[16] Pneumococcal polysaccharide vaccines consist of purified pneumococcal polysaccharides that induce a restricted IgG response and do not recruit T cells or generate memory B cells.[17] For pneumococcal conjugate vaccines, the polysaccharides are covalently bound to an immunogenic carrier protein. Peptides from the carrier proteins interact with T cells via major histocompatibility complex (MHC) Class 2 receptors on antigen presenting cells, recruiting T cell responses and promoting B cell differentiation into memory B cells.[16,18,19] Immunosuppressive treatments in SOT recipients are primarily targeted to cellular immunity, however both cellular and humoral immune responses may be reduced to varying degrees. Hence, in order to enhance functionality and longevity of antibody responses,[16] the ability to

induce T cell responses and create immunological memory suggest that the conjugate pneumococcal vaccination may offer advantages over the polysaccharide vaccine.[6,20]

Laboratory measurement of pneumococcal vaccine responses

Clinical outcomes in efficacy studies of pneumococcal vaccination include IPD (such as bloodstream infection or meningitis), non-invasive pneumococcal disease (such as pneumonia) and death.[21,22] The majority of studies of pneumococcal vaccination in SOT recipients have not examined clinical outcomes, but rather the surrogate endpoint of laboratory seroresponses to pneumococcal vaccination.[23-42] The most frequently used method is quantification of serotype-specific immunoglobulin concentrations pre- and post-vaccination. Functional antibody responses can be measured by opsonophagocytic assays (OPA). OPA may be particularly important in SOT recipients as these assays measure the ability of the antibodies to opsonise and kill pneumococci, which may be affected by the immunosuppression used in transplantation.[43-46] Studies in SOT recipients have examined both antibody titres and opsonophagocytic assay titres.[6,20,36,41]

There is reported discordance between between antibody concentrations and opsonic concentrations.[42]

Laboratory correlates of clinical protection against invasive pneumococcal disease

Controversy exists regarding the optimal laboratory cut-offs and clinical correlates of protection for pneumococcal vaccination. International recommendations are based predominantly on expert opinion due to insufficient and conflicting data.[47-56] The majority of studies have used a pneumococcal IgG cut-off of 0.35 μg/mL[51-54,56] and an opsonophagocytic cut-off of 1:8. There are no studies specifically examining the clinical corelates or seroprotection for either the polysaccharide or conjugate pneumococcal vaccine in SOT recipients. For 23-valent pneumococcal polysaccharide vaccines, the American Academy of Asthma, Allergy and Immunology defines a protective response

to each pneumococcal serotype as a titre equal to or greater than 1.3 μg/mL antibody.[47] The studies from which this value is derived were performed in heterogenous populations, a number of which were children.[48-50,55,56] Only one study includes high-risk adults[50] and there are no studies in SOT recipients.

For the conjugate pneumococcal vaccines, based on studies in children, an anti-capsular polysaccharide antibody concentration between 0.20 and 0.35 μg/mL aggregated across all seven serotypes is suggested as the correlate of clinical protection against IPD; however, recent evidence suggests that this should be higher.[51-54,56]

Evidence to support the use of pneumococcal polysaccharide vaccination in SOT recipients

In immunocompetent adult patients, pneumococcal polysaccharide vaccination has demonstrated benefit in reducing vaccine serotype IPD but the evidence for an effect on non-invasive pneumococcal diseases is less certain.[57-60] The recommendation to administer 23-valent pneumococcal polysaccharide vaccine to SOT recipients is based on effectiveness data derived from observational studies in HIV patients which have demonstrated reduced pneumococcal bacteraemia and mortality.[61-64] Importantly, one study performed in Uganda found an increase in pneumococcal disease in vaccine recipients.[61]

There are no randomised studies in SOT recipients that examine clinical protection against IPD following pneumococcal polysaccharide vaccination, however there are a number of observational studies that examine seroresponses.[23-42] Most of these studies have small numbers and were performed over two decades ago, before the era of modern immunosuppression. Over two-thirds of the studies were performed assessing kidney transplant recipients, with only four assessing heart transplant recipients, one assessing liver transplant recipients and none in other transplanted organ groups. Overall, the majority of these studies show that serotype-specific and functional

antibodies can be generated following 23-valent pneumococcal polysaccharide vaccination and the levels are comparable with healthy controls. In the absence of randomised controlled data, Cho et al. developed a mathematical model to estimate the effectiveness of PPV23 in SOT recipients. In this model, the effectiveness of PPV23 was estimated at 25% (95% CI 0–50%) for invasive pneumococcal disease. This is based on estimates that vaccination in SOT recipients has one-third less efficacy than in HIV positive patients.[65]

Evidence to support pneumococcal conjugate vaccination in SOT recipients

Similar to pneumococcal polysaccharide vaccination, the recommendations for pneumococcal conjugate vaccination in immunocompromised patients are based on efficacy estimates derived from studies in HIV infected patients.[66]

A large study analysing 18 years of IPD surveillance in Canada that included 149 adult solid organ or bone marrow transplant recipients reported that IPD rates declined in adults in response to the introduction of 7-valent pneumococcal conjugate vaccination in children.[8] Rates of IPD due to 7-valent pneumococcal conjugate vaccine serotypes decreased at approximately the same rate in immunocompetent and immunocompromised adults, with parallel increases in non-vaccine serotypes. Serotypes not included in any vaccine were more common in immunocompromised patients compared with immunocompetent cases. Sangil et al. analysed 799 IPD episodes and found a reduction in IPD since pneumococcal conjugate vaccine licensure, both in the general population and the immunosuppressed.[4] Of 189 immunosuppressed patients, there were no SOT recipients included.

A systematic review of serologic vaccination response after SOT transplantation was conducted in 2013.[67] Nine studies in adult and paediatric patients examining pneumococcal vaccination were included. The seroresponse rate from these studies was above 50%, with a summary estimate of 83% (95% CI 83–93%) with substantial

heterogeneity. This heterogeneity related to vaccine type, vaccine schedules, serological testing and definitions of response rates. Importantly, the response rate may be overestimated as the authors accepted the serological response to a single antigen as a positive response while others suggest responses to multiple antigens is required.[67]

The best evidence examining the seroresponses of pneumococcal vaccination are randomised controlled trials performed in kidney and liver transplant recipients. Kumar et al. performed a randomised study in adult kidney transplant recipients and found no difference in seroresponses between 23-valent pneumococcal polysaccharide vaccination and 7-valent pneumococcal conjugate vaccination.[6] Kumar et al. and McCashland et al. demonstrated that pneumococcal antibodies post 7-valent pneumococcal conjugate vaccination were not durable.[20,32] Table 2 summarises adult studies of pneumococcal conjugate vaccination in liver, heart/lung and kidney transplant recipients.[23-42]

Factors associated with pneumococcal vaccine responses

Immunocompromise has been associated with poor response to pneumococcal vaccination[59] but other specific host factors that attenuate seroconversion are not well defined. Unlike influenza vaccination where the use of mycophenolate mofetil reduces vaccine seroresponses,[68] Kumar et al. have not found a correlation between the type of immunosuppression and 7-valent pneumococcal conjugate vaccine seroresponses in either liver or kidney transplant recipients, in whom this drug is widely used.[6,36] Gattringer et al. did not find an association of 7-valent pneumococcal conjugate vaccination response and age, sex, time from transplantation or immunosuppression.[39]

A strategy to improve seroresponses of pneumococcal vaccination is prime boosting, whereby pneumococcal conjugate vaccination is followed by 23-valent pneumococcal polysaccharide vaccination aiming to enhance T cell dependent responses.[69-72] There are several studies in immunocompetent patients and in other immunocompromised

patients, such as HIV, Hodgkin's lymphoma and sickle cell disease, to support the use of a prime boost strategy to increase serotype-specific functional antibody concentrations.[17,73-75] Evidence to support prime boosting in SOT recipients is weak. Goldblatt et al. found no benefit could be observed after a second vaccination with either 23-valent pneumococcal polysaccharide vaccine or 7-valent pneumococcal conjugate vaccine in adult SOT recipients receiving a 7-valent pneumococcal vaccine six months previously.[76] Gattringer et al. could not demonstrate any benefit of a booster vaccine six to eight weeks after the first 7-valent pneumococcal vaccination in liver, heart or lung transplant recipients.[39] Kumar et al. performed a randomised controlled trial in liver transplant recipients whereby PPV23 was compared with PCV7 followed eight weeks later by PPV23. There was no difference in seroresponses between the groups.[36] Tobudic et al. performed a randomised control trial in kidney transplant recipients whereby PPV23 was compared with PCV7 followed one year later by PPV23. There was no difference in seroresponses between the groups.[41]

Hyporesponsiveness has been reported with both polysaccharide and conjugate pneumococcal vaccines in adults and pediatric patients.[33,77] Musher et al. reported that middle aged or older adults who received the 23-valent pneumococcal polysaccharide vaccine within a year of prior vaccination had almost no response to the revaccination, although IgG levels increased in proportion to the time elapsed after the first vaccination.[78] Blumberg et al. demonstrated that when heart transplant recipients received pneumococcal polysacchardie vaccination, those previously vaccinated with pneumococcal polysaccharide vaccine had increased seroresponses compared to heart transplant recipients undergoing a primary vaccination course. This suggests 'boosting' rather than hyporesponsiveness but the study was very small.[33]

Although the literature to support the optimal pneumococcal vaccine schedule in SOT recipients is limited and not clearly supportive of a particular regimen, most guidelines recommend vaccination with pneumococcal conjugate vaccine followed by a

pneumococcal polysaccharide vaccine, with booster doses of polysaccharide vaccine every five years.[1,2]

Safety of pneumococcal vaccines in SOT recipients

A key safety issue to consider when recommending vaccination to SOT recipients is the potential of triggering allograft rejection. It is hypothesised that vaccination could stimulate graft rejection through stimulation of alloreactive T and B cells.[80] This is particularly relevant to the pneumococcal conjugated vaccines that are specifically bioengineered to increase immune activation. A recent case report described a link between 13-valent pneumococcal conjugate vaccine and immune thrombocytopenic purpura which may have involved antibody production driven by T helper cells reacting to platelet surface glycoproteins.[81] There is limited literature specifically examining the development of de novo human leukocyte antigen antibodies or graft rejection in SOT recipients following pneumococcal vaccination, however there are no studies that suggest an increased rate of rejection.[6,20,30,36,41,82] Additionally, a recent systematic review of SOT recipients found no clear association between vaccination of all types (including pneumococcal) and allograft rejection or de novo donor-specific antibody formation.[83] Table 3 summarises studies examining pneumococcal vaccination and allograft rejection.

Conclusions

SOT recipients are at high risk of invasive pneumococcal disease and its attendant morbidity and mortality. Pneumococcal vaccination represents an important strategy in the preventive care of transplant recipients but in many centres, coverage is suboptimal.

For pneumococcal polysaccharide vaccine, there are fewer than 30 studies that specifically examine serological outcomes in SOT recipients. Most of these studies included fewer than 50 participants and the majority were performed in kidney

transplant recipients. Most of the studies had small control groups. Overall, although pneumococcal polysaccharide vaccination resulted in measurable seroresponses, these were reduced compared with healthy controls.

The quality of evidence for pneumococcal conjugate vaccination in adult SOT recipients is better than that for polysaccharide vaccination. While there are fewer studies (less than 10), they are well-conducted randomised controlled trials. The trials were performed in liver and kidney transplant recipients, with the largest study including 113 liver transplant recipients. Overall, these studies demonstrated that pneumococcal conjugate vaccination resulted in measurable seroresponses. Of concern, however, is that there was no clear enhancement of responses from prime boosting with the polysaccharide vaccine and the responses were not durable.

There are very few studies examining clinical outcomes for either pneumococcal polysaccharide or conjugate vaccines, however cohort data suggests that pneumococcal conjugate vaccines reduce IPD. Pneumococcal conjugate vaccination is safe and well tolerated in SOT recipients and there does not appear to be evidence of an increased incidence of allograft rejection.

Further research is required in SOT recipients to identify the optimal timing, sequence, dosing and clinical outcomes for these very important vaccines.

Conflict of interest

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Table 1. Incidence of invasive pneumococcal disease in adult SOT recipients

Population	First author and year of publication	Incidence of invasive pneumococcal disease
General population	Shigayeva 2016[8]	4.8 per 100,000 person/years
	Kumar 2007[7]	11.5 per 100,000 person/years
SOT recipients overall	Shigayeva 2016[8]	195 per 100,000 person/years##
	Kumar 2007[7]	146 per 100,000 transplanted patients/year
Kidney transplant recipients	Kumar 2007[7]	104 per 100,000 transplanted patients/year
Lung transplant recipients	Kumar 2007[7]	239 per 100,000 transplanted patients/year
Heart transplant recipients	Kumar 2007[7]	0 per 100,000 transplanted patients/year
	Amber 1990[11]	3600 per 100,000 person/years**
Liver transplant recipients	Kumar 2007[7]	354 per 100,000 transplanted patients/year
Pancreas transplant recipients	Kumar 2007[7]	0 per 100,000 transplanted patients/year

Includes solid organ and bone marrow transplant recipients

** All pneumococcal infections, not just invasive pneumococcal disease

Table 2. Studies examining seroresponses following pneumococcal vaccination in SOT recipients

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
Silberman 1980[23]	Prospective controlled	Kidney transplant recipients (27) Controls (17)	PPV14, single dose	IgG seroresponse (13 serotypes) and 4-fold rise in titre measured pre-vaccination and post vaccination	No difference between kidney transplant recipients and controls	9 patients had minor reactions and 5 patients had systemic symptoms temporally but not causally related to the vaccine
Cosio 1981[24]	Prospective controlled	Kidney transplant recipients (25: 11 non-splenectomised and 14 splenectomised) Controls (14) Chronic renal failure dialysis patients (7)	PPV14, single dose	IgG seroresponse to 12 serotypes Seroresponse defined as ≥ 2-fold increase in antibody concentration and a post-vaccination value of ≥200ng N/mL Measured at pre-vaccination and 3 to 6 weeks post vaccination	80% kidney transplant recipients, 93% controls and 43% chronic renal failure dialysis patients No difference between splenectomised vs non-splenectomised	3 patients had local pain 3 patients had erythema No effect on serum creatinine
Rytel 1986[25]	Prospective controlled	Kidney transplant recipients Chronic renal failure dialysis patients Controls	PPV, single dose	IgG seroresponse measured pre-vaccination and 1 month 1 year, 2, and 3.5 years post vaccination	No difference between kidney transplant recipients and controls at 1 month Chronic renal failure dialysis patients lower than kidney transplant recipients and controls at 1, 2 and 3.5 years	

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
Linnemann 1981 and 1986[26,27]	Prospective controlled	Kidney transplant recipients Chronic renal failure dialysis patients Controls	PPV, single dose Re-vaccinated 2 years following primary PPV vaccination	IgG seroresponse measured pre-vaccination and post vaccination	Kidney transplant recipients and chronic renal failure dialysis patients lower than controls Re-vaccination of kidney transplant recipients resulted in a 2-fold increase in antibody titres but the levels did not reach those seen after primary vaccination	
Arnold 1985[28]	Prospective uncontrolled	Kidney transplant recipients (75: 32 non-splenectomised and 43 splenectomised)	PPV14	OPA (2 serotypes) measured at pre-vaccination, 1 and 6 months post vaccination	50–84% kidney transplant recipients No difference between splenectomised vs non-splenectomised	None reported
Dengler 1996[29]	Prospective controlled	Heart transplant recipients (16) Liver transplant recipients (15) Controls (23)	PPV23, single dose	IgG seroresponse (9 serotypes) Measured pre-vaccination and post-vaccination	No difference between heart transplant recipients, liver transplant recipients and controls	
Dengler 1998[30]	Prospective controlled	Heart transplant recipients (16) Controls (23)	PPV23, single dose	IgG seroresponse Seroresponse defined as ≥ 4-fold increase in antibody concentration and a post-vaccination value of ≥ 1000 U/mL	94% heart transplant recipients No difference between heart transplant recipients and controls	44% local pain, erythema and swelling
Kazanciouglu 2000[31]	Prospective uncontrolled	Kidney transplant recipients (21)	PPV23, single dose	IgG seroresponse	100% kidney transplant recipients	

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
McCashland 2000[32]	Prospective controlled	Chronic liver disease who went on to liver transplant (25) Controls (13)	PCV7, single dose	IgG seroresponse to 2 serotypes Seroresponse defined as ≥ 5-fold increase in antibody concentration Measured pre-vaccination and 3 months post-transplant	Antibody titres declined to pre-vaccination levels rapidly in the first few months post-transplant	Not reported
Blumberg 2001[33]	Prospective controlled	Heart transplant recipients (35) Controls (35)	PPV14 or PPV23, single dose	IgG seroresponse (5 serotypes) Measured at a mean of 9 weeks post-vaccination	91% heart transplant recipients Heart transplant recipients lower than controls Heart transplant recipients previously vaccinated with polysaccharide vaccine had a higher mean antibody titre for 4 of 5 serotypes when compared with heart transplant recipients not previously vaccinated	No adverse effects reported

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
Kumar 2003 and 2007[6,20]	Randomised controlled	Kidney transplant recipients (60)	PPV23 single dose vs PCV7 single dose	IgG seroresponse to at least one serotype and OPA (7 serotypes) measured pre-vaccination, 8 weeks and 3 years post-vaccination Seroresponse defined as ≥ 2-fold increase in antibody concentration and a post-vaccination value of ≥ 1ug/mL OPA 4-fold rise and absolute value > 1:8	Seroresponse: PPV23 vs PCV7 (53% vs 73%) OPA: PPV23 vs PCV7 30–57% vs 37–53% Antibody titres declined significantly at 3 years for 6 of 7 serotypes No difference in durability was found in patients who had received PPV23 vs PCV7 The only factor predictive of response durability was a strong multi-serotype response	No difference in local and systemic reactions between the groups Mean creatinine was similar at baseline but higher at 8 weeks for patients who received PPV23 There was no difference between the groups at a6 months
Sarmiento 2006[34]	Prospective uncontrolled	Heart transplant recipients (32)	PPV23, single dose	IgG seroresponse (23 serotypes) Measured pre-vaccination and at various time points until 18 months post-transplant	Progressive decline in antibody titres during the first year after heart transplant	One patient developed invasive pneumococcal disease
Willcocks 2007[35]	Randomised controlled (calcineurin inhibitor based immunosuppression vs sirolimus based immunosuppression)	Kidney transplant recipients (23) Liver transplant recipients (9)	PPV23, single dose	IgG (7 serotypes) pre-vaccination and post vaccination Seroprotection defined as post-vaccination antibody concentration of ≥0.35 μg/mL	Antibody titres increased significantly all 7 serotypes No difference between patients taking calcineurin inhibitors and those taking sirolimus	None reported

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
Kumar 2008[36]	Randomised controlled	Liver transplant recipients (113)	PCV7 single dose vs PCV7 single dose followed 8 weeks later by PPV23	IgG seroresponse to at least 1 serotype and OPA (7 serotypes) measured pre-vaccination and 8 weeks post-vaccination Seroresponse defined as ≥ 2-fold increase in antibody concentration and a post-vaccination value of plus ≥ 1ug/mL OPA 4-fold rise and absolute value > 1:8	PCV7 vs PCV7 followed by PPV23 (91% vs 86%) No difference in antibody titres and OPA between the arms	After the first vaccination, more patients had an adverse reaction to PCV7 than placebo After the second vaccination (PPV23 in both groups) there were no differences in adverse reactions between primed and unprimed groups
Pourfarzani 2008[37]	Prospective controlled	Kidney transplant recipients (37) Chronic renal failure dialysis patients (14)	PPV23, single dose	IgG seroresponse measured pre-vaccination, 4 weeks, 6 months and 12 months post-vaccination	96%, 94% and 85% kidney transplant recipients 96% at 4 weeks, 6 months and 12 months No difference between kidney transplant recipients, chronic renal failure dialysis patients and controls	None reported
Lindemann 2010[38]	Prospective Published controls	Kidney transplant recipients (43)	PPV23, single dose	IgG seroresponse (9 serotypes) Measured pre-vaccination and post-vaccination	91% kidney transplant recipients No difference between kidney transplant recipients and controls	Not reported

First author and year	Design	Participants	Vaccine and schedule	Measurement of seroresponse	Post-vaccination seroresponse	Safety
Gattringer 2011[39]	Retrospective uncontrolled	Heart transplant recipients (14) Lung transplant recipients (12)	PCV7 followed 8 weeks later by PPV23	IgG seroresponse (10 serotypes) Seroresponse defined as ≥ 2-fold increase in antibody concentration	26–61% heart and lung transplant recipients No benefit from PPV23 dose	Not reported
Lindemann 2012[40]	Prospective uncontrolled	Kidney transplant recipients (49)	PPV23, single dose	IgG seroresponse measured pre-vaccination and 1 and 15 months post-vaccination	77% of antibody titres remained detectable at 15 months	Not reported
Tobudic 2012[41]	Randomised controlled	Kidney transplant recipients (80)	PCV7 vs PCV7 followed 12 months later by PPV23	IgG seroresponse and OPA (10 serotypes) measured pre-vaccination and 8 weeks after PCV7 and PPV23 Seroresponse defined as ≥ 2-fold increase in antibody concentration and a post-vaccination value of ≥ 1 μg/mL	Lower antibody and OPA titre to at least one serotype in patients who received PCV7 alone vs patients who received PCV7 plus PPV23 (77% vs 93%)	Minor reactions equal in both groups
Fishman 2017[42]	Prospective uncontrolled	Kidney transplant recipients (30)	Not recorded	IgG and OPA (serotype 19A) measured at 3 points 6–48 months following transplant Seroprotection defined as a post-vaccination antibody concentration of IgG ≥ 0.35μg/mL and OPA > 1:8	OPA and IgG titres do not correlate well using current values for protective immunity	Not reported

PPV: pneumococcal polysaccharide vaccine, PCV: pneumococcal conjugate vaccine, OPA: opsonophagocytic assay

Table 3. Studies examining allograft rejection following pneumococcal vaccination in SOT recipients

First author and year	Design	Participants (type of organ transplant)	Vaccine and schedule	Monitoring of allograft rejection	Results
Dengler 1998[30]	Prospective controlled	Heart transplant recipients (16) Controls (23)	PPV23, single dose	Clinical rejection requiring corticosteroids	No increased episodes of rejection
Kumar 2003 and 2007[6,20]	Randomised controlled	Kidney transplant recipients (60)	PPV23 single dose vs PCV7 single dose	Serum creatinine and clinical rejection measured at 8 weeks and 6 months	No episodes of clinical rejection Serum creatinine was higher in PPV23 group at 8 weeks but no different at 6 months
Kumar 2008[36]	Randomised controlled	Liver transplant recipients (113)	PCV7 single dose vs PCV7 single dose followed 8 weeks later by PPV23	Biopsy-proven acute rejection that required treatment with corticosteroids measured at 6 months	1 (1.8%) in PCV7 alone arm vs 5.3% in prime boost arm (p = 0.33)
Tobudic 2012[41]	Randomised controlled	Kidney transplant recipients (80)	PCV7 vs PCV7 followed 12 months later by PPV23	Clinical rejection	No rejection
Lindemann 2013[82]	Prospective uncontrolled	Kidney transplant recipients (49)	PPV23, single dose	HLA class I and II and MHC class I–related chain antibodies measured and 1 and 15 months	No increase in anti-HLA antibody titres

PPV: pneumococcal polysaccharide vaccine, PCV: pneumococcal conjugate vaccine, HLA: human leukocyte antigen, MHC: major histocompatibility complex

3.5 Seroresponses and safety of 13-valent pneumococcal conjugate vaccination in kidney transplant recipients

HIGHLIGHTS

- 45 kidney transplant recipients were vaccinated with a single dose of 13-valent conjugated pneumococcal vaccine.
- There was a modest increase in anti-pneumococcal IgG and functional antibody titres post vaccination.
- No patients developed de novo anti-HLA antibodies or rejection.

PUBLICATION

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Seroresponses and safety of 13-valent pneumococcal conjugate vaccination in kidney transplant recipients

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Abstract

Background: Conjugated pneumococcal vaccine is recommended for kidney transplant recipients, however, their immunogenicity and potential to trigger allograft rejection though generation of de novo anti-human leukocyte antigen antibodies has not been well studied.

Methods: Clinically stable kidney transplant recipients participated in a prospective cohort study and received a single dose of 13-valent conjugate pneumococcal vaccine. Anti-pneumococcal IgG was measured for the 13 vaccine serotypes pre and post vaccination and functional anti-pneumococcal IgG for 4 serotypes post vaccination. Anti-human leukocyte antigen antibodies antibodies were measured before and after vaccination. Kidney transplant recipients were followed clinically for 12 months for episodes of allograft rejection or invasive pneumococcal disease.

Results: Forty-five kidney transplant recipients participated. Median days between pre and post vaccination serology was 27 (range 21-59). Post vaccination, there was a median 1.1 to 1.7-fold increase in anti-pneumococcal IgG antibody concentrations for all 13 serotypes. Kidney transplant recipients displayed a functional antibody titer ≥1:8 for a median of 3 of the 4 serotypes. Post vaccination, there were no de novo anti-human leukocyte antigen antibodies, no episodes of biopsy proven rejection or invasive pneumococcal disease.

Conclusion: A single dose of 13-valent conjugate pneumococcal vaccine elicits increased titers and breadth of functional anti-pneumococcal antibodies in kidney transplant recipients without stimulating rejection or donor-specific antibodies.

KEYWORDS

kidney transplantation, luminex technology, pneumococcal antigen, serotype-specific antibody response

1 | INTRODUCTION

Pneumococcal vaccination is widely recommended for solid organ transplant recipients to prevent community acquired pneumonia and invasive pneumococcal disease (IPD).[1,2] The incidence of invasive pneumococcal disease is estimated to be up to 41 times higher in transplant recipients compared with the general population,[3,4] with rates in kidney transplant recipients of 104 per 100 000 transplanted patients per year.[5] There is also an increased risk of mortality from pneumococcal pneumonia and invasive pneumococcal disease compared with the general population.[6] Case fatality rates in transplant recipients range from 10% in young adults to 21% in those over 65 years.[6]

The main virulence factors of *Streptococcus pneumoniae* are the capsular polysaccharides, which can inhibit phagocytosis and antibodies against the polysaccharides protect against invasive infection.[7] Antibodies against pneumococcal capsular polysaccharides are generated through colonization or disease[8] however, these antibodies can decline, either quantitatively or functionally over time. Pneumococcal vaccination focuses on inducing or boosting serotype-specific antibody concentration with the additional advantage of conjugate vaccination of T-cell recruitment potentially resulting in improved functional antibody.[8]

Two types of pneumococcal vaccines are currently licensed and available for routine use: the pneumococcal polysaccharide vaccines (PPV) and pneumococcal conjugate vaccines (PCV). PPV consists of purified pneumococcal polysaccharides that act as T-cell independent type 2 antigens. These antigens induce a restricted IgG response and poor generation of memory B cells.[9] In contrast, PCV was developed to enhance immunogenicity by covalent conjugation to carrier proteins. Peptides from the carrier proteins interact with T cells via Major Histocompatibility Complex (MHC) Class 2 receptors on antigen presenting cells, recruiting T-cell responses against the conjugated polysaccharide antigens. T helper cells can promote B-cell differentiation into antibody producing plasma calls or memory B cells.[10,11] The generation of this immunologic memory may be crucial in solid organ transplant recipients, in whom immunity to pneumococcus wanes quickly after polysaccharide vaccination. Lindemann et al[12] demonstrated a 3-fold decrease in antibody titers 2 years following PPV23 vaccination in kidney transplant recipients.

In immunocompetent patients, evidence for the effectiveness of pneumococcal polysaccharide vaccine in reducing IPD is poor.[13,14] A Cochrane review of 23-valent pneumococcal polysaccharide vaccine demonstrated an efficacy of 74% in protecting against vaccine serotype IPD. There was no effect in protection against all cause pneumonia and mortality.[15] In contrast, pneumococcal conjugate vaccines have been shown to prevent vaccine type bacteremic and non-bacteremic pneumonia, and invasive pneumococcal disease.[16] Current international guidelines[1] recommend both PPV and PCV for kidney transplant recipients, however, immunogenicity and safety data are scarce. Laboratory outcomes following pneumococcal vaccination can be tested via serotype-specific IgG concentrations or functional antibody concentration, measured by opsonophagocytic assay (OPA).[17-20] Serotype-specific antibody concentration identifies and quantifies the presence of pneumococcal antibody while OPA provides information as to whether these antibodies are capable of opsonizing and killing pneumococci. OPA are particularly important to assess in immunocompromised patients or those with pre-existing immunity as there may be discordance between antibody concentration and opsonic concentrations.[21]

A key safety issues to consider when recommending vaccination to solid organ transplant recipients is the risk that vaccination may trigger allograft rejection. It is hypothesized that vaccination could stimulate graft rejection through stimulation of alloreactive T and B cells.[22] This is particularly relevant to adjuvanted PCVs that are specifically bioengineered to increase immune activation. There have not been any studies specifically examining the development of de novo HLA antibodies nor graft rejection in solid organ transplant recipients vaccinated with PCVs.

The aims of this study were to examine serological and functional responses to the 13-valet conjugated pneumococcal vaccine (PCV13) among kidney transplant recipients and determine if PCV13 vaccination is associated with the development of de novo donor-specific antibodies (DSA) or allograft rejection. The hypothesis of this study was that PCV13 was immunogenic and safe in kidney transplant recipients.

2 | MATERIALS AND METHODS

2.1 | Setting

A prospective cohort study, assessing seroresponses of kidney transplant recipients was performed at Monash Health, a tertiary referral center in Victoria, Australia. Monash Health is a 1500-bed academic health service that performs approximately 90 kidney transplants per year and has 850 kidney transplant recipients who receive ongoing follow-up care. Investigators attempted to contact all kidney transplant recpients by post, to offer vaccination with the PCV13 vaccination and an invitation to participate in the study. Because of the financial cost of laboratory testing for this study, the first 58 kidney transplant recipients consenting patients were then enrolled in the study at a subsequent transplant clinic visit. The study was approved by the human research ethics committee of Monash Health (13085A) and written informed consent was obtained from all participants.

2.2 | Inclusion and exclusion criteria

Kidney transplant recipients were eligible to participate if they were aged ≥18 years, were at least 3 months post transplant and had not received pneumococcal conjugate vaccine previously. Kidney transplant recipients with a known allergy to pneumococcal vaccine, recently augmented immunosuppression to treat rejection, or infectious illness just prior to or at the time of the study were excluded.

2.3 | Data collection

Patient demographics, comorbidities, cause of end-stage kidney disease, and medication use including current and previous

immunosuppressive drug regimen were assessed at vaccination. Kidney transplant function was assessed by the glomerular filtration rate estimated (eGFR) using the CKD-Epi formula. Kidney transplant recipients were specifically asked if they had previously received pneumococcal polysaccharide vaccine and results were recorded as yes, no or unknown. Additional documentation to corroborate receipt of vaccination was sought in the medical record.

2.4 | Vaccination procedures

Participants attended the hospital's outpatient vaccination clinic where they received a single 0.5 mL intramuscular dose of PCV13 (Prevnar-13™/Prevernar-13™, Pfizer).

The vaccine contained polysaccharides from pneumococcal serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F individually conjugated to a non-toxic diphtheria toxin cross-reactive material CRM_{197} protein. The vaccine contained 2.2 μg of each polysaccharide (except for serotype 6B (4.4 μg), along with 5.0 mmol/L succinate buffer, 0.85% sodium chloride, 0.02% polysorbate 80, and 0.125 mg of aluminum as aluminum phosphate adjuvant per 0.5-mL dose.[16] Blood samples was collected immediately prior to vaccination and at 1 month post vaccination to assess seroresponses and immediately prior to vaccination and at 3-6 months post vaccination for DSA assessment. Patients were asked if they developed any adverse reactions such as pain and swelling, fever or rash following vaccine administration.

2.5 | Blood collection

Blood samples were tested for anti-PCV13 antibodies. Kidney transplant recipients underwent venepuncture just prior to vaccination, 1 month post vaccination for anti-PCV antibodies and then 3 months later for anti-HLA antibodies.

2.5.1 | Anti-pneumococcal antibody assays-IgG to PCV13

IgG antibody to capsular polysaccharides from all 13 vaccine *Streptococcus pneumoniae* serotypes were measured by standard Enzyme-linked immunosorbent assay (ELISA) assay after serum samples were absorbed to neutralize antibody to cell wall polysaccharides. Serotype-specific anti-pneumococcal IgG was measured for the serotypes in PCV13 using a previously published modified ELISA method[17] and the international reference serum, 007sp (FDA/CBER).[17] Results are reported in μg/mL of serotype-specific IgG. Pre and post vaccination samples were tested in the same run.

2.5.2 | Anti-pneumococcal antibody assays—Opsonophagoytic assay to PCV13

Functional serotype-specific anti-pneumococcal IgG was measured post vaccination for 4 serotypes (1,4,9v, and 23F) in PCV13 using a multiplexed opsonophagocytic assay.[18] The serotypes for OPA assay were selected based on the most immunogenic serotype from the ELISA results for each multiplexed OPA. The antibiotic resistance of the bacteria used in each multiplexed assay were as follows: Optochin resistant (serotypes 4,18c,7f, and 3), spectinomycin resistant (serotypes 6b, 19f, and 1), streptomycin resistant (serotypes 5, 9v, and 14), and trimethoprim resistant (serotype 6a,19a, and 23f). Serial dilutions of heat inactivated infant sera (IgG remains intact) were incubated with cultured HL-60 phagocytic cells (American Type Culture Collection, Manassas, VA, US), rabbit complement (Pel-Freez, Arkansas, USA) and a mix of cultured antibiotic resistant streptococcus pneumoniae. After 45 minutes, the serial dilutions were plated to selective THYE agar plates. At 24 hours, the number of colonies per dilution was measured using a ProtoCol 3 colony counter (SynopticsLtd, UK). A control serum sample, a complement control (no serum) and a bacterial control (no complement) were included in each assay. Results were recorded as an opsonic titer, which is the reciprocal of the last serum dilution with at least 50% killing when compared to the average growth in complement control wells. An OPA ≥8 was accepted as a positive response.[18]

2.5.3 | Anti-HLA antibodies

Sera from a subset of patients (n = 15) were assessed for de novo anti-HLA antibodies. Cost precluded testing all patients and hence to reduce variability the subgroup was selected by excluding patients with eGFR < 30 mL/min/1.73 m^2 and age >65 which are known associates of reduced vaccine responses.[23] Five control Kidney transplant recipients, who did not receive vaccination, had sera collected at the same time points. Anti-HLA antibody assays were performed by the Victorian Transplantation and Immunogenetics Service (Parkville, Australia) using Luminex class I and II single antigen beads (One Lambda, Canoga Park, CA, USA).

2.6 | Outcome definitions

Seroprotection for PCV13 was defined as a geometric mean titer of ≥1.0 μg/mL.[24] Baseline seroprotection for PCV13 was defined as a geometric mean titer of ≥1.0 μg/mL before vaccination. Seroconversion for PCV13 was defined as a geometric mean titer of ≥1.0 μg/mL plus a two-fold rise in titer[24]

2.6.1 | Clinical outcomes

Kidney transplant recipients were reviewed in outpatient clinics every 3 months for 12 months post vaccination. Investigators were notified when kidney transplant recipients were admitted to hospital and the reasons for admission were determined. Additionally, at the end of the follow-up period a chart review was conducted for all participants to ensure no clinical events had been missed.

Severe proven pneumococcal infection was defined as an infection requiring admission whereby patient had clinical symptoms plus *Streptococcus pneumoniae* isolated from the clinical site of infection. All rejection episodes were biopsy proven. Allograft biopsies were

performed on the basis of a sustained rise in serum creatinine or for surveillance at 12 months post transplantation, where relevant.

2.7 | Statistical analysis

Median and interquartile ranges were used to describe continuous variables when the data were not normally distributed. Number and percentages were used to describe categorical variables. IgG antibodies to each polysaccharide antigen were quantified pre vaccination and post vaccination and geometric mean titers were calculated. Change in antibody concentrations pre vaccination and post vaccination were determined by Wilcoxon signed-rank testing. Statistical significance was set at a *P*-value of .05. Analyses were conducted using Stata Version 15 (College Station, Texas, USA) and GraphPad Prism Version 7 (GraphPad Software, La Jolla, California, USA).

3 | RESULTS

3.1 | Characteristics of participants

Fifty-eight (7%) of a possible 850 kidney transplant recipients consented to this study and received PCV13 vaccination. Of the 58 subject who completed pre vaccination testing, 45 completed both pre and post vaccination serological and OPA testing. The median time between pre vaccination and post vaccination serological testing was 27 (Range 21-59) days. The baseline demographics of the 45 subjects who completed all testing are presented in Table 1. The median age was 56.1 (47.0-63.9) and 60% were male. The median time from transplant was 2.24 years (1.1-5.7). A combination of tacrolimus plus mycophenolate plus prednisolone was the most common immunosuppressive regimen in 81.0%. All participants received basiliximab as induction therapy. The participants in this study were similar in age and other demographic details to the cohort of kidney transplant recipients at our institution who were not vaccinated as part of the study.[25] No subjects had previously received a pneumococcal conjugate vaccine, however, 27 (77%) had previously been vaccinated with PPV23 (Merck & Co., Inc.).

TABLE 1 Demographics

Characteristic	n (%) or median (IQR)
Age (y)	56.1 (47.0-63.9)
Sex	
Male	27 (60)
Ethnicity	
Caucasian	32 (71)
Asian	7 (16)
Other	6 (13)
Cause of ESKF	
Diabetes	11 (24)
IgA	6 (13)
Glomerulonephritis	4 (9)
PCKD	9 (20)
Reflux	3 (7)
Hypertension	2 (5)
Vascultitis	4 (9)
Other	6 (13)
Number of previous grafts	
0	42 (93)
≥1	3 (7)
Transplant duration (y)	2.2 (1.1-5.7)
Medications	
Tacrolimus	38 (84)
Mycophenolate	38 (84)
Prednisolone	42 (93)
mTOR inhibitor	3 (7)
Tacrolimus level (μg/l)	4.9 (4.0-5.5)
Mycophenolate dose (mg/d)	1080 (1000-1500)
eGFR (mL/min/1.73 m^2)	48.9 (41.0-68.4)
Rejection episode[a]	1 (2)
Prior PPV23 vaccination	33 (73)

ESRF, end-stage kidney failure; IQR, interquartile range; KTRs, kidney transplant recipients; mTOR inhibitor, Mammalian Target of rapamycin inhibitor; PCKDm, polycystic kidney disease; PCV13, 13-valent pneumococcal conjugate vaccine; PPV23, 23 valent polysaccharide pneumococcal vaccine.

[a]Rejection episode requiring corticosteroid therapy and occurring 12 to 3 mo prior to study enrolment.

3.2 | Seroresponses to PCV13

Data on the seroresponses to each serotypes are presented in table 2 and figures 1 and 2. Overall the number of serotypes for which patients had seroprotection significantly increased from a median of 7 (IQR 3.0-10.0) pre vaccination to 9 (IQR 4.0-11.0) post vaccination ($P < .001$). The median fold increase in anti-pneumococcal IgG antibody concentrations ranged from 1.1- to 1.7-fold across all 13 serotypes (Figure 1). For kidney transplant recipients with and without baseline seroprotection, the median fold increase in anti-pneumococcal IgG antibody concentrations ranged from 1.0- to 2.0-fold and 1.1- to 3.4-fold, respectively. Seroconversion differed by serotype with the greatest responses to 19A and 18C. The serotypes with the least response were 3 and 14. Figure 2 describes the percentage of subjects achieving seroconversion post-PCV13 vaccination for each serotype. Overall, 12 (27%) subjects seroconverted to zero serotypes, 26 (58%) seroconverted from 1 to 8 serotypes, and 7 (16%) seroconverted to ≥9 serotypes.

Four weeks post vaccination, kidney transplant recipients displayed an OPA titer ≥1:8 for 3 of the 4 serotypes tested (Table 2). For kidney transplant recipients without baseline seroprotection, there was an OPA titer of ≥1:8 for 2 of the 4 serotypes and for kidney transplant recipients with baseline seroprotection, there was an OPA titer of ≥1:8 for 4 of the 4 serotypes.

3.2.1 | Adverse events

Of the 15 patients (35.7%) who underwent anti-HLA antibody testing, 12 (80.0%) had pre-existing anti-HLA antibodies in pre-vaccination sera and in 5 (33.3%) patients these included DSA (Table 3). After vaccination, no new DSA were detected in any patient (0 of 15, 95%CI 0-22%) and there was no significant change in DSA mean fluorescence intensity for those with pre-existing DSA. Five patients had 1 to 3 additional anti-HLA Abs detected post vaccination without a common HLA antigen target. In 3 patients with pre-existing non-DSA anti-HLA antibodies 1 or more antibodies were no longer present on post vaccination sera (Table 3). No previously unsensitized patient developed any de novo anti-HLA antibodies. A similar pattern was observed in the non-vaccinated control transplant patients with 1 of 5 developing a new non-DSA antibody.

There were no cases of biopsy proven rejection at 12 months post vaccination.

Pneumococcal vaccination was well tolerated by 44 patients (97.7%). One patient developed arthralgia of hands, shoulders, knees, ankles, and feet 24 hours following vaccination. This patient did not require hospital admission and symptoms resolved spontaneously after 48 hours. At 12 months after PCV13 vaccination, no vaccine recipients had developed documented, microbiologically proven pneumococcal infections.

4 | DISCUSSION

This study examines seroresponse rates of PCV 13 in a solid organ transplant population. Here we demonstrate that a single dose of PCV13 elicits an increase in antibody concentrations for all pneumococcal serotypes and an increase in the number of serotypes with protective antibody titers. OPAs on selected serotypes suggested that the antibodies were functional for 3 of the 4 serotypes. The vaccine was well tolerated and did not result in graft rejection or the development of donor-specific antibodies in the subset tested. Given the increased risk of pneumococcal infection and its attendant high case fatality rate in KTRs, this study supports guideline recommendations for the use of PCV13 in this immunocompromized group.[1,2]

There have been limited studies examining anti-HLA antibodies and allograft rejection following pneumococcal vaccination and ours is the only study, to our knowledge, that has specifically examined the quantification of anti-HLA antibodies following PCV13 vaccination in solid organ transplant recipients. Lindemann et al[26] demonstrated that there was no increased anti-HLA antibody production following vaccination with a pneumococcal polysaccharide vaccine. Pneumococcal polysaccharide vaccine is a T-cell independent vaccine in contrast to PCV, which induces T-cell responses and is specifically designed to stimulate more robust immune activation. Theoretically, the risk of de novo antibody generation would be higher with adjuvated PCV than PPV owing to non-specific effects of the adjuvant leading to alloimmune stimulation. Increased anti-HLA antibody production and allograft rejection has been reported in renal transplant recipients vaccinated with adjuvanted influenza vaccine,[27] however several other studies did not corroborate this concern.[28,29] Studies examining allograft rejection following PCV7 vaccination were in keeping with our study with no increase in rejection.[26, 30–32]The studies examining PCV7 vaccination, however, did not assess anti-HLA antibody production.[26, 30–32] Our findings that DSA are not induced with adjuvanted pneumococcal vaccination provide reassurance but further studies with larger patient numbers are required for verification.

All subjects demonstrated a serological response to PCV13, however, overall the immunogenicity was poor. Only 16% achieved seroconversion to greater than 9 serotypes. There have been few studies of PCVs in solid organ transplant recipients and a lack of standardization of definitions between studies makes them difficult to compare.[31-35] Kumar et al[31] performed a randomized controlled trial whereby PPV23 was compared with PCV7 followed by PPV23 in kidney transplant recipients. There were 30 kidney transplant recipients in each arm. 73% kidney transplant recipients who received PCV7 followed by PPV23 had an antibody response to at least 1 serotype and 37%-53% had OPA responses to individual serotypes at 8 weeks. Notably, there was no difference in seroresponses between the arms. Three years following vaccination, geometric mean titers declined significantly for 6 of 7 serotypes.[36] A similar study performed in liver transplant recipients found that 81% of those who received PCV7 had antibody and OPA responses to at least 1 serotype at 8 weeks.[32] Tobrudic et al performed a randomized controlled trial whereby PCV7 was compared to PCV7 followed 1 year later by PPV23 vaccine. There were 40 kidney transplant recipients in each arm. 77% of kidney transplant recipients who received PCV7 vs 93% kidney transplant recipients who received PCV7 followed by PPV23 had antibody and OPA response to at least 1 serotype at 8 weeks. It is important to note that the serological cut off of 1μg/mL used in this study was the same as the cut off used in our study but substantially higher than that used in other studies, which makes the immunogenicity of the vaccine in this cohort appear inferior. International recommendations for the serological cut offs for clinical correlates of protection are based on a limited data.[37-40] In particular, for pneumococcal conjugate vaccines, the anti-capsular polysaccharide antibody concentration and OPAs that correlate with clinical protection are uncertain.[41] For PCV7, an anti-capsular polysaccharide antibody concentration of 0.35 μg/mL aggregated across all 7 serotypes is recommended as the correlate of clinical protection. However, more recent data suggest this cut off should be higher.[41] Andrews et al[41] measured serotype-specific antibody concentration in infants after 2 doses of either PCV7 or PCV13 and linked these to a registry of invasive pneumococcal disease. The aggregate correlate of protection against invasive pneumococcal disease for PCV7 and PCV13 was 0.59 μg/mL and 0.98, respectively. To further complicate the situation, the amount of antibody required to prevent clinical diseases differs according to serotype, as well as site of clinical pneumococcal infection.[41,42,44] A recent study found that OPA and IgG

TABLE 2 Seroresponses to the 13-valent pneumococcal conjugate vaccination

Serotype	1	3	4	5	6A	6B
Geometric mean titre μg/mL, median (IQR) n = 45						
Pre-vaccination	0.75 (0.52-1.1)	0.45 (0.33-0.61)	0.37 (0.25-0.69)	0.78 (0.58-1.00)	1.20 (0.94-2.04)	1.26 (0.89-1.70)
Post-vaccination	1.49 (1.00-2.01)	0.80 (0.44-0.83)	1.00 (0.67-1.49)	1.57 (1.06-2.33)	2.09 (1.47-2.91)	2.06 (1.46-2.91)
Opsonophagocytic assay μg/mL, median (IQR) n = 45						
	8.00 (4.00-152.5)		4.00 (4.00-46.00)			

antibody assays do not correlate well using current values for protective immunity against the pneumococcus in immunosuppressed transplant recipients.[45]

This study showed that both the antibody and OPA responses 4 weeks post PCV-13 vaccination were substantially lower than healthy controls of similar ages.[16,46] Bryant et al examined serological responses to PCV13 among healthy controls aged 60-64 and found responses were more than twice and OPA responses 15-500 geometric mean titers times that of our patients. Importantly, these patients were given a primary pneumococcal vaccine course compared with our cohort in which 3 quarters had previous exposure to PPV. In our study, a single dose of PCV13 was administered because of the number of patients with PPV vaccination within the last 5 years and because it is not recommended to re-dose owing to potential hyporesponsiveness.[32] The data regarding the effect of prior vaccination are conflicting. Blumberg et al[47] demonstrated that when heart transplant recipients received PPV vaccination, those previously vaccinated with PPV had increased seroresponse compared to heart transplant recipients undergoing primary pneumococcal vaccination course. Differing PCV dosing regimens, prior vaccination and the timing between PPV23 make comparison between studies challenging.[32-34] To further complicate matters, for a number of patients in our study we found limited records of previous pneumococcal vaccination. This represents a limitation of our study as we do not have accurate information about length of time between previous PPV23 and the current PCV13, as there may have been recall bias.

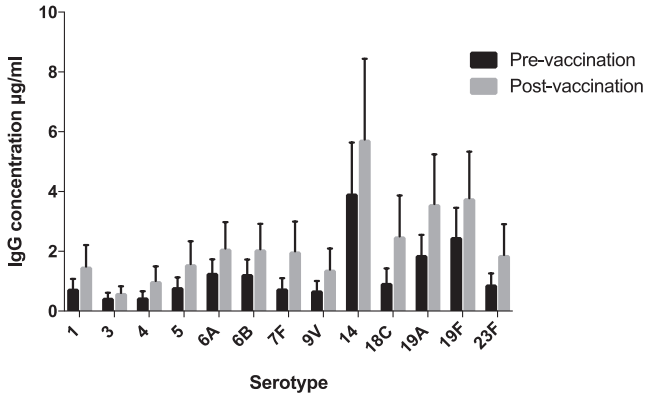

FIGURE 1 Pneumococcal antibody concentrations in 45 kidneys transplant recipients pre and 4 week post vaccination with 13-valent conjugate pneumococcal vaccine. Geometric mean and 95% confidence intervals are given separately for 13 serotypes of capsular polysaccharides. All 13 pre and post pairs $P > .001$

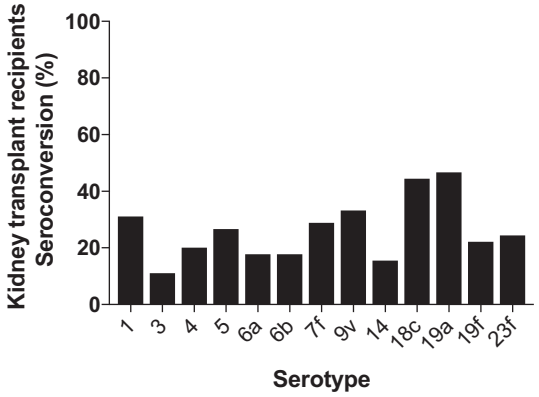

FIGURE 2 Percentage of kidney transplant recipients achieving seroconversion post 13-valent pneumococcal conjugate vaccine according to serotype. Seroconversion was defined as a ≥2-fold rise in pre-vaccination titer and a serotype-specific antibody concentration of ≥1.0 μg/mL

This is a single center, non-randomized study. The small sample size may not be representative of the total population and is not large enough to provide definitive evidence regarding de novo antibodies to HLA following PCV13 vaccination. The results of this study may not apply to pediatric patients or recipients of other types of solid organs as these groups were not included. Infections such as pneumonia that were managed as an outpatient were not captured.

The low numbers meant that it was not possible to perform multivariable analysis to identify risk factors associated with poor seroresponses. Immunocompromize has been associated with poor response to pneumococcal vaccination[15] but other specific host factors that decrease seroconversion are not well defined. Unlike influenza vaccination whereby the use of mycophenolate mofetil reduces vaccine efficacy,[23] Kumar et al found no association between type of immunosuppression and PCV7 vaccine efficacy in liver or kidney transplant recipients.[31,32] Gattinger et al[34] could not find an association between PCV vaccine response and age, sex, time from transplantation or immunosuppression. The absence of a concurrent control group makes it difficult to interpret the results.

In summary, this study demonstrated that kidney transplant recipients mount significant seroresponses to PCV13 but they appear

7F	9V	14	18C	19A	19F	23F
0.75 (0.52-1.10)	0.69 (0.48-1.00)	3.93 (2.74-5.60)	0.94 (0.62-1.42)	1.87 (1.37-2.55)	2.48 (1.78-3.45)	0.99 (0.63-1.25)
1.99 (1.33-2.99)	1.38 (0.91-2.09)	5.74 (3.90-8.44)	2.50 (1.61-3.80)	3.58 (2.44-5.24)	3.77 (2.67-5.33)	1.87 (1.12-2.90)
	102.00 (4.00-882.0)					11.00 (4.00-138.00)

TABLE 3 Development of anti-HLA antibodies in PCV13 vaccinated kidney transplant recipients n = 15

Patient number	Number of anti-HLA antibodies pre-vaccination	Number of anti-HLA antibodies post vaccination	De novo non-DSA anti-HLA antibodies
1	4	4	
2	4	6	DP28 and DP20
3	2	1	
4	2	2	
5	8	8	
6	10	10	B47 (Cw15 lost)
7	6	5	(DP5 lost)
8	5	5	
9	3	3	
10	5	8	B15, DP5, and DP11
11	5	5	
12	41	16	B76 (26 others lost)
13-15	0	0	0
Total	95	73	

HLA, Human Leukocyte antigen.

blunted compared to reports in healthy controls. As the participants in this study only received a single dose of PCV13, it is possible the seroresponses could be improved with a booster dose or prime boosting with PPV23, as recommended by guidelines. Future research in larger transplant cohorts could analyze factors related to vaccine efficacy and the optimal dosing strategies. The vaccine is safe and did not trigger the development of anti-HLA antibodies or allograft rejection. Our data support guideline recommendations for PCV vaccination of kidney transplant recipients. Further research could examine the efficacy and safety of PCV in larger populations of kidney transplant recipients through multicenter studies or using registry data.

DISCLOSURE

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Chapter 4:
Cellular immune biomarkers and infections

4.1 Introduction

This section explores the association between biomarkers of the cellular immune system and infectious outcomes in kidney transplant recipients.

T lymphocytes are the key effector cells of the cellular immune system and provide host defence against pathogens by responding to specific antigens, with rapid proliferation of T lymphocytes into effector cells that secrete cytokines for direct killing and stimulating antibody production.[1] All of the patients enrolled in this study were taking iatrogenic immunosuppression directly targeted towards depressing these T cell responses. It is postulated that certain agents are stronger inhibitors of T cell proliferative responses than others; however, clinical data to support this is limited.[2]

Several studies that have found an association between reduced T cell numbers (absolute CD4+ and CD8+ count) and all-cause infections, as well as opportunistic infections.[3,4] Two case-controlled studies that compared kidney transplant recipients with PCP to matched recipients without PCP found that absolute CD4+ cell numbers and lymphocyte counts were significantly reduced in PCP-infected kidney transplant recipients.[5,6] Stratification of patients according to CD4+ cell number nadir has been examined in lung transplant recipients. This study only included viral opportunistic infections and demonstrated that a CD4+ cell nadir less than 200 cells/mm^3 within the first three months of transplant was associated with an increased risk of viral opportunistic infections as well as poor T cell recovery.[7]

The measurement of T cell subsets is done in most immunology laboratories. They are well recognised, easy to interpret, standardised tests. In contrast, T cell proliferation assays are complicated laboratory tests to perform. They are time consuming and there are multiple steps in which errors and variations can occur. There is not a single methodology that is accepted as an international standard for T cell proliferation assays although, in recent years, there has been a move away from the previous 'gold standard'

assay using radioactive iodine labeled cells. In our study, in order to quantify T cell proliferation, we used 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled cells. CFSE is a non-radioactive dye that strongly binds to intracellular proteins such that it cannot egress from the cell and so is halved with each cell division.[8] Lymphocyte proliferation can be monitored by flow cytometry in up to eight divisions, before it is decreased to the background fluorescence of unlabeled cells.

There is no data that examines the use of CFSE proliferative responses and their association with infections in kidney transplant recipients.

In this section, the investigators wanted to determine whether measuring functional T cell proliferative responses offered any advantage over measuring peripheral blood T lymphocyte subsets to stratify patients at high risk of infection.

The hypothesis was that functional T cell proliferative tests were more likely to be able to discriminate patients who are 'over-immunosuppressed' as a result of their medications, and therefore at high risk of developing infection in the follow-up period.

Section 4.2 in this chapter is represented by the manuscript "The measurement of cellular immune competence and infection risk in kidney transplant recipients". This has not yet but is intended to be submitted for publication.

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4.2 The measurement of cellular immune competence and infections in kidney transplant recipients

HIGHLIGHTS

- 10% of stable kidney transplant recipients had T cell proliferative responses below the normal range for healthy controls.
- 24% of stable kidney transplant recipients had CD4+ cell number below the normal range for healthy controls.
- T lymphocyte proliferation to phytohaemagglutinin stimulation does not predict severe infection.
- Absolute CD4+ cell number predicts severe infection over two years follow-up.

ABSTRACT

The objective of this prospective study was to compare the ability of T lymphocyte proliferative assays and the quantitation of T lymphocyte subsets to predict severe infections in stable kidney transplant recipients.

In 2015, a cohort of 123 kidney transplant recipients underwent baseline measurement of CD4+ and CD8+ cell numbers and in vitro T cell responses to phytohaemagglutinin (PHA). The value of these biomarkers was estimated for predicting the development of severe infection within two years.

The median patient age was 56.1 (48.4–64.0) years, time from transplant was 4.1 (1.4–7.5) years and 85% were taking a combination of tacrolimus, mycophenolate and prednisolone. Forty-six (37%) patients developed infection requiring hospitalisation within the two-year follow-up period. After adjustment for age, transplant duration, estimated glomerular filtration rate and mycophenolate use, logistic regression for first severe infection demonstrated that increasing CD4+ cell number was more strongly associated with protection from infection (OR 0.84 per 100 cells/mL increase, 95% CI 0.74–0.95, $p = 0.006$) than increasing T lymphocyte proliferation (OR 0.99 per 1% increase, 95% CI 0.97–1.01, $p = 0.238$) or increasing CD8+ cell number (OR 0.82 per 100 cells/mL, 95% CI 0.67–1.02, $p = 0.079$). CD4+ cells are a primary target for current transplant immunosuppression and drug-induced reduction in number or function are associated with infection risk. This study shows that quantitation of circulating CD4+ cells is more predictive of infection risk than measurement of T cell proliferative function.

MANUSCRIPT

Introduction

Deficiencies of cellular immunity are a recognised risk factor for infections in kidney transplant recipients.[1] An effective cellular immune response relies on rapid expansion in the number of T lymphocytes in response to pathogenic challenge and can be quantified in vitro by measurement of T cell proliferative capacity. In human immunodeficiency virus infections, quantitative loss of CD4+ T cells occurs relatively late after seroconversion; however, loss or reduction of T cell proliferative capacity to in vitro stimulation can be detected much earlier.[2-5] In solid organ transplant recipients, there is limited data that compares quantification of the components of T cell protective immunity and their association with infectious outcomes[6] and no data to suggest whether measuring T cell functional capacity offers any advantage over measurement of absolute T cell numbers.

Lymphocyte proliferative function can be quantified by stimulating fluorescently labelled cells with mitogen and then measuring the uptake of the fluorescent dye, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), into daughter cells using flow cytometry. CFSE creates strong covalent bonds and does not diffuse out of the cell. The fluorescence of each labeled cell is divided equally between daughter cells, allowing the number of cell divisions to be determined.[7]

In this study, we report on T cell proliferative function for a cohort of Australian kidney transplant recipients who underwent cross-sectional testing of immune biomarkers then prospective follow-up for infectious outcomes.[8] We have previously reported associations between innate immune functional testing and infection but we were particularly interested in cellular immune competence, as immunosuppression following kidney transplantation is predominately directed towards blunting T cell responses.[8-12] Calcineurin inhibitors, mycophenolate mofetil (MMF) and mammalian

target of rapamycin inhibitors (mTORi) target different phases of T lymphocyte activation, however all inhibit T cell proliferation to some degree.[8-12]

The aim of this study was to compare the ability of circulating T lymphocyte proliferative responses and T lymphocyte subset numbers to predict severe infection in kidney transplant recipients.

Methods

Study design and setting

This study prospectively compared lymphoproliferative responses and lymphocyte subsets in 123 stable adult kidney transplant recipients. Patients underwent baseline testing and were then followed for two years for the development of severe infection, defined as hospital admission for infection. All severe infections were confirmed in real time by an infectious diseases physician, as described previously.[8] Specific infection types were defined according to internationally recognised definitions.[13-15]

Laboratory methods
T lymphocyte proliferative responses to phytohaemagglutinin

Peripheral blood was collected into tubes containing lithium heparin anticoagulant three to six hours after the patients had taken their usual immunosuppressive medication. The whole blood was mixed 1:1 with PBS and underwent separation using a ficoll gradient to remove serum and plasma and isolate peripheral blood mononuclear cells (PBMCs), which were used as the source of T lymphocytes for this study. The PBMCs were labelled with CFSE (Molecular Probes, Invitrogen) according to methods described elsewhere.[7] Fluorescent dye-labelled lymphocytes were cultured at a concentration of 2×10^6/mL in sterile RPMI (Invitrogen) supplemented with 10% foetal calf serum, penicillin and streptomycin (ICN Biomedicals) and glutamine RPMI in a total volume of 200µL in 96 well U-bottomed plates. PBMCs were stimulated with a range of doses (0.5–5 µg/mL) of phytohaemagglutinin (PHA, Wellcome, UK). Positive and

negative controls were included. After six days of incubation at 37°C and 5% CO_2, collected cells were stained with propidium iodide to determine dead cells, which were excluded from the analysis. They were then stained with anti-CD45 and anti-CD3. The cells were analysed on the Navios flow cytometer (Beckman Coulter, California, US), and a minimum of 10,000 events in a lymphocyte gate were analysed. Percentages of CD3+ CFSE proliferating cells were evaluated simultaneously by gating on CD3+ cells. Net percentages of CFSE were calculated by subtracting the positive control values (percentage proliferation of CFSE stained, PHA stimulated sample minus the percentage proliferation of CFSE stained, unstimulated sample). The PHA dose of 0.5µg/mL was included in the analyses as this dose had maximal separation of patients and controls.

T cell lymphocyte numbers

Peripheral blood T lymphocyte subsets (CD3+4+ and CD3+8+) were measured using flow cytometry.

Normal ranges of healthy controls

The normal ranges were derived using the 5^{th} to 95^{th} percentile measurements from a random cohort of staff controls who were well, with no known medical, inflammatory or infectious conditions.

The normal ranges were CD4+ cell number (389–1569 cells/mL), CD8+ cell number (168–894 cells/mL) and T cell proliferation to PHA (32–89%).

Statistical analysis

The Mann-Whitney U test was used to assess differences for two continuous non-parametric variables. Dichotomous variables were analysed using Fisher's exact tests or chi squared. Logistic regression was performed to determine predictors of first severe infection. Variables were put into the multivariable logistic regression model if they had a p value < 0.2 on univariable analysis. Age was included regardless of the univariable

analysis. *p* values < 0.05 were considered significant. Analyses were performed using Stata (Version 15, College Station, Texas, US) and Graph Pad Prism (Version 7, La Jolla, California, US).

Results

The median age was 56.1 (48.4–64.0) years, 63% were male, the median time from transplant was 4.1 (1.4–7.5) years and 81% of patients were taking a combination of tacrolimus, mycophenolate and prednisolone. No patients had received anti-thymocyte globulin within the 12 months prior to study entry or at any stage.

Severe infections

Over the two-year follow-up period, 46 (37%) patients developed severe infection requiring hospitalisation.

T cell proliferative responses to PHA

Representative examples of CFSE analysis are depicted in Figure 1A. There was no difference in the ability to respond to mitogen stimulation between healthy controls and the patient population as a whole: 79.9% (IQR 63.3–89.4) and 84.0% (IQR 61–86) ($p = 0.351$), respectively (Figure 1B). Twelve patients (10%) had T cell proliferation to PHA below the 5th centile for healthy controls. There was no significant difference in T lymphocyte proliferation at study entry between those who did and did not develop infection (Figure 1C).

T lymphocyte subsets and proportions

CD4+ cell number was the most commonly reduced cellular biomarker: 24 (20%) patients had a CD4+ cell count below the 5th percentile for healthy controls. Seventeen (14%) had a CD8+ cell number less than the 5th percentile of healthy controls. The median CD4+ cell number at study entry was lower among patients with severe infections compared with patients who did not develop severe infection: 558 cells/mL

(IQR 377–836) versus 797 cells/mL (IQR 471–1157) (p = 0.0045). There was no difference in median CD8+ cell number, CD4+ cell percentage or CD8+ cell percentage among patients with or without severe infection. The risk of severe infection was related to CD4+ cell number. The risk of infection increased for patients with CD4+ cell numbers below 750 cells/mL. Forty-seven percent of patients (32 of 68) developed severe infection had CD4+ counts below 750 cells/mL compared with 25% (14 of 55) with > 750 cells/mL (p = 0.002).

The relationship between T lymphocyte subsets, T lymphocyte proliferation and first severe infection

When the CD4+ count was within the normal range for healthy controls (n = 99), the percentage of patients who developed severe infection was similar whether the T cell proliferation to PHA was normal (31 of 93, 33%) or reduced (3 of 6, 50%). When the CD4+ count was below the 5th percentile for healthy controls (n = 24), the percentage of patients who developed severe infection was similar whether the T cell proliferation to PHA was normal (3 of 6, 50.0%) or below the 5th percentile for healthy controls (9 of 18, 50.0%).

First severe infection prediction models

Increasing T cell proliferation to PHA was not associated with severe infection on univariate (OR 0.99 per 1% increase, 95% CI 0.97–1.00, p = 0.107) or multivariate analysis (OR 0.99 per 1% increase, 95% CI 0.97–1.00, p = 0.211).

On univariate analysis, increasing CD4+ number (OR 0.84 per increase of 100 cells/mL, 95% CI 0.76–0.94, p = 0.003) and increasing CD8+ number (OR 0.82 per increase of 100 cells/mL, 95% CI 0.67–0.99, p = 0.042) were associated with a lower likelihood of severe infection. On multivariate analysis, only CD4+ number (OR 0.83 per increase of 100 cells/mL, 95% CI 0.72–0.95, p = 0.010) remained significantly associated with a reduced likelihood of severe infection.

The only other variable associated with severe infection in both the univariate and multivariate analysis was estimated glomerular filtration rate (eGFR) (OR 0.97 per increase of 10 mL/min/m^3, 95% CI 0.95–0.99, p = 0.018) (Table 1).

Discussion

The key finding of our study was that assessment of T cell proliferation did not offer an advantage over measurement of CD4+ cell counts for the prediction of severe infection in a cohort of renal transplant recipients. T cell proliferative assays are more technically challenging to perform, time consuming and expensive than CD4+ testing. Measurement of CD4+ cell counts may therefore be useful for clinical immune monitoring of kidney transplant recipients.

Ours is the first study to examine the association between T lymphocyte proliferation and infection using CFSE uptake in kidney transplant recipients. Valor et al. used this methodology to perform a small study (n = 12) in heart transplant recipients that demonstrated lower proliferative responses to mitogen in those that developed infection compared to those who did not.[6] Two studies have incorporated T cell proliferation results into an immune score in kidney transplant recipients but, like our study, found that the proliferative assays alone could not stratify infection risk.[16,17]
A number of studies have examined T cell proliferative responses in kidney transplant recipients but they have not correlated the results with infective outcomes. T cell proliferative assays derived from whole blood have reported substantially lower proliferation than those using isolated PBMCs, presumably because autologous plasma contains trace amounts of circulating immunosuppressive agents, which are known to inhibit T cell proliferative responses in nanomolar amounts.[17,18] Mycophenolate use is reported to be a strong inhibitor of T cell proliferation.[18-21] In our study, an association between mycophenolate use and infection approached statistical significance. Other biomarkers of T cell function are well described, such as soluble CD30[22-25] or

intracellular concentrations of adenosine triphosphate in stimulated CD4+ cells.[26-28] However, none of these tests have proven a predictive relationship with infection in SOT recipients.[18-20,29]

Our study demonstrated that CD4+ cell number is a significant predictor of severe infection and that the lower the CD4+ count, the higher the infective risk. For a drop of every 100 cells/mL, there was a 16% increase in the risk of severe infection, with 46% of patients developing a severe infection when the CD4+ count was less than 750 cells/mL. The analysis and monitoring of absolute CD4+ cell number and CD4+cell nadir have been shown in a number of studies to predict infection after kidney transplantation.[30-33] An absolute CD4+ cell number less than 200 cells/mL is predictive of opportunistic infections[30-33] and quantification of CD4+ cell number has been proposed as a monitoring strategy for duration of PCP prophylaxis.[34,35]

The reason that CD4+ cell number but not proliferative function predicts infection requires further investigation. It is possible that although proliferative function is targeted by immunosuppressive medications, the capacity to participate in host defence is preserved to some extent and it is only when the numbers of cells drop below a certain threshold that the capacity to kill organisms becomes clinically relevant. However, our small study did not find that reduced CD4+ cell count changed the risk of infection associated with reduced proliferative capacity. On univariable analysis, CD8+ cell number was predictive of infections, suggesting that cytotoxic T cells are important in host defence against infections but proportionally less so than CD4+ cells. However, the small sample size may have led to type II error and an underestimation of the predictive value of both CD8+ and proliferative capacity on infectious risk.

This study was performed at a single transplant centre and requires validation in other cohorts. We used a polyclonal mitogen for the proliferative assays. Further studies could add antigen stimulation, as this requires different effector pathways and may be a

more sensitive test of T cell functional ability.[6] Furthermore, there may be a disconnect between the proliferative capacity tested in vivo in this study and the proliferative capacity in vivo.

We have identified that the measurement of CD4+ cell number was superior to T cell proliferative responses for the prediction of severe infection in kidney transplant recipients. Considering there is data supporting the use of CD4+ counts for immune monitoring, and that in contrast to T cell proliferative assays it is a simple and inexpensive test, we would recommend that measurement of CD4+ counts be incorporated into the care of kidney transplant recipients to assess their risk of infection.

Authorship

Claire Dendle, William R. Mulley, Kevan R. Polkinghorne, Poh-Yi Gan, John Kanellis and Stephen Holdsworth participated in research design and preparation of the manuscript.

Claire Dendle, William R. Mulley and Poh-Yi Gan participated in the performance of the research.

Claire Dendle and Kevan R. Polkinghorne participated in the data analysis.

Disclosure

The authors declare no conflicts of interest.

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Table 1A. Univariable logistic regression analysis for predictors of first severe infection (n = 123)

	OR†	95% CI‡	*p* value
Age (per 10 year increase)	1.13	0.84–1.52	0.850
Sex (female)	0.88	0.41–1.89	0.748
Transplant duration (per year increase)	0.95	0.88–1.02	0.199
Number of previous grafts (per transplant)	0.69	0.33–1.45	0.335
MMF use	0.44	0.16–1.16	0.098
eGFR§ (per 10 mL/min/m^3 increase)	0.70	0.58–0.84	**< 0.001**
T cell proliferation to PHA stimulation (per 1%)	0.98	0.97–1.00	0.107
CD4+ cell number (per 100 cells/mL)	0.85	0.76–0.95	**0.003**
CD4+ cell percent (per 1%)	0.99	0.97–1.02	0.746
CD8+ cell number (per 100 cells/mL)	0.81	0.67–0.99	**0.042**
CD8+ cell percent (per 1%)	1.00	0.97–1.03	0.949

† OR: odds ratio, ‡ CI: confidence interval, MMF: mycophenolate mofetil, § eGFR: estimated glomerular filtration rate, PHA: phytohaemagglutinin

Bold indicates *p* value < 0.05

Table 1B. Multivariable logistic regression analysis for predictors of first severe infection (n = 123)

	OR†	95% CI‡	*p* value
T cell proliferation model			
Age (per 10-year increase)	0.87	0.61–1.25	0.480
Transplant duration (per year increase)	0.96	0.88–1.05	0.439
MMF use	3.13	0.87–11.34	0.082
eGFR§ (per 10 mL/min/m^3 increase)	0.67	0.54–0.82	**< 0.001**
T cell proliferation to PHA stimulation (per 1%)	0.99	0.97–1.01	0.238
CD4+ cell number model			
Age (per 10 year increase)	0.79	0.54–1.17	0.248
Transplant duration (per year increase)	0.96	0.86–1.05	0.369
MMF use	3.24	0.87–11.93	0.077
eGFR§ (per 10 mL/min/m^3)	0.67	0.54–0.82	**< 0.001**
CD4+ cell number (per 100 cells/mL)	0.84	0.74–0.95	**0.006**
CD8+ cell number model			
Age (per 10 years)	0.84	0.57–1.23	0.380
Transplant duration (per year)	0.97	0.88–1.06	0.500
MMF use	3.61	0.97–13.50	0.056
eGFR§ (per 10 mL/min/m^3)	0.67	0.55–0.83	**< 0.001**
CD8+ cell number (per 100 cells/mL)	0.82	0.67–1.02	0.079

† OR: odds ratio, ‡ CI: confidence interval, MMF: mycophenolate mofetil, § eGFR: estimated glomerular filtration rate, PHA: phytohaemagglutinin

Bold indicates *p* value < 0.05

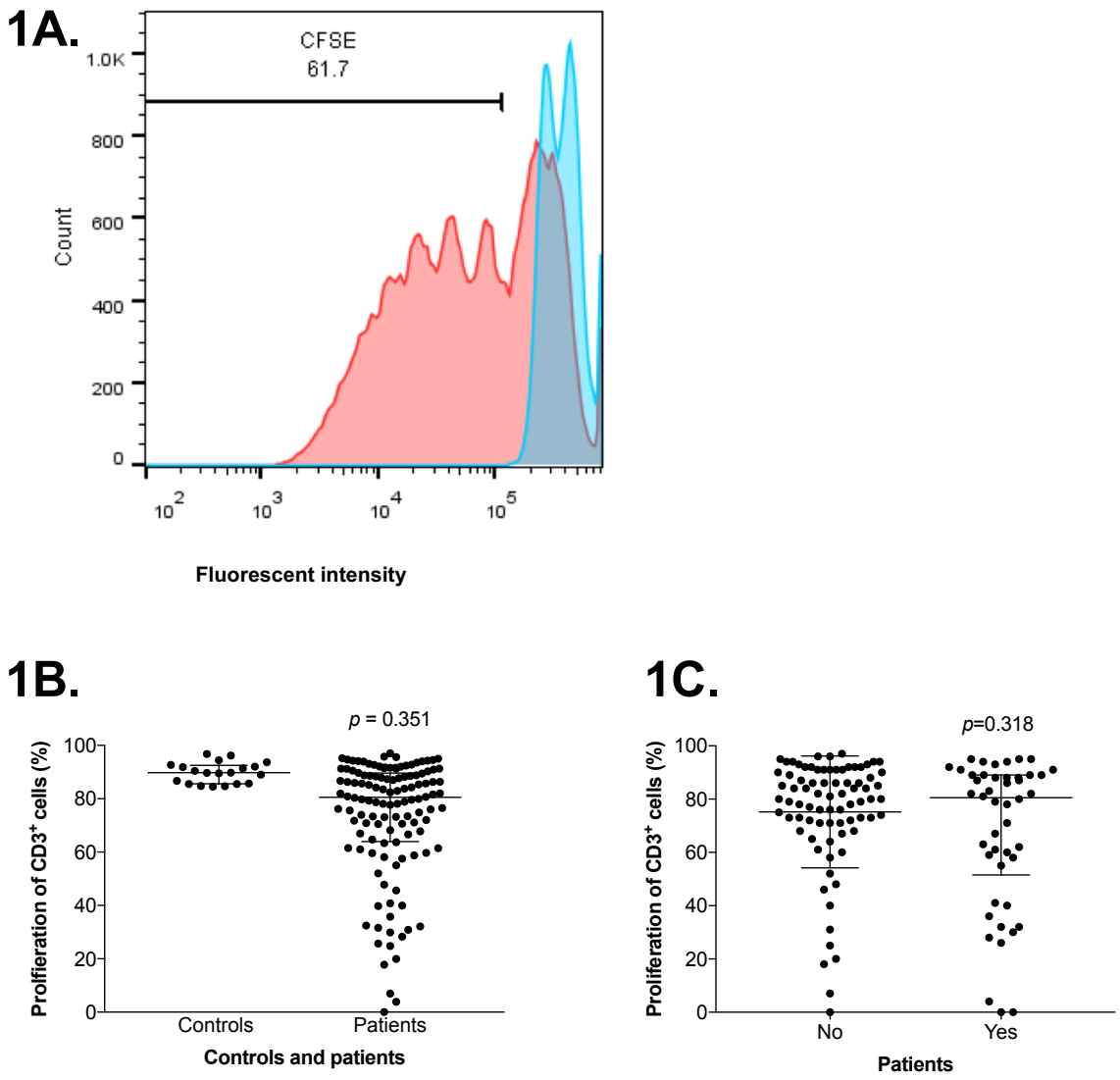

Figure 1. T lymphocyte proliferation to polyclonal mitogen in kidney transplant recipients

1A. Representative example of a kidney transplant patient showing peripheral blood mononuclear cells (PMBCs) labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and then stimulated with or without phytohaemagglutinin (PHA) to evaluate CD3+ proliferation by CSFE dilution. Blue shaded area shows division peaks of unstimulated PBMCs labelled with CFSE. CD3+ proliferation to unstimulated patient PBMCs is (0.49%). Red shaded area shows division peaks following PHA stimulation of PBMCs labelled with CFSE. Five distinct peaks are visible, reflecting five cell divisions. Percentage CD3+ proliferation to stimulated patient PBMCs is 61.7%.
1B. Comparison of the percentage proliferation of CD3+ cells to PHA stimulation between healthy controls and kidney transplant patients.
1C. Comparison of the percentage proliferation of CD3+ cells to PHA stimulation at study entry in kidney transplant patients who did and did not develop severe infection over the following two years.

Chapter 5:
A composite clinical and immune biomarker score and infections

5.1 Introduction

The data reported in this thesis so far have demonstrated an association between severe infections and reduced B cell numbers, reduced absolute CD4+ cell count and reduced natural killer cell cytotoxic function in kidney transplant recipients. The final study of this thesis compares the predictive value of these tests and also determines whether the ability to predict infection was improved if biomarkers were combined rather than taken in isolation. Natural killer cell cytotoxic function was the biomarker most strongly predictive of severe infection. However, natural killer cell cytotoxic function is not a test that is widely available as it requires isolation of peripheral blood mononuclear cells as well as maintenance of a target cell line. It was decided to exclude NK cytotoxic function from this analysis and focus on more readily accessible and well-validated biomarkers.

There are only a few published studies that examine composite scores of immune biomarkers to predict infection.[1-6] The study on which this section of the thesis is based was performed by Blazik et al. and examined five immune parameters including T cell proliferation (using radioactive thymidine), neutrophil phagocytosis and generation of reactive oxygen species, CD4+ cell number and immunoglobulins. They found that none of the individual biomarkers alone were predictive of infection; however, when incorporated into a composite score, the score was predictive of hospital admission with infection. In their study, each biomarker was equally weighted, which differs from our statistical methodology.[1] A more recent study of a composite immune score in 100 heart transplant patients in Spain found the score to be predictive of infection.[3]

The outcomes selected for this study were all severe infections, opportunistic infections and recurrent infections (two or more severe infections) in order to represent a broad range of infectious outcomes, considering that both innate and adaptive biomarkers were included in the score. There were 166 kidney transplant recipients included in this

study, which represent a larger analysis group than used for NK cytotoxic function testing, T cell proliferation and influenza and pneumococcal vaccination responses, as almost all patients were able to undertake peripheral blood testing.

The hypothesis was that a composite score of immune biomarkers would be superior to the individual biomarkers alone, as host defence requires multiple mechanisms and if patients were deficient in more than one area, they would be more susceptible to infection.

Section 5.2 is represented by the manuscript "A simple score can identify kidney transplant recipients at high risk of severe infections over the following two years". This manuscript has been submitted to *Transplant International* for publication**.**

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5.2 A simple score can identify kidney transplant recipients at high risk of severe infections over the following two years

HIGHLIGHTS

- Over two years, 59 (34%) kidney transplant recipients developed severe infection.
- A group of patients at very high risk of infection could be prospectively identified by a point score.
- Predictive variables included in the score were CD4+ and NK cell number, eGFR and mycophenolate use.
- The point score's area under the receiver operating curve for first severe infection was 0.75 (95% CI 0.67–0.83).

SUBMITTED MANUSCRIPT

Dendle C, Polkinghorne KR, Mulley WR, Gan PY, Kanellis J, Stuart RL, Thursky K, Holdsworth SR. A simple score can identify kidney transplant recipients at high risk of severe infections over the following two years.

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ABSTRACT

Introduction: The aim of this study was to determine whether a composite score of simple immune biomarkers and clinical characteristics could predict severe infections in kidney transplant recipients.

Methods: We conducted a prospective study of 168 stable kidney transplant recipients who underwent measurement of lymphocyte subsets, immunoglobulins and renal function at baseline and were followed for two years for the development of any severe infections, defined as infection requiring hospitalisation. A point score – the Level of Immunosuppression Score – was developed to predict severe infection based on logistic regression analysis of factors in baseline testing.

Results: Fifty-nine (35%) patients developed severe infection, 36 (21%) had two or more severe infections and three (2%) died of infection. A group of 19 (11%) patients had the highest predicted infectious risk (> 60%), as predicted by the score. Predictive variables were mycophenolate use, graft function, CD4+ and natural killer cell number. The Level of Immunosuppression Score had an area under the receiver operating curve of 0.75 (95% CI 0.67–0.83).

Conclusions: Our Level of Immunosuppression Score for predicting the development of severe infection over two years has sufficient prognostic accuracy for identification of high-risk patients. This data can inform research that examines strategies to reduce the risks of infection.

MANUSCRIPT

Introduction

Hospitalisation with infection remains an important cause of morbidity and mortality in solid organ transplant recipients.[1,2] The risk of infection post-transplant is determined by the balance between the net state of immunosuppression, epidemiological exposure to infectious agents and prophylactic strategies. An episode of severe infection generally indicates that the level of immunosuppression is too high for a particular patient. While most transplant units offer standardised immunosuppressive regimens, these can have markedly heterogeneous immunological effects on patients; biomarkers are needed to stratify patients into those at the highest risk of infection.

We performed a study whereby we tested immune biomarkers and their association with different infectious outcomes in kidney transplant recipients. We found that natural killer cell (NK) cytotoxic function was predictive of infection.[3] However, NK cell cytotoxic function is a specialised test, thus limiting its implementation into routine clinical practice. We therefore wanted to determine whether other cheaper and more readily available tests of immune function could also predict severe infection.

The aim of this current study was to determine whether a composite score of simple immune biomarkers along with specific clinically important characteristics could predict severe infections in kidney transplant recipients. We hypothesised reduced lymphocyte subsets (T cell, B cell and NK cell counts) combined with kidney transplant function would identify patients at high risk of infection.

Methods

We conducted a prospective, single-centre cohort study to evaluate the performance of a composite immune score to identify transplant recipients at risk of severe infection during two years of follow-up.

Ethical statement

The study was approved by the human research ethics committee of Monash Health (Number 13085). Written informed consent was obtained from all participants.

Setting

This prospective cohort study was performed at Monash Health, a large health service in Australia that performs approximately 90 kidney transplants per year.

Patients

In total 168 patients were enrolled in the study between April and August 2015. All kidney transplant recipients who were greater than three months post-transplant were invited to participate. Patients were excluded from the study if they were ≤ 18 years of age if they had received increased immunosuppression to treat rejection within three months. All patients underwent measurement of immune biomarkers at study entry and were followed for two years for the development of any severe infections. Baseline clinical characteristics were determined and included age, gender, ethnicity, cause of the end-stage kidney disease, duration from transplant (years), number of previous transplants, current immunosuppressive regimens, mycophenolate dose, tacrolimus level, cytomegalovirus donor-recipient serostatus and estimated glomerular filtration rate (eGFR). The eGFR was calculated by the CKD-EPI formula.[4]

Baseline laboratory testing

The biomarkers selected for inclusion in this study were based on outcome data from our cohort[3] as well as published literature demonstrating an association between the biomarkers and infections.[5] The biomarkers included for analysis in this study were CD45+, CD4+, CD8+, CD19+, CD56±16± (NK) cell numbers and immunoglobulin concentrations.

Lymphocyte subsets

Lymphocyte subset testing was performed on freshly collected peripheral blood. Fluorochrome-conjugated monoclonal antibodies were obtained from Beckman Coulter (Brea, California, US) and cells were acquired on an FC500 flow cytometer (Beckman Coulter). Total white cells were identified by the immunophenotyped CD45+, T cells were identified by the immunophenotype CD3+/CD4+ and CD3+/CD8+, B cells were identified by the immunophenotype CD3–/CD19+ and NK cells were identified by the immunophenotype CD56±16± after gating on lymphocytes by forward and side scatter characteristics. Absolute numbers were calculated using the lymphocyte count provided by full blood examination.

Immunoglobulins

Immunoglobulin isotype (IgA, IgG, IgM) concentrations in serum were measured by Southern Cross Pathology (Dade Behring Dimension Clinical Chemistry System).

Infectious outcomes

The primary outcome was severe infection, defined as any infection requiring admission to hospital. The secondary outcomes were opportunistic infection and recurrent infection. Opportunistic infection was defined as those due to intracellular bacteria (mycobacteria spp., *Nocardia* spp., *Legionella* spp. and *Listeria monocytogenes*), herpes viruses (cytomegalovirus [CMV], herpes simplex virus [HSV] and varicella-zoster virus [VZV] and Epstein-Barr virus–related post-transplant lymphoproliferative disease), yeasts (*Candida* spp. and *Cryptococcus* spp.), moulds, *Pneumocystis jirovecii* (PCP) and parasites (*Toxoplasma gondii* and *Leishmania* spp.).[6] Polyoma virus was included under the definition of opportunistic infections. It was the only infection in this study that was included whereby none of the patients were hospitalised as a direct result of the infections. Polyoma virus was only included under the definition of opportunistic infection, and not included as a “severe infection”. The definition of

polyoma virus included both BK virus and John Cunningham virus. Polyoma virus replication was defined by increasing polyoma viral loads. Probable polyoma virus disease was defined as viral replication > 104 copies per mL or together with compatible symptoms and signs of viral syndrome or organ disease, but without histological confirmation. Proven polyoma virus disease was defined as evidence of virus replication plus corresponding specific histopathology.(7,8) Patients underwent regular polyoma virus screening through polyoma virus specific polymerase chain reaction testing on plasma. Recurrent infection was defined as two or more episodes of severe infections requiring admission after the date of enrolment. All recurrent infections were new infections, rather than relapse of previously diagnosed infections.

Details of all severe infections were collected. When a patient was hospitalised with infection, study investigators were notified, at which time a specialist infectious diseases physician assessed the patient clinically to determine the site of infection and review the microbiological investigations. In addition, all patients underwent three-monthly clinical reviews at a specialty transplant clinic, where an assessment of infectious episodes requiring admission was performed to ensure all severe infections were captured.

Infectious episodes were classified as microbiologically defined (a microorganism related to the clinical presentation was isolated) or clinically defined (no micro-organism related to the clinical presentation was isolated but a clinical diagnosis of the site of infection could be determined by the study investigators). Fever without focus was defined as a febrile patient, with no microorganism related to the clinical presentation isolated and no site of infection able to be determined.

CMV infection was defined as virus isolation, or detection of viral nucleic acid in any body fluid or tissue specimen.[9] Fungal infections and bloodstream infections and sepsis were defined by internationally recognised criteria.[10-12]

Each episode of infection was classified according to source: bloodstream infection, upper respiratory tract, lower respiratory tract, cardiovascular, gastrointestinal, urogenital, neurological, skin and soft tissue, bone and joint, other, device related or line related, and source unknown.

Statistical analysis

We assumed an expected rate of severe infection of 20% based on previous studies from our centre and other reported rates.[13-15] The minimum required sample size for an area under the receiver operating characteristic (ROC) curve of 0.80 was 64 patients assuming a 90% power and an alpha at 0.05.

Variables with a normal distribution were presented as mean ± standard deviation; variables with a skewed distribution were presented as medians and interquartile range. Chi-square or Fischer's exact test were used to determine whether or not an association existed between severe infection and categorical variables. Mann-Whitney U test was used to assess differences between non-parametric continuous variables in patients with and without severe infection.

Logistic regression was used to evaluate immune biomarkers as potential predictors for the first episode of severe infection. Clinical and immune biomarker variables were entered into univariable models. Variables that demonstrated a significant association ($p \leq 0.05$) with the development of severe infection were included in the multivariable model. Age, time from transplant, mycophenolate use and eGFR were included in all multivariable models due to their known influence on infection risk.[16,17]

A point score tool was derived from the multivariable logistic regression modelling. Variables that demonstrated a significant association ($p \leq 0.05$) with a coefficient with an absolute value equal or greater than 0.1 were put into the Level of Immunosuppression Score. The level of immunosuppression scores were created by

multiplying each estimate by four and rounding the values to one decimal place for continuous variables and to the nearest integer for categorical variables.[18] In the case of eGFR, CD4+ cell number and NK cell number, a decreasing score was associated with an increasing risk of infection and the resulting score was negative (i.e. subtracted). A constant of 18 was added to each individual derived score in order to scale the final total Level of Immunosuppression Score in the range of 0–20, where an increasing score was associated with a higher risk of infection. Level of immunosuppression scores were categorised to identify subgroups at higher risks and the proportion of patients with severe infection was summarised for these subgroups. ROCs were calculated from the logistic regression models.

All tests were two-sided with a p value < 0.05 considered significant. All statistical analyses were conducted using Stata (Version 15, College Station, Texas, US) and Graph Pad Prism (Version 7, La Jolla, California, US).

Results

Characteristics of kidney transplant recipients

One hundred and sixty-eight of a possible 850 (20%) kidney transplant recipients accepted the offer to participate in this study, 114 (13%) declined to participate, 50 (6%) met exclusion criteria and 519 (61%) did not respond to the invitation. Two patients did not undertake blood testing, leaving 166 (20%) included in the analysis.

The median age was 56.1 (47.1–63.9) years and 106 (63%) were male. The median time from transplant was 4.1 (1.6–7.8) years. Most patients (81%) were taking tacrolimus, mycophenolate and prednisolone maintenance immunosuppression. No patients had received anti-thymocyte globulin within the 12 months prior to study entry. Of the 168 participants, 157 (93%) had received annual seasonal influenza vaccination for a minimum of two years prior to study entry and annually during the study follow-up period. Forty-five (27%) received 13-valent pneumococcal conjugate vaccination after

study entry. We were unable to definitely determine the prevalence and type of other vaccinations prior to study entry due to inadequate documentation in the medical record. All patients were followed for two years. The clinical characteristics and immune biomarkers are presented in Table 1. The patients in this study were similar in age and other demographic details to the cohort of kidney transplant recipients at our institution.[19]

Description of severe infections

Fifty-nine (35%) of the 168 patients had at least one severe infective episode, with a total of 141 episodes, during the two-year follow-up. Of the patients with severe infections, 36 (21%) had recurrent infection (two or more infections).

Of the 141 episodes of severe infection, 68 (48.2%) were microbiologically proven, 72 (51.1%) were clinically defined and one (0.7%) was fever without focus.

The microbiology proven infections included 33 (48%) bacterial, 29 (43%) viral, five (7%) fungal and one (2%) parasitic. *Escherichia coli* was the most frequently detected bacteria causing severe infection.

The most common source of infection was respiratory, accounting for 53 (38%) severe infections. There were eight (6%) episodes of bloodstream infection and 10 (7%) episodes of sepsis.

The median length of stay for severe infection was 5.0 days (IQR 2.0 to 215.5); 17 (12%) admissions required intensive care. At the end of the follow-up period, 115 (82%) participants were cured of their infections and 23 (16%) were not cured.

Seven (9%) participants died during the follow-up period, three (2%) directly attributable to infection.

Sixty-nine (48.9%) of the 141 infections were defined as opportunistic, occurring in 28 (17%) patients. There was one *Nocardia* spp. pulmonary infection. Viral infections were the predominant microbiologically proven opportunistic infection, with one VZV, three EBV, seven CMV, one HSV encephalitis, one disseminated adenovirus infections and 51 cases of polyoma virus (42 polyoma virus replication, four probable polyoma virus disease and five proven polyoma virus disease). There were five fungal infections (three cases of proven invasive pulmonary aspergillosis and one case of disseminated cryptococcosis) and one probable fungal infection (*Pneumocystis jirovecii* pneumonia, PCP). There was one case of disseminated microsporidiosis.

Description of rejection episodes

Thirteen patients (8%) experienced a rejection episode after study entry. Of the 13 rejection episodes, 12 were chronic antibody-mediated rejection and one was chronic antibody medicated rejection plus cellular rejection. Of the 168 patients, 104 (62%) had neither severe infection nor rejection, 51 (30%) developed severe infection alone, five (3%) developed rejection alone and eight (5%) developed severe infection and rejection.

Logistic regression for first severe infection

Results of the logistic regression models for first severe infection are presented in Table 2. On univariable logistic regression, a decreasing CD4+ number was associated with an increased risk of infection (per each decrease of 100 cells/mL, the odds ratio [OR] was 0.85, 95% CI 0.77–0.94, $p = 0.002$) and a decreasing NK number was also associated with an increased risk of infection (per each decrease of 100 cells/mL, OR 0.71, 95% CI 0.51 to 0.99, $p = 0.044$).

Multivariable logistic regression demonstrated that the only clinical variable statistically associated with development of infection was mycophenolate use. The biomarker variables that were statistically associated with development of infection

were decreasing eGFR (per 10 mL/min/m^2 decrease) (OR 0.70, 95% CI 0.58–0.84, $p < 0.0001$), decreasing numbers of CD4+ cells (OR 0.86, 95% CI 0.77–0.96, $p < 0.0001$) and decreasing NK cells (OR 0.64, 95% CI 0.43–0.93, $p = 0.019$). They all remained predictive of an increased likelihood of severe infection in the multivariable model. Immunoglobulin concentrations were not significantly associated with severe infections.

Calculation of the Level of Immunosuppression Score

The Level of Immunosuppression Score was calculated by converting coefficients in Table 2. Decreasing CD4+ cell number, decreasing NK cell number, decreasing eGFR and MMF use increased the risk of severe infection, whereas age and time from transplant were not sufficiently strongly predictive to warrant a point score. The individual risk factors that received points were eGFR (–1.2 points), mycophenolate use (6 points), CD4+ (–0.5 points) and NK number (–1.7 points). A constant of 18 was added to each score with maximal score of 20, suggesting global immune impairment. Table 3 describes predictions for the Level of Immunosuppression Score and theoretical examples.

The baseline Level of Immunosuppression Score demonstrated a linear increase in the probability of severe infection, such that when the score was above 15, there was an 0.6 probability of hospitalisation with infection in the next two years. For every one point increase in the Level of Immunosuppression Score, the risk of infection increased (OR 1.27, 95% CI 1.16–1.40, $p = 0.0001$) (Figure 1). The ROC for first severe infection according to the Level of Immunosuppression Score demonstrated moderate to good predictive ability with an AUROC curve of 0.75 (95% CI 0.67–0.83).

The median Level of Immunosuppression Score was 9.9 (IQR 6.5–13.5). The Level of Immunosuppression Score categorised according to ranges and the proportions of patients with infection is presented in Table 4. The Level of Immunosuppression Score

had a threshold effect for the first severe infection. Severe infections occurred in three of 25 (12%) of patients with a score of 0–5, 13 of 61 (21%) of patients with a score of 5.1–10, 27 of 61 (44%) of patients with a score of 10.1–15 and 16 of 19 (84%) of patients with a score of 16.1–20.

The baseline increasing Level of Immunosuppression Score was also significantly predictive of opportunistic infection (OR 1.25, 95% CI 1.12–1.40, $p < 0.0001$) and recurrent infections (OR 1.39, 95% CI 1.23–1.58, $p < 0.0001$).

Discussion

A baseline score of simple biomarkers and clinical characteristics could predict hospitalisation with severe infection over the next two years in kidney transplant recipients. This data can be used to help clinicians identify patients at high risk of infection, and can help inform trials examining strategies to reduce infections in transplant recipients.

The score consisted of four components: CD4+ cell number, natural killer cell number, eGFR and mycophenolate use. Each of the predictors in the score have independently been associated with infection in other studies but ours is the first to combine them. CD4+ cell count is associated with opportunistic and all-cause infections.[6,13,20-22] There is emerging evidence of an association between NK number and infections (such as fungal infections) in SOT recipients.[23-27] Interestingly, we found NK cell cytotoxic function as superior to the measurement of NK cell number.[3]

A poorly functioning graft, as evidenced by reduced eGFR, was a powerful risk factor for severe infection in this study. It may be that patients with poor eGFR had been exposed to increased immunosuppression as treatment for chronic rejection. We controlled for this relationship by recruiting only patients with stable graft function who had not received increased immunosuppression in the past three months and including a past

history of rejection and exposure to immunosuppressants in the univariable logistic regression. None of these factors were significantly associated with severe infections; however, this may represent a type II statistical error. Another possibility is that uraemia itself may predispose to infection above and beyond that from iatrogenic transplant immunosuppression. Infections are one of the leading causes for increased morbidity and mortality among patients with chronic kidney disease.[28] Uraemia directly or indirectly affects the function of a number of immune cells.[29] For example, defective phagocytosis can be caused by uremic toxins, iron overload and anaemia of renal disease.[30] Reduced eGFR in kidney transplant recipients has also been associated with poor vaccine responsiveness.[31] These factors may in part explain why reduced eGFR was an important risk factor for severe infection in our study.[16]

Mycophenolate use had previously been shown to be associated with increased risk of infection.[32]

Only a few studies have examined the use of a composite immune score in SOT recipients, but these are difficult to compare directly with ours.[13-15,23,26,33] The majority of these studies have been performed in the early post-transplant period, whereas our study specifically examined patients who were stable on immunosuppression and some months or years post-transplant (median time from transplant 4.1 years). Quantifying the net state of immunosuppression with the Level of Immunosuppression Score at this time is useful for clinicians in two circumstances. Firstly, if the patient is 'over-immunosuppressed', with a high Level of Immunosuppression Score and high infectious risk, it might be worthwhile considering whether the risk of infection outweighs the risk of rejection at an individual patient level. Secondly, when a patient with a high Level of Immunosuppression Score is admitted with an active infection, an understanding of the degrees of immunosuppression may help clinicians to decide on the empirical antimicrobial management and diagnostic pathways.

A large proportion of patients (35%) were hospitalised with severe infection over the two-year follow-up period, which is substantially higher than the rate in the general population[34] and highlights the need to address infection prevention in our own institution. The rate of infections is similar to that reported elsewhere in the transplant literature, suggesting infection is common and there is an unmet need to identify patients at highest risk. In this study, we carefully followed the consequences of these infections. Three patients died as a direct result of infection but the impact was broader in terms of lengthy hospitalisations and intensive care admissions. Infection risk is clearly an important issue in the management of transplant patients at our own institution and it is probable that with the demographics of transplant recipients, with older recipients and the use of more marginal donors, it will continue to be a key factor in transplant patients' care.

This study was performed at single transplant centre and although prospective, the score needs validation in other kidney transplant cohorts. Importantly, we did not simultaneously examine rates of rejection, which would be necessary in any study looking to reduce immunosuppression.[35] We only recorded infectious episodes for patients to our institution; therefore, the number of infections may be underestimated, since patients may have been admitted elsewhere or had an infection that did not require hospitalisation.

The strengths of this study relate to the prospective design, the careful identification and diagnosis of infectious outcomes and the simplicity of biomarkers tested. We showed that readily available clinical features and biomarkers at a single point in time can be used in a score to identify a subgroup of patients at very high risk of severe infection in the next two years.

Further research could be directed to not only validating this data but also using these findings to inform clinical trials examining strategies to reduce the risk of infections.

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Table 1. Clinical, demographic immunological characteristics of kidney transplant recipients

Characteristic		Number (%) or median (interquartile range)
Age (years)		54.6 (47.1–63.9)
Sex	Male	106 (63)
Ethnicity	Caucasian	106 (63)
	Asian	20 (12)
	Other	42 (25)
Cause of ESRF	Diabetes	37 (22)
	Glomerulonephritis	31 (18)
	PCKD	18 (11)
	Other	
No. of previous transplants	0	150 (89)
	≥ 1	18 (11)
Transplant duration (years)		4.1 (1.6–7.8)
Medications	Tacrolimus	137 (81)
	Mycophenolate	140 (83)
	Azathioprine	22 (13)
	Prednisolone	144 (86)
	mTORi	11 (6)
Tacrolimus level (µg/l)		4.6 (3.5–5.5)
MMF dose (mg/day)		1375 (1000–1500)
eGFR mL/min/m^2		54.9 (41.0–73.2)
CMV donor/recipient serostatus	D-R-	21 (12)
	D-R+	60 (36)
	D+R+	68 (40)
	D+R-	19 (11)
CD45+ number cells/mL		7.2 (6.0–8.8)
CD4+number cells/mL		683 (427–986)
CD8+ number cells/mL		344 (244–520)
CD4+8+ ratio		1.9 (1.2–3.0)
CD19+ number cells/mL		103 (61–212)
NK number cells/mL		140.0 (77.5–211.5)
IgG g/L		8.8 (7.15–15.6)
IgM g/L		0.8 (0.5–1.1)
IgA g/L		1.7 (1.0–2.7)

IQR = interquartile range, ESRF = end-stage renal failure, IgA = IgA nephropathy, PCKD = polycystic kidney disease, MMF = mycophenolate, mTORi = mammalian target of rapamycin inhibitor, eGFR = estimated glomerular filtration rate, CMV = cytomegalovirus, D = donor, R = recipient

Table 2. Logistic regression for first severe infection

	Univariable analysis			Multivariable analysis			
Variables	**OR**	**95% CI**	***p* value**	**Coefficient**	**OR**	**95% CI**	***p* value**
Age (per 10 years)	1.18	0.90–1.52	0.236	–0.12	1.00	0.72–1.39	0.978
Sex (male)	0.77	0.38–1.56	0.473				
Transplant duration (per year)	0.95	0.89–1.02	0.167	–0.19	0.96	0.88–1.04	0.368
Number of previous transplants (per 1 increase)	0.71	0.35–1.47	0.365				
Mycophenolate use	2.62	0.93–7.40	0.068	1.44	3.86	1.06–13.94	**0.039**
Tacrolimus use	1.10	0.46–2.67	0.818				
Prednisolone use	1.31	0.47–3.64	0.599				
mTORi use	0.91	0.23–3.80	0.901				
Tacrolimus level	1.12	0.94–1.35	0.179				
History of rejection	1.13	0.49–2.62	0.760				
eGFR (per 10 mL/min/m^2)	0.74	0.61–0.87	**0.001**	–0.30	0.73	0.60–0.88	**0.001**
Total white cell number (per 1 increase)	1.01	0.89–1.15	0.861				
CD4+ cell number (per 100 increase)	0.85	0.77–0.94	**0.002**	–0.13	0.86	0.77–0.95	**0.007**
CD4+ cell % (per 1% increase)	0.99	0.97–1.02	0.925				
CD8+ cell number (per 100 increase)	0.89	0.77–1.01	0.880				
CD8+ cell % (per 1% increase)	1.00	0.97–1.03	0.818				
CD19+ cell number (per 100 increase)	0.81	0.62–1.04	0.110				
CD19+ cell % (per 1% increase)	1.00	0.97–1.04	0.739				
NK number (per 100 increase)	0.71	0.51–0.99	**0.044**	–0.42	0.64	0.44–0.93	**0.022**

	Univariable analysis			Multivariable analysis			
Variables	**OR**	**95% CI**	***p* value**	**Coefficient**	**OR**	**95% CI**	***p* value**
NK cell % (per 1% increase)	0.99	0.96–1.03	0.698				
IgG concentration (per 1 increase)	0.96	0.87–1.07	0.502				
IgM concentration (per 1 increase)	1.10	0.65–1.85	0.714				
IgA concentration (per 1 increase)	0.87	0.65–1.150	0.322				

Bold indicates *p* value < 0.05

Table 3. Predictions for the two-year risk of severe infection using the Level of Immunosuppression Score

Predictive variable	Level of Immunosuppression Score	Examples		
		Three hypothetical kidney transplant recipients		
Characteristics of hypothetical patients		MMF use eGFR 90 mL/min/m^2 CD4+ 800 cells/mL NK 500 cells/mL	MMF use eGFR 50 mL/min/m^2 CD4+ 500 cells/mL NK 300 cells/mL	MMF use eGFR 30 mL/min/m^2 CD4+ 200 cells/mL NK 50 cells/mL
Mycophenolate use	6	6	6	6
eGFR per 10 mL/min/m^2	–1.2	–10.8	–6	–3.6
CD4+ per 100 cells/mL	–0.5	–4	–5	–1
NK per 100 cells/mL	–1.7	–8.5	–8.5	–0.85
Summative total points		**–17.3**	**–13.5**	**0.55**
Final Level of Immunosuppression Score (addition of constant of 18)		**0.7**	**4.5**	**18.55**

Level of Immunosuppression Score derived by multiplying each coefficient from multivariable analysis by 4, summing the values and adding a constant of 18.

Normal ranges for the immune biomarkers were derived from cuts offs derived using 5th to 95th percentile measurements from a random cohort of staff controls who were well, with no known medical, inflammatory or infectious conditions.

The normal range of CD4+ cell number for healthy controls (5th–95th centile) at our institution was 389–1569 cells/mL.

The normal range of NK number for healthy controls (5th–95th centile) at our institution was 61–776 cells/mL.

Table 4. Level of Immunosuppression Score ranges and the observed number of patients, eGFR, CD4+, NK cell number and the number admitted with severe infection per range

Level of Immunosuppression Score range	Number (%) of patients	eGFR 10 mL/min/m²	CD4+ number cells/mL	NK number cells/mL	Number (%) of patients with severe infection
0–5	25	76.7 (62.1–89.2)	951 (750–1308)	189 (115–287)	3 (12)
5.1–10	61	67.1 (51.0–81.4)	839 (603–1067)	167 (97–223)	13 (21)
10.1–15	61	46.8 (37.0–59.0)	531 (357–766)	130 (74–190)	27 (44)
15.1–20	19	27.7 (20.5–36.0)	379 (281–585)	62 (33–113)	16 (84)

Level of Immunosuppression Score derived by multiplying each coefficient from multivariable analysis by 4, summing the values and adding a constant of 18.

Results are presented as median and interquartile range.

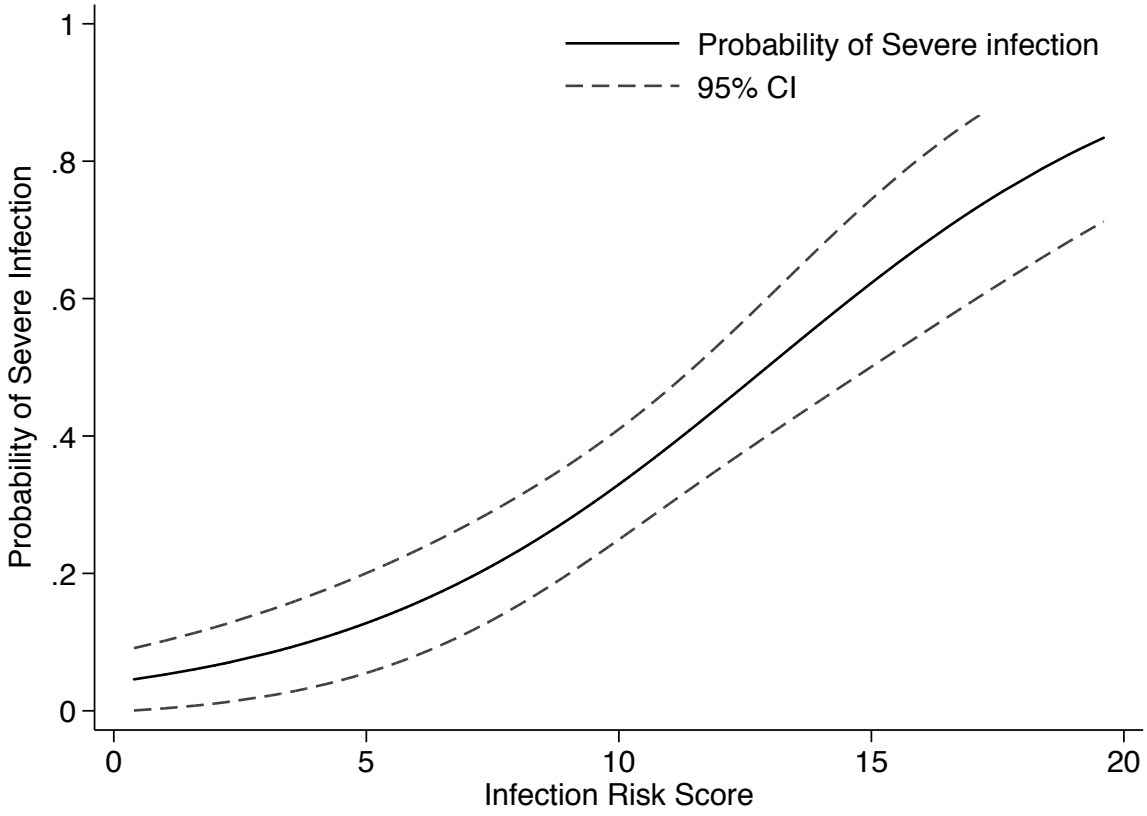

Figure 1. The probability of developing severe infection in the next two years according to the Level of Immunosuppression Score

Chapter 6:
Summary and implications for clinical practice

6.1 Summary of research findings

This thesis examined a cohort of 168 stable kidney transplant recipients who underwent comprehensive assessment of their immune biomarkers at study entry and were then followed prospectively for two years for development of any infections requiring hospital admission.

There are four key findings of this thesis:

- The first finding was that immune biomarkers can be measured in stable kidney transplant patients to identify patients at high risk of developing severe infection. The immune biomarkers most strongly associated with infections were CD4+ cell number, CD19+ cell number, NK number and NK cytotoxic function. NK cell cytotoxic function was the biomarker most strongly predictive of severe infection.
- The second finding was that a composite score of biomarkers and clinical characteristics was more predictive of severe infections than each biomarker alone.
- The third finding was that influenza vaccination resulted in poor seroresponses in kidney transplant recipients and hence could not be used as a diagnostic tool to stratify those patients with increased immunosuppression who were at risk of infection. Importantly, influenza vaccine was safe and not associated with the development of anti-HLA antibodies or rejection.
- The fourth finding was that a single dose of the 13-valent conjugate pneumococcal vaccine elicited increased titres and breadth of functional anti-pneumococcal antibodies. Similar to influenza vaccination, it was not associated with the development of anti-HLA antibodies or stimulation of rejection.

6.2 Comparison with previous research

There are numerous studies that examine immune biomarkers to predict infections in SOT recipients. A substantial amount of research and development has been directed towards finding biomarkers to quantify the “net state of immunocompromise”. However, most of these studies have not identified a single biomarker or combination of

biomarkers that have been translated into standard clinical practice or been incorporated into transplant guidelines.[1-6]

In terms of vaccine research, there are multiple studies examining seroresponses following influenza vaccination in SOT recipients. The meta-analysis brings together the safety data from all vaccine studies in this population. This is the first study to use influenza vaccine seroresponses as a biomarker to help stratify patients at risk of infections.

With regard to pneumococcal conjugate vaccine in SOT recipients, there are three randomised controlled trials in which 7-valent pneumococcal conjugate vaccine was used.[7-9] This is the first study to examine 13-valent pneumococcal conjugate vaccine; however, it is difficult to compared directly with other studies due to differing study methodologies.

6.3 Implications of findings

Immunosuppression has made SOT successful. However, it comes with unavoidable adverse side effects including enhanced infection and cancer susceptibility. The focus of this research was on the absolute risk of serious infection in stable, well kidney transplant recipients and the capacity to stratify this risk. Having found simple ways of predicting at-risk patients, it must then be determined what to do to minimise this risk. The tempting solution is to reduce immunosuppression; however, this raises the important risk of rejection. Research is being done to try, as we have done, to stratify risk by the use of biomarkers and clinical observation. These tools could help determine the risk of reduction of immunosuppression. The obvious first steps in responding to the knowledge of who is at highest risk of infection is close surveillance, rapid and aggressive treatment of infection, optimal prophylaxis including effective vaccination and antimicrobial prophylaxis.

As outlined in the introduction, two steps are required to address this question. Data from this thesis has added to the body of knowledge for the first step – to identify biomarkers to measure the net state of immunocompromise. In this thesis, we have demonstrated that certain biomarkers can identify a subgroup of patients who are at high risk of infection. This thesis has not addressed the second question, which is to test interventions to reduce infection in the group of patients identified at high risk by these biomarkers. This study did not examine rejection as a primary endpoint and does not provided evidence that immunosuppression should be weaned in these high-risk patients. Without dedicated studies in this area, reduction of immunosuppression may place graft function at risk.

Compared with other data in this field, the cohort of 168 patients was relatively large and the demographics and clinical characteristics of our kidney transplant population was representative of the kidney transplant population nationally.[10] This suggests that the findings may be generalisable to other Australian and international kidney transplant cohorts.

Ours is the first study to examine NK cytotoxic function and its association with infection in any SOT group. If the predictive value of NK cytotoxic function is validated in other studies, this test could become a tool for clinicians to help assess their transplant patients' infectious risk. Currently this is not a standard test in most immunology laboratories but, rather, an assay used in reference laboratories. Standardisation of this assay may be required, as well as further studies to more precisely define the sensitivity of this assay to predict the likelihood of imminent serious infection.

Ours is also the first study to identify that CD19+ cell number is associated with sino-pulmonary infection in kidney transplant patients. We have also shown that a reduced CD19+ cell number is associated with a higher risk of infection than reduced IgG

concentration, and that when reduced IgG concentration is combined with reduced CD19+ cell number, the risk of sino-pulmonary infection increased substantially.

CD4+ cell number has previously been shown to be an important predictor of infection in kidney transplant recipients, which is supported by our data. However, we have added to this body of evidence by demonstrating that measurement of CD4+ cell number is superior to measurement of other T cell subsets or T cell proliferation in its ability to predict infection. This has implications for practice, suggesting that CD4+ cell numbers alone can obviate the need for more complex lymphocyte subset analyses or proliferative assays to stratify patients according to infectious risk.

Ours is one of the few studies to show that a composite score combining clinical and immune biomarkers (CD4+ and natural killer cell number as well as mycophenolate use and graft function) can be used to give clinicians information about the future risk of infections.

The findings presented in this thesis require validation in other cohorts before translation into clinical practice. If validation is forthcoming, we could have more confidence that these simple biomarkers offer a robust, practical way to identify a subgroup of patients at high risk of being admitted with infection. For this group, careful attention to infection prevention would be important. With regard to vaccination, this study confirmed that influenza and pneumococcal vaccine were immunologically safe in kidney transplant recipients and supported the use of pneumococcal conjugate vaccine, from a seroresponse and safety perspective, in this group. This would be an important vaccination to include in infection prevention for high-risk patients.

One of the most powerful ways in which to reduce the risk of infection is to reduce iatrogenic immunosuppression; however, the most important consequence of reducing immunosuppression is stimulating allograft rejection.

In our study, failing graft function (as evidenced by reduced eGFR) was a powerful and consistent risk factor for infection regardless of the biomarker being measured or the infectious outcome being examined. Our hypothesis was that patients with a failing graft as a group are likely to have been exposed to increased immunosuppression as treatment for chronic rejection. These studies have not yet been done in our cohort but may provide another stratifying instrument. Additionally, all patients in our study were stable immunologically, in that they did not receive increased immunosuppression in the three months prior to study entry. Patients with significant graft rejection are also likely to have reduced eGFR. Uraemia itself is associated with immuno-incompetence and this patient group is also at risk of infections. It may be possible to develop biomarker analysis to predict infection susceptibility in this group. A failing graft as a risk factor for infection is currently not well documented in the literature.

6.4 Limitations

A number of elements of the study design have limited the conclusions that can be drawn from this work. It was performed at a single centre, so validation studies could significantly strengthen the value of our results.

Only 20% of the kidney transplant cohort were recruited to this study, raising the potential for selection bias. Despite being one of the largest studies of its kind, some of the outcomes were approaching statistical significance, so a larger study would increase the sensitivity of our results. Similarly, larger studies would have less risk of type II error.

The inclusion criteria restricted the study population to patients at least at their third transplant month. The highest risk of infection post kidney transplant is in the first three months post-transplant so this time period was not included.

Furthermore, the immune biomarkers were performed cross-sectionally; hence, patients were at different time points post-transplant. The lack of prospective monitoring of the kinetics of different immune biomarkers, such as peripheral blood lymphocyte subpopulations or serum IgG levels at later time points, was a limitation of the study design. This has implications, as the overall amount of immunosuppression and the infectious risk changes according to the time from transplant. The median time from transplant was 4.1 (1.6–7.8) years and such large heterogeneity in post-transplant follow-up might hamper the interpretation of results. Future research could follow patients longitudinally from pre-transplant through the post-transplant period.

The findings from the study revealed that NK function is a good predictor of infection risk. The mechanisms of NK dysfunction in these patients will now become important in better understanding the role and function of NK cells in host defence.

6.5 Future research and direct extensions

Direct extensions of this thesis might include:

- Validation studies. Successful validation of the data from this study will largely depend on the type of immunosuppressive strategy used, adjustment for confounders and difference in antimicrobial prophylaxis strategies.[11]
- Long-term prospective studies starting from pre-transplantation.
- Optimal care of identified high-risk patients. In terms of vaccination research, further studies are needed to inform optimal vaccine regimens that enhance seroresponses for both influenza and pneumococcal conjugate vaccine.
- Studies examining the risk of other adverse outcomes, principally cancer.
- The application of these studies to other immunosuppressed patients; that is, immunosuppression for autoimmune disease.
- Studies examining careful reduction of immunosuppression; that is, personalised immunosuppression.

6.6 Conclusions

By analysing a cohort of kidney transplant recipients and prospectively defining the number and nature of severe infections in a two-year follow-up, we were able to find immune biomarkers that could identify a subgroup of patients who were at high risk of developing severe infection. Strengths of our study include the numerous immunological markers tested and the ability of the immune biomarkers to predict infection even after adjustment for clinical variables. With the exception of NK function, the biomarkers we identified are simple and easy to test in most transplant centres. NK cell cytotoxic function was more complicated to perform but also the most powerful immune biomarker to predict severe infection. We hope that the findings from this research can be validated in other studies and provide useful data for clinicians seeking to find patients at high risk to become a group receiving a high level of surveillance and prophylactic care.

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Appendices

Appendix 1

Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis

Appendix 2

Occupational *Legionella pneumophila* exposure in a street sweeper with a renal transplant

Appendix 3

Confirmed microsporidial graft infection in a HIV-negative renal transplant recipient: A case report and review of the literature

Appendix 4

Infection is an independent predictor of death in diffuse large B cell lymphoma

Appendix 5

Disseminated enteroviral infection associated with obinutuzumab

Appendix 6

An analysis of the thromboembolic outcomes of 2472 splenectomized individuals

Appendix 1:
Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis

Mulley WR, Dendle C, Ling JEH, Knight SR.

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J Heart Lung Transplant. 2018 Jul;37(7):844–852.

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Does vaccination in solid-organ transplant recipients result in adverse immunologic sequelae? A systematic review and meta-analysis

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KEYWORDS:
donor specific antibodies;
allograft rejection;
anti-hla antibodies;
allograft failure;
influenza vaccination

BACKGROUND: Clinical guidelines recommend vaccinations for solid-organ transplant recipients. However, concern exists that vaccination may stimulate adverse alloimmune responses.

METHODS: We systematically reviewed the published literature regarding this aspect of vaccine safety. Electronic databases were searched for interventional and observational studies assessing de novo donor-specific antibodies (DSA) and rejection episodes after vaccination against infectious pathogens. Graft loss was also assessed. A meta-analysis was conducted for prospective, controlled studies. PRISMA reporting guidelines were followed.

RESULTS: Ninety studies (15,645 vaccinated patients and 42,924 control patients) were included. Twelve studies included control groups. The incidence of de novo DSA (14 studies) was 23 of 1,244 patients (1.85%) at 21 to 94 days. The incidence of rejection (83 studies) was 107 episodes in 5,116 patients (2.1%) at 0.7 to 6 months. Meta-analysis of prospective controlled studies ($n = 8$) showed no increased rejection risk with vaccination compared with no vaccination (RR 1.12, 95% CI 0.75 to 1.70). This finding was supported by data from 3 registry analyses.

CONCLUSIONS: Although the current evidence lacks high-quality, controlled studies, the currently available data provide reassurance that clinicians should recommend appropriate vaccination for their transplant patients as the risk of de novo DSA and rejection is relatively low.

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Solid-organ transplant recipients are at increased risk of infection due to the effects of immunosuppression required to prevent rejection,[1–3] compounded by immunosuppressive effects of organ failure and chronic disease.[4] In addition, infections in transplant recipients are, on average, more severe than in the general population.[1–3] Strategies to prevent infection, such as vaccination, are therefore important. Vaccinations for influenza and other infections are recommended by clinical guidelines[1–3,5]; however, there are concerns that vaccination may trigger development of donor-specific anti-human leukocyte (HLA) antibodies (DSA) and/or allograft rejection.[6–8]

It is proposed that vaccination could lead to the generation of T- and B-cell responses to vaccine antigens that directly cross-react with alloantigens such as is thought

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to occur with some viral infections.[3,9] In addition, innate immune responses to vaccination, including cytokine release, may stimulate previously quiescent alloreactive memory responses.[3,9] A third mechanistic possibility is that adjuvants used in some vaccines may lead to non-specific immunostimulating effects, including against the allograft.[7] Indeed, reports of increased rates of rejection and DSA formation after the use of vaccines containing the ASO3 adjuvant during the 2009 H1N1 influenza pandemic heightened concerns about the safety of adjuvanted vaccines.[7,8]

We undertook a systematic review to explore these aspects of vaccine safety. Our primary study question was: Does vaccination of solid-organ transplant recipients lead to an increased incidence of de novo DSA and rejection episodes?

Methods

This review was registered with National Institute for Health Research PROSPERO system and conducted using a pre-determined protocol (Prospero Registration No. CRD42017065578). It is reported in accordance with the PRISMA (Preferred Reporting Items in Systematic Reviews and Meta-analysis) statement.[10]

Eligibility

Studies examining vaccination in solid-organ transplant recipients were included. Cellular transplants (pancreatic islet and hematopoietic stem cell) were excluded to reduce clinical heterogeneity. All vaccination routes and regimens against infectious pathogens administered post-transplantation were included. Studies examining cellular or immunotherapy vaccines were excluded. Studies in patients with underlying immunocompromise from conditions such as human immunodeficiency virus (HIV) or malignancy were excluded. The outcome measures of interest were de novo DSA, allograft rejection, or allograft loss. De novo DSA were defined as DSA that were negative on pre-vaccination testing but positive on post-vaccination testing. Rejection episodes were defined as any episode of rejection occurring post-vaccination, excluding patients with rejection at the time of vaccination.

We were most interested in controlled studies comparing vaccinated and unvaccinated patients, but, being aware that few such studies exist, we included case–control studies and case series with ≥10 participants. There were no limitations on publication status or date nor language.

Search

We searched Cochrane Central Register of Controlled Trials, MEDLINE, EMBASE, and The Transplant Library, from inception until April 9, 2017. We also reviewed article reference lists for additional studies. Literature searches included keywords and free text terms for solid-organ transplantation, vaccination, and the outcomes of interest.

Study selection and data collection

Title and abstract of studies returned from the search were screened independently by 2 authors, and potentially eligible studies proceeded to full manuscript review. Manuscript inclusion was determined independently by 2 authors using pre-determined criteria. Conflicting decisions were resolved by consensus. Where more than 1 manuscript reported data from the same study, the first full publication was used unless unique data were presented. Data were extracted onto pre-formed sheets independently by 2 authors. Data were compared and differences resolved by consensus.

Risk of bias

Risk of bias was assessed, independently by 2 authors, at the outcome level rather than the study level using the tool of Downs and Black, simplified at item 27, as previously reported.[11,12] This tool was chosen as it can be applied to randomized and non-randomized studies. Risk of bias assessment was not attempted in abstract-only publications due to a lack of detail.

The quality of evidence across all studies was assessed using GRADE criteria.[13] Assessment of publication bias was not possible due to the low number of controlled studies, but the clinicaltrials.gov registry was searched to identify unpublished data.

Summary measures

Most included studies were uncontrolled, preventing meta-analysis for all outcomes. Instead, the incidence is presented per 100 patients/time of follow-up calculated from studies with uniform follow-up times for all patients. Meta-analysis of risk ratios for rejection used a random effects model, using STATA version 15 (StataCorp, College Station, TX). Statistical heterogeneity was measured by the I^2 test for proportion of variation occurring beyond chance. Subgroup analyses were planned for de novo DSA and rejection by vaccine type, adjuvanted and non-adjuvanted vaccines, and transplant type, but this was precluded by the limited number of controlled studies. Instead, these factors were examined in a linear regression model of rejection rates.

Results

The search returned 2,243 citations, which yielded 82 unique studies for inclusion (Figure 1).[6–9,14–91]

Characteristics of included studies

The 82 included studies were considered as 90 unique studies due to dual vaccine arms in 8 studies. Characteristics of each study are summarized in Table S1 (refer to Supplementary Material available online at www.jhltonline.org/).

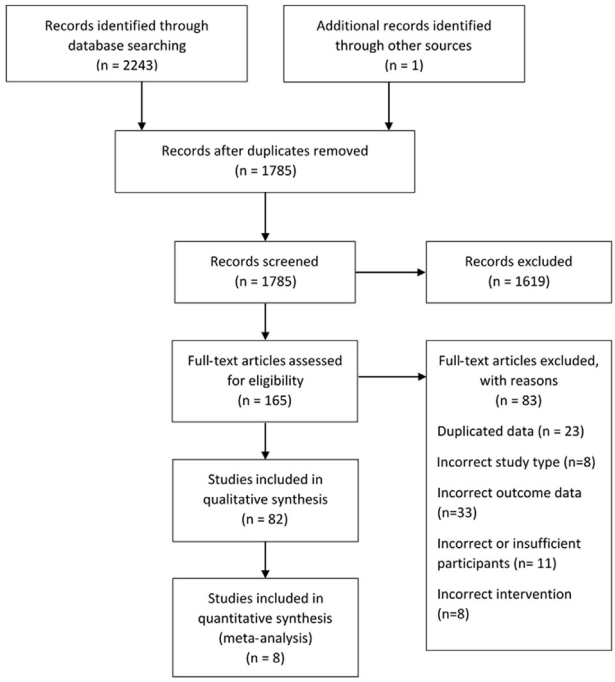

Figure 1 PRISMA flow diagram of study eligibility assessment.

The studies included 15,645 vaccinated patients and 42,924 control patients. The total numbers were heavily influenced by the registry analysis of Hurst et al, which included 9,678 vaccinated patients and 42,052 controls.[39] The remaining 89 studies contributed 5,967 vaccinated and 872 control patients. Only 12 studies had a relevant control group.[6–8,20,24,35,39,45,57,59,65,73] The study designs were: uncontrolled series ($n = 73$); cohort ($n = 6$); retrospective series ($n = 5$); registry analysis ($n = 3$); and randomized, controlled trial ($n = 3$) (see Table S1 online). The primary aims of the included studies were: vaccine efficacy ($n = 44$); efficacy and rejection ($n = 27$); efficacy and alloimmune responses ($n = 7$); and alloimmune responses without efficacy ($n = 12$).

Vaccinated patients had received kidney (13,123 patients in 45 studies), liver (1,384 patients in 15 studies), heart (605 patients in 9 studies), lung (421 patients in 5 studies), and a mixture (16 studies) of transplants. Immunosuppressive regimens were mentioned for 13,716 vaccinated patients. Most (11,776) received a calcineurin inhibitor. Mycophenolate was used by 10,058 patients, prednisolone by 2,196, azathioprine by 600, and, although mammalian target-of-rapamycin (mTOR) inhibitors were used by 8,063, 96% of this usage was from the registry analysis of Hurst et al.[39]

Most studies ($n = 52$) included adult patients, with a slight majority (58.9%) of patients being male. Most vaccines studied related to influenza ($n = 68$), with pneumococcus the next most frequent ($n = 7$). Vaccines contained adjuvant in 28 studies, no adjuvant in 28 studies, and not described in 34. Eight prospective studies contained a control arm, with 7 using no vaccine as the comparator and 1 study using a placebo control.[48] A summary of results is provided in Table 1.

Risk of bias within studies

The median Downs and Black risk of bias score was 17 (range 6 to 22) (see Table S1 online). Quality was assessed as poor in 18% (score 0 to 14), fair in 76% (score 15 to 19), and good in 6% (score 20 to 25) of studies. No study had excellent methodologic quality. The domains where risk of bias was highest were: external validity; internal validity; and statistical power (see Table S2 online).

De novo DSA

Fourteen studies examined vaccination and de novo DSA generation, and all were uncontrolled (Table 2). All studies ultimately employed single-antigen beads, but only 9 proceeded to this step if mixed screening beads were positive. A positive test was reporter-defined and equated to a mean fluorescence intensity of $\geq$1,000 ($n = 5$), $>$300 normalized ($n = 1$), any positive ($n = 2$), and not stated ($n = 6$). The median incidence of de novo DSA was 0 (interquartile range [IQR] 0 to 3). In total, 23 de novo DSA among 1,244 patients were identified (1.85 per 100 patients at 21 to 94 days post-vaccination). One study each for lung and heart and 3 for a mixture of transplant recipients identified no de novo DSA, with all de novo DSA encountered in studies of kidney transplant recipients (Table 2).

Twelve studies reported on non–donor-specific, anti-HLA antibodies, with 26 identified among 1,068 patients (2.4% at 21 to 56 days post-vaccination). There were 2 controlled studies, including Katerinis et al,[7] who described a non-significant increased risk of de novo anti-HLA antibodies (relative risk [RR] 2.17, 95% confidence interval [CI] 0.99 to 4.76) in vaccinated relative to control patients. Lindemann et al used a novel score to detect changes in reactivity to HLA and MICA screening beads after 23-valent pneumococcal polysaccharide vaccination.[55] Their results were consistent with no overall effect.

The included studies did not demonstrate an increased risk of de novo DSA or non-DSA anti-HLA antibodies after vaccination. Controlled studies are required to enhance the evidence base in this area.

Rejection

Eighty-three studies reported on rejection post-vaccination. The follow-up period post-vaccination varied between 0.7 and 6 months. Excluding registry analyses, there were 107 rejection episodes in 5,116 patients (2.1% at 0.7 to 6 months) (see Table S3 online). Forty-six studies reported no rejection episodes. The median incidence was 0 (IQR 0 to 0.79) rejection episode per 100 patient-months of follow-up (see Table S3 online). By transplant type, studies reported rejection rates (per 100 patient-months) of: heart ($n = 8$) 1.68 (IQR 0.79 to 8.99); lung ($n = 4$) 0.82 (IQR 0.79 to 4.06); liver ($n = 13$) 0.29 (IQR 0 to 0.89); and kidney ($n = 37$) 0 (IQR 0 to 0.16).

Four studies compared rejection and vaccination using retrospective methodologies. A single-center review described a reduced rate of rejection among vaccinated

Table 1 Summary of Results

Outcome	Studies [*n* (pts vaccine/control)]	Incidence post-vaccination (pts)	RR (95% CI)	Quality of evidence
De novo DSA				
All studies	15 (1,284)	1.86 per 100 at 21 to 94 days		Low
Controlled studies	0		NA	Nil
De novo anti-HLA Abs				
All studies	11 (1,046)	2.4 per 100 at 21 to 56 days		Low
Controlled studies	1 (151/131)		2.17 (0.99 to 4.76)	Low
De novo rejection				
All studies[a]	83 (5,172)	2.1 per 100 at 0.7 to 6 months		Low
Controlled studies	9 (239/402)		1.12 (0.75 to 1.70)	Moderate
Graft loss				
All studies[b]	6 (507)	1.5 per 100 at 6 to 12 months		Low
Controlled studies	0		NA	Nil

Abs, antibodies; CI, confidence interval; DSA, donor specific antibodies; HLA, human leukocyte antigen; NA, not applicable; pts, patients; RR, relative risk.

[a]Does not include results of 4 retrospective studies (3 registry analyses and 1 single-center chart review; see Results for details of these studies).

[b]Does not include results from 1 registry analysis (see Results for details of this study).

compared with non-vaccinated patients.[73] Two registry analyses, using a "self-controlled case-series method," demonstrated no increased risk of rejection after trivalent seasonal influenza or adjuvanted H1N1 pandemic influenza vaccination.[20,24] Hurst et al performed a large registry analysis (51,730 patients of whom 9,678 had received vaccination) linking the United States Renal Data System (USRDS) to claims for influenza vaccination.[39] Vaccination was associated with an adjusted odds ratio for rejection of 1.00 (95% CI 0.88 to 1.14, $p = 0.965$). However, a temporal relationship between vaccination and rejection could not be determined. Further, that study is subject to the previously reported limitations of USRDS analyses including potential underreporting of rejection, which may have led to inaccurate or underestimated measures of the true rejection rate.[39,92]

Eight studies (5 cohort and 3 randomized, controlled trials) included control groups.[6,8,35,45,48,57,59,65] One trial was considered as 2 separate studies due to inclusion of 2 different vaccine arms.[59] Transplant types were heart ($n = 5$), liver ($n = 3$), and a mixed cohort ($n = 1$). The vaccines targets were influenza (seasonal [$n = 6$] or pandemic H1N1 [$n = 1$]), pneumococcus ($n = 1$), or papillomavirus ($n = 1$). Study details are presented in Tables S1 and S3 online. None of the studies demonstrated an increased relative risk of rejection. The combined estimate of rejection risk post-vaccination by meta-analysis was RR 1.12 (95% CI 0.75 to 1.70, $p = 0.6$) (Figure 2). No significant statistical heterogeneity was detected ($I^2 = 4.5\%$, $p = 0.4$).

Multivariable linear regression of rejection rates adjusted for transplant type did not identify any increased risk by vaccine target or use of adjuvanted vaccines (Table 3). The rejection episodes reported were predominantly cellular without evidence of a significant rate of antibody-mediated rejection (see Table S3 online).

Table 2 Studies Examining De Novo Donor Specific Antibodies After Vaccination

Study (first author, year)	Transplant type	Vaccine	Method	Incidence of de novo DSA
Kumar, 2010	Lung	Influenza	Screen/SAg	0 of 59 (0%) at 56 days
Kimball, 2000	Heart	Influenza	Screen/SAg	0 of 29 (0%) at 21 days
Danziger-Isakov, 2010	Mixture	Influenza	Screen/SAg	0 of 17 (0%) at 94 days
Vermeiren, 2014	Mixture	H1N1 + influenza	Screen/SAg	0 of 169 (0%) at 28 days
Baluch, 2013	Mixture	Influenza	Screen/SAg	0 of 229 (0%) at 30 days
Mujtaba, 2015	Kidney	H1N1 + influenza	SAg	0 of 47 (0%) at 28 days
Mujtaba, 2013	Kidney	H1N1 + influenza	SAg	0 of 57 (0%) at 50 days
Kumar, 2016	Kidney	Influenza	SAg	0 of 34 (0%) at 30 days
Rinaldi, 2014	Kidney	Influenza	Screen/SAg	0 of 81 (0%) at 21 days
LeCorre, 2012	Kidney	H1N1	SAg	1 of 121 (0.82%) at 21 days
Fairhead, 2012	Kidney	H1N1	Screen/SAg	3 of 124 (2.4%) at 30 days
Candon, 2009	Kidney	Influenza	SAg	3 of 66 (4.55%) at 30 days
Brakemeier, 2012	Kidney	H1N1	Screen/SAg	3 of 60 (5%) variable follow-up
Katerinis, 2011	Kidney	H1N1	Screen/SAg	13 of 151 (8.60%) at 42 days
Total				23 of 1,244 (1.85%) at 21 to 94 days

DSA, donor specific antibodies; H1N1, influenza A H1N1 vaccine; SAg, single human leukocyte antigen (HLA) beads; screen, multiple HLA-coated screening beads.

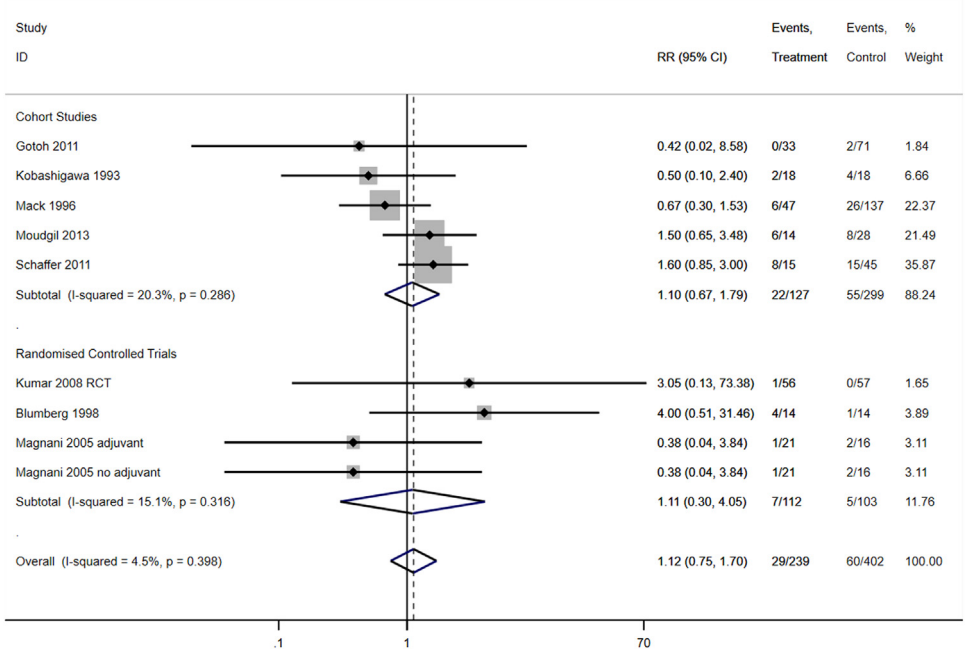

Figure 2 Forest plot of controlled studies assessing rejection post-vaccination. Controlled studies are divided into cohort studies and randomized, controlled trials with relative risk (RR) and 95% confidence intervals represented by the boxed black diamonds and lines, respectively. The larger open diamonds represent the summary RR for the study type subgroups and all the studies combined. An RR = 1 represents no difference in the incidence of rejection between the vaccinated and non-vaccinated patients. The test for statistical heterogeneity between studies (I^2) was shown to be consistent, with no significant heterogeneity.

The included studies did not demonstrate an increased risk of rejection after vaccination. The controlled studies increase the quality of evidence in this area, but further larger, controlled studies are needed, particularly for lung and kidney transplant recipients.

Graft loss

Seven studies examined graft loss post-vaccination (Table 4).[15,17,26,30,31,39,44] Six non-controlled studies reported 8 graft losses among 507 patients (1.5%) at 6 to 12 months post-vaccination.[15,17,26,30,31,44] Two studies reported no graft losses.[30,44] Three graft losses due to death (2 cardiovascular and 1 liver failure) with graft function by 6 months after influenza vaccination in a mixed transplant cohort were reported.[15] In kidney transplant recipients, separate studies reported 2 kidney graft losses from chronic rejection (1 known pre-vaccination) by 12 months after diphtheria and tetanus vaccination,[26] 2 kidney graft losses within 12 months of H1N1 influenza vaccination,[31] and 1 graft loss due to acute antibody-mediated rejection at 10 weeks post-vaccination for H1N1 influenza.[17] Hurst et al's USRDS analysis reported a

Table 3 Multivariable Linear Regression of Study Factors Associated With Rejection, Adjusted for Transplant Type

Factor	Coefficient (95% CI)	*p*-value
Adjuvant		
No	Reference	
Yes	2.39 (−4.17 to 8.94)	0.5
Not stated	2.63 (−3.31 to 8.57)	0.4
Vaccine		
H1N1 ref	Reference	
Seasonal trivalent influenza	-4.14 (−10.27 to 2.00)	0.2
Seasonal bivalent influenza	−4.10 (−16.51 to 8.31)	0.5
Influenza NOS	−3.27 (−22.43 to 15.89)	0.7
Hepatitis B	−5.08 (−16.29 to 6.13)	0.4
Tetanus/diphtheria	−8.30 (−20.88 to 4.28)	0.2
Pneumococcus CV	−4.10 (−23.27 to 15.06)	0.7
Pneumococcus PS	−2.34 (−12.01 to 7.34)	0.6
Papillomavirus	1.22 (−12.27 to 14.72)	0.9
Varicella	−3.02 (−22.78 to 16.74)	0.9
Measles	−4.94 (−24.74 to 14.85)	0.6

CV, conjugate; H1N1, influenza A H1N1 vaccine; NOS, not otherwise specified; PS, polysaccharide.

Table 4 Graft Loss After Vaccination

Study (first author, year)	Vaccine	Transplant type	Follow-up (months)	Graft loss
Kimball, 2000	Influenza	Heart	6	0 of 29
Felldin, 2012	H1N1	Mixture	12	0 of 82
Brakemeier, 2012	H1N1	Kidney	Approx 6	1 of 60
Enke, 1997	Tet and dip	Kidney	12	2 of 42
Fernandez-Ruiz, 2015	H1N1	Kidney	12	2 of 65
Baluch, 2013	Influenza	Mixture	6	3 of 229
Hurst, 2011	Influenza	Kidney	—[a]	HR 0.77

HR, hazard ratio; Tet and Dip, tetanus and diptheria.
[a]Mean 3.2 ± 1.9 years follow-up from transplantation. Only included patients who were vaccinated in their first post-transplant year.

reduced adjusted hazard ratio (0.77, 95% CI 0.69 to 0.85, $p = 0.001$) for allograft loss and death (0.82, 95% CI 0.76 to 0.89, $p = 0.001$) in kidney transplant recipients receiving seasonal influenza vaccination within their first post-transplant year.[39]

The included studies did not demonstrate an increased risk of graft loss after vaccination. The evidence quality is reduced by a lack of controlled studies. There are no data for lung and liver transplant recipients.

Risk of bias across studies

The quality of evidence was very low for de novo DSA, graft loss, and rejection across all studies (see Table S4 online). However, it was moderate for rejection assessed by controlled studies. The major factor reducing overall quality was a lack of control groups, which impacted on limitations and precision criteria. There was some indirectness in detecting rejection, as not all were biopsy-proven. Despite this, studies were quite consistent in the reported incidence of outcomes. No unpublished, relevant controlled interventional studies were detected to suggest serious publication bias.

Discussion

Clinical guidelines recommend vaccinating transplant recipients against a variety of pathogens.[1–3] Live-pathogen vaccines are not currently recommended and the safety of vaccines containing immune adjuvants is unclear.[1–3,6–8] Guideline recommendations are based on the premise that the potential benefits of vaccination outweigh risks in this population.[1–3] This is the first systematic review to assess de novo DSA and rejection episodes after vaccination in solid-organ transplant recipients. The major findings of this review are that there is a deficit of high-quality, controlled studies assessing these outcomes; however, the evidence that is available suggests a low overall incidence of post-vaccination de novo DSA and rejection episodes. In addition, rejection was not increased in patients receiving vaccination compared with non-vaccinated patients.

The purpose of vaccination is to generate immune memory, including antibody production against potentially harmful pathogens. Transplant recipients are immunosuppressed to prevent similar processes taking place against alloantigens. A concern exists that vaccination may trigger alloantibody in addition to responses against the desired pathogenic target, either through cross-reactivity with pathogen antigens or through non-specific immune activation.[3,9] Nine of 14 studies included in this review reported no de novo DSA formation after vaccination. The combined rate of de novo DSA formation if extended to 12 months was approximately 7 to 22 per 100. This is higher than the reported annual background rates in heart, liver, and kidney transplant recipients of between 2.5% and 5%, but within range for lung transplant recipients of up to 17%, albeit with most formed in the first post-transplant year.[93–99] These differences may relate to differences in immunologic risk, time since transplant, immunosuppressive regimen, or detection methodologies and cut-off values, and require non-vaccinated controls to account for background rates. In addition, although pre- and post-vaccination samples were tested to define de novo DSA, memory responses with fluctuations in DSA intensity over time cannot be entirely excluded. The only controlled study examined non-donor-specific anti-HLA antibody formation and reported a non-significant increase in HLA sensitization with vaccination.[7] Taken together, the evidence suggests a low risk of de novo DSA formation with vaccination, but the evidence quality is low given that most studies were uncontrolled.

Rejection was reported by most included studies, but this was commonly indicated as an adverse event in efficacy studies, rather than the primary outcome, and was given limited space in the published article. Approximately half the studies reported no rejection episodes during the follow-up period. Two studies indicated higher incidences of rejection, both in heart transplant recipients after influenza vaccination.[8] The higher rates may be explained by significant pre-vaccination rejection rates in the studied populations and the use of protocolized biopsies soon after vaccination. The incidence of any rejection was not increased in vaccinated patients in this study, but the incidence of moderate to severe rejection was increased in the study by Schaffer et al.[8] The remainder of studies reported relatively modest rejection rates consistent with reported registry annual background rates, within the first 5 post-transplant years, of approximately 8.6% for lung, 9% for heart, 4% to 15% for liver, and 3.6% to 6.4% for kidney.[100] Indeed, when combining controlled studies, vaccination was not associated with an increased rejection risk. A large number of studies contributed evidence to this review outcome with a high level of consistency of results

despite significant clinical heterogeneity in transplant type, vaccine type, and timing of outcome assessment. Combined with the moderate quality of evidence for studies contributing to the meta-analysis, this provides reassurance that vaccination does not appear to increase rejection in solid-organ transplant recipients represented by the included studies. In addition, adjuvanted vaccines did not appear to have an increased incidence of rejection, suggesting their relative safety in this respect.

There were limited data on the association between vaccination and allograft loss. This is likely due to the short follow-up time in most studies. Hurst et al, however, provided significant reassurance from their large registry analysis, that vaccination against influenza in the first post-transplant year is associated with a reduced risk of allograft loss in the medium term.[39]

Limitations

The findings of this review are limited by a lack of high-quality. controlled studies, which reduces the overall evidence quality to very low for DSA formation and rejection incidence. In addition, it is quite possible that major differences exist in these outcomes by transplant type or vaccine type that could not be explored separately due to the limited number of controlled studies, nor could immunosuppressive regimen be controlled for. The primary focus of most included studies was vaccine efficacy; hence, our outcomes of interest may not have been sought as assiduously as possible. Differences exist between studies in methodologies used to detect DSA and rejection episodes, which may account for a degree of heterogeneity in reported rates.

Conclusions

Many studies have contributed evidence regarding rejection without a strong signal for an increased incidence after vaccination. A smaller number of studies have contributed evidence for DSA formation without a strong signal of an increased incidence after vaccination. In this review we have provided reassurance that use of clinical guideline recommendations to vaccinate transplant recipients is relatively safe from an alloimmune stimulation perspective. Further high-quality, controlled studies with rejection and de novo DSA formation as primary outcome measures would add significantly to this field.

Disclosure statement

The authors have no conflicts of interest to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at www.jhltonline.org/.

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Appendix 2:
Occupational *Legionella pneumophila* exposure in a street sweeper with a renal transplant

Tedjaseputra A, Manzoor M, Dendle C, Kanellis J.

Occupational Legionella pneumophila exposure

in a street sweeper with a renal transplant.

Nephrology (Carlton). 2018 May;23(5):493–494.

OCCUPATIONAL *Legionella pneumophila* EXPOSURE IN A STREET SWEEPER WITH A RENAL TRANSPLANT

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INSTRUCTIVE CASE

A 46-year-old street sweeper presented with a 5-day history of shortness of breath, non-productive cough and fevers. He underwent renal transplantation 21 months earlier for end-stage renal failure due to IgA nephropathy. His immunosuppression included tacrolimus, mycophenolate and prednisolone. He took trimethoprim-sulfamethoxazole for *Pneumocystis jirovecii* prophylaxis. Notably, he had returned

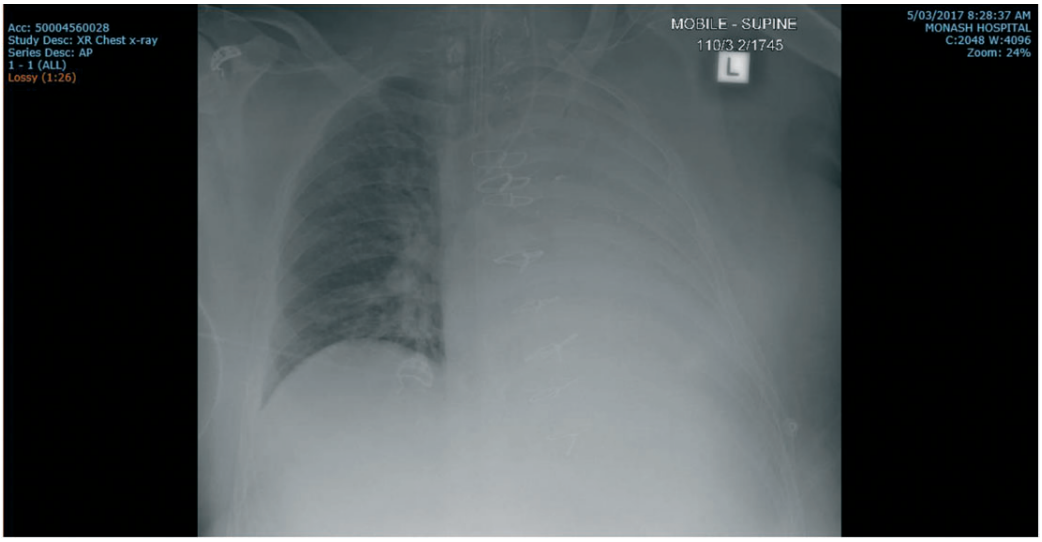

Fig. 1 Chest X-ray of our patient – severe *Legionella* pneumonia with complete wipe out of left lung is noted.

to cleaning local streets 2 months earlier (January 2017) by operating a street sweeper vehicle. Five days before symptom onset, whilst cleaning the vacuum on a wet day, damp mud splashed onto his face resulting in aspiration of a substantial volume of mud water. He had not recently been hospitalized or visited shopping centres, spas or swimming pools.

On arrival, he was febrile, tachypnoeic (40 breaths/min) and hypoxic (SpO_2 86% on 50% FiO_2). Blood tests demonstrated raised serum Cr (258 μmol/L), thrombocytopaenia (106×10^9/L) and a highly elevated C-reactive protein (397 g/L). Chest X-ray revealed a complete wipe out of the left lung field (Fig. 1), and a diagnosis of severe community-acquired pneumonia was made. Given his clinical history and immunosuppression, broad antimicrobial cover was administered with piperacillin-tazobactam, azithromycin and ciprofloxacin. Urgent bronchoscopy was performed and *Legionella pneumophila* serogroup 1 was cultured from broncho-alveolar lavage. Antimicrobial therapy was rationalized; azithromycin was given for 21 days with good effects. He was subsequently discharged 2 weeks post-admission with minimal residual infiltrate on his chest X-ray.

Legionella is an important cause of community-acquired pneumonia.[1] In Australia, *Legionella* infection follows a predictable trend, with the highest incidence reported in summer–autumn.[2] Annually, it peaks in March–April, because of the combination of warmer days with occasional heavy rains, which allows *Legionella* to replicate in aquatic reservoirs.[2] Most infections are transmitted via aerosol spread of contaminated water systems. Rarely, direct environmental inoculation can occur, as was the apparent case in our patient, deduced from the timing of his exposure.[1]

Impaired cell-mediated immunity, specifically CD_4 T cell-mediated activation of macrophages, from immunosuppression underpins the susceptibility of renal transplant recipients to *Legionella*.[1] A major adaptive mechanism of *Legionella* is its inhibition of phagosome–lysosome fusion, allowing its survival and replication within macrophages.[1] In this context, a higher incidence (~10-fold) and mortality (approximately threefold) from *Legionella* infection has been reported in the renal transplant cohort compared with the general population.[3–5]

In conclusion, we report a case where direct, occupation-related, environmental exposure of *Legionella pneumophila* has resulted in severe community-acquired pneumonia. Given the known seasonality and transmission patterns (including unusual ones) of *Legionella*, this episode may have been prevented. Clinicians caring for renal transplant recipients should regularly update their socio-occupational history and offer occupation-specific advice for infection prevention, especially at high-risk periods (e.g. the use of facemasks when street cleaning in March–April in Australia).

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Appendix 3:
Confirmed microsporidial graft infection in a HIV-negative renal transplant recipient: A case report and review of the literature

Brown M, Longano A, Dendle C, Polkinghorne KR, Kanellis J.

Confirmed microsporidial graft infection in a HIV-negative renal transplant recipient:

A case report and review of the literature.

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CASE REPORT

WILEY

Confirmed microsporidial graft infection in a HIV-negative renal transplant recipient: A case report and review of the literature

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Abstract

Microsporidia are intracellular organisms most commonly known to cause opportunistic infection in patients with human immunodeficiency virus (HIV). There have been several case reports of infection in solid organ and bone marrow transplant recipients. Here, we report a case of a non-HIV–infected renal transplant patient with microsporidiosis of the renal tract associated with acute graft dysfunction. We also review the literature of 12 previously reported cases of microsporidiosis in patients with renal transplants who had described graft involvement. We review the pattern of illness as well as the common renal biopsy features when microsporidial infection is associated with renal graft infection.

KEYWORDS

graft infection, microsporidiosis, renal transplant

1 | INTRODUCTION

Microsporidia are obligate intracellular spore-forming organisms, which have recently been reclassified from protozoa to fungi.[1] They are primarily known to cause opportunistic infection in patients affected by human immunodeficiency virus (HIV); however, there have been several case reports of infection in recipients of solid organ and bone marrow transplants.[2-13] We report a case of a non-HIV–infected renal transplant patient with microsporidiosis of the renal tract causing acute deterioration in graft function.

2 | HISTORY, EXAMINATION AND MANAGEMENT

The patient was a 50-year-old female, who emigrated from Vietnam to Australia in 2008.

She had a background history of end-stage kidney failure (ESKF) secondary to IgA nephropathy, rheumatoid arthritis, rheumatic aortic and mitral valve regurgitation, benign breast cysts, and hypertension. Her rheumatoid arthritis was anti-CCP negative and had previously been managed with methotrexate, sulfasalazine, and plaquenil. She was weaned off all treatment between 2011 and 2012 with no signs of ongoing disease activity.

Hemodialysis was commenced in November 2014 and she subsequently received a living-related kidney transplant from her sister in April 2015. The transplant was a 3/6 HLA mismatch with no donor-specific HLA antibodies noted. She received standard immunosuppression with basiliximab, tacrolimus, mycophenolate, and prednisolone. Initial graft function was excellent with the creatinine falling to 94 umol/L (Normal range(N)= 55-105 umol/L) in the first week. Her initial recovery was uneventful, and she was discharged from hospital 7 days post-transplant.

At 2 weeks post-transplant, her creatinine rose to 135 umol/L and a transplant biopsy was performed. This was reported as normal and showed no features of acute rejection or calcineurin-inhibitor–related damage. The serum creatinine subsequently settled to 110-120 umol/L with no additional intervention. A protocol biopsy performed 13 weeks post-transplant showed focal interstitial changes suggestive of possible drug-induced interstitial nephritis but no evidence of rejection. In this setting, she was changed from pantoprazole to ranitidine with no changes to her immunosuppressive

TABLE 1 Summary of previously described microsporidial infection with kidney graft involvement

Article	Age+ Gender + Transplant(s)	Baseline IS	Recent Rejection (+Treatment)	Time from transplant to diagnosis (wk)	Key symptoms in addition to fever
Our Case	50F Kidney	Basiliximab, Tac, MMF and Pred	AbMR (treated with MP and PEx)	16	Headaches, vertigo
Hocevar et al 2014[2]	NS Kidney	Tac, MMF and Pred	Rejection (corticosteroids and ATG)	9	Myalgia
	NS Kidney (same donor as above)	Tac, MMF and Pred	Rejection (MP)	10	Myalgia, joint pains
Ladapo et al 2014[3]	13F Kidney	Basiliximab, Tac, Pred, and AZA	CMR (MP and change AZA to MMF)	12	Intermittent diarrhea
	13F Kidney (same donor as above)	AZA others not specified	CMR (ATG, and AZA changed to MMF)	16	Diarrhea
Nagpal et al 2013[4]	68F Kidney	MMF, Tac, and Pred	CMR (MP + ATG)	26	Cough
George et al 2012[5]	57M Kidney	Tac, MMF and Pred	Vascular rejection (IV MP+ anti-thymocyte globulin)	60	Hypoxia
Talabani et al 2010[6]	38F Kidney	Anti-thymocyte globulin, MMF and Cyclosporine A	No	6	Abdominal pain, anorexia
Carlson et al 2004[7]	43M Simultaneous kidney pancreas transplant	Daclizumab, Tac, MMF and Pred	No	8	Abdominal pain, diarrhea
Mahmood et al 2003[8]	45F Kidney	Not specified	Rejection (MP)	8	Keratoconjunctivitis, neurological involvement
Mohindra et al 2002[9]	45F Kidney	Not specified	Two episodes of rejection (MP and muromona-CD3)	8	Graft tenderness, keratoconjunctivitis
Gamboa-Dominguez et al 2002[10]	42M Kidney	Rapamycin, Cyclosporine A and Steroid	Rejection (MP and increased oral pred)	21	Cough, thoracic pain, weakness, diarrhea, keratitis, decreased level of consciousness
Latib et al 2001[11]	39F Kidney (2nd graft)	Cyclosporine, Aza, and Pred	IV MP	16	Nil

AbMR, antibody-mediated rejection; ABZ, albendazole; AZA, azathioprine; BD, twice daily; CMR, cell-mediated rejection; CMV, cytomegalovirus; GIT, gastrointestinal tract; IS, immunosuppression; MMF, Mycophenolate; MP, methylprednisolone; NS, not specified; PEx, Plasma Exchange; Pred, prednisolone; Tac, Tacrolimus; UTI, urinary tract infection.

regime. There was also a small single non-necrotizing granuloma of unclear significance seen on this biopsy.

Sixteen weeks post-transplant, the patient was admitted with 1-week history of vertigo, headaches, and fevers. She underwent extensive investigation including cerebral computer tomography scan, lumbar puncture, and cerebral magnetic resonance imaging. These were performed to rule out stroke, meningitis/encephalitis, and post-transplant lymphoproliferative disorder, respectively. None of these revealed a cause for her symptoms. A nasopharyngeal aspirate was positive for picornavirus and a diagnosis of vestibular neuritis was made. Her fevers initially settled, but then recurred and she was found to have influenza B by way of polymerase chain reaction (PCR) testing on repeat nasopharyngeal aspirate, not evident on the aspirate done eleven days prior. She also developed leukopenia with mixed neutropenia and lymphopenia. The nadir white cell count was 1.9 × 10^9/L (N = 4.0-11.0) with neutrophil count of 1.3 × 10^9

Laboratory/radiology findings	Species if known	Organs involved	Treatment	Outcome
Neutropenia, Graft dysfunction	*Encephalitozoon cuniculi*	Kidney	ABZ 400 mg BD for 1 mo	Alive with stable graft function
Neutropenia, graft dysfunction	*E. cuniculi*	Kidney	Broad spectrum antimicrobials	Died Diagnosed postmortem
Thrombocytopenia, leukopenia	*E. cuniculi*	Kidney	ABZ 400 mg BD for 6 mo total therapy	Alive and well
Leukopenia, anemia, acute graft dysfunction	*E. cuniculi*	Kidney	ABZ 400 mg BD for more than 12 mo Reduced IS	Alive and well
Graft dysfunction	*E. cuniculi*	Kidney	ABZ 400 mg BD for more than 12 mo Reduced IS	Alive and well
Anemia, leukopenia, acute graft dysfunction	*E. cuniculi*	Lung and kidney	ABZ 400 mg BD for 6 mo MMF ceased	Alive with stable graft function
Pancytopenia, graft dysfunction	Encephalitozoon species	Lung and kidney	ABZ (dose NS) with plan for lifelong Reduced IS	Alive with stable graft function
Anemia, leukopenia, graft dysfunction	*E. cuniculi*	Lung and kidney	ABZ 400 mg BD for 1 mo then 400 mg daily for 9 mo, MMF changed to AZA	Alive with stable graft function
Graft dysfunction, lung infiltrates	Encephalitozoon species	Liver, kidneys (native and graft), heart, brain, GIT, and omentum	Broad-spectrum antimicrobials	Died Diagnosis confirmed postmortem
Graft dysfunction,	*E. cuniculi*	Kidney, Conjunctiva, lungs, and GIT	Antimicrobial therapy not specified further	Died Diagnosis confirmed postmortem
Chest infiltrate	*E. cuniculi*	Kidney, conjunctiva, GIT, lungs and brain	ABZ 400 mg PO BD, Fumagillin eye drops discontinued after one mo due to thrombocytopenia Reduced IS	Initially improved then deteriorated with seizures and died
Graft dysfunction	*E. cuniculi*	GIT, liver, and skin	ABZ 400 mg daily for two wk deceased IS, plasmapheresis	Alive on hemodialysis
Acute graft dysfunction	Presumed *E. intestinalis*	Kidney	ABZ 400 mg BD more than 12 mo	Alive with stable graft function

(N=2.0-8.0) and lymphocyte count of 0.3 × 10^9 (N=1.0-4.0), 3 weeks after admission. Mycophenolate was ceased and tacrolimus doses were adjusted aiming for a target serum level of 3-5 ng/mL. She developed acute graft dysfunction during her admission with a creatinine rise to 210umol/L; however, this was thought to be due to a decreased volume state. Her creatinine had improved to 173 umol/L when she was discharged, but her kidney function rapidly deteriorated soon after and she had recurrent fevers at home along with night sweats. She was readmitted less than a week later with a creatinine of 273umol/L.

A repeat transplant biopsy was performed (20 weeks post-transplant). This demonstrated two distinct abnormalities. The first was that of moderate-to-marked acute humoral rejection with ptc1 and C4d3 scores as per the Banff criteria.[14] The second finding was a prominent granulomatous interstitial nephritis. Ziehl-Neelsen staining was undertaken given the clinical history and revealed

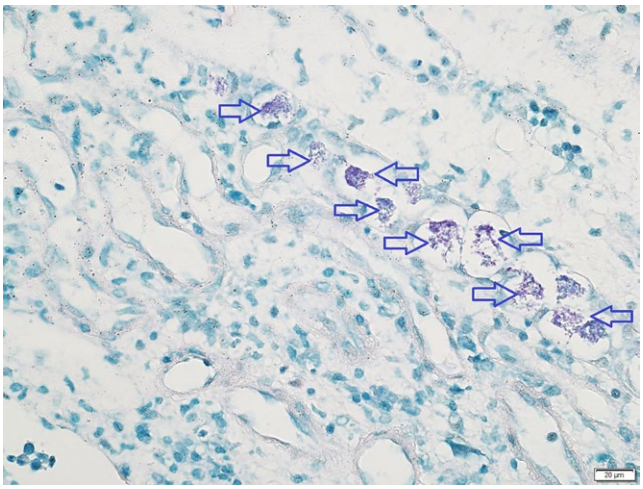

FIGURE 1 Zhiel-Neelsen stain showing ZN-positive cocci in tubular cells (arrows)

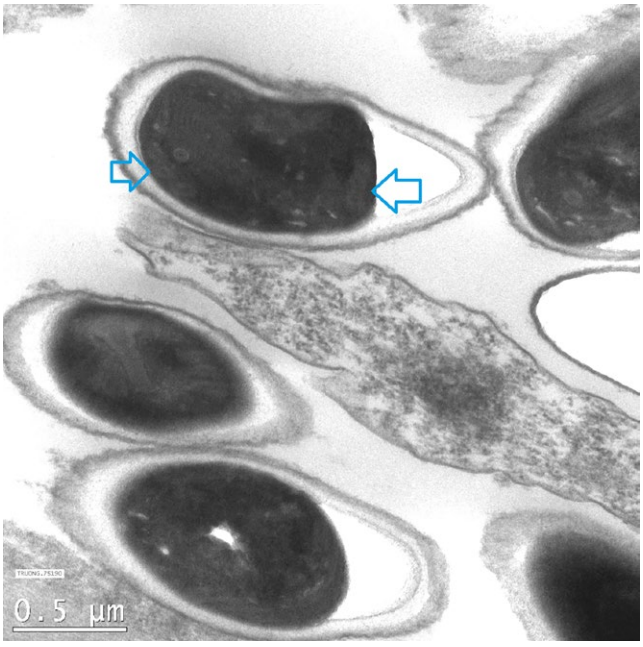

FIGURE 2 Electron microscopy showing microsporidial spores with coiled polar tube (arrows)

multiple small rounded structures suggestive of microsporidia (see Figure 1). Electron microscopy showed organisms with a single row of coiled polar tube typical for microspordia infection (see Figure 2). Microsporidial spores were identified on a modified trichrome strain of the urine. Microsporidial DNA was detected by PCR testing of the urine. PCR was performed using primers targeting SSU—rRNA gene. A PCR product of 250-270 bp represented a positive result for microsporidia species. Sequencing of amplicon was performed with MicF and MicR primers. *Encephalitozoon cuniculi* was identified.[15] PCR was not performed on the kidney biopsy specimen and was negative from the feces, blood, and cerebrospinal fluid. Based on the findings from light and electron microscopy, in conjunction with the urine PCR, it was determined that the diagnosis of microsporidial infection was confirmed.

The patient was treated with both pulse methylprednisolone and plasma exchange for the rejection and albendazole for the microsporidial infection. She was given 400 mg of albendazole orally twice daily for 4 weeks. Her serum creatinine settled to between 160 and 180 umol/L over the next 6 weeks.

A repeat transplant biopsy was performed 18 weeks post diagnosis. This demonstrated ongoing but decreased acute humoral rejection and complete resolution of the previously noted granulomatous interstitial nephritis. The Ziehl-Neelsen stain was negative for microsporidia.

3 | DISCUSSION

There are approximately 150 genera and more than 1300 species of microsporidia. Infection most commonly occurs in those with human immunodeficiency virus (HIV). The most common pathogenic species are Enterocytozoon bieneusi, Encephalitozoon intestinalis, *E. cuniculi*, and *E. helem*.[4]

The most common manifestation of microsporidial infection in humans is diarrhea, which can be acute and self-limited or become chronic, especially in those who are immunosuppressed. Diarrhea is particularly common with the *E. bieneusi* species. Microsporidia can also cause disseminated infection including renal, respiratory, ophthalmologic, and CNS involvement in immunosuppressed patients.[2,13,16] Cases of disseminated infection are more commonly described with Encephalitozoon species. In our case, it is difficult to separate the contribution of her other infections and the microsporidia to her acute febrile illness. It is also unclear whether the leukopenia contributed to the microsporidia induced insterstitial nephritis or if the microsporidial infection itself contributed to the leukopenia. Of note, the earlier transplant biopsy performed at 13 weeks showed no evidence of microsporidia spores although the presence of a single non-necrotizing granuloma may have been an early sign of microsporidial infection.

Microsporidia typically enter the host through the intestinal or respiratory tract with fecal-oral transmission thought to be the primary mechanism.[5] However, transmission from an infected donor has been described in solid organ transplant with one report of three different recipients being affected by microsporidia from a single common donor [2] and another report of two pediatric renal transplant recipients from the same donor developing microsporidial infection.[3] Protective immunity against *E. cuniculi* is primarily dependant on the cellular immune response and it seems that, in humans, infection requires simultaneous $CD4^+$ and $CD8^+$ T-cell depletion.[17] At the time of her diagnosis, our patient had a CD4 count of 212 *10^6 (N=389-1569) or 51% (N-31-59) and a CD8 count of 100*10^6 (N=168-894) or 24% (N=12-42).

Several cases of microsporidial infection in non-HIV–infected recipients of solid organ and bone marrow transplants have been reported worldwide.[16] Of the reported cases, 12 describe kidney transplant involvement (see Table 1). Where species identification was possible, *E. cuniculi* was the most commonly reported (confirmed in 9/12 cases). Other cases were due to other or undifferentiated encephalitozoon species. Ten cases specifically describe acute graft dysfunction as a feature of the presenting illness. Other common features include: fever, neutropenia, myalgia, keratoconjunctivitis, cough, and lung infiltrates.

In 9 of the 12 cases, the patient had coexistent infection found during workup. Common coexisting infections were cytomegalovirus (in 5/9 cases) and urinary tract infection (in 3/9 cases). Other infections reported were brucellosis,[2] BK viremia,[2] tuberculosis [3] clostridium difficile,[5] aspergillous,[7] and oral herpetic infection.[10]

The onset of symptoms varied between 6 and 60 weeks post-transplant; however, the majority (10/12) occurred within 6 months post-transplant. Ten of the 12 reported cases were treated for rejection prior to the diagnosis of microsporidial infection. The findings of associated treatment for rejection and relatively early post-transplant infection suggest correlation with periods of high immunosuppression. Another possibility is that microsporidial infection causes biopsy changes which can be confused with rejection. Across the previously described cases, the commonly described biopsy features include tubulitis, tubular necrosis, granuloma formation, acute and/or chronic interstitial nephritis, and microsporidial spores. The glomeruli are usually spared.[3,4,11]

Of the 12 reported cases, in 4 of these, the patient died. Three of those did not have the diagnosis of microsporidia confirmed until after death. Most patients were treated with albendazole and a reduction in immunosuppression. Considering all previously reported cases treatment with albendazole is largely effective for infection with Encephalitozoon species but less effective in *E. bieneusi* infections.[2,4,5] Systemic fumagillin has been used in these cases with some success; however, it has significant side effects including bone marrow toxicity, abdominal pain, vomiting, and hyperlipasemia.[4,5,18] There has been one case report of probable fumagillin-associated meningoencephalitis after treating *E. bieneusi* infection in a renal transplant recipient.[12] The optimal duration of therapy is unknown, however, in several of the cases reported prolonged therapy was required.

4 | CONCLUSION

Microsporidiosis should be considered in transplant patients presenting with fever and signs of graft dysfunction, especially when associated with interstitial nephritis or granulomas. It requires a high index of suspicion for diagnosis and may be an underrecognized cause of morbidity and mortality in renal transplant recipients.

AUTHOR CONTRIBUTIONS

M. Brown drafted the article and completed literature review; A. Longano provided figures and biopsy analysis and revised the article; C. Dendle involved in laboratory and microbiologic diagnosis and revised the article; K. Polkinghorne involved in the concept of the report, and revised the article; J. Kanellis revised and approved the article.

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Appendix 4:
Infection is an independent predictor of death in diffuse large B cell lymphoma

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Infection is an Independent Predictor of Death in Diffuse Large B Cell Lymphoma

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To identify risk factors for infection in patients with diffuse large B cell lymphoma (DLBCL) undergoing rituximab, cyclophosphamide, vincristine, adriamycin and prednisolone (R-CHOP) treatment. All patients with DLBCL who received R-CHOP from 2004–2014 in a tertiary Australian hospital were identified and information collected from hospital admission data, laboratory results and medical record review. Infection was defined as hospitalisation with an ICD-10-AM diagnostic code for infection. Risk factors for infection and association between infection and survival were modelled using Cox proportional hazards regression. Over the 10-year period there were 325 patients; 191 (58.8%) males, median age 66 years. 206 (63.4%) patients experienced ≥1 infection. Independent predictors of infection were Charlson comorbidity index score (hazard ratio [HR] 3.60, p = 0.002), Eastern Cooperative Oncology Group (ECOG) performance status (HR 2.09 p = <0.001) and neutropenia (HR 2.46, p = <0.001). 99 (31%) patients died. Infection was an independent predictor of survival (HR 3.27, p = <0.001, as were age (HR 2.49, p = 0.001), Charlson comorbidity index (HR 4.34, p = <0.001), ECOG performance status (HR 4.33, p = 0.045) and neutropenia (HR 1.95, p = 0.047). Infections are common and infection itself is an independent predictor of survival. Patients at highest risk of infection and death are those with multiple comorbidities, poor performance status and neutropenia.

Non-Hodgkin Lymphoma is one of the most common adult malignancies[1] and diffuse large B cell lymphoma (DLBCL) the most frequent histological subtype[2]. Treatment with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) is currently standard of care for DLBCL, with three year overall survival ranging from 50 to >95% depending upon prognostic variables[3]. Infection is a common cause of morbidity and mortality with neutropenic fever occurring in 10–20% of patients treated for lymphoma[4–13]. However there is limited information on the risk factors and impact of infection among patients treated for DLBCL. The ability to define a high-risk subset of patients may be useful for targeted application of preventative therapies.

The aim of this study was to determine the incidence, risk factors and timing of infections in patients with DLBCL treated with R-CHOP and R-CHOP-like chemotherapy, and to explore the association between infection and overall survival.

Results

Description of patient cohort. Over the 10-year period there were 325 patients with DLBCL who received R-CHOP or R-CHOP-like chemotherapy with curative intent. Median follow up of surviving patients was 2.54 years (IQR 1.11, 4.93).

Demographic details are outlined in Table 1. There were 191 (58.8%) males and the median age at diagnosis was 67.0 years. The most common Charlson comorbidity score was 0–2 in 270 (83.1%) and the most common

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Characteristic		Number	Percent
Age in years (n = 325)		66	IQR −55.8–77.1
Sex (n = 325)	Female	134	41.3
	Male	191	58.8
Charleson comorbidity score (n = 325)	0–2	270	83.1
	3–5	48	14.8
	6+	7	2.2
ECOG status (n = 288)	0	73	25.4
	1	111	38.5
	2	81	28.1
	3	22	7.6
	4	1	0.4
Stage (n = 310)	1	32	10.3
	2	86	27.7
	3	42	13.6
	4	150	48.4
NCCN IPI (n = 315)	Low risk	26	8.0
	Low intermediate	97	29.9
	High Intermediate	113	34.8
	High	79	24.3
Chemotherapy type (n = 325)	R-CHOP 21	286	90.8
	R-CHOP 14	20	6.2
	R-CEOP	4	1.2
	R-CVP	3	0.9
	R-CODOXM/IVAC	3	0.9
	Other combinations of rituximab, cyclophosphamide, vincristine or prednisolone	9	2.7
No. of chemotherapy cycles (n = 316)	1	6	1.9
	2	14	4.3
	3	16	4.9
	4	37	11.4
	5	9	2.8
	6	219	67.4
	>6	15	4.7
Creatinine (n = 320)	Normal	208	64.0
	Raised	112	34.5

Table 1. DEMOGRAPHICS. ECOG = Eastern Cooperative Oncology Group performance status point scale. NCCN-IPI = International Prognostic Index. R-CHOP-21: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone administered every 21 days. R-CHOP-14: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone administered every 14 days. R-CEOP: rituximab, cyclophosphamide, etoposide, vincristine and prednisolone. R-CVP: rituximab, cyclophosphamide, vincristine and prednisolone. R-CODOXM/IVAC: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone, cytarabine, methotrexate.

ECOG status at diagnosis was 1 in 111 (38.5%). The most common stage at diagnosis was stage IV in 150 (48.4%). The median number of R-CHOP chemotherapy cycles was six. 112 (34.5%) had a raised creatinine at baseline.

Description of infections. 206 patients (63.4%) patients experienced at least one infection with a single admission in 82 patients (25.2%), two in 50 (15.4%), three in 32 (9.9%) and four or more presentations in 42 (12.9%). Of the 206 patients with an infection, 25 (3.7%) required ICU admission and 19 (2.8%) required mechanical ventilation. The median time from the first day of chemotherapy to first infection was 85 days (IQR 52–134).

Overall, there were 3732 admissions recorded with 517 (13.9%) infections. The site of infection was recorded in 322 (62.3%) (Table 2). The most common sites of infection were lower respiratory tract (40.7%), skin and soft tissue (18.7%) and blood stream infection (15.1%).

A diagnostic code specifying a microbiological organism was reported in 375 (72.5%) of the 517 infection episodes. Bacteria accounted for 186 (49.6%), viruses for 117 (31.2%) and fungi for 72 (19.2%). Within the bacterial category, there were 50 (13.6%) blood stream isolates. Of the blood stream isolates, gram-negative bacteria were the most common isolates, accounting for 39 (10.6%) and *E. coli* was the most frequently isolated gram-negative blood stream isolate. Of the blood stream isolates, *Staphylococcus* species and *Streptococcus* species accounted for

Blood stream infection	50 (15.1%)
Lower respiratory tract	135 (40.7%)
Upper respiratory tract	30 (9.0%)
Cardiovascular	8 (2.4%)
Gastrointestinal	6 (1.8%)
Urogenital	6 (1.8%)
Neurological	3 (0.9%)
Skin and Soft tissue	62 (18.7%)
Bone and Joint	2 (0.6%)
Other	1 (0.3%)
Device or line related	24 (7.2%)

Table 2. SITES OF INFECTION – All infectious episodes (n = 332).

Class of organism	Organism	Number of isolates n (%)
Bacterial (Gram-positive organisms) 47	*Staphylococcus aureus*	11 (2.9)
	Coagulase negative Staphylococcal spp.	25 (6.7)
	Streptococcus pneumoniae	1 (0.3)
	Other Streptococcal spp.	10 (2.7)
Bacterial (Gram-negative organisms)	*Escherichia coli*	15 (4.0)
	Klebsiella spp.	12 (3.2)
	Pseudomonas spp.	12 (3.2)
	Campylobacter spp.	3 (0.8)
	Other gram negative bacteria	57 (15.2)
Bacterial other	Bacteria other	19 (5.1)
	Clostridium difficile	14 (3.7)
Mycobacterial	*Mycobacterium tuberculosis*	5 (1.3)
	Mycobacterium spp. other	2 (0.5)
Viral	*Herpes spp.*	10 (2.7)
	Varicella zoster virus	10 (2.7)
	Hepatitis B	49 (13.1)
	Hepatitis C	38 (10.1)
	HIV	2 (0.5)
	Influenza	3 (0.8)
	Viral infection other	5 (1.3)
Fungal	*Candida spp.*	59 (15.7)
	Aspergillus spp.	2 (0.5)
	Other fungal spp.	2 (0.5)
	Pneumocystitis jirovecci	9 (2.4)

Table 3. MICROBIOLOGICALLY CONFIRMED INFECTIONS n = 375.

10 (2.7%) and 1 (0.3%), respectively. Of all bacterial isolates, gram-negative bacteria accounted for 99 (27.0%), gram-postive for 47 (12.8%) and other bacteria for 33 (9%). There were seven patients with *Mycobacterium* species, five (1.4%) with tuberculosis and two (0.5%) with other mycobacteria. Of the viral isolates, herpes viruses accounted for 20 (17.1%), hepatitis B for 49 (41.9%), hepatitis C for 38 (32.5%) and HIV for 2 (1.7%). Within the fungal category, *Candida* species accounted for 59 (81.9%) however 39 (54.1%) were oral candidiasis. *Aspergillus* species for 2 (2.7%), other fungi for 2 (2.7%) and *Pneumocystis jirovecci* for 9 (12.5%) infections (Table 3).

Neutropenia was identified in 218 (5.8%) of 3732 admissions. Of admissions in which the patient was neutropenic, 59 (27.1%) had an associated infection code compared with 253 (11.3%) of 2227 (59.8%) admissions where the patient was not neutropenic.

Prior administration of pegfilgrastim was identified in 1439 (38.5%) admissions. Of those patients who received pegfilgrastim, 141 (9.7%) had an associated infection code compared to 376 (16.4%) of 2293 (62.5%) patients who did not receive pegfilgrastim.

Predictors of an infectious episode. The results of the regression analysis of factors associated with an infectious episode are shown in Table 4.

After adjustment for all other model covariates, factors which remained significant predictors of infection in the multivariable analysis included Charlson comorbidity score three or greater (reference category score of 2 or less), ECOG status of one, two, three or four (with zero the reference category), and NCCN-IPI low/

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	95% CI p value	Hazard Ratio	95% CI p value
Age	<65 years	1.00		1.00	
	>65 years	1.23	1.02 to 1.47 p = 0.025	0.96	0.77 to 1.18 p = 0.69
Sex	Female	1.00		1.00	
	Male	0.97	0.81 to 1.17 p = 0.78	0.95	0.77 to 1.19 p = 0.69
Charlson Comorbidity Score	1–2	1.00		1.00	
	3–5	3.60	2.88 to 4.51 p = <0.001	2.16	1.71 to 2.74 p = <0.001
	6+	5.35	3.47 to 8.26 p = <0.001	3.91	2.43 to 6.28 p = <0.001
ECOG	0	1.00		1.00	
	1	2.44	1.77 to 3.37 p = <0.001	2.09	1.46 to 3.01 p = <0.001
	2	4.58	3.33 to 6.30 p = <0.001	3.33	2.22 to 5.04 p = <0.001
	3 and 4	5.95	3.89 to 9.10 p = <0.001	3.36	1.99 to 5.66 p = <0.001
Stage	1	1.00		1.00	
	2	1.69	1.11 to 2.58 p = 0.013	1.78	1.11 to 2.84 p = 0.017
	3	2.18	1.41 to 3.40 p = 0.001	1.88	1.12 to 3.17 p = 0.017
	4	2.22	1.49 to 3.30 p = <0.001	1.71	1.04 to 2.82 p = 0.36
NCCN IPI	Low risk	1.00		1.00	
	Low intermediate	2.87	1.51 to 5.47 p = 0.001	4.19	1.45 to 12.07 p = 0.008
	High intermediate	4.87	2.58 to 9.18 p = <0.001	3.99	1.29 to 12.34 p = 0.016
	High	5.47	2.88 to 10.41 p = <0.001	3.69	1.12 to 12.14 p = 0.032
Number of chemotherapy cycles	1–2	1.00			
	3–4	0.65	0.40 to 1.05 p = 0.081	1.21	0.71 to 2.04 p = 0.48
	5–6	0.62	0.40 to 0.96 p = 0.035	0.91	0.57 to 1.45 p = 0.69
	>6	1.22	0.73 to 2.05 p = 0.444	1.41	0.81 to 2.44 p = 0.22
Creatinine	Normal	1.00		1.00	
	Raised	1.60	1.31 to 1.90 p = <0.001	1.06	0.84 to 1.33 p = 0.64
Neutropenia within 48 hours of admission with infection	No	1.00		1.00	
	Yes	2.68	2.10 to 3.41 p = <0.001	2.46	1.91 to 3.17 p = <0.001
Pegfilgrastim w/I 21 days of admission with infection	No	1.00		1.00	
	Yes	0.60	0.40 to 0.74 p = <0.001	0.71	0.57 to 0.88 p = 0.002

Table 4. Regression analysis of the factors associated with infection in all study patients (n = 325). ECOG = Eastern Cooperative Oncology Group performance status point scale. NCCN-IPI = International Prognostic Index.

intermediate or greater (reference category low). Neutropenia within 48 hours of admission was also associated with an increased risk of infection (compared with neutrophil count $>1 \times 10^9$/L within the 48 hours prior). The use of pegfilgrastim in the preceding 21 days was associated with a reduced risk of infection (compared with no use of pegfilgrastim in the preceding 21 days).

The regression analysis for predictors of infection was also performed including only patients who received R-CHOP on a 21 day cycle and excluding patients who received R-CHOP like therapy or R-CHOP on a 14 day cycle. See Table 5.

Overall Survival. Over the 10 year study period, 99 (30.5%) of the 325 patients died. For those who died, the median time from diagnosis to death was 273 days (129–636 days). The cause of death was progressive lymphoma in 58 (58.6%), infection in 12 (12.1%), another cancer in five (5.5%), liver failure in four (4.4%), other in seven (7.1%) and unknown in 11 (11.1%).

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	95% CI p value	Hazard Ratio	95% CI p value
Age	<65 years	1.00		1.00	
	>65 years	1.25	1.03 to 1.51 p = 0.023	0.52	0.74 to 1.16 p = 0.69
Sex	Female	1.00		1.00	
	Male	0.97	0.80 to 1.18 p = 0.81	1.00	0.81 to 1.24 p = 0.93
Charlson Comorbidity Score	1–2	1.00		1.00	
	3–5	3.55	2.79 to 4.52 p = <0.001	1.93	1.49 to 2.50 p = <0.001
	6+	5.65	3.49 to 9.12 p = <0.001	4.26	2.55 to 7.11 p = <0.001
ECOG	0	1.00		1.00	
	1	2.28	1.64 to 3.18 p = <0.001	1.95	1.33 to 2.86 p = 0.001
	2	4.30	3.09 to 5.99 p = <0.001	3.36	2.16 to 5.21 p = <0.001
	3 and 4	5.95	3.89 to 9.10 p = <0.001	3.36	1.99 to 5.66 p = <0.001
Stage	1	1.00		1.00	
	2	1.8	1.16 to 2.93 p = <0.001	1.37	0.82 to 2.29 p = 0.228
	3	2.49	1.54 to 4.02 p = <0.001	1.57	0.90 to 2.71 p = 0.110
	4	2.51	1.61 to 3.89 p = 0.001	1.36	0.79 to 3.17 p = 0.261
NCCN IPI	Low risk	1.00		1.00	
	Low intermediate	4.55	1.85 to 11.17 p = 0.001	3.12	1.24 to 7.85 p = 0.015
	High intermediate	7.88	3.24 to 19.16 p = <0.0001	3.06	1.14 to 8.20 p = 0.026
	High	8.95	3.65 to 21.94 p = <0.0001	3.83	0.99 to 8.09 p = 0.052
Number of chemotherapy cycles	1–2	1.00		1.00	
	3–4	0.60	0.32 to 1.11 0.106	1.02	0.53 to 1.93 p = 0.94
	5–6	0.67	.38 to 1.17 p = 0.163	0.77	0.43 to 1.37 p = 0.37
	>6	1.41	0.74 to 2.67 p = 0.284	1.23	0.64 to 2.44 p = 0.51
Creatinine	Normal	1.00		1.00	
	Raised	1.55	1.28 to 1.89 p = <0.0001	1.10	0.85 to 1.42 p = 0.48
Neutropenia within 48 hours of admission with infection	No	1.00		1.00	
	Yes	4.92	2.82 to 8.59 p = <0.0001	2.68	2.05 to 3.51 p = <0.0001
Pegfilgrastim w/I 21 days of admission with infection	No	1.00		1.00	
	Yes	0.67	0.44 to 0.69 p = <0.001	0.71	0.53 to 0.85 p = 0.001

Table 5. Regression analysis of the factors associated with infection in patients who received R-CHOP 21 (n = 286). ECOG = Eastern Cooperative Oncology Group performance status point scale. NCCN-IPI = International Prognostic Index.

The results of regression analysis of the factors associated with overall survival are shown in Table 6.

After adjustment for all other model covariates, factors which remained significant predictors of overall survival in the multivariable analysis were age, Charlson comorbidity score of three or greater (reference category 2 or less), ECOG status of one, three or four (with zero reference category), and an infectious episode (Fig. 1). Chemotherapy cycle number greater than or equal to three was associated with a reduced risk of death compared with cycle number one and two. The presence of neutropenia was associated with reduced survival (adjusted HR 1.95; 95% CI, 1.01–3.78; p = 0.047) compared with no neutropenia.

The regression analysis for predictors of survival was also performed including only patients who received R-CHOP on a 21 day cycle and excluding patients who received R-CHOP like therapy or R-CHOP on a 14 day cycle. See Table 7.

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	95% CI p value	Hazard Ratio	95% CI p value
Age	<65 years	1.00		1.00	
	>65 years	2.50	1.64 to 3.82 p = <0.001	2.49	1.42 to 4.35 p = 0.001
Sex	Female	1.00		1.00	
	1.03	0.70 to 1.52 p = 0.887	1.01	0.66 to 1.56 p = 0.85	1.03
Charlson Comorbidity Score	1–2	1.00		1.00	
	3–5	4.54	2.70 to 7.63 p = <0.001	4.34	2.00 to 6.33 p = <0.001
	6+	11.26	5.77 to 21.97 p = <0.001	7.36	3.38 to 16.00 p = <0.001
ECOG	0	1.00		1.00	
	1	4.33	1.81 to 10.34 p = <0.001	2.61	1.02 to 6.66 p = 0.045
	2	8.47	3.56 to 20.13 p = <0.001	2.41	0.84 to 6.95 p = 0.10
	3 and 4	19.83	7.49 to 52.64 p = <0.001	7.16	2.04 to 25.06 p = 0.002
Stage	1	1.00		1.00	
	2	1.27	0.54 to 2.95 p = 0.583	1.70	0.68 to 4.30 p = 0.25
	3	1.59	0.64 to 3.94 p = 0.318	1.50	0.51 to 4.41 p = 0.45
	4	2.23	1.02 to 4.87 p = 0.046	1.90	0.71 to 5.10 p = 0.19
NCCN IPI	Low risk	1.00		1.00	
	Low intermediate	7.16	0.97 to 52.72 p = 0.053	2.56	0.31 to 20.76 p = 0.280
	High intermediate	9.66	1.32 to 70.44 p = 0.025	2.87	0.32 to 25.90 p = 0.34
	High	18.18	2.49 to 132.54 p = 0.004	4.27	0.43 to 42.38 p = 0.21
Number of chemotherapy cycles	1–2	1.00		1.00	
	3–4	0.18	0.10 to 0.34 p = <0.001	0.39	0.18 to 0.84 p = 0.016
	5–6	0.09	0.05 to 0.15 p = <0.001	0.13	0.06 to 0.25 p = <0.001
	>6	0.09	0.03 to 0.27 p = <0.001	0.10	0.03 to 0.36 p = <0.001
Creatinine	Normal	1.00		1.00	
	Raised	1.23	0.82 to 1.84 p = 0.318	0.31	0.43 to 1.30 p = 0.31
Neutropenia within 48 hours of admission with infection	No	1.00		1.00	
	Yes	4.45	2.61 to 7.60 p = <0.001	1.95	1.01 to 3.78 p = 0.047
Pegfilgrastim w/I 21 days of admission with infection	No	1.00		1.00	
	Yes	0.69	0.43 to 1.10 p = 0.124	0.8	0.58 to 1.65 p = 0.58
Admission with infection	No	1.00		No	1.00
	Yes	5.08	3.46 to 7.49 p = <0.001	3.27	2.03 to 5.27 p = <0.001

Table 6. Regression analysis of the factors associated with survival in all study patients (n = 325). ECOG = Eastern Cooperative Oncology Group performance status point scale. NCCN-IPI = International Prognostic Index.

Discussion

The most notable findings of this study are that infections are common among DLBCL patients receiving R-CHOP and R-CHOP-like chemotherapy and that an infection was associated with reduced overall survival.

The rate of infection in admitted episodes in our population was 63% and of the patients that experienced an infectious episode, 60% experienced multiple episodes. This rate is higher than in other reports, and may be explained by the study design, which included all DLBCL patients undergoing therapy, compared with carefully selected patient populations that are included in clinical trials. Data from observational cohorts have demonstrated higher rates of infection compared with randomised controlled trials with reported rates ranging from 10

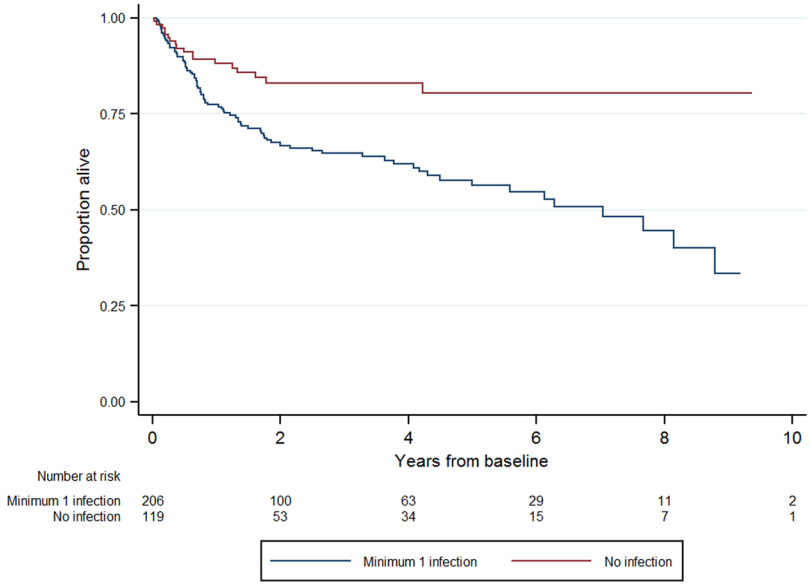

Figure 1. Survival analysis of DLBCL patients who had at least one infectious episode compared with those who did not have an infectious episode.

to 42%[6–13]. Our higher rates may also be due to differing definitions of infection and/or data collection methods. For example, the risk of an episode of neutropenic fever during R-CHOP chemotherapy has been reported as 19%[4,5] however non-neutropenic infective episodes were not documented. In this study the definition of neutropenia was 1.0×10^9/L, while another common definition is 0.5×10^9/L. This may be another explanation for why the neutropenia infection rate on our study was higher than reported elsewhere. Despite differences in definitions, our study suggests rates of infection for DLBCL may be higher in a real world setting and that infection prevention is a key strategy in the supportive management of DLBCL. The results from this study may inform the use of infection prevention strategies, including which patients are most likely to benefit. This study identified patients at high-risk of infection, highlighted the highest risk period during R-CHOP therapy, and provided data on the most common types of infections. Patients with newly diagnosed DLBCL who are at highest risk of infection are those who, have multiple comorbidities, poor performance status and an advanced risk NCCN-IPI. The presence of multiple comorbidities and poor performance status were also predictors of earlier death. Findings from previous studies that describe predictors of infection are inconsistent and use heterogeneous definitions, making it difficult for clinicians to accurately predict the risk of infection in their patients. In the pre-rituximab era, Lyman *et al.* constructed a predictive model that demonstrated, age, LDH, albumin, neutropenia and bone marrow involvement predicted hospitalisation for life threatening neutropenia fever[13]. Pettengell *et al.* found that older age, low albumin, previous chemotherapy and recent infection were predictive of neutropenia fever in cycle one[14].

In our study, patients were more likely to die from all causes during their first two cycles of chemotherapy compared with subsequent cycles, which is consistent with other studies in lymphoma patients[14,15]. This suggests that preventative measures could be maximised early in the R-CHOP treatment course, rather than instituted after infection has occurred.

Current strategies to prevent infection include patient education, vaccination, and antimicrobial prophylaxis. In this study, the leading site of infection was the lower respiratory tract. *Streptococcus pneumo*niae is known to cause the majority of these infections[16] however studies regarding the efficacy of vaccination before the commencement of R-CHOP are lacking. Further research is required to examine the optimal timing, efficacy and clinical outcomes are of pneumococcal vaccination specifically in patients receiving R-CHOP.

In terms of the use of antimicrobial prophylaxis, it is difficult to draw conclusions or make firm recommendations based on the microbiological data acquired through clinical coding data, as non-clinically relevant isolates may have been included. Fungal infections accounted for 19% of infections, which is substantially higher than in other literature[17]. In this study, 2.5% had *Pneumocystis jirovecci*, which is below the 3.5% rate for which prophylaxis is recommended according to Australian national consensus guidelines[18].

The use of growth factors, such as pegfilgrastim, to reduce the impact of neutropenia is also used to prevent infections. This study confirmed that neutropenia was a strong a predictor of an infectious episode and was associated with reduced survival. The use of pegfilgrastim was also independently associated with a reduction in the risk of an infectious episode. Interestingly, pegfilgrastim use had no significant effect on survival. This is consistent with other studies that have demonstrated reduced risk of severe neutropenia and neutropenia fever with colony stimulating factors but no effect on mortality[15,19]. Importantly, as our study was a retrospective cohort study, the use of pegfilgrastim was not random and may be a surrogate measure of other factors. International guidelines[4,20,21] recommend primary prophylaxis with colony stimulating factors when the incidence of neutropenia fever is greater than 20% for the chemotherapy regimen. In lymphoma specifically, it is suggested to administer primary prophylaxis in patients older than 65 with comorbidities[4]. Our study would support this recommendation.

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	95% CI p value	Hazard Ratio	95% CI p value
Age	<65 years	1.00		1.00	
	>65 years	2.21	1.42 to 3.42 p = <0.001	1.32	0.75 to 2.30 p = 0.328
Sex	Female	1.00		1.00	
	Male	1.19	0.77 to 1.82 p = 0.421	1.49	0.91 to 2.44 p = 0.112
Charlson Comorbidity Score	1–2	1.00		1.00	
	3–5	5.14	3.03 to 8.74 p = <0.001	4.12	2.32 to 7.33 p = <0.0001
	6+	13.88	7.03 to 27.41 p = <0.001	11.02	4.99 to 24.34 p = <0.0001
ECOG	0	1.00		1.00	
	1	4.07	1.69 to 9.78 p = <0.002	2.69	1.00 to 7.26 p = 0.050
	2	8.30	3.46 to 19.89 p = <0.001	2.97	1.27 to 12.41 p = 0.018
	3 and 4	19.83	7.49 to 52.64 p = <0.001	7.16	2.04 to 25.06 p = 0.002
Stage	1	1.00		1.00	
	2	1.29	0.51 to 3.24 p = 0.583	1.25	0.44 to 3.54 p = 0.665
	3	1.56	0.58 to 4.16 p = 0.37	1.17	0.365 to 3.80 p = 0.78
	4	2.43	1.04 to 5.66 p = 0.039	1.22	0.399 to 3.73 p = 0.72
NCCN IPI	Low risk			1.00	
	Low intermediate	1.00		1.00	
	High intermediate	1.35	0.80 to 2.27 p = 0.256	0.88	0.39 to 1.99 p = 0.76
	High	2.61	1.54 to 4.40 p = <0.001	1.82	0.63 to 5.22 p = 0.26
Number of chemotherapy cycles	1–2	1.00			
	3–4	0.13	0.062 to 0.27 p = <0.0001	0.27	0.11 to 0.68 p = 0.005
	5–6	0.08	0.04 to 0.15 p = <0.0001	0.09	0.041 to 0.19 p = <0.0001
	>6	0.10	0.03 to 0.34 p = <0.0001	0.11	0.03 to 0.40 p = <0.0001
Creatinine	Normal	1.00		1.00	
	Raised	1.27	0.82 to 1.96 p = 0.270	0.79	0.44 to 1.43 p = 0.45
Neutropenia within 48 hours of admission with infection	No	1.00		1.00	
	Yes	4.92	2.82 to 8.58 p = <0.0001	3.15	1.66 to 5.96 p = <0.0001
Pegfilgrastim w/I 21 days of admission with infection	No	1.00		1.00	
	Yes	0.82	0.50 to 1.10 p = 0.449	1.26	0.72 to 2.21 p = 0.40
Admission with infection	No	1.00		No	1.00
	Yes	1.63	1.94 to 6.65 p = <0.0001	3.27	1.00 to 2.63 p = <0.046

Table 7. Regression analysis of the factors associated with survival in patients who received R-CHOP 21 (n = 286). ECOG = Eastern Cooperative Oncology Group performance status point scale. NCCN-IPI = International Prognostic Index.

The main limitations of this study were that it was performed at a single centre, was retrospective, and relied upon administrative datasets. Use of an administrative dataset may result in missing data or misclassification, which could inaccurately represent the number and type of infections. In addition, patients with infections not requiring admission to hospital are not included. This may underestimate the rate of infections as well as influence whether infection is a predictor of survival when these infections are included. Relying on ICD-10 diagnostic codes to classify the types of organisms may be misleading. Nonetheless, this study represents one of the largest cohorts of DLBCL patients and one of the few studies in real world setting.

Our study has identified a subset of patients at high risk of infection and death and some possible strategies to mitigate this risk. Further research could be directed towards prospectively studying preventative strategies in high-risk patients as identified in this study, with a view to developing preventative strategies that are personalised, targeted and effective.

Methods

Study design and setting. A retrospective cohort study was performed at a Monash Health, a 2000 bed academic health service in Melbourne, Australia. All patients with a new diagnosis of DLBCL who received R-CHOP or R-CHOP-like chemotherapy over a 10-year period between 2004 and 2014 were identified using hospital admission data and medical record review.

Data sources. Demographic data collected from medical records included age, sex, lymphoma diagnosis details (including date, stage and type), Eastern Cooperative Oncology Group (ECOG) performance status classified on a five-point scale[22], and International Prognostic Index (NCCN-IPI)[3, 23]. Details on chemotherapy regimen, number of cycles, date of death or last follow up was obtained from the medical record.

Data on all hospital admissions for each patient was obtained from the clinical information services, and included admission and discharge dates, diagnostic codes (classified according to the Australian modification of the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10-AM) and procedure codes (classified according to the Australian Classification of Health Interventions)[24].

The use of colony stimulating factors was obtained from the pharmacy information system. Pathology results were obtained from the pathology laboratory information system.

Definitions. Co-morbidities were identified using the ICD-10-AM diagnostic codes in admission data and classified according to the Charlson comorbidity index[25].

The absolute neutrophil count, 48 hours before or after the first day of each admission episode, was identified where available. If the neutrophil count within 48 hours of the admission and including the day of admission was less than 1.0×10^9/L and the admission contained an infectious code, then this was defined as infection with neutropenia. If the neutrophil count within 48 hours of the admission was greater than 1.0×10^9/L and the admission contained an infectious code, then this was defined as infection without neutropenia. If the neutrophil count within 48 hours of the admission was less than 1.0×10^9/L and the admission did not contain an infectious code, then this was defined as neutropenia without infection.

For each admission, the use of pegfilgrastim as primary or secondary prophylaxis within 21 days of the first day of the admission was recorded. At our institution pegfligrastim is used for primary prophylaxis in DLBCL patients aged 65 years or older. Filgrastim use was not considered in the analysis of factors associated with infection, as in our institution it is more frequently administered to patients with established infection rather than as prophylaxis.

Infectious outcomes. Infectious episodes were defined as any hospitalization after the date of DLBCL diagnosis with an infection code recorded in the hospital admission data.

Each infection was classified according to body site; blood stream infection (BSI), upper respiratory tract, lower respiratory tract, cardiovascular, gastrointestinal, urogenital, neurological, skin and soft tissue, bone and joint, other, device or line related and source unknown.

For each infection, intensive care unit (ICU) admission, ICU length of stay and the timing of infection in relation to first diagnosis of DLCBL were identified using the admission data.

Statistical analysis. Descriptive statistics were used for incidence of infection, types of infection and changes over time. Categorical variables were summarized using frequency and percentage. Continuous variables were summarized using mean and standard deviation (SD) or median and inter-quartile range (IQR) as appropriate.

Conditional risk set time-to-event modeling for multiple failure time data was used to determine possible clinical predictors of infection using episodes of infection as the evaluable outcome. In this model, subjects were permitted to contribute multiple events (infection episodes) to the analysis. Due to the multiplicity of events, the model considers the entire time period at risk of infection (period of patient follow-up) for the specified outcome of interest, rather than censoring a patient at the first observed infection event. In the survival analysis, the proportion of patients with neutropenia and infection was compared to the proportion of patients with neutropenia without infection and the proportion of patients with pegfilgrastim use and infection was compared to the proportion of patients with pegfilgrastim use without infection.

A Cox proportional hazards regression was used to investigate predictors of mortality. For both models, hazard proportionality was analyzed using analysis of scaled Schoenfeld residuals. For all analyses $p < 0.05$ was considered significant. All analyses were performed using Stata version 14, (StataCorp Inc., College Station, TX, USA).

The project was approved by the Monash Health Human Research Ethics Committee. All methods were carried out in accordance with relevant guidelines and regulations.

Data Availability. The datasets generated during and analysed during the current study are not publicly available due to patient confidentiality but are available from the corresponding author on reasonable request.

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Author Contributions

C.D., M.G. and Z.M. performed the research. C.D., M.G. and Z.M. and S.O. designed the research study. T.S. analysed the data. C.D., Z.M., T.K., R.S., K.T. and wrote the paper.

Additional Information

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Appendix 5:
Disseminated enteroviral infection associated with obinutuzumab

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Disseminated enteroviral infection associated with obinutuzumab.

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Disseminated Enteroviral Infection Associated with Obinutuzumab

Claire Dendle, Michael Gilbertson, Tony M. Korman, Vera Golder, Eric Morand, Stephen Opat

Two cases of disseminated enteroviral infection occurred in patients who received the CD20 monoclonal antibody obinutuzumab. Clinical features included hepatitis, edema, and a dermatomyositis-like syndrome. These manifestations may be unfamiliar to clinicians and are possibly responsive to intravenous immunoglobulin. Clinicians should remain vigilant for enteroviral infections in patients receiving obinutuzumab.

Viral, fungal, and bacterial infections (*1,2*) and a recent case of enteroviral meningoencephalitis (*3*) associated with obinutuzumab use have been described. Early recognition is critical because the infection can be effectively treated with intravenous immunoglobulin (IVIg).We report 2 cases of disseminated enteroviral infections in patients in Australia treated for lymphoma with the CD20 monoclonal antibody (mAb) obinutuzumab. Clinical features, including hepatitis, edema, and a dermatomyositis-like syndrome, were similar to those mentioned in the original descriptions of disseminated enteroviral infections in children with X-linked agammaglobulinemia (XLA) (*4,5*).

Case Reports

Case 1

During summer 2014, a 63-year-old woman with symptomatic high tumor burden follicular lymphoma achieved a complete clinical and radiologic response to induction treatment with 6 cycles of bendamustine and obinutuzumab, then began maintenance therapy with obinutuzumab for 8 weeks. Eleven months after she began taking obinutuzumab, the patient sought treatment for 4 weeks of fatigue, myalgias, muscle tenderness, and leg edema without fever. Peripheral blood lymphocyte count was 0.52×10^9 cells/L (reference range $1–4 \times 10^9$ cells/L), and lactate dehydrogenase was 354 IU/L (reference range 100–200 IU/L); serum creatine kinase and inflammatory markers were within reference ranges. Immunoglobulin levels were also within reference ranges: IgG 10.2 g/L, IgM 0.3 g/L, and IgA 1.3 g/L. The patient had moderately impaired liver function and was hypoalbuminemic without evidence of renal protein loss. Magnetic resonance imaging of the thighs showed diffuse inflammatory changes involving subcutaneous tissues, fascia, and musculature (Figure). Results of tests to determine possible causes of muscle pathologic changes were negative; tests included those for autoantibodies, HIV antibodies, thyroid function, and PCR for respiratory viruses (including influenza) and herpesvirus. Bone marrow biopsy results indicated no evidence of lymphoma. Muscle histopathologic findings from a biopsy of the quadriceps showed features of an inflammatory myopathy (interstitial edema, perivascular lymphocytic cuffing, and degenerating fibers) consistent with the features of early dermatomyositis. Reverse transcription PCR of the muscle tissue indicated enterovirus RNA. Reverse transcription PCR also detected enterovirus RNA in plasma, nasopharyngeal, and fecal specimens. Viral protein 1 gene obtained from RNA extracted from muscle was sequenced, and we identified the virus as echovirus 6. When we ceased treatment with obinutuzumab and gave the patient 0.8 g/kg IVIg, her symptoms rapidly improved. Results from a repeat plasma enterovirus PCR 11 days after initiation of IVIg were negative.

Case 2

During summer 2014, a 35-year-old woman with symptomatic follicular lymphoma achieved a complete clinical and radiological response to induction treatment with 6 cycles of bendamustine and obinutuzumab; she subsequently took obinutuzumab for an additional 8 weeks. Twelve months after she began taking obinutuzumab, she sought treatment for fever, headaches, and myalgias. Peripheral blood lymphocyte count was 0.40×10^9 cells/L (*1,2,4,5*). Cerebrospinal fluid was acellular, but we detected enterovirus in cerebrospinal fluid and feces by using PCR. Sequencing of the PCR product was unsuccessful, and we could not identify the enterovirus strain. Immunoglobulin levels were at the lower end of the reference ranges: IgG 7.9 g/L (reference range 7.5–15.6 g/L), IgM 0.6 g/L (reference range 0.5–3.0 g/L), and IgA 1.5 g/L (reference range 0.8–4.5 g/L). Results of liver function tests were initially normal, but liver function deteriorated after 2 weeks. Peak level of bilirubin was 86 μmol/L (reference range 0–20 μmol/L), of alanine aminotransferase was 1,419 IU/L (reference range 7–56 UI/L), of alkaline phosphatase was 117

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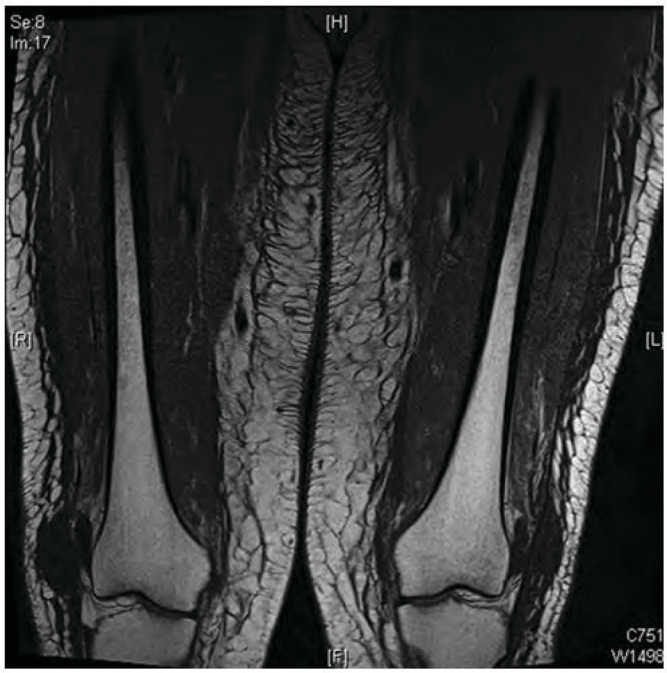

Figure. Magnetic resonance image of 63-year-old woman in Australia with disseminated enteroviral infection that manifested after she received obinutuzumab for lymphoma. Image shows patient's thighs and diffuse inflammatory changes involving subcutaneous tissues, fascia, and musculature.

U/L (reference range 30–120 U/L), and of albumin was 28 g/L (reference range 35–45 g/L); international normalized ratio peaked at 2.0 (reference range 0.8–1.2). Results of liver biopsy showed active hepatitis. Results of tests to determine possible causes of hepatitis and encephalitis were negative; the tests included those for autoantibodies, HIV antibodies, thyroid function, and PCR for respiratory viruses (including influenza) and herpesvirus. Bacterial and fungal cultures were negative. Obinutuzumab was ceased, and the patient was treated with 0.8 g/kg IVIg. All clinical and laboratory features rapidly improved.

Conclusions

Anti-CD20 mABs such as rituximab are now standard of care for treatment of B-cell lymphoma in combination with chemotherapy. The US Food and Drug Administration approved obinutuzumab in September 2013 for use in chronic lymphocytic leukemia, but indications for use probably will expand. Obinutuzumab is glycoengineered to cause more profound and rapid B-cell depletion than rituximab, elicited by subtle differences in the orientation of binding to the CD20 molecule between the 2 drugs (*6*). As a result of these binding differences, compared with rituximab, obinutuzumab has superior induction of apoptosis, natural killer cell activation, and antibody-dependent cytotoxicity but less complement-dependent toxicity (*6*).This mechanism may also explain the differences in susceptibility to, and patterns of, enteroviral infections associated with obinutuzumab, resulting in a phenotype similar to XLA (*5*).

Antibodies are the main form of defense against enteroviruses (*7*), and severe, chronic, and disseminated enteroviral infections are generally limited to neonates or patients with profound B-cell deficiencies (XLA or hematopoietic stem cell transplantation). During the 1970s and 1980s, reports described the clinical manifestation of disseminated enterovirus infection in children with XLA (*4,5*) and demonstrated that IVIg is an effective therapy for disseminated enterovirus infection (*7,8*). Since then, reports of disseminated enteroviral infections have been uncommon. Enteroviral infection has not featured prominently among patients with partial B-cell or immunoglobulin deficiencies, such as patients with chronic variable immunodeficiency (*7*). Immunoglobulin levels of the 2 patients in our study were within reference ranges, but analysis of lymphocyte subsets was not performed. Both patients received the combination of obinutuzumab and bendamustine; it is possible that an association exists between the 2 drugs that results in increased host susceptibility to disseminated enteroviral infection.

The clinical features described in most cases of disseminated enteroviral infections relate to chronic meningoencephalitis (*2,5*). However, several reports describe a dermatomyositis-like syndrome with edema and hepatitis that responded to IVIg (*5*); this syndrome is strikingly similar to the cases reported here. Enteroviral infections (coxsackieviruses and echoviruses) also have been implicated in the pathogenesis of myositis (*9*). Enterovirus PCR was positive from the muscle biopsy of the patient in our report, suggesting that the virus had a direct role in pathogenesis of the myositis.

Reports of enteroviral infections associated with rituximab use since its introduction have been rare, in contrast to obibutuzumab, for which a case of enteroviral meningencephalitis has been reported (*2*,*3*). Of the 11 cases of enteroviral infection associated with rituximab use, 8 were meningoencephalitis and 2 were myocarditis (*2*,*10*–*12*). To our knowledge, enteroviral infection has not previously been associated with rituximab use in patients who also had hepatitis, dermatomyositis, and edema, as in the cases we report and those associated with XLA (*5*).

Future studies could define susceptibility to enteroviruses through the effect of obinutuzumab on B-cell and immunoglobulin function and host defense against enteroviral infections. It would be clinically useful to identify biomarkers or clinical predictors of disseminated infection. Future research might also focus on the development of a screening strategy for enteroviral infections followed by prophylactic or preemptive therapy with IVIg.

The clinical manifestation of disseminated enteroviral infections, particularly those similar to dermatomyositis, may be unfamiliar to clinicians caring for adults because

most experience of the illness is in children and there have been few reports in recent years. Given the therapeutic response to IVIg in the cases we report, enteroviral infection and the use of IVIg therapy should be considered in patients treated with obinutuzumab who develop atypical clinical features of organ inflammation.

S.O. has received speakers' fees and clinical research funding from Roche.

Dr. Dendle is an infectious diseases physician at Monash Health and researcher at Monash University. She specializes in immunocompromised hosts and is especially interested in infections in patients with hematologic malignancies and who have undergone transplantation.

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Appendix 6:
An analysis of the thromboembolic outcomes of 2472 splenectomized individuals

Dendle C, Spelman T, Sundararajan V, Chunilal S, Woolley I.

An analysis of the thromboembolic outcomes of 2472 splenectomized individuals.

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To the editor:

An analysis of the thromboembolic outcomes of 2472 splenectomized individuals

The diseases associated with the reason for splenectomy play an important role in the rate of infections in splenectomized patients.[1] Our hypothesis was that the same reasoning would apply to the rates of venous thromboembolism (VTE) after splenectomy and that these findings could inform future preventive care.

All patients undergoing a splenectomy in Victoria, Australia, were identified by using linked hospital discharge data with International Classification of Diseases, 10th Revision, Australian Modification (ICD-10-AM) diagnostic codes.[2] The sampling frame included patients between July 1, 1998, and December 31, 2006, who were ≥15 years of age. This sampling frame was selected to ensure adequate numbers of patients in each of the splenectomy indication groups for statistical power. Splenectomy indications were divided into 6 mutually exclusive groups, and patients who had multiple indications were ordered hierarchically: (1) trauma, (2) therapeutic malignant (planned; malignant disease such as lymphoma or leukemia), (3) therapeutic hematologic (planned; hematologic disease such as idiopathic thrombocytopenic purpura [ITP] or hemolytic anemia), (4) therapeutic other (local infections or congenital abnormalities of the spleen), (5) iatrogenic malignant (unplanned; unintended accompaniment to surgery for malignant disease), and (6) iatrogenic nonmalignant (unplanned; unintended consequence of surgery for nonmalignant disease).

VTE was divided by using ICD-10-AM codes into lower-extremity acute deep vein thrombosis (DVT), pulmonary embolism (PE), and portal vein thrombosis (PVT). This classification was based on studies using similar coding methodologies.[3] The groups were not mutually exclusive. Patients who had VTE that occurred in the first 30 days after splenectomy were excluded to avoid inclusion of complications potentially resulting from the primary surgery or related admission. Incidence rates of first VTE were calculated for sex, age group, and indication for splenectomy. Multivariate Cox proportional hazards regression models were fitted to compute hazard ratios adjusted for age, sex, and indication for splenectomy. Hazards proportionality was assessed by using analysis of scaled Schoenfeld residuals. All reported P values were two-tailed and, for each analysis, $P < .05$ was considered significant. We used Stata, version 131.0 (STATA,

Table 1. Adjusted rates of first VTE in splenectomized patients

		Events		Incidence rates per 100 person-years		Unadjusted (univariable)			Adjusted (multivariable)		
	No. of patients	No.	%	Value	95% CI	HR	95% CI	P	HR	95% CI	P
Sex											
Male	1325	68	5.1	1.17	0.92 to 1.48	1			1		
Female	1147	74	6.5	1.45	1.15 to 1.82	1.25	0.90 to 1.74	.179	1.17	0.84 to 1.63	.366
Age group (y)											
<50	947	27	2.9	0.62	0.42 to 0.90	1			1		
50+	1525	115	7.5	1.75	1.46 to 2.11	2.75	1.81 to 4.18	<.001	2.57	1.62 to 4.09	<.001
Splenectomy indication											
Therapeutic malignancy	269	21	7.8	1.74	1.14 to 2.67	2.23	1.23 to 4.06	.008	1.32	0.70 to 2.49	.395
Therapeutic hematologic	583	36	6.2	1.33	0.96 to 1.85	1.75	1.03 to 2.98	.038	1.28	0.74 to 2.22	.372
Therapeutic other	138	8	5.8	1.35	0.67 to 2.70	1.68	0.75 to 3.76	.211	1.38	0.61 to 3.12	.438
Iatrogenic noncancer	350	16	4.6	1.01	0.62 to 1.64	1.30	0.68 to 2.47	.426	0.80	0.41 to 1.57	.514
Iatrogenic cancer	497	39	7.9	1.89	1.38 to 2.58	2.32	1.38 to 3.91	.002	1.30	0.73 to 2.30	.371
Trauma	635	22	3.5	0.79	0.52 to 1.20	1			1		

HR, hazard ratio.

College Station, TX) for analysis. The Victorian Department of Health granted ethics approval for this study.

In all, 2472 patients underwent splenectomy over the 8-year period with 8236 person-years of follow-up (mean follow-up, 3.3 years per person). Of those patients, 1147 (46.4%) were female and 1525 (61.7%) were aged ≥50 years. The indications for splenectomy are described in Table 1.

A total of 142 splenectomized patients (5.74%) had a VTE requiring hospitalization; the incidence of first VTE was 1.30 per 100 person-years (95% confidence interval [CI], 1.10 to 1.53) (Table 1). Sites of VTEs included lower-extremity DVT (95; 66.43%), PE (60; 41.96%), and PVT (11; 7.69%).

Age was the most important risk factor for VTE, with a hazard ratio of 2.76 when splenectomized patients younger than age 50 years were compared with those age 50 years or older.

There was no statistical difference in the rate of VTE according to indication for splenectomy. When patients splenectomized for trauma were compared with patients who had other indications, there was no difference in hazard of VTE (Table 1).

Our VTE cumulative incidence of 5.74% is consistent with that of other large studies of splenectomized patients that used similar methodologies; Boyle et al[3] found cumulative incidence of 4.4% for DVT and PE and 1.7% for PVT in 1762 splenectomized patients with ITP. Our VTE rate of 1.30 per 100 person-years is similar to that in a Danish study with a rate of VTE between 1.03 and 1.90 per 100 person-years.[4]

The finding of no significant difference in the rate of VTE between those splenectomized for trauma and those splenectomized for therapeutic hematologic reasons (such as ITP) differs from the findings of previous studies.[3] Boyle et al[3] reported that patients splenectomized for ITP have higher rates of VTE than nonsplenectomized ITP patients. Our findings suggest that splenectomy per se might increase the risk of VTE above that which is posed by the indication for splenectomy.

The weaknesses of this study are the lack of a disease-specific control group, the broad splenectomy indication categories, and the possibility that VTE may have been missed if patients were not hospitalized.

The strength of this study is the large number of splenectomized patients. This study provides new evidence that the risk of VTE following splenectomy does not differ between patients splenectomized for trauma and those splenectomized for hematologic reasons. These findings may have implications for practice regarding targeted administration of thromboembolic prophylaxis.

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