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Immunological Manipulation to Prevent or Treat Relapse of Haematological Malignancies after Allogeneic Stem Cell Transplantation

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Abstract

Allogeneic haematopoietic stem cell transplantation (alloSCT) is a potent form of immunotherapy and an important treatment modality for patients with haematological malignancies. While alloSCT is a curative therapy for many patients, relapse remains a significant cause of treatment failure in a considerable proportion of patients. There is therefore an unmet need to improve the outcomes of patients after alloSCT by identifying patients at high risk of relapse and to develop effective strategies to prevent relapse in these patients. In addition, the outcome of patients with relapsed haematological malignancies after alloSCT remains poor and there is significant scope to develop novel strategies to treat relapse after alloSCT.

In this thesis I investigate immunological biomarkers of relapse and explore novel strategies to prevent and treat relapse of haematological malignancies after alloSCT through two investigator initiated clinical trials. I describe that donor/recipient T-cell chimerism is significantly associated with relapse after both myeloablative and non-myeloablative alloSCT in a continuous fashion, however its utility is limited by poor sensitivity. In addition, there remains a subgroup of patients with low-risk disease who can remain in long-term remission despite mixed T-cell chimerism.

In another piece of work, I explore the dynamics of T-cell receptor (TCR) repertoire reconstitution post-alloSCT and demonstrate that contrary to previous reports, a restricted TCR repertoire is not associated with early relapse of acute myeloid leukaemia (AML) after alloSCT. As an interesting aside, TCR diversity is significantly and adversely impacted by early cytomegalovirus (CMV) viremia and this effect persists late post-transplant.

Therapeutic strategies to prevent relapse of haematological malignancies after alloSCT are required and in this context I explore the feasibility of low-dose lenalidomide to prevent relapse of AML and myelodysplastic syndromes (MDS) post-alloSCT. Preliminary results of this investigator-initiated phase 1 dose escalation study, which remains open to recruitment, have demonstrated that to date lenalidomide 5mg twice per week commencing at day 40 post-alloSCT is safe and tolerable.

Finally, I describe the safety and efficacy of nivolumab, an inhibitor of the programmed death 1 (PD-1) receptor, for the treatment of relapsed haematological malignancies after alloSCT. In this investigator-initiated clinical trial, nivolumab induced highly potent alloimmune responses with complete remissions observed in highly refractory haematological malignancies albeit with a considerable risk of GVHD. Importantly, immunophenotypic and gene expression markers of T-cell activation may be able to identify patients who are primed to develop GVHD following nivolumab treatment and therefore guide the use of this powerful treatment strategy to those patients least likely to develop significant toxicities.

Declaration

This thesis comprises my original work towards the degree of Doctor of Philosophy

Due acknowledgement has been made in the text to all other material used

The thesis is fewer than 100,000 words in length, exclusive of tables, figures and references

Signed

Eric Youwei Wong

Preface

I acknowledge that the following work described in this thesis was carried out in collaboration with others:

- Chapter 6. The utility of T-cell chimerism as a biomarker of relapse following allogeneic stem cell transplantation for haematological malignancies: T-cell separation performed by Barbara Hockridge and Janis Boyd. Institutional clinical database curated by Kate Mason and Jenny Collins. Statistical advice provided by Alexandra Gorelik
- Chapter 7. Clinical determinants of T-cell receptor diversity after allogeneic haematopoietic stem cell transplantation: TCRβ sequencing performed by Piers Blombery, Georgina Ryland, Jerick Guinto, Yamuna Kankanige
- Chapter 8. MicroLEN: Micro-dose lenalidomide as maintenance therapy post-alloSCT for patients with acute myeloid leukaemia or myelodysplastic syndromes at high risk of relapse: Protocol design performed by myself in collaboration with David Ritchie and Travis Perera
- Chapter 9. NIVALLO: Nivolumab treatment for relapsed or residual haematological malignancies after allogeneic haematopoietic stem cell transplantation: Protocol design performed by myself in collaboration with David Ritchie and Andrew Grigg. T-cell gene expression profiling performed by myself in collaboration with Mandy Ludford-Menting

I assess that my contribution towards the work encompassed within this thesis is in excess of 95%.

The following components of this thesis have been published in peer-reviewed journals:

- **Wong E**, Davis JE, Grigg A, Szer J, Ritchie D. Strategies to enhance the graft versus tumour effect after allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant 2019; 54(2):175-189
  - I conceived the concept, reviewed the literature, wrote the manuscript and prepared all tables and figures.
  - Davis J, Grigg A, Szer J and Ritchie D reviewed the manuscript
- **Wong E**, Mason K, Collins J, Hockridge B, Boyd J, Gorelik A, Szer J, Ritchie D. Prognostic limitations of donor T cell chimerism after myeloablative allogeneic stem cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. Biol Blood Marrow Transplant 2017;840-844
  - I conceived the concept, collated the data, analysed the data, wrote the manuscript and prepared all tables.
  - Mason K and Collins J curated the institutional clinical database
  - Hockridge B and Boyd J performed T cell separation
  - Gorelik A provided statistical advice
  - Szer J and Ritchie D reviewed the manuscript

This thesis also includes data presented within the following published paper of which I was the principal author:

- **Wong E**, Davis J, Koldej R, Szer J, Grigg A, Ritchie D. Nivolumab induces dynamic alterations in CD8 T-cell function and TIM-3 expression when used to treat relapsed acute myeloid leukemia after allogeneic stem cell transplantation. Leuk Lymphoma 2019. doi: 10.1080/10428194.2019.1648803. [Epub ahead of print]

- I designed and conducted the clinical trial, performed the experiments, analysed the data, wrote the manuscript and prepared all figures.
- Davis J and Koldej R provided experiment design advice
- Szer J, Grigg A and Ritchie D reviewed the manuscript

This thesis references data published in the following paper of which I was a contributing author:

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    - *Mastaglio S and Wong E contributed equally to this work
    - I analysed the data and wrote the manuscript
    - Mastaglio S analysed the data and contributed to the writing of the manuscript
    - Perera T analysed the data
    - Ripley J performed the experiments
    - Blombery P performed molecular characterisation of leukemia
    - Smyth MJ, Koldej R, Ritchie D conceived the study hypothesis and reviewed the manuscript

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    - Published by Bone Marrow Transplantation 2019
- Materials and methods

- Unpublished material not submitted for publication
- The utility of T-cell chimerism as a biomarker of relapse following allogeneic stem cell transplantation for haematological malignancies
    - Published in part in Biology of Blood and Marrow Transplantation 2017
- Clinical determinants of T-cell receptor diversity after allogeneic haematopoietic stem cell transplantation
    - Submitted for publication, under review
- MicroLEN: Micro-dose lenalidomide as maintenance therapy post-alloSCT for patients with acute myeloid leukaemia or myelodysplastic syndromes at high risk of relapse
    - Unpublished material not submitted for publication
- NIVALLO: Pilot study of the tolerability of nivolumab for relapsed or residual haematological malignancies after allogeneic haematopoietic stem cell transplantation
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3 Abbreviations

ALL	Acute lymphoblastic leukaemia
alloSCT	Allogeneic haematopoietic stem cell transplant
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
AUC	Area under the curve
BiTE	Bispecific T cell engager
BM	Bone marrow
BuCy	Busulfan cyclophosphamide
CAR	Chimeric antigen receptor
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
CTL-EX	Exhausted cytotoxic T-lymphocyte
CyTBI	Cyclophosphamide total body irradiation
D100chim	Day 100 T-cell chimerism
DLI	Donor lymphocyte infusion
DLT	Dose limiting toxicity
DRI	Disease risk index
EBV	Epstein barr virus
FCS	Foetal calf serum
FluHDCy	Fludarabine high-dose cyclophosphamide
FluLDCy	Fludarabine low-dose cyclophosphamide

FluMel	Fludarabine melphalan
G-CSF	Granulocyte colony stimulating factor
GVHD	Graft versus host disease
GVT	Graft versus tumour effect
GZMA/GZMB	Granzyme A/B
HCT-CI	Haematopoietic stem cell transplant comorbidity index
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
IFNG	Interferon gamma
irAE	Immune related adverse effect
JSD	Jensen-Shannon Divergence
KGF	Keratinocyte growth factor
KIR	Killer immunoglobulin receptor
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MM	Myeloma
MPN	Myeloproliferative neoplasm
MRD	Measurable residual disease
MTD	Maximum tolerated dose
MUD	Matched unrelated donor
NHL	Non-hodgkin lymphoma

NK	Natural killer
NMA	Non-myeloablative
NPV	Negative predictive value
NRM	Non-relapse mortality
ORR	Overall response rate
OS	Overall survival
PB	Peripheral blood
PD-1	Programmed death 1
PFS	Progression free survival
PPV	Positive predictive value
PR3	Proteinase 3
PRF	Perforin
RFS	Relapse free survival
RIC	Reduced intensity conditioning
ROC	Receiver operating characteristic
ST2	Suppression of tumourigenicity 2
Tcm	T cell central memory
TCR	T cell receptor
Tem	T cell effector memory
TEMRA	T cell terminal effector memory
TNF	Tumour necrosis factor
Treg	Regulatory T cell
WIS	Withdrawal of immunosuppression
WT1	Wilms tumour 1

4 Introduction

While allogeneic haematopoietic stem cell transplantation (alloSCT) is a potentially curative therapy for many haematological malignancies by inducing an immunological graft-versus-tumour (GVT) effect, cancer relapse is the most significant cause of treatment failure and mortality[1, 2]. Current strategies to treat post-transplant relapse including immunosuppression withdrawal and donor leukocyte infusions have low efficacy and are associated with significant toxicity including high rates of graft versus host disease (GVHD)[3]. Novel therapies are therefore required to enhance the GVT response to prevent and treat cancer relapse. In this review we consider the mechanisms that limit the efficacy of the T- and NK-cell mediated GVT effect including factors that impair cellular immune reconstitution and promote T- and NK-cell immune evasion. Following this, we explore novel approaches that may allow us to prevent and treat cancer relapse by enhancing the potency of the GVT effect

4.1 The cellular basis of the graft versus tumour effect

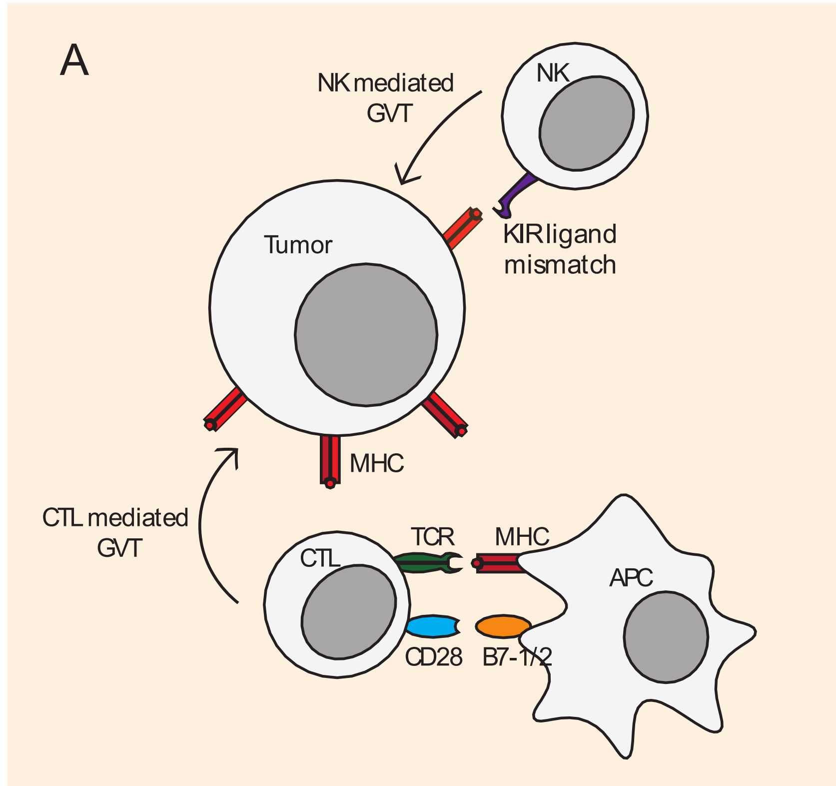
Early evidence pointing to the critical role of the donor-derived GVT effect in maintaining remission following alloSCT was derived from observation of the ability of donor lymphocyte infusions to achieve durable remission in relapsed chronic myeloid leukaemia after alloSCT, the association between chronic graft versus host disease (GVHD) with reduced relapse rate and demonstrable survival benefit, and that T-cell depletion reduced GVHD but was associated with increased leukemia relapse[4, 5]. Subsequent work in murine models of transplantation as well as clinical and translational research data have in part dissected the cellular mechanisms of the GVT effect. An effective GVT response requires the establishment of donor-derived immunity, the presence of anti-tumour effector populations, access of those populations

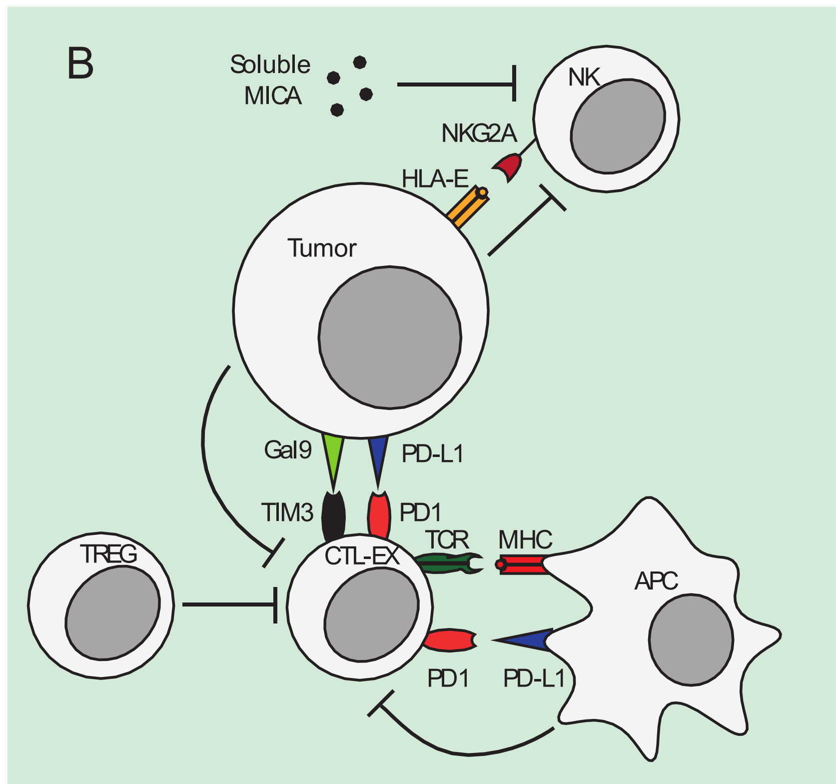
to the tumour site and sensitivity of the tumour to immune-mediated clearance. The GVT response is primarily mediated by donor CD4+ and CD8+ T-cells (Figure 3.1). Although cytotoxic CD8+ T cells are the main effectors of the GVT effect, CD4+ T cells also mediate a crucial role in alloreactivity by producing cytokines including IFNγ and TNFα, by providing help for cytotoxic T cell functions, and recruitment of NK-cells to tumour targets[6]. The critical role of CD4+ T cells in augmenting the GVT response was demonstrated in murine models whereby CD8+ T cell depleted marrow transplantation was able to result in low rates of GVHD but an effective GVT response[7]. In HLA-matched alloSCT, CD4+ and CD8+ T cells recognise minor histocompatibility antigens presented on host antigen presenting cells (APC), or directly presented by tumour cells with APC functions. Minor histocompatibility antigens may be tissue restricted, such as those selectively expressed by haematopoietic cells and these (e.g. HA-1, HA-2, HB-1 and BCL2A1) are ideal candidates for a haematopoietic malignancy-specific GVT effect[8]. However, many minor histocompatibility antigens are widely expressed and T-cell recognition of these antigens are likely responsible for the inextricable link between GVT and GVHD[9]. Graft-versus-tumour effects may also be directed against leukaemia associated antigens including Wilms Tumour 1 (WT1) or proteinase 3 (PR3) that may be overexpressed by leukemia. Following allorecognition, costimulatory interactions (CD28 and CD80/CD86) in the presence of the cytokine storm that ensues from T-cell activation and augmented by the pro-inflammatory environment induced by chemotherapy conditioning or infection promotes T-cell proliferation and cytotoxicity mediated either directly via the perforin/granzyme pathway or Fas/Fas ligand induced tumour apoptosis[10].

Evidence for the importance of NK-cell alloreactivity in mediating GVT responses post-alloSCT comes from reports of early NK-cell reconstitution being associated with reduced relapse[11] and that inhibitory KIR ligand incompatibility is associated with reduced relapse after haploidentical alloSCT. Early work by Ruggeri *et al*. demonstrated that KIR-ligand mismatch in the graft-versus-host direction mediated potent anti-leukemia cytotoxicity in-vivo in human AML-engrafted mice, and also abrogated GVHD by eliminating recipient antigen presenting cells[12]. Consistent with this, the authors also demonstrated a significant benefit of KIR-ligand incompatibility on relapse rate (3% for KIR ligand incompatible donors vs 47% for KIR ligand compatible) and event-free survival in patients with AML who underwent T-cell depleted HLA-haploidentical alloSCT[12, 13]. In HLA-matched alloSCT, the significance of NK-cell mediated alloreactivity is less clear, with some reports suggesting favourable leukemia free survival and reduced relapse in donor-recipient combinations mismatched for inhibitory KIRs[14]. Recent reports have also suggested a favourable impact of the presence of specific donor KIR haplotypes or donor activating KIR[15, 16]. In addition, the previously noted association between cytomegalovirus (CMV) reactivation and decreased relapse post-alloSCT may be partly attributed to virus-driven expansion of $CD56^{dim}CD57+$ NK cells with high expression of the activating receptor NKG2C, which are then also able to mediate a GVT effect[17]. Overall, there is evidence to suggest that NK-cell mediated alloreactivity does play a role in mediating a GVT effect, however the relative importance of this compared to T-cell alloreactivity is likely to be dynamic (greater impact early post-alloSCT prior to T-cell reconstitution) and dependent on disease type, HLA disparity (greater in haploidentical transplants than HLA-matched or single antigen mismatched transplants), as well as extent of T-cell depletion[18, 19].

Figure 4.1 Mechanisms of T and NK-cell immune escape after alloSCT

A) The GVT effect is mediated by donor T-cells which recognise mismatched minor histocompatibility antigens or leukemia-associate antigens presented by antigen presenting cells or directly by tumour cells in addition to co-stimulation signals. Donor NK-cells also contribute to the GVT by KIR-ligand mismatch and the balance of activating and inhibitory NK-receptor ligand signals. **B)** The T- and NK-cell GVT effects may be inhibited by a myriad of suppressive cellular interactions including induction of T-cell exhaustion by PD-1/PD-L1 interactions, Tregs, immunosuppressive microenvironments, and soluble factors (e.g. MICA) which downregulate NK-cell responses. CTL, cytotoxic T-lymphocyte; CTL-EX, exhausted cytotoxic T-lymphocyte; APC, antigen presenting cell.


A
NK mediated
GVT
NK
Tumor
KIR ligand
mismatch
MHC
CTL mediated
GVT
CTL
TCR
MHC
APC
CD28
B7-1/2


B
Soluble
MICA
NK
NKG2A
HLA-E
Tumor
Gal9
PD-L1
TIM3
PD1
TCR
MHC
TREG
CTL-EX
APC
PD1
PD-L1

4.2 Mechanisms of immune evasion after allogeneic transplantation

Despite the potency of the T and NK-cell mediated GVT effect, relapse of the primary haematological malignancy occurs in a significant proportion of patients post-transplant, including up to 50% of patients with high-risk AML. Our understanding of the precise mechanisms of disease relapse are incomplete, however in some patients relapse may represent escape from the immunological T- and NK-cell mediated GVT effect (Figure 4.1). Immune escape following alloSCT may be considered to fall into 3 broad categories: immunological immaturity due to the time course of immune reconstitution[20], failure of T- and NK cells to recognise and respond to tumour antigens due to antigen downregulation[21, 22] and progressive T-cell exhaustion consequent to chronic exposure to alloantigen expressed by recipient non-hematopoietic cells[23-25].

4.2.1 Relapse in an environment of immunological immaturity

In the first few months following alloSCT, the potency of the GVT effect is limited by deficiencies in the absolute numbers of effector cells, lack of persistence of tumour specific effector T cells, lack of CD4+ T cell help, and functionally immature NK cells[26, 27]. These factors are secondary to the inherent dynamics of immune reconstitution following conditioning therapy and are exacerbated by the exogenous impact of immunosuppressive medications and the prohibitive effects of GVHD and infection on immune reconstitution[28-30]. In recipients of T-cell replete grafts, the number of circulating CD8+ T cells returns to normal within 3 months post-alloSCT, whereas the CD4+ subset only returns to the normal range by 6 months post-transplant[31]. Impaired CD4+ and CD8+ T-cell reconstitution due to T-cell depletion or immunosuppression to treat GVHD adversely impacts overall survival and may contribute to an increased risk of relapse[32, 33].

In contrast to T-cell reconstitution, NK cell reconstitution occurs rapidly following alloSCT and precedes CD4+ and CD8+ T-cell recovery[32, 34]. Despite numerical reconstitution, NK cells are functionally immature in the first few months post-transplant. Early recovering NK-cells are derived predominantly from engrafted donor progenitors and harbour an immature CD56^{bright}NKG2A+KIR- phenotype[26, 35]. The increased expression of the inhibitory receptor NKG2A and decreased expression of the activating receptor NKp30 seen in early reconstituting NK cells contributes to an impaired GVT that can be restored *in-vitro* by blocking NKG2A[26]. NK-cells also demonstrate impaired degranulation and interferon gamma production within the first few months post-alloSCT that limits their anti-tumour effect[36].

4.2.2 Tumour antigen downregulation

The GVT response is dependent on effector cell recognition of alloantigen. In haploidentical alloSCT, alloreactive T cells against mismatched HLA haplotypes provide a potent GVT effect. However, loss of mismatched HLA alleles through genomic uniparental disomy or reduced surface expression may facilitate immune escape[22]. Loss of mismatched HLA was observed at the time of AML or MDS relapse in 33% of patients following myeloablative haploidentical alloSCT and resulted in reduced proliferation and cytotoxicity of donor T-cells against relapsed leukaemia[21, 37]. In contrast to haploidentical alloSCT, HLA-loss relapses are significantly less frequent following mismatched unrelated donor (MMUD) or HLA-matched alloSCT, suggesting that HLA-loss relapse is a consequence of immune selection pressure[38-40]. HLA-loss relapse has been most characterised in acute leukaemia, and it remains to be seen if this mechanism of immune escape occurs in other haematological malignancies after allograft.

Antigen downregulation may also contribute to evasion from NK-cell mediated attack. The ability of NK cells to mediate cytotoxicity is determined by the balance of activating and inhibitory receptor ligand interactions[18]. In HLA-matched alloSCT, the inhibitory role of NK cell interaction with self-MHC may be overcome by activating NK receptor ligands such as ULBP[18]. However, haematological cancers may evade NK cell cytotoxicity by reducing expression of these ligands[41]. Alternatively, cancer cells may shed activating ligands, particularly MHC class I chain-related gene A and B peptides (MICA and MICB), which results in downregulation of the activating receptor NKG2D on NK cells and T cells[42]. In a retrospective analysis, elevated serum MICA levels were associated with an increased risk of disease relapse post-allograft[43].

4.2.3 T-cell exhaustion and immunosuppressive tumour microenvironments

T-cell exhaustion is a state of cellular dysfunction induced by chronic antigen exposure and immunosuppressive cytokines (IL-10, TGF-β), and is functionally characterised by decreased proliferation and cytotoxicity and failure to control malignancy[23-25, 44]. Exhausted CD8+ T cells progressively express one or more inhibitory receptors including PD-1, LAG-3 and TIM-3 which have an additive effect to repress T-cell function[25, 44, 45]. T-cell exhaustion following alloSCT is in part driven by alloantigen (HLA and minor histocompatibility alloantigen) expression on recipient non-haematopoietic cells[46-48]. Alloantigen expression on host epithelium upregulates PD-1 on donor CD8+ T cells which results in impaired cytotoxicity that may be reversed by PD-1 blockade[46]. Exhausted T-cells expressing PD-1 have been observed prior to and at the time of AML relapse following alloSCT, and demonstrate functional impairment including failure to proliferate in the presence of cognate antigen[49, 50]. *In-vitro* antibody

blockade of PD-1 significantly enhanced leukaemia-specific CD8+ T cell proliferation suggesting that therapeutic inhibition of PD-1 signalling can restore T-cell functionality and enhance the GVT effect[49].

The cancer microenvironment may also induce T-cell exhaustion by upregulation of PD-L1. Tissue expression of PD-L1 may create local niches of T-cell exhaustion favouring tumour escape[51, 52]. In addition, the cancer microenvironment may release soluble factors that upregulate immunosuppressive factors on tumour associated macrophages, promote apoptosis of tumour-reactive effector cells and recruit immunosuppressive regulatory T-cells (Tregs)[53-57]. Tregs post-allograft may be induced from peripheral CD4+ conventional T-cells in the presence of transforming growth factor beta (TGF-β) producing an early pool of Tregs and a later pool derived from the thymus (natural Tregs), and act to suppress effector T-cells and contribute to immune tolerance[58]. Antigen presenting cells, fibroblasts and endothelial cells release the chemokine CXCL12 which recruits Tregs to sites of lymphoma and abrogates GVT responses[59]. Blockade of CXCL12/CXCR4 interactions improved survival in mice harbouring B-cell lymphoma following allograft by blocking Treg recruitment toward lymphoma sites[59].

4.3 Current strategies to treat post-transplant relapse and their limitations

Current strategies to treat relapse of haematological malignancies after alloSCT include withdrawal of immunosuppression (WIS) particularly for early post-transplant relapses, donor lymphocyte infusions (DLI), chemoradiotherapy in combination with WIS or DLI, or a minority of patients may be candidates for second alloSCT. There are no prospective clinical trials to suggest benefit of one modality over another, however there

is some retrospective cohort data to suggest that some form of immunotherapy, particularly donor-derived cellular therapy, may be beneficial compared to chemoradiotherapy alone[1]. Withdrawal of immunosuppression alone may be able to induce remissions in patients with relapsed malignancies that demonstrate a high degree of sensitivity to the GVT effect such as chronic myeloid leukemia and indolent non-Hodgkin lymphoma, however is usually insufficient in patients with aggressive malignancies including acute myeloid leukemia or aggressive non-Hodgkin lymphoma[60-63]. Meaningful disease responses to WIS are also invariably accompanied by GVHD, which demonstrates the close inter-relationship between the beneficial GVT response and GVHD[61]. Donor-lymphocyte infusions may be used either pre-emptively in the context of mixed donor-chimerism to boost the GVT effect to prevent relapse, as well as treat relapse post-alloSCT. The efficacy of DLI for post-alloSCT relapse is greatest in those conditions with a high degree of intrinsic sensitivity to the GVT effect, low tumour burden, and tumour kinetics that permit the establishment of a beneficial immunological effect, including relapsed CML and low grade lymphomas[61]. Response rates to DLI alone are lower in relapsed AML post-alloSCT, and as such DLI are often combined with other therapies including chemotherapy or hypomethylating agents to enhance response, and indeed there may be a synergistic effect of the combination of DLI with azacitidine or decitabine via upregulation of tumour associated antigen expression[64, 65]. The major toxicity of DLI is GVHD which occurs in up to 60% of patients, and patients who receive DLI within 6 months of alloSCT are at particularly high risk[65]. Second alloSCT may be a potential therapeutic strategy for only a minority of patients primarily due to toxicity. Non-relapse mortality approaches 50% following a second transplant, although this may be partly mitigated by reduced intensity conditioning[66, 67]. As a consequence of both high toxicity in combination with the

inherent selection of patients with intrinsically more resistant disease, survival outcomes post second alloSCT remain dismal[68]. The role, therefore of second alloSCT should therefore be limited to fit patients without rapidly progressive disease or large tumour burdens, with both patient and physician cognisant of the high likelihood of treatment failure. The timing of post-transplant relapse is also prognostically important and should be taken into account when determining post-transplant therapeutic strategies. The majority of patients who relapse do so within 1 year post-transplant; in a large retrospective analysis of 1338 patients who underwent alloSCT for AML, the median time to relapse from alloSCT was 5.5months[1]. Patients who relapse within 6 months post-alloSCT have a 5 year survival of less than 5%, and these patients are therefore unlikely to benefit from a second alloSCT.

Overall, the efficacy of current immunotherapeutic strategies post-alloSCT are likely to be limited by the same factors which promoted relapse in the first place, namely poor intrinsic sensitivity to a non-augmented GVT response as well as immune evasion via antigen downregulation or immunosuppressive and/or exhaustion promoting microenvironments. It is therefore no surprise that many patients either do not respond to these strategies or such responses are short-lived, and underscores the need to develop strategies that target mechanisms of immune evasion.

4.4 How to build a more effective graft versus tumour response

Our understanding of the mechanisms of immune evasion after alloSCT highlights avenues of opportunity to enhance the potency of the GVT effect in order to effectively treat post-transplant relapse. With the development of new immunotherapies we now have an array of potential tools to manipulate the allogeneic immune response.

Therefore, we may be able to view the engrafted allogeneic immune system as a foundation upon which further immunological ‘building blocks’ may be added to construct a potent, non-exhausted allogeneic immune response that addresses the key mechanisms of immune escape. In the following discussion, we consider 3 building blocks to optimise the GVT effect: enhancing immunological potency, reversing T cell exhaustion and refocusing the GVT response towards cancer (Table 4.1).

Table 4.1 Selected clinical studies of post-transplantation immune therapies

Strategy	**Reference**	**Study Design**	**Study Size**	**Relapse Prevention /Treatment**	**Timing of Post-Transplant Therapy after AlloSCT**	**Disease Subtype**	**Results**
Enhancing Immunological Potency							
IL-7	Perales 2012[69]	Phase I	12 patients	Prevention	60-210 days	Myeloid (AML, MDS, CML)	Increase in CD4+ and CD8+ effector memory T-cells following IL-7 No change in frequency of recent thymic emigrants 1 patient developed GVHD
KGF	Levine 2008[70] Rizwan 2011[71]	Phase II randomised (KGF vs placebo)	100	Prevention	Pre-SCT up to 3 weeks post-SCT	All subtypes	No difference in OS No difference in acute or chronic GVHD No difference in lymphocyte recovery post-alloSCT
	Saber 2016[72]	Retrospective case control	816	Prevention	Not stated	All subtypes	No difference in GVHD, TRM or relapse
Lenalidomide	Sockel 2012[73] LENMAINT	Phase II single arm Lenalidomide 10mg/d d1-21	10	Prevention	2-4 months	AML or MDS with del(5q)	Trial terminated due to toxicity 60% acute GVHD grade 3-4 within 2 cycles
	Kneppers 2011[74] HOVON 76	Phase II single arm Lenalidomide 10mg/d d1-21	38	Prevention	1-6 months	Myeloma	53% grade 2-4 acute GVHD or extensive chronic GVHD Median GVHD onset 18 days after lenalidomide commencement 37% maintained best myeloma response or improved response

							Phenotypic T-cell activation (increased HLA-DR expression)
	Khouri 2017[75]	Phase I Randomised to lenalidomide versus immunosuppression withdrawal/rituximab/DLI	17	Treatment	90-100 days	CLL	Maximum tolerated dose 10mg/d Lenalidomide arm: 38% ORR; 43% grade 2-4 acute GVHD Immunomodulation alone arm: 66% ORR; 11% grade 2-4 acute GVHD
Adoptive NK-cell infusion	Ciurea 2017[76]	Phase I IL-21 expanded donor NK cells after haploidentical alloSCT and PTCy	13	Prevention	Days -2, 8, 28	Myeloid (AML, MDS, CML)	11 patients received NK cell product 54% grade 1-2 acute GVHD (no grade 3-4 acute GVHD or chronic GVHD) 1 year OS 92% and DFS 85%
	Shaffer 2016[77]	Phase II Haploidentical NK cell infusion	8	Treatment	3.5 months	Myeloid (AML, MDS, CML)	No GVHD 38% ORR, 25% CR
	Passweg 2004[78]	Phase I NK cell infusion after haploidentical SCT	5	Prevention	3-24 months	Myeloid (AML, CML)	No GVHD Increase in donor chimerism in 40% of patients
Reversal of T-cell Exhaustion							
CTLA-4 inhibition	Davids 2016[79]	Phase I/Ib Ipilimumab 3 or 10mg/kg every 3 weeks for 4 doses	22	Treatment	6-63 months	All subtypes	35% immune related adverse events or GVHD Among 10mg/kg cohort: 23% CR, 9% PR 1 year OS 49%

PD-1 inhibition	Haverkos 2017[80]	Retrospective cohort study Nivolumab (3mg/kg every 2weeks) or pembrolizumab (200mg every 3 weeks)	31	Treatment	5-108 months	Lymphoid	ORR 77%; 50% CR, 27% PR 55% GVHD (including 29% grade 3-4 acute GVHD or severe chronic GVHD) Median PFS 19 months
	Herbeaux 2017[81]	Retrospective cohort study Nivolumab (dose not specified)	20	Treatment	2-111 months	Hodgkin lymphoma	30% acute GVHD (including 25% grade 3-4 acute GVHD) 90% ORR; 40% CR, 50% PR 1 year OS 79%, PFS 58%
Refocusing the GVT Response towards Cancer							
Bi-specific T cell engagers (BiTE)	Kantarjian 2017[82]	Phase III randomised Blinatumomab versus chemotherapy	140 had prior alloSCT	Treatment	Not stated	B-ALL	40% CR in patients with prior allograft No documentation of GVHD
	Martinelli 2017[83]	Phase II	20 had prior alloSCT	Treatment	Not stated	B-ALL (Philadelphia chromosome positive)	25% CR No documentation of GVHD
Allogeneic CAR T-cells	Maude 2014[84]	Phase I Anti-CD19 CAR with 4-1BB costimulation domain Recipient derived product	18 had prior alloSCT	Treatment	Not stated	B-ALL	Entire cohort: 90% CR at 1 month 6 month EFS 67%, OS 78% No GVHD
	Lee 2015[85]	Phase I Anti-CD19 CAR with CD28 costimulation domain	8 had prior alloSCT	Treatment	Not stated	Lymphoid (CD19 positive)	50% CR Entire cohort: 51.6% OS at median follow-up of 10 months

		Recipient derived product					No GVHD
	Kochenderder 2013[86] Brudno 2016[87]	Phase I dose escalation Anti-CD19 CAR with CD28 costimulation domain Donor derived product	20	Treatment	2-25 months after last DLI	Lymphoid (CD19 positive)	No new onset acute GVHD after CAR T cell infusion B-ALL: 80% ORR (all CR) All patients: 40% ORR
	Cruz 2013[88]	Phase I Anti-CD19 CAR and virus specific T cells Donor derived product	8	Treatment	3-33 months	Lymphoid (CD19 positive)	No new GVHD after CAR T cell infusion 25% ORR (1 CR in B-ALL; 1 PR in Richter's transformed CLL)
	Kebriaei 2016[89]	Phase I Anti-CD19 CAR with CD28 costimulation domain Donor derived product	19	Prevention	Day 43-84	Lymphoid (CD19 positive)	16% GVHD (2 acute GVHD, 1 chronic GVHD)
Cancer vaccines	Burkhardt 2013[90]	Phase I Whole tumour cell vaccine	18	Prevention	Day 30-45	CLL	39% acute GVHD (no difference compared with historical controls) 72% remain in CR
	Rousseau 2006[91]	Phase I Whole tumour cell vaccine	10	Prevention	10 days to 6 months after IS cessation	AML or B-ALL	No GVHD developed after vaccination Increase in CD4+ and CD8+ T cells after vaccination 8 patients remain disease free 27-62 months after vaccination
	Franssen 2017[92]	Phase I	9	Treatment	Not stated	Myeloma	No GVHD No clinical responses

		Minor histocompatibility antigen loaded DC vaccine in combination with DLI					
	Shah 2016[93]	Phase I WT-1 peptide loaded donor derived dendritic cell vaccine in combination with DLI Relapsed haematological malignancies after alloSCT	5	Treatment	1-12 months	All subtypes	20% acute GVHD (grade 1 skin) No clinical response
	Maeda 2013[94]	Phase I WT-1 peptide vaccine	9	Prevention or treatment	2-60 months	All subtypes	11% acute GVHD 33% (2 out of 6) of patients who were not in remission prior to vaccination achieved CR

4.4.1 Building block 1: enhancing immunological potency

A potential strategy to enhance the early GVT effect is to accelerate T and NK-cell numerical and functional reconstitution. Stimulation of T-cell maturation and proliferation using IL-7 has shown promise in murine models of transplantation by accelerating thymic recovery and T-cell proliferation[95-98]. In a phase I dose-escalation study, recombinant human interleukin (IL)-7 following T-cell depleted alloSCT increased CD4+ and CD8+ effector memory T-cells by enhancing peripheral T-cell proliferation, however there was no observed impact on thymopoiesis[69]. Recombinant IL-7 was safe in this T-cell depleted cohort, with only 1 patient developing GVHD. Similarly, IL-15 stimulates the proliferation and activation of NK and CD8+ T cells[99]. Exogenous IL-15 following T-cell depleted transplants improved CD8+ T- and NK cell reconstitution and enhanced NK cell cytotoxicity in mice, which enhanced GVT efficacy[100, 101]. Limiting the utility of post-transplant exogenous IL-15 is the potential to stimulate GVHD particularly following T-cell replete alloSCT[102]. The safety and efficacy of IL-15 post-transplant is yet to be investigated in clinical trials.

An adjunct to cytokine therapy may be to use adoptive cellular therapy of T and NK cell subsets that are lacking in the early post-transplant period. For example, post-transplant NK cell infusions may curtail the depletion of alloreactive NK cells following post-transplant cyclophosphamide[103]. Clinical trials have varied in the timing of post-transplant NK cell infusion, however overall adoptive NK cell therapy post-haploidentical alloSCT appears to be safe and well tolerated with a low rate of GVHD[76, 78]. A recent phase 1 trial used three infusions of *ex-vivo* expanded haploidentical NK cells between day -2 and 28 and demonstrated enhanced NK cell reconstitution compared with historical controls[76]. In this patient cohort with high risk myeloid

malignancies there was an impressively low rate of disease relapse (7%) and virus reactivation.

Beyond immune reconstitution, strategies to enhance the overall potency of the GVT effect may reduce the risk of disease relapse. Lenalidomide has pleiotropic immunomodulatory effects including stimulation of T-cell proliferation and has been investigated in post-alloSCT maintenance strategies[104, 105]. Sockel *et al.* conducted a phase II trial of lenalidomide 10mg daily for up to 12 months in patients with AML or high risk MDS with del(5q) following alloSCT[73]. Lenalidomide was commenced at a median 2.5 months post allograft. This study was ceased prematurely due to a high incidence of grade 3-4 acute GVHD (60% of patients within 2 months of lenalidomide). Similarly, a high incidence of acute GVHD was reported using lenalidomide maintenance (10mg daily, commencing median 12 weeks post alloSCT) after tandem autologous followed by non-myeloablative alloSCT for patients with myeloma[74]. Fifty-three percent of patients developed grade 2-4 acute GVHD or extensive chronic GVHD. It can be concluded from these studies that lenalidomide at a dose of 10mg daily as an early post-transplant maintenance strategy is not tolerable, however it remains unknown if smaller doses in carefully selected patients may have a more acceptable safety profile whilst augmenting GVT responses in patients with high risk malignancies.

The example of post-allograft lenalidomide demonstrates the difficulty in separating an augmented GVT response from the simultaneous promotion of GVHD[106]. An important lesson from the post-transplant lenalidomide trials discussed above is that the timing of post-transplant stimulation is likely to be a significant factor in the likelihood of initiating GVHD. The commencement of lenalidomide within the first 3 months post-

alloSCT, as in these studies, is associated with very high rates of GVHD; however, a retrospective analysis of lenalidomide treatment in 52 patients with relapsed myeloma after alloSCT commencing a median of 24 months post-alloSCT had a lower, albeit still moderate, rate of acute of chronic GVHD (31%), suggesting that post-transplant maintenance therapies that non-selectively enhance GVT may be safer in later post-alloSCT relapses[107]. Key in moving the field forward may be to identify patients who are close to the maximal tolerable point of non-specific alloreactivity post-transplantation. Plasma suppression of tumourigenicity 2 (ST2) is a promising biomarker that is predictive of non-relapse mortality and steroid refractory acute GVHD[108]. In two independent patient cohorts, high plasma ST2 on day 14 after alloSCT was significantly associated with increased non-relapse mortality (NRM) and was superior to conventional risk factors including recipient age, conditioning intensity, donor source and degree of HLA match[108]. Likewise, a 2 biomarker algorithm comprising ST2 and REG3α at day 7 post alloSCT was predictive of 6 month NRM and GVHD-related mortality[109]. Validation of these and other biomarkers at other time points and prior to immunotherapies may help to identify patients at lower risk of GVHD and more likely to benefit from strategies to enhance the GVT effect.

4.4.2 Building block 2: reversal of T-cell exhaustion

Pre-clinical evidence that T-cell exhaustion and immune evasion following alloSCT is mediated in part by co-inhibitory signals suggests that GVT responses may be augmented following alloSCT with the use of checkpoint inhibitors; however this optimism is tempered by recognition that the same pathways are responsible for protection against GVHD[110-112]. In particular, the timing of checkpoint blockade post-alloSCT is critical, as the risk of GVHD is likely to be significantly higher in the early

post-transplant period (e.g. within 6 months post-alloSCT) due to the lack of immune tolerance. Early phase clinical trials have demonstrated the potential utility of CTLA-4 blockade with ipilimumab. A phase I study treated 22 patients with relapsed haematological malignancies after alloSCT with ipilimumab (4 induction doses followed by maintenance for 60 weeks)[79]. The median time from alloSCT to treatment with ipilimumab was 675 days, with the earliest patient being treated 198 days post-alloSCT. The overall response rate was 32%, including 23% of patients who achieved complete remission. Immune related adverse events and GVHD were seen in 27% of patients[79]. Patients who responded uniformly had a prior history of GVHD, fewer CD4+ Tregs and a greater percentage of peripheral blood effector memory T cells compared with non-responders, suggesting that an existing immune system that is primed for alloreactivity favours response to CTLA4 inhibition.

Tentative steps have also been taken to explore the safety and efficacy of PD-1 inhibitors post-alloSCT, with reports at this time limited to retrospective series[81, 113, 114]. Table 4.1 describes the outcomes of two series of patients with relapsed lymphoma post-alloSCT who received pembrolizumab or nivolumab. On balance, these early reports provide some proof of principle that PD-1 blockade post-allograft is a potent therapeutic tool to enhance alloreactivity, albeit at the cost of GVHD. While there is some suggestion that careful patient selection may mitigate the risk of severe GVHD (i.e. patients without prior GVHD being at considerably less risk of developing GVHD following PD-1 inhibitors), questions remain regarding the optimal dose and timing of PD-1 blockade either to prevent or treat post-allograft relapse and is the subject of prospective trials (ClinicalTrials.gov NCT03146468 and NCT01822509). In the series by Herbeaux and colleagues, earlier treatment with nivolumab post-alloSCT appeared to

increase the risk of nivolumab-induced GVHD (GVHD group median time from alloSCT to nivolumab 7.5months vs no GVHD group 28.5months)[81]. Confirmation of this trend in larger series is required, however this early data suggests that checkpoint blockade early post-transplant (e.g. within the first 3-6 months after alloSCT) may be poorly tolerated. While most reports have used a fixed-dose approach, variable dosing of PD-1 inhibitors may allow titration of effect according to the risk of GVHD[115]. We have recently treated 3 patients for relapsed Hodgkin lymphoma following alloSCT, each with active GVHD, with dose-adjusted pembrolizumab (25-100mg) given at infrequent intervals (two received just one dose). All patients had a flare of GVHD, which was controlled by systemic immunosuppression in two patients, and achieved either complete or durable partial remission from lymphoma (unpublished data).

4.4.3 Building block 3: refocusing the GVT response towards cancer cells

So far we have discussed means of enhancing the strength of the emergent GVT response and reversing exhaustion in a patient-tailored approach informed by novel biomarkers. Another strategy to enhance the GVT effect is to direct the highly potent, non-exhausted allogeneic immune system towards residual tumour. Strategies to focus the GVT response towards cancer antigens include cancer vaccines, bi-specific T and NK-cell engager molecules and chimeric antigen receptor T-cells. The concept of vaccination against cancer comprises numerous possible strategies including whole tumour vaccines, dendritic cell vaccination, or peptide vaccines[116]. Phase I clinical trials of tumour cell vaccines have reported a low rate of GVHD, but their efficacy is uncertain[90, 91, 117]. Cancer peptide vaccines offer the advantage of broad applicability and scalability, and in a phase 1 clinical trial using a WT1 peptide vaccine, two patients with demonstrable disease prior to immunisation developed sustained clinical

responses[94]. While these results highlight the promise of vaccine therapy after alloSCT, most work has been performed in small trials and requires further demonstration of efficacy in larger cohorts.

A direct approach to enforce immune versus cancer cell interaction is through the use of bi-specific T-cell engager molecules (BiTEs). Blinatumomab is a BiTE that bridges the invariant CD3ε TCR subunit on T-cells with CD19 on normal and malignant B-cells, leading to target cell cytolysis. In the phase III randomised trial of blinatumomab versus chemotherapy in B-cell acute lymphoblastic leukaemia (B-ALL), 34% of patients had a prior allograft, and in this subgroup blinatumomab induced a significantly greater rate of complete remission compared with chemotherapy alone (40.4% vs 10.9%; OR 5.6)[82]. It is pertinent to note that there have been occasional reports of GVHD in patients treated with blinatumomab after alloSCT, likely a result of cytokine release and bystander activation of allogeneic T-cells against non-CD19 bearing host cells[118, 119]. Given the efficacy of blinatumomab in reasserting a CD19-focused GVT effect, its use as a maintenance strategy to prevent disease relapse following alloSCT is an attractive strategy, and is currently being investigated (ClinicalTrials.gov NCT02807883, NCT03114865, NCT02790515).

Cellular therapy with autologous T-cells transduced to express chimeric antigen receptors (CARs) or antigen-specific TCRs have demonstrated impressive results against haematological malignancies. The majority of CAR constructs have been directed against the CD19 antigen, and hence the majority of clinical experience is against B-ALL and B-cell non-Hodgkin lymphoma (B-NHL). Early clinical trials of CD19 CAR T-cells for relapsed B-ALL or B-NHL included in their cohorts some

patients with relapsed disease after alloSCT. Maude et al. reported their experience using a CD19-directed CAR T-cell product with a 4-1BB co-stimulatory domain in 30 paediatric and adult patients, of which 18 had relapsed disease after a prior alloSCT[84]. The CAR T-cell product in this study was recipient-derived, and in alloSCT recipients, the median donor chimerism at the time of leukapheresis was 100% (range 68% to 100%). The complete response rate was 90% in the entire cohort, and there was no significant difference in response between patients who had a prior alloSCT and this who had not. Importantly, in patients treated with CAR T-cells after alloSCT, there were no cases of GVHD observed. Similarly, Lee et al. reported a phase I dose escalation study of recipient derived CD19 CAR T-cells with a CD28 costimulatory domain in patients with relapsed B-ALL or B-NHL[85]. Of the 21 patients enrolled, 8 patients had prior alloSCT, although inclusion into the study required a minimum of 100 days post transplant and no current evidence of GVHD or need for immunosuppression. In the post-alloSCT subgroup, 4 patients achieved CR, including 3 patients with MRD negative CR. Similar to the report by Maude et al., there were no cases of GVHD observed. These reports and others demonstrate that recipient-derived CD19-directed CAR T-cells are highly effective in achieving complete responses in highly refractory B-cell malignancies in the post-alloSCT setting without causing GVHD. A disadvantage of recipient-derived CAR T-cells is the time taken for product manufacture, requiring interim chemotherapy or disease stability for several weeks. A potential solution is the use of donor-derived allogeneic CAR T-cells for an ‘off-the-shelf’ product that can be readily available without the delay required for leukapheresis and product manufacture. In the post-alloSCT setting, donor-derived CAR T-cells have been explored in patients with high risk disease (e.g. MRD positivity) to prevent relapse, as well as to treat post-transplant relapses. The distinction between recipient-

derived and donor-derived CAR T-cells in the post-alloSCT setting is likely to be important, as the infusion of non-tolerised T-cells in a donor-derived product may result in a greater risk of GVHD. Several groups have evaluated the safety and efficacy of donor-derived CAR T-cells for the treatment of relapsed B-cell malignancies after alloSCT. In a phase I dose escalation study, donor-derived CD19-directed CAR T-cells were administered to 20 patients with persistent or relapsed CD19-positive malignancies after alloSCT[86, 87]. Patients were not administered lymphodepleting chemotherapy prior to CAR T-cell infusion. The overall response rate was 40%, including 4 out of 5 patients with B-ALL achieving a MRD negative CR. Response rates were lower in patients with B-NHL. There were no observed cases of acute GVHD, however 2 patients developed either new onset chronic GVHD or worsening of pre-existing chronic GVHD following CAR T-cell infusion. Kebriaei et al. reported a phase I study of donor-derived CD19-specific CAR T-cells produced using the sleeping beauty transposon/transposase system for patients with high risk B-ALL or B-NHL to prevent relapse after autologous or alloSCT. In the alloSCT subgroup, CAR T-cells were administered without additional lymphodepletion at a median of 64 days after alloSCT, however all patients remained on GVHD prophylaxis at the time of CAR T cell infusion. Overall survival and PFS at 1 year compared favourably to a historical cohort and 3 patients developed GVHD (2 acute, 1 chronic). These early phase trials demonstrate the feasibility and low rates of GVHD following donor-derived allogeneic CAR T-cells, and while the efficacy of this approach particularly in the relapse prevention setting requires an appropriately controlled clinical trial, they are nonetheless promising. The low rate of GVHD observed with this approach may be due to functional exhaustion and activation-induced cell death due to simultaneous activation of T-cells through the TCR and the CAR construct[120]. As a result, in a murine transplant

model, allogeneic donor-derived CAR T-cells demonstrated poor persistence after infusion, a finding recapitulated in the aforementioned phase I trials[84, 85]. As a note of caution, Ghosh et al. also demonstrated that the occurrence of GVHD post allogeneic CAR T cell infusion was dependent on T-cell dose and the burden of disease, and that at high effector-to-target ratios, allogeneic CAR T cells mediated significant acute GVHD[120].

4.5 Final thoughts

There is both the need and significant opportunity to enhance the GVT effect after alloSCT in order to treat cancer relapse. With an increasing armamentarium of immunotherapy we have an opportunity to build upon the immunological foundation established by allogeneic transplantation. We have conceptualised the opportunities for therapeutic intervention as the 3 building blocks of enhancing immunological potency, reversing T cell exhaustion and refocusing the GVT towards cancer cells. The optimal timing of these interventions and the potential to combine multiple strategies are yet to be determined. Moreover, the deliverability of some of these strategies is likely to be impacted by the timing of post-transplant relapse; the majority of patients who relapse post-transplant do so within the first year after alloSCT, and it is also within this timeframe, and in particular within the first 3 to 6 months post-alloSCT that patients are at greatest risk of developing GVHD from immune stimulation for example by lenalidomide or checkpoint inhibitors. Therefore, a patient-specific approach taking into account the timing of post-transplant relapse, the identification and validation of biomarkers that are able to identify patients at greater risk of developing GVHD, and an understanding of the potential mechanisms of tumour escape will be required rather than

a universal approach to post-transplant immunomodulation in order to prevent and treat relapse.

5 Research Aims

The aim of my research is to improve the outcome of patients at high-risk of relapse of haematological malignancies post-alloSCT by investigating predictive biomarkers of relapse and developing novel strategies to enhance the allogeneic graft versus tumour response to prevent and treat relapse after alloSCT. In this thesis, I have therefore investigated four key topics:

1. The utility of T-cell chimerism as a biomarker to predict relapse of haematological malignancies after alloSCT
2. Clinical determinants of T-cell receptor diversity after alloSCT for AML and impact on relapse
3. Micro-dose lenalidomide as maintenance therapy post-alloSCT for patients with AML and myelodysplastic syndromes at high risk of relapse
4. The safety of nivolumab as treatment of relapsed or residual haematological malignancies after alloSCT

6 Methods

6.1 Haematopoietic chimerism analysis

Donor-recipient chimerism analysis was performed using peripheral blood samples collected in EDTA at day 100 post-alloSCT, 1 year post-alloSCT and prior to successive donor lymphocyte infusions (DLI). T-cell populations were separated using CD3+ immunomagnetic cell separation with density gradient centrifugation (RosetteSep; StemCell Technologies, Vancouver, CA). Polymerase chain reaction (PCR) was performed using oligonucleotide primers specific for short tandem repeats that were identified pretransplant to distinguish donor from recipient DNA (Invitrogen, ThermoFisher Scientific Inc. Waltham, MA). Products were analysed using a DNA Sequencer (Applied Biosystems, Foster City, CA) and chimerism results were expressed as the percentage of donor-specific DNA present.

6.2 Statistical considerations for chimerism analyses

Associations between T-cell chimerism and relapse, non-relapse mortality (NRM), relapse-free survival (RFS), overall survival (OS), acute and chronic GVHD were examined in both univariate and multivariate analyses using the Cox proportional hazards model for RFS and OS, and the model of Fine and Gray for relapse with NRM as a competing risk and vice versa. Similarly, the composite outcome of death or relapse was a competing risk for the cumulative incidence of acute and chronic GVHD. In these analyses, chimerism was treated in turn as a continuous variable as well as an ordinate variable with pre-specified increments of 10% between 55% and 95%. Variables identified to be statistically significant with $\alpha<0.05$ in univariate analyses were then used in multivariate models.

The predictive utility of T-cell chimerism for post-transplant relapse and survival was evaluated by assessing its sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for these outcomes. This was performed due to the limitations of evaluation of statistical associations in describing the utility of a prognostic tool. The underlying reason for this is the distinction between association and classification, which has been well described in previously[121]. In brief, the ideal prognostic tool will be able to categorise patients precisely into two groups with evens (for example relapse) and those without events (no relapse) with little or no overlap in the distribution of values of the predictive biomarkers between the two groups. However, such accurate classification requires odds ratios and hazards ratios that are rarely seen in analyses of statistical association, providing an explanation for why many purported biomarkers do not actually prove to be useful in clinical practice. Rather, the conventional method of assessment of diagnostic or prognostic utility is evaluation of sensitivity and specificity, both of which are reflected in the receiver operating characteristic curve (ROC) and area under the curve (AUC) which is represented numerically as the c-statistic.

6.3 T-cell receptor diversity

Details of the methods used for TCRβ gene sequencing and analysis including VDJ recombination patterns, repertoire overlap and diversity are described in the results chapter, which has been presented in publication format and is currently under peer review.

In order to evaluate the impact of TCR diversity at day 100 post-transplant on early relapse, a propensity score matching approach was used to identify suitable controls (patients without relapse) that were matched for key pre-transplant criteria for each

patient who developed early relapse. The reason for this was to minimise the potential impact of confounding factors including use of T-cell depletion, donor and recipient age, donor type, conditioning regimen intensity and time post-transplant. Importantly, this approach recognises the inherent heterogeneity of the cohort of patients with relapse post-transplant. Rather than attempting in vain to obtain a more homogeneous cohort of patients with relapse which would likely be nigh on impossible due to the innumerable combinations and permutations that characterise both the selection of transplant technique and patient characteristics, this technique therefore attempts to establish a suitably matched cohort with similar baseline characteristics in a manner analogous to how randomisation in a prospective clinical trial balances potential confounding factors between two comparator arms. Propensity score matching was performed using the nearest neighbour method with one-to-one matching in the package MatchIt in R[122].

6.4 MicroLEN and NIVALLO human research ethics and research governance approvals and role of funding bodies

Detailed descriptions of both MicroLEN and NIVALLO clinical trials including study design, participant inclusion and exclusion criteria, investigation product and treatment strategy and clinical trial endpoints are described in the respective results chapters. Both clinical trials were approved by the Human Research Ethics Committee of the Royal Melbourne Hospital and were conducted in accordance with good clinical practice guidelines and in accordance with the declaration of Helsinki. The study sponsor for both trials was Melbourne Health. The MicroLEN trial was supported by funding and provision of lenalidomide by Celgene and funding from Snowdome Foundation. MicroLEN is registered in the Australian New Zealand clinical trials registry (ANZCTR registration number ACTRN12619000556145). The NIVALLO trial was supported

financially and with by provision of nivolumab by Bristol-Myers-Squibb (BMS), who reviewed the study protocol prior to commencement however did not have direct input into the study design or data interpretation. This study was registered prospectively in local and international clinical trial registries (ClinicalTrials.gov identifier NCT03146468 and ANZCTR registration number ACTRN12617000473369).

6.5 Clinical trial design in the context of GVHD risk

An important note in the design of each of these trials was the need to consider that strategies to augment the allogeneic GVT effect may also simultaneously provoke GVHD. This narrow therapeutic index of alloimmune reactivity post-alloSCT has been well established in strategies ranging from immunosuppression withdrawal, donor lymphocyte infusions and also published experience with lenalidomide at considerably higher doses than used in the MicroLEN study[73, 74]. In this context, both MicroLEN and NIVALLO incorporated conservative recruitment strategies with re-assessment of safety outcomes including particular attention to GVHD risk at pre-specified timepoints during each trial. In the MicroLEN trial and as will be described in detail along with the complete study design, within each dose level of four patients, two patients were required to complete at least two cycles of lenalidomide with no more than one patient experiencing a dose limiting toxicity before recruitment of a further two patients to the same dose level. In the NIVALLO trial recruitment was opened to an initial three patients. As a pre-specified requirement for study continuation, once three patients had completed a minimum of two cycles and completed assessment of adverse events and grade 2 or higher GVHD was observed in no more than one patient, recruitment of a further three patients was permitted. Once six patients had received at least one dose of

nivolumab, a planned safety and data monitoring committee (SDMC) meeting was convened to discuss recruitment of the remaining eight patients in light of existing data.

6.6 Peripheral blood and tissue sampling for correlative analyses

MicroLEN and NIVALLO both included peripheral blood and tissue sampling at pre-specified time points in order to examine the immunological impact of treatment with lenalidomide and nivolumab respectively. Samples were cryopreserved for batch analysis. PBMC were isolated by Ficoll separation and frozen down in FCS+10% DMSO for cryopreservation at -80oC until required. The schedule of collection timepoints for each trial are described in Tables 6.1 and 6.2.

Table 6.1. MicroLEN peripheral blood and bone marrow collection schedule

Timepoint	Peripheral blood (80mL EDTA and 10mL serum tube)	Bone marrow aspirate (10mL in EDTA)
Pre-transplant	x	x
Pre-cycle 1 lenalidomide	x	
Pre-cycle 3	x	
Pre-cycle 5	x	
Pre-cycle 7	x	
Post-cycle 12	x	
Relapse	x	x

Table 6.2 NIVALLO: Correlative sample collection schedule

Test Sample	C1D1	C1D3	C1D7	C2D1	C2D7	C3D1	C3D7	C4D1	C5D1	C9D1	C13D1	C24D14	Relapse
PB EDTA 30mL	x	x	x	x	x	x	x	x	x	x	x	x	x
PB SST 10mL	x	x	x	x	x	x	x	x	x	x	x	x	x
Tumour sample (biopsy/ BM)	x												x

6.7 T- and NK-cell immunophenotyping

Cryopreserved peripheral blood samples obtained at specified study timepoints were thawed and used to perform detailed characterisation of T- and NK-cell phenotype, activation and co-inhibitory receptor expression. A target of 1-2x10^6 cells were resuspended in 100µL of FACS buffer (phosphate buffered saline [PBS] with 2% foetal calf serum [FCS]) for immunophenotyping experiments noting that in a small proportion of patients significantly fewer cells were available for analysis. Staining antibodies used for T- and NK-cell analyses in microLEN and NIVALLO studies are listed in Tables 6.3 to 6.5. Cells were incubated with antibodies for 30 minutes on ice prior to washing with FACS buffer and fixed with paraformaldehyde solution (PBS 2% paraformaldehyde) prior to acquistion on a BD LSRFortessa 5 laser flow cytometer. Analysis was performed using FlowJo (BD Biosciences) software.

Cell populations were gated on single, viable lymphocytes. T-cell memory subsets were defined as follows: naïve (Tn: CCR7+CD45RA+), central memory (Tcm: CCR7+CD45RA-), effector memory (Tem: CCR7-CD45RA-), terminally differentiated effectors (TEMRA: CCR7-CD45RA+)(Figure 6.1). Regulatory T-cells were defined as CD4+CD25+CD127low/-. T-cell expression of CD38 and/or HLA-DR were analysed as markers of cellular activation. Expression of co-inhibitory receptors (PD-1, TIM3, LAG3) which are upregulated following T-cell encounter with antigen and also as a phenotypic marker of T-cell exhaustion following chronic antigen stimulation in the context of chronic infection or malignancy were also analysed.

Natural killer cell maturation was analysed using expression of CD56 and CD16 as previously defined with particular attention to CD56brightCD16- and CD56dimCD16+ subsets (Figure 6.2)[35]. NK cell receptor expression was also investigated including killer immunoglobulin receptors (KIR), NKG2A and NKG2D, the latter of particular interest in patients with AML as previously published work has demonstrated that AML blasts express a heterogeneous range of NK receptor ligands and the balance of inhibitory and activating ligands impacts on prognosis, suggesting a role of NK-cell surveillance in maintaining remission (Appendix 12.1)[123]. The impact, therefore, of low dose lenalidomide on NK cell receptor expression post-alloSCT in patients with AML and MDS was therefore of particular interest.

6.8 T-cell intracellular cytokine production

T-cell intracellular cytokine production was ascertained by stimulating $1x10^6$ peripheral blood mononuclear cells with anti-CD2/3/28 microbeads (Miltenyi Biotec) in T-cell medium (comprising RPMI with 10% foetal calf serum, 2nM L-glutamine, 1% penicillin streptomycin solution, 1% sodium pyruvate, 1% non-essential amino acids, 1% HEPES and 0.1% 2-mercaptoethanol)in the presence of protein transport inhibitors (BD GolgiStop 0.7µL per mL cell culture and GolgiPlug 1µL/mL, BD Biosciences) and incubation at 37 degrees Celsius for 4 hours. Following stimulation, cells were stained with antibodies to surface antigens and fixed and permeabilised (BD Fixation/Permeabilisation kit, as per manufacturer's instructions). Permeabilised cells were incubated with anti-cytokine antibodies for 30 minutes prior to resuspension in FACS buffer and flow cytometry analysis. Surface and intracellular antibodies in this panel are listed in Table 6.6.

6.9 Statistical analysis of flow cytometry data

Flow cytometry data were expressed as percentage of positive cells and comparisons between unpaired groups were performed using the Mann-Whitney U test. Comparisons of paired samples were analysed using the Wilcoxon matched-pairs signed rank test.

Table 6.3 MicroLEN T-cell immunophenotype panel

Antibody specificity	Fluorochrome	Isotype	Clone	Source	Dilution
CD3	BUV496	IgG1	UCHT1	BD	1/200
CD4	BUV395	IgG1	RPA-T4	BD	1/200
CD8	BUV805	IgG1	SK1	BD	1/200
HLA-DR	APC-H7	IgG2a	G46-6	BD	1/50
CD127	AF647	IgG1	HIL-7R-M21	BD	1/50
CD25	BB515	IgG1	11A9	BD	1/25
CXCR3	BB700	IgG1	1C6	BD	1/50
CD45RA	PE-Cy7	IgG2b	HI100	BD	1/200
CCR6	PE	IgG1	11A9	BD	1/50
CCR4	BV605	IgG1	1G1	BD	1/50
PD-1	BV786	IgG1	EH12.1	BD	1/50
TIM3	BV650	IgG1	7D3	BD	1/50
CCR7	BV421	IgG2a	150503	BD	1/100
Fixable Viability Stain 700				BD	1/500

Table 6.4 MicroLEN NK-cell immunophenotype panel

Antibody specificity	Fluorochrome	Isotype	Clone	Source	Dilution
CD3	BUV496	IgG1	UCHT1	BD	1/25
CD4	BUV395	IgG1	RPA-T4	BD	1/200
CD8	BUV805	IgG1	SK1	BD	1/200
CD45RA	APC-H7	IgG2b	HI100	BD	1/100
CD16	BV480	IgG1	3G8	BD	1/50
CCR7	BB515	IgG2a	3D12	BD	1/25
NKG2D	BV786	IgG1	1D11	BD	1/50
NKG2A	APC	IgG2b	Z199	Beckman Coulter	1/25
TIM3	BV650	IgG1	7D3	BD	1/50
TIGIT	PE-Dazzle	IgG2a	A15153G	Biolegend	1/50
KIR2D	PE	IgG1	NKVFS1	Miltenyi Biotec	1/50
KIR3DL1/DL2	PE	IgG1	5.133	Miltenyi Biotec	1/50
CD56	BV421	IgG2b	NCAM16.2	BD	1/100
Fixable Viability Stain 700				BD	1/500

Table 6.5 NIVALLO T-cell immunophenotype panel

Antibody specificity	Fluorochrome	Isotype	Clone	Source	Dilution
CD3	BUV395	IgG1	UCHT1	BD	1/100
CD4	BV510	IgG1	SK3	BD	1/100
CD8	BUV805	IgG1	SK1	BD	1/200
HLA-DR	PE-Cy7	IgG2a	G46-6	BD	1/200
CD38	BUV496	IgG1	HIT2	BD	1/100
CCR7	PE	IgG2a	3D12	BD	1/200
CD45RA	BB515	IgG2b	HI100	BD	1/100
PD1	BV786	IgG1	EH12.1	BD	1/50
TIM3	BV421	IgG1	7D3	BD	1/50
LAG3	AF647	IgG1	T47-530	BD	1/100
Fixable Viability Stain 780				BD	1/500

Table 6.6 NIVALLO intracellular cytokine panel

Antibody specificity	Fluorochrome	Isotype	Clone	Source	Dilution
CD3	BUV395	IgG1	UCHT1	BD	1/100
CD4	BV510	IgG1	SK3	BD	1/100
CD8	BUV805	IgG1	SK1	BD	1/200
CD45RA	BB515	IgG2b	HI100	BD	1/100
CCR7	PE	IgG2a	3D12	BD	1/200
IL2	BV650	IgG2a	MQ1-17H12	BD	1/100
IFNγ	BV786	IgG1	4S.B3	BD	1/100
TNFα	APC	IgG1	MAb11	BD	1/100

Figure 6.1 T-cell memory subsets gating strategy

Representative flow cytometry plots demonstrating T-cell gating strategy from one patient peripheral blood sample

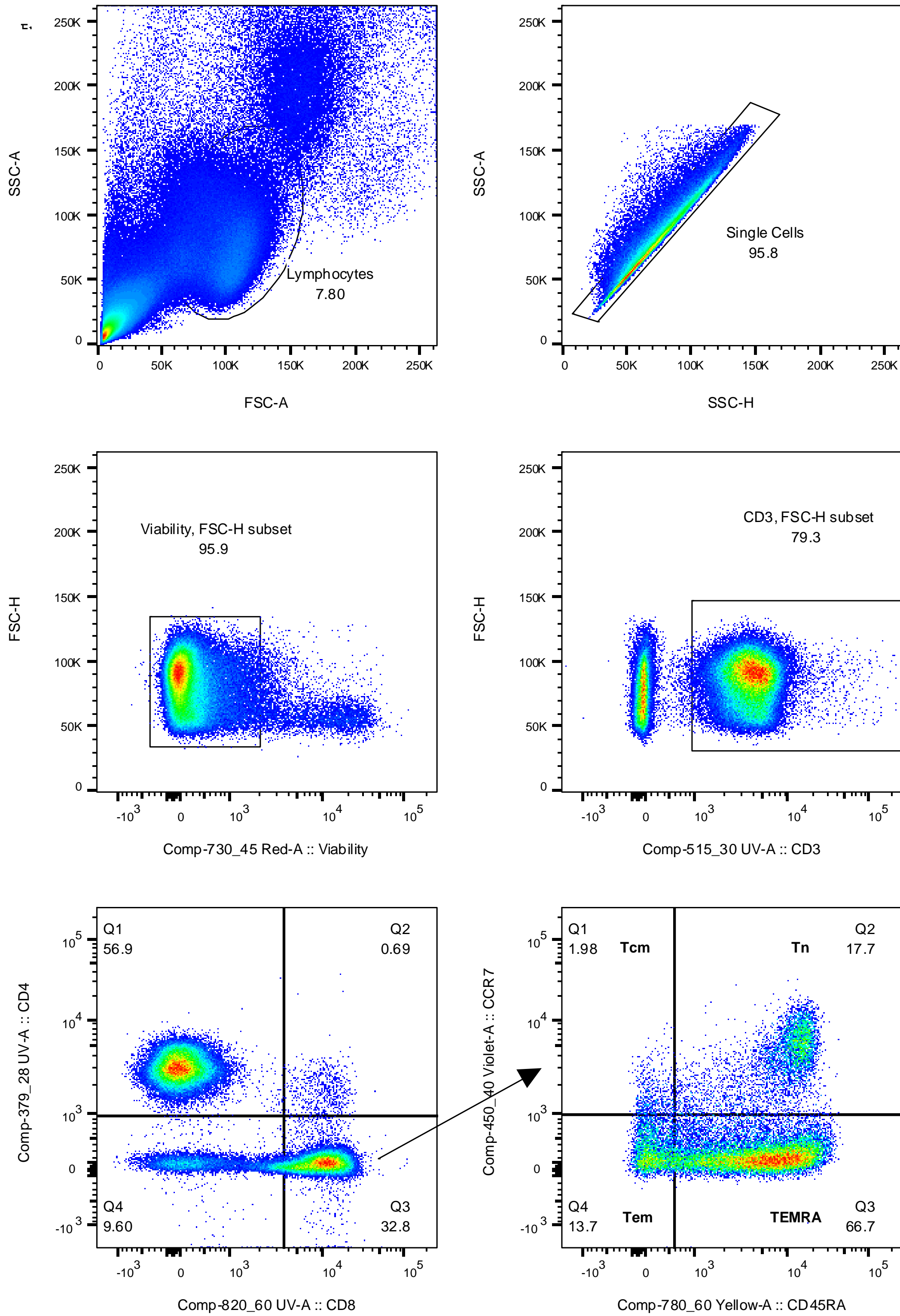
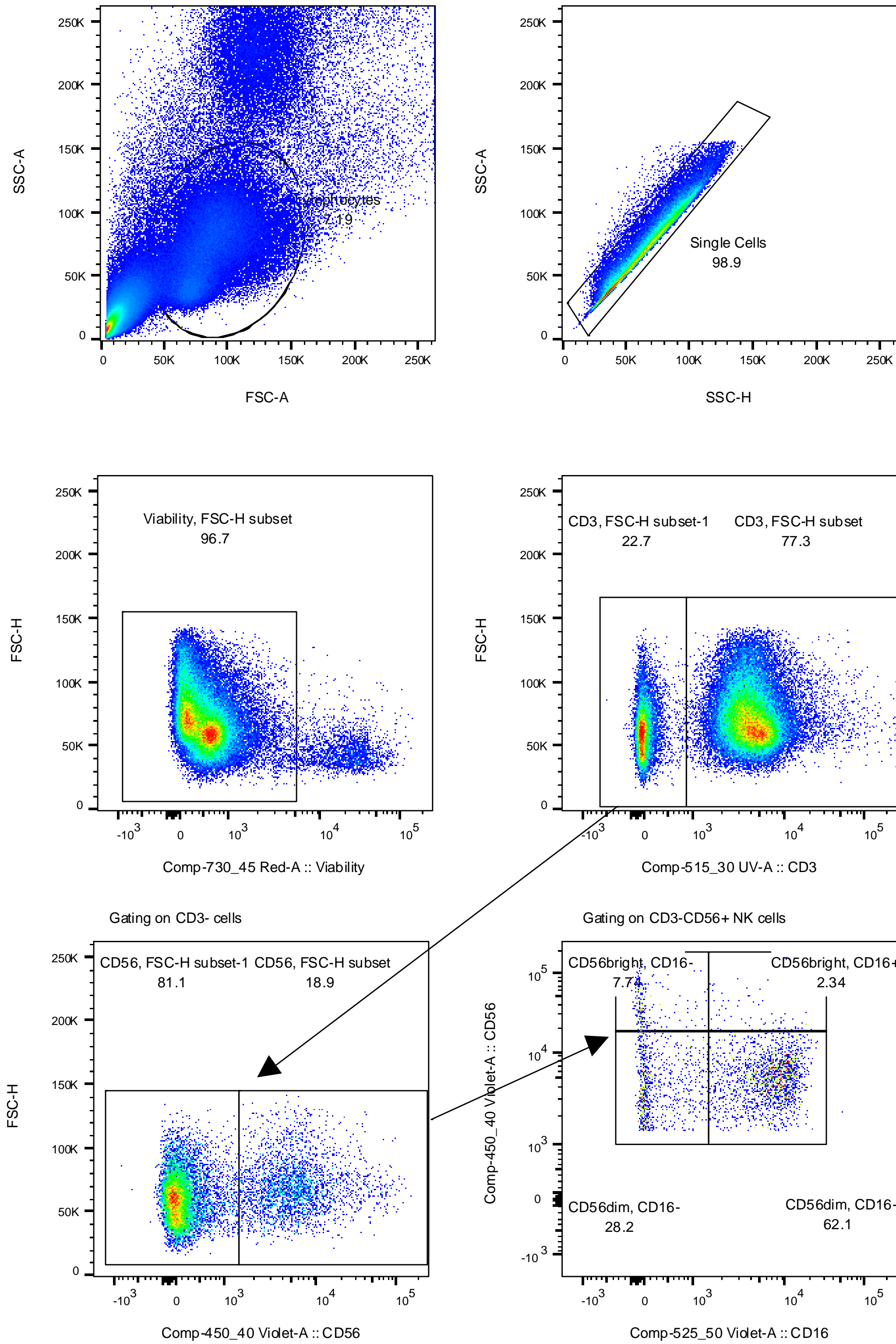


Figure 6.2 NK-cell gating strategy

Representative flow cytometry plots demonstrating NK-cell gating strategy from one patient peripheral blood sample



6.10 Gene expression profiling

CD8+ T-cells were isolated from cryopreserved peripheral blood samples by magnetic labelling and negative selection in magnetic columns (CD8+ T cell isolation kit and LS columns, Miltenyi Biotec). CD8+ T cell enrichment was confirmed by flow cytometry, demonstrating 90% purity following negative selection. Cell lysis and RNA extraction was performed using the ReliaPrep RNA Cell Miniprep System (Promega), according to the manufacturer's instructions. Purified RNA was amplified using the nCounter low RNA input amplification kit (Nanostring) to generate sufficient input RNA for downstream gene expression profiling. Briefly, 1ng of mRNA at a concentration of 0.25ng/µL was converted to cDNA followed by addition of primers for pre-specified gene targets and amplification over 8 cycles in a thermocycler. Following amplification, 5ng input RNA was used for gene expression profiling using the nCounter Immunology V2 Panel (Human V2, Nanostring), a multiplex gene expression platform analysing 594 genes including cytokines, cytokine receptors, chemokine ligands and receptors, inhibitory and activating checkpoint receptors and ligands, interferons and their receptors and KIR family genes. Gene expression data normalisation was performed using the geometric mean expression of internal housekeeping genes with counts above 20. Analysis was performed using nSolver 4.0 software (Nanostring).

6.11 Statistical analysis of gene expression data

Gene expression data were expressed as log2 normalised counts. Univariate analyses of normalised gene expression data were performed using *t*-tests for 12 pre-specified RNA targets of particular interest including CD45RA, CD45RO, CTLA4, GZMA, GZMB, PRF1, TIM3, LAG3, PDCD1, TNF, IFNG, IL2. These genes were selected to specifically examine the impact of nivolumab treatment on genes related to T-cell naïve

and memory subset differentiation (CD45RA and CD45RO), cytokine and cytotoxic granule production (GZMA, GZMB, PRF1, IL2, TNF, IFNG), and expression of co-inhibitory checkpoints (PDCD1, TIM3, LAG3). Differential gene expression was analysed for the entire cohort of 594 RNA targets and expressed as $\log_2$ fold-change in expression calculated using the following formula:

$$(\text{arithmetic mean of } \log_2 \text{ counts of gene 'A' in group 1}) - (\text{arithmetic mean of } \log_2 \text{ counts of gene 'A' in group 2})$$

t-tests were applied to differential gene expression comparisons between two groups. The Benjamini-Yekutieli (B-Y) calculation with a false discovery rate of 0.05 was performed to assess for statistical significance where multiple comparisons were performed for gene expression data. Note that B-Y calculations for expression levels of the 12 pre-specified genes therefore required a lower threshold for determination of statistical significance as compared with the complete set of 594 genes due to fewer comparisons being performed.

6.12 Control samples for flow cytometry and gene expression comparisons

MicroLEN and NIVALLO were single arm studies. In MicroLEN, patient immunology analyses were compared with both healthy patient samples and contemporaneous non-trial patients who underwent alloSCT for AML and MDS who did not receive post-transplant lenalidomide or early immunosuppression withdrawal. These latter samples were obtained from an institutional ethics approved tissue biobank. In the NIVALLO study flow cytometry data was compared with normal patient controls.

7 The utility of T-cell chimerism as a predictive biomarker of relapse following allogeneic stem cell transplantation for haematological malignancies

7.1 Introduction

Mixed donor/recipient haematopoietic chimerism, defined as the co-existence of recipient and donor-derived cell populations, is common following reduced intensity and non-myeloablative conditioned (collectively referred to hereafter as RIC) allogeneic haematopoietic stem cell transplants (alloSCT), particularly within the first 3 to 6 months post-transplant. While some patients spontaneously convert to complete donor haematopoiesis, others may require post-transplant immune modulation either with accelerated withdrawal of immunosuppression or donor lymphocyte infusions (DLI) in order to do so.

Haematopoietic chimerism post-alloSCT may be measured in either unsorted whole blood samples or in specific cell lineages, most commonly T-cell, myeloid or CD34 expressing cell fractions. Several reports have described that mixed donor/recipient T-cell chimerism as early as day 30 or day 100 post-transplant has adverse prognostic impact in patients receiving alloSCT for haematological malignancies via an increased risk of relapse[124-129]. While described following both myeloablative and RIC conditioning regimens, it is particularly more prevalent and also likely to be more significant in the latter where there is greater reliance on the graft-versus-tumour effect to maintain remission[130].

Most reports have analysed T-cell chimerism as a static measure and have not taken into account the impact of post-transplant immune manipulation strategies such as

accelerated immunosuppression withdrawal or DLI and the results of serial chimerism analyses following these interventions. As such, the long-term outcome of patients with initially mixed donor/recipient T-cell chimerism with subsequent conversion to complete donor haematopoiesis compared with those with stable mixed chimerism remains unclear. Furthermore, while it is recognised that there is a small subset of patients who are able to remain in long-term remission with stable mixed T-cell chimerism, the characteristics of patients in ongoing remission despite mixed chimerism have not been well defined[130]. This is clinically relevant as aggressive strategies to improve donor T cell engraftment may not be required for long-term disease control in specific patient subgroups[131].

A related issue is the characterisation of T-cell chimerism with respect to its sensitivity and specificity for post-transplant relapse. Although biomarker research is frequently reported in terms of statistical associations with the outcome of interest, these analyses are usually inadequate to describe the ability of a biomarker to accurately distinguish between patients who will relapse and those who will not[121]. The sensitivity and specificity of T-cell chimerism for relapse post-alloSCT has not been described.

In order to address the issues aforementioned, this chapter describes investigations into the utility of T-cell chimerism at day 100 post-transplant as a predictor of post-transplant relapse. These analyses are separated into firstly patients receiving reduced-intensity (RIC) or non-myeloablative (NMA) conditioning, with an interrogation of the impact of immunosuppression withdrawal or DLI as a means of salvaging low T-cell chimerism and an attempt to identify the characteristics of patients in whom persistent low mixed T-cell chimerism was compatible with long-term remission. The second part

of this results chapter describes an analysis of T-cell chimerism in patients undergoing myeloablative conditioning (MAC) with a specific focus on AML and MDS and a description of the sensitivity and specificity of day 100 T-cell chimerism to predict relapse in this homogeneous cohort. This latter analysis of chimerism in MAC alloSCT has been published in the peer reviewed journal Biology of Blood and Marrow Transplantation where I am the first author and performed the majority of the work in design of the study, analysis and manuscript preparation[124].

7.2 Part 1. Mixed donor/recipient T-cell chimerism after reduced intensity and non-myeloablative allogeneic haematopoietic stem cell transplantation and impact on long-term outcomes

7.2.1 Patients and methods

Adult patients (≥ 18 years) who underwent RIC alloSCT for a haematological malignancy at the Royal Melbourne Hospital between 2000 and 2016 were included in this retrospective analysis. Patients who had undergone a prior alloSCT and those who relapsed or died prior to day 100 post-alloSCT were excluded. Peripheral blood or bone marrow grafts were used from HLA-matched sibling or unrelated donors matched at a minimum of 8/10 loci at HLA-A, B, C, DRB1 and DQB1. The conditioning regimens used were: fludarabine 125mg/m^2 in combination with melphalan 140mg/m^2 (FluMel); fludarabine 125mg/m^2 and high-dose cyclophosphamide 120mg/kg (FluHDCy); and fludarabine 90mg/m^2 in combination with low-dose cyclophosphamide 2250mg/m^2 (FluLDCy). FluMel was considered a reduced-intensity regimen, while the fludarabine and cyclophosphamide combinations were considered non-myeloablative regimens as per consensus definitions of conditioning intensity[132]. Graft versus host disease

(GVHD) prophylaxis consisted of cyclosporin 3mg/kg intravenously (i.v) commencing day -1, with transition to an equivalent oral dose upon recommencement of oral intake, and short course methotrexate 15mg/m^2 day 1 followed by 10mg/m^2 days 3, 6 and 11. AlloSCT recipients from sibling donors also received prednisolone 0.5mg/kg/d days 14 to 34 decreasing to 0.25mg/kg/d days 35 to 48[133]. Patients receiving transplants from unrelated donors received anti-thymocyte globulin 4.5mg/kg pre-alloSCT in three divided doses (Thymoglobulin; Genzyme Corporation, Cambridge, MA).

Cyclosporin Withdrawal and Donor Lymphocyte Infusions

Cyclosporin withdrawal was commenced from day 100 or earlier depending on physician discretion due to perceived risk of relapse or the presence of mixed T-cell chimerism. DLI were prepared either from excess peripheral blood stem cell product at the time of initial donation and subsequently cryopreserved until needed or collected as a second request for steady-state peripheral blood lymphocytes from the same donor as the alloSCT. Donor lymphocytes were fractionated into graded aliquots according to CD3 T-cell content, beginning with 1x10^6 CD3 T-cells per kg of recipient body weight with subsequent doses of 5x10^6/kg, 10x10^6/kg, 50x10^6/kg and 100x10^6/kg or until all cells were used. Administration of DLI was according to physician discretion however was typically prescribed for patients with persistent low mixed chimerism, defined as donor T-cell chimerism $<75\%$, beyond day 100 post-alloSCT despite cessation of immunosuppression. Patients received DLI at four-week intervals in the absence of GVHD until complete donor chimerism was obtained or all product was used. DLI dose and schedule was identical for products from sibling and unrelated donors.

Chimerism Analysis

Donor-recipient chimerism analysis was performed using peripheral blood samples collected in EDTA at day 100 post-alloSCT, 1 year post-alloSCT and prior to successive DLI. T-cell populations were separated using CD3+ immunomagnetic cell separation with density gradient centrifugation (RosetteSep; StemCell Technologies, Vancouver, CA). Polymerase chain reaction (PCR) was performed using oligonucleotide primers specific for short tandem repeats that were identified pretransplant to distinguish donor from recipient DNA (Invitrogen, ThermoFisher Scientific Inc. Waltham, MA). Products were analysed using a DNA Sequencer (Applied Biosystems, Foster City, CA) and chimerism results were expressed as the percentage of donor-specific DNA present. Low mixed T-cell chimerism was defined as the presence of <75% donor-derived cells, the threshold at which all patients underwent expedited immunosuppression withdrawal and/or initiation of DLI. Very low chimerism was defined as <25%, and graft failure was defined as T-cell chimerism <5%.

Endpoints and Statistical Considerations

The impact of day 100 T-cell chimerism on the clinical endpoints of overall survival (OS) and relapse free survival (RFS) were investigated using Cox proportional hazard models with chimerism as a continuous independent variable. The association between chimerism and the cumulative incidence of relapse was investigated with non-relapse mortality (NRM) as a competing risk. Similarly, the composite outcome of death or relapse was a competing risk for the cumulative incidence of acute (aGVHD) or chronic GVHD (cGVHD). Variables in addition to chimerism analysed for univariate associations with these outcomes included age, disease-risk index (DRI)[134], primary malignancy category (lymphoid or myeloid), donor type, use of T-cell depletion,

conditioning intensity (RIC or NMA) and graft source (peripheral blood or bone marrow). Variables found to be statistically significant in univariate analyses were included in multivariable models. Significant level was set at $\alpha<0.05$. Comparisons of day 100 T-cell chimerism between conditioning regimens was performed using the Kruskall-Wallis test and Mann-Whitney U test. Outcomes following immunosuppression withdrawal or DLI in the setting of low mixed chimerism at day 100 are presented descriptively due to the small size of this subgroup. The impact of primary malignancy category (lymphoid versus myeloid) was pre-specified as a key variable for interrogation of outcomes following low mixed T-cell chimerism. This study was approved by the Melbourne Health institutional human research ethics committee. All participants had previously provided informed consent for collection of transplant-related data for research purposes.

7.2.2 Results

There were one-hundred and ninety-seven patients who received alloSCT in the analysis period with available day 100 T-cell chimerism results, of which 9 patients were excluded due to relapse prior to day 100, leaving 188 patients in the final analysis (Table 7.1). Overall, myeloid neoplasms comprised 48% of primary indications for transplant; the remainder were lymphoid neoplasms. The distribution of DRI was similar between patients with myeloid (high or very high DRI: 21%) and lymphoid neoplasms (high or very high DRI: 15%). FluMel was the most common conditioning regimen used (60%). A greater proportion of patients with myeloid neoplasms received FluMel (74%) compared with 46% transplanted for lymphoid neoplasms.

Table 7.1 Patient characteristics

Characteristic	Value
Recipient age, median (range)	54 (19-69)
Recipient sex: male/female	103/75
Haematological malignancy: n (%)	
AML	69 (37)
MDS	12 (6)
MPN	8 (4)
CML	2 (1)
CLL	17 (9)
NHL	42 (22)
MM	22 (12)
HL	13 (7)
ALL	3 (2)
DRI: n (%)	
low/intermediate	154 (82)
High/very high	34 (18)
Donor relation: n (%)	
Matched sibling	133 (71)
Matched unrelated	55 (29)
T-cell depletion: n (%)	48 (26)
Conditioning regimen: n (%)	
Fludarabine/melphalan	112 (60)
Fludarabine/cyclophosphamide (high dose)	46 (24)
Fludarabine/cyclophosphamide (low dose)	30 (16)
Graft type: n (%)	
PB	176 (94)
BM	12 (6)

MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; CML, chronic myeloid leukaemia; CLL, chronic lymphocytic leukaemia; NHL, non-Hodgkin lymphoma; MM, myeloma; HL, Hodgkin lymphoma; ALL, acute lymphoblastic leukaemia; DRI, disease risk index; PB, peripheral blood; BM, bone marrow.

Figure 7.1 Day 100 T-cell chimerism by conditioning regimen

*** denotes $P<0.0001$; n.s, not significant

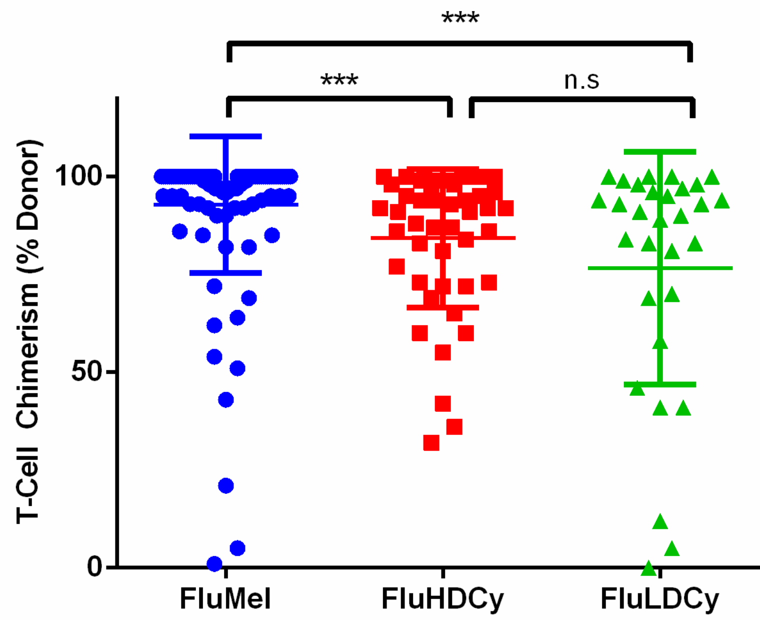


Table 7.2 T-cell chimerism by conditioning regimen and T cell depletion

	Day 100 T-cell chimerism, median (IQR)		
	FluMel	**FluHDCy**	**FluLDCy**
T-cell replete	100 (95-100)	91 (72.5-97)	93 (70-98)
T-cell depletion	100 (95.3-100)	92 (77-100)	83 (5-95)

Variables impacting on day 100 T-cell chimerism

Conditioning regimen

The median T-cell chimerism at day 100 post-alloSCT was 97% (interquartile range [IQR] 87-100%). Thirty-one (16%) patients had low mixed T-cell chimerism, and 6 patients (3%) had very low mixed chimerism. There were significant differences in chimerism according to conditioning regimen used. Patients who received FluMel had statistically significant greater day 100 chimerism (median 100%, IQR 95-100%) compared with FluHDCy (91.5%, IQR 74-98%; $P<0.0001$) and FluLDCy (90.5%, IQR 69.3-96.8%; $P<0.0001$) (Figure 7.1). There was no significant difference between patients who received FluHDCy and FluLDCy.

T-cell depletion

Among patients who received FluMel or FluHDCy , there was no significant difference in chimerism among patients who received T-cell depletion and those who did not (Table 7.2). For the lowest intensity FluLDCy group, the median chimerism at day 100 was numerically lower in patients who received T-cell depletion compared to those who did not (83% vs 93%), and there was a significantly greater proportion of patients with very low chimerism (defined as <25%) at day 100 in patients who received T-cell depletion (43% vs 0%; $P<0.01$).

Impact of T-cell Chimerism on Transplant Outcomes

Graft loss

In the FluLDCy group, two patients (7%) with myeloma developed secondary graft loss with T-cell chimerism less than 5% at day 100. Both had T-cell depletion and had undergone a previous autograft a considerable time previously (211 days and 2382 days

respectively). No patient developed secondary graft loss in the 23 patients who received FluLDCy without T-cell depletion.

Relapse

The association between T-cell chimerism and relapse, NRM, RFS, OS and incidence of chronic GVHD was assessed in univariate (Table 7.3) and multivariable models. Increasing chimerism was the only independent variable significantly associated with reduced relapse of the primary haematological malignancy (HR 0.98, 95% CI 0.97-1.00; *P*=0.01). Conditioning regimen, T-cell depletion, disease histology (myeloid versus lymphoid) and DRI were not significantly associated. Despite this, DRI and conditioning regimen were included in a multivariable model together with T-cell chimerism due to previously being demonstrated in other cohorts to be associated with post-transplant relapse[134]. In this model, T-cell chimerism was the only variable that remained independently associated with relapse (HR 0.98, 95% CI 0.97-1.00; *P*=0.03).

Table 7.3 Univariate associations with transplant outcomes

DRI, disease risk index; FluCy, fludarabine/cyclophosphamide; FluMel, fludarabine/melphalan; PB, peripheral blood; BM, bone marrow; NRM, non-relapse mortality; OS, overall survival; RFS, relapse free survival; GVHD, graft versus host disease.

Variable	**Relapse**			**NRM**			**OS**			**RFS**			**Chronic GVHD**		
	HR	**95%CI**	***P***	**HR**	**95%CI**	***P***	**HR**	**95%CI**	***P***	**HR**	**95%CI**	***P***	**HR**	**95%CI**	***P***
Age	1.00	0.97-1.02	0.88	1.03	0.98-1.09	0.26	1.02	0.99-1.05	0.18	1.01	0.98-1.03	0.60	0.99	0.98-1.01	0.49
Sex (F)	0.83	0.46-1.51	0.54	1.13	0.43-2.95	0.80	0.88	0.48-1.61	0.58	0.84	0.50-1.41	0.51	0.77	0.53-1.12	0.17
DRI															
Low/Int	Ref			Ref			Ref			Ref			Ref		
High/Very High	1.49	0.75-2.98	0.26	2.67	1.02-7.04	**0.05**	1.61	0.81-3.20	0.18	2.00	1.12-3.55	**0.02**	0.97	0.59-1.60	0.89
Disease															
Lymphoid	Ref			Ref			Ref			Ref			Ref		
Myeloid	0.82	0.46-1.49	0.52	1.07	0.42-2.69	0.89	1.29	0.72-2.31	0.4	0.87	0.52-1.46	0.60	0.72	0.50-1.05	0.09

Donor															
Sibling	Ref			Ref			Ref			Ref			Ref		
Unrelated	1.25	0.64-2.45	0.51	3.24	1.25-8.39	**0.02**	2.85	1.51-5.37	**0.001**	1.86	1.07-3.23	**0.03**	0.33	0.20-0.55	**<0.001**
T-Cell Depletion	1.41	0.71-2.81	0.32	1.9	0.68-5.29	0.22	2.53	0.13-5.09	**0.009**	1.69	0.93-3.07	0.09	0.33	0.19-0.57	**<0.001**
Conditioning															
FluCy	Ref			Ref			Ref			Ref			Ref		
FluMel	0.80	0.44-1.44	0.45	2.35	0.77-7.19	0.13	1.67	0.69-4.02	0.25	1.01	0.50-2.03	0.97	1.52	1.03-2.23	**0.03**
Graft															
PB	Ref			Ref			Ref			Ref			Ref		
BM	0.68	0.18-2.59	0.57	2.41	0.55-10.6	0.24	0.41	0.43-4.60	0.57	1.18	0.43-3.27	0.75	1.7	0.23-1.47	0.26
T-Cell Chimerism	0.98	0.97-1.00	**0.01**	1.07	1.01-1.13	**0.03**	1.00	0.98-1.02	0.94	0.99	0.98-1.00	0.13	1.03	1.02-1.04	**<0.001**

Non-relapse mortality

Increasing T-cell chimerism was also associated with increased NRM after day 100 post-transplant (HR 1.07, 95% CI 1.01-1.13; *P*=0.03) on univariate analysis, as were transplantation from an unrelated donor (HR 3.24, 95% CI 1.25-8.39; *P*=0.02) and DRI (HR 2.67, 95% CI 1.02-7.04; *P*=0.047). On multivariable analysis taking into account donor, DRI, patient age and conditioning regimen, chimerism remained independently associated with NRM (HR 1.05, 95% CI 1.01-1.10; *P*=0.03).

Chronic GVHD

The increase in NRM seen with increasing chimerism was accompanied by a significant association between increasing chimerism and the incidence of chronic GVHD (HR 1.03, 95% CI 1.02-1.04; *P*<0.001). Transplantation from an unrelated donor (HR 0.33, 95% CI 0.20-0.55; *P*<0.0001) was associated with reduced chronic GVHD due to an interaction with the exclusive use of T-cell depletion in unrelated donor transplants. FluMel conditioning was also associated with increased chronic GVHD (HR 1.52, 95% CI 1.03-2.23; *P*=0.03). On multivariable analysis, chimerism remained independently associated with chronic GVHD (HR 1.03, 95% CI 1.01-1.04; *P*<0.001), as did transplantation from an unrelated donor (HR 0.34, 95% CI 0.2-0.57; *P*<0.001).

Survival

There was no significant impact of the degree of T-cell chimerism at day 100 with overall survival or relapse free survival, likely secondary to the competing outcomes of reduced relapse and increased NRM.

Impact of interventions on outcomes following low mixed T-cell chimerism at day 100

I investigated relapse outcomes following low mixed T-cell chimerism at day 100, and whether this was impacted by the nature of the underlying haematological malignancy. My hypothesis was that patients with myeloid neoplasms, of which the predominant subgroup was AML, would require a greater degree of donor T-cell engraftment to facilitate a greater graft-versus-tumour effect to maintain remission, as compared with lymphoid neoplasms for which a degree of low mixed chimerism may be sufficient for disease control. Twelve patients (13%) transplanted for myeloid malignancies had low mixed chimerism at day 100. One patient underwent a second alloSCT and was excluded from further analysis. The remaining 11 patients underwent immunosuppression withdrawal and one patient received additional DLI (Figure 7.2). Of these, 7 (64%) had a subsequent improvement in chimerism to >75% at a median of 94 days following their day 100 result (IQR 62-220 days). There was a significant difference in median day 100 chimerism between this group and patients who did not increase chimerism to >75% (69% vs 31%; P=0.006). Five of the 7 patients have remained in long-term remission, while 2 relapsed 876 days and 1348 days later. Two of 4 patients with persistent low mixed chimerism have remained in long-term remission; one was transplanted for AML with intermediate risk cytogenetics in CR1 and one for chronic myeloid leukaemia in accelerated phase at presentation. Both of these patients had low or intermediate DRI. Of the 11 patients with low mixed chimerism at day 100 who received immunomodulation therapy, 3 (27%) patients developed chronic GVHD, all of whom had an increase in chimerism to >75% (Figure 7.2B). Two patients who developed severe chronic GVHD following immunomodulation have remained free of disease relapse; one patient with mild chronic GVHD relapsed despite achieving a subsequent donor chimerism result of 97%.

Nineteen patients (20%) transplanted for lymphoid malignancies had low mixed T-cell chimerism at day 100 post-alloSCT (Figure 7.3A). All received immunosuppression withdrawal and 5 received additional DLI. Eleven (58%) patients achieved a subsequent chimerism result >75% following immunomodulation therapy, of whom 8 patients have remained in long-term remission and 3 patients have relapsed. Eight (42%) patients had persistent low mixed T-cell chimerism despite immunomodulation; of these, 3 relapsed and 5 remain in remission after a median of 29 months of follow-up. Of these 5 patients, two patients were transplanted for CLL, two for follicular lymphoma, and one for mantle cell lymphoma in first complete remission. All of these patients had either low or intermediate DRI, and had received either FluLDCy or FluHDCy as conditioning,

As in the myeloid cohort, lymphoid malignancy patients who increased their chimerism to greater than 75% following immunomodulation already had significantly greater baseline chimerism at day 100 compared with those who did not subsequently increase (median 65% vs 34%; $P<0.001$). Chronic GVHD developed in 7 of the 19 (37%) patients in this cohort (Figure 7.3B); all cases of GVHD was accompanied by an increase of T-cell chimerism to >75% following immunomodulation. There was no significant difference in the incidence of relapse among patients who developed GVHD post immunomodulation compared with those who did not (29% vs 42%, $P=0.57$).

Figure 7.2 Outcomes following mixed chimerism: myeloid neoplasms

Outcomes according to post-immunomodulation chimerism response (**A**) or development of subsequent GVHD (**B**). (Patient numbers in parentheses)

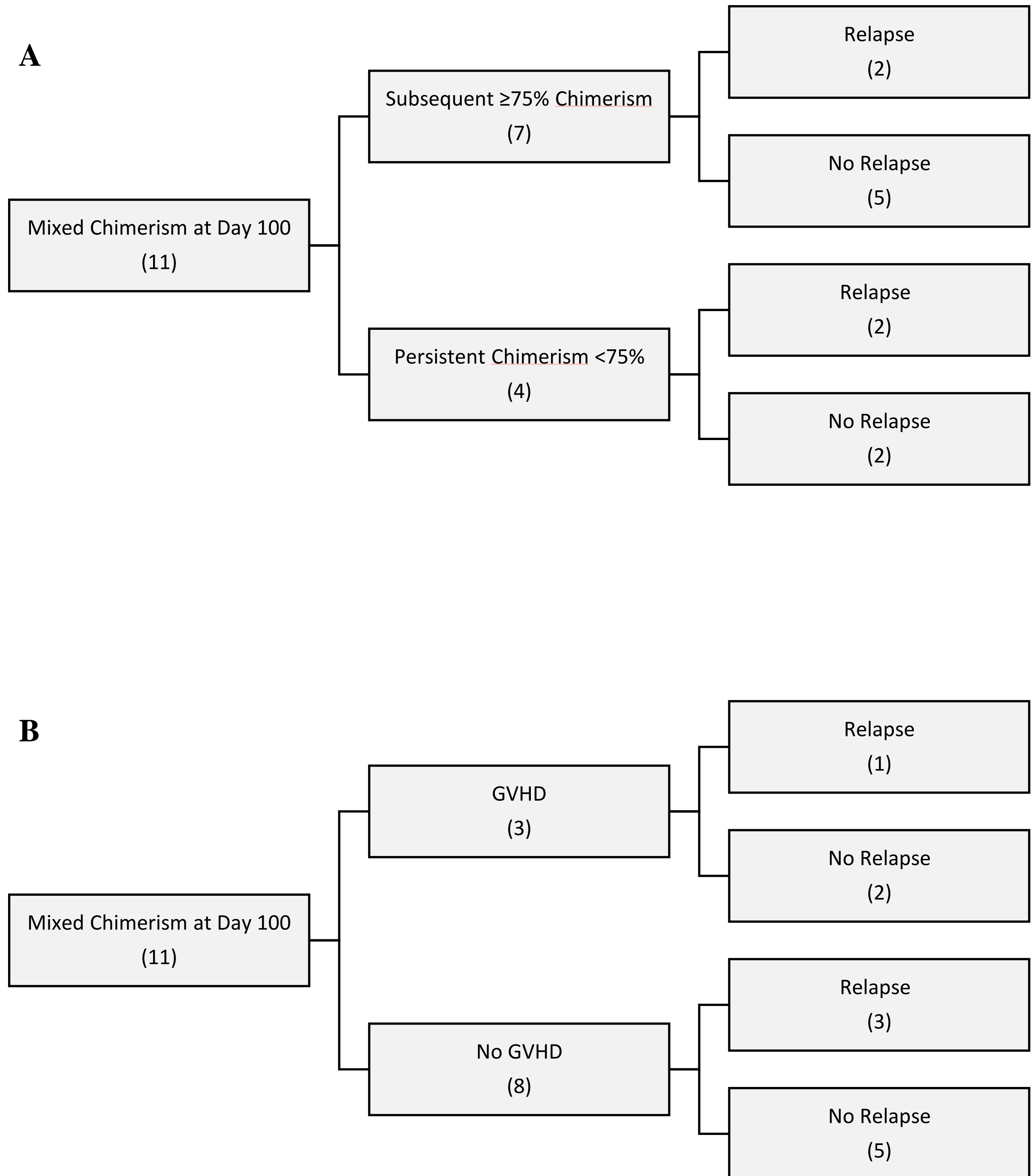
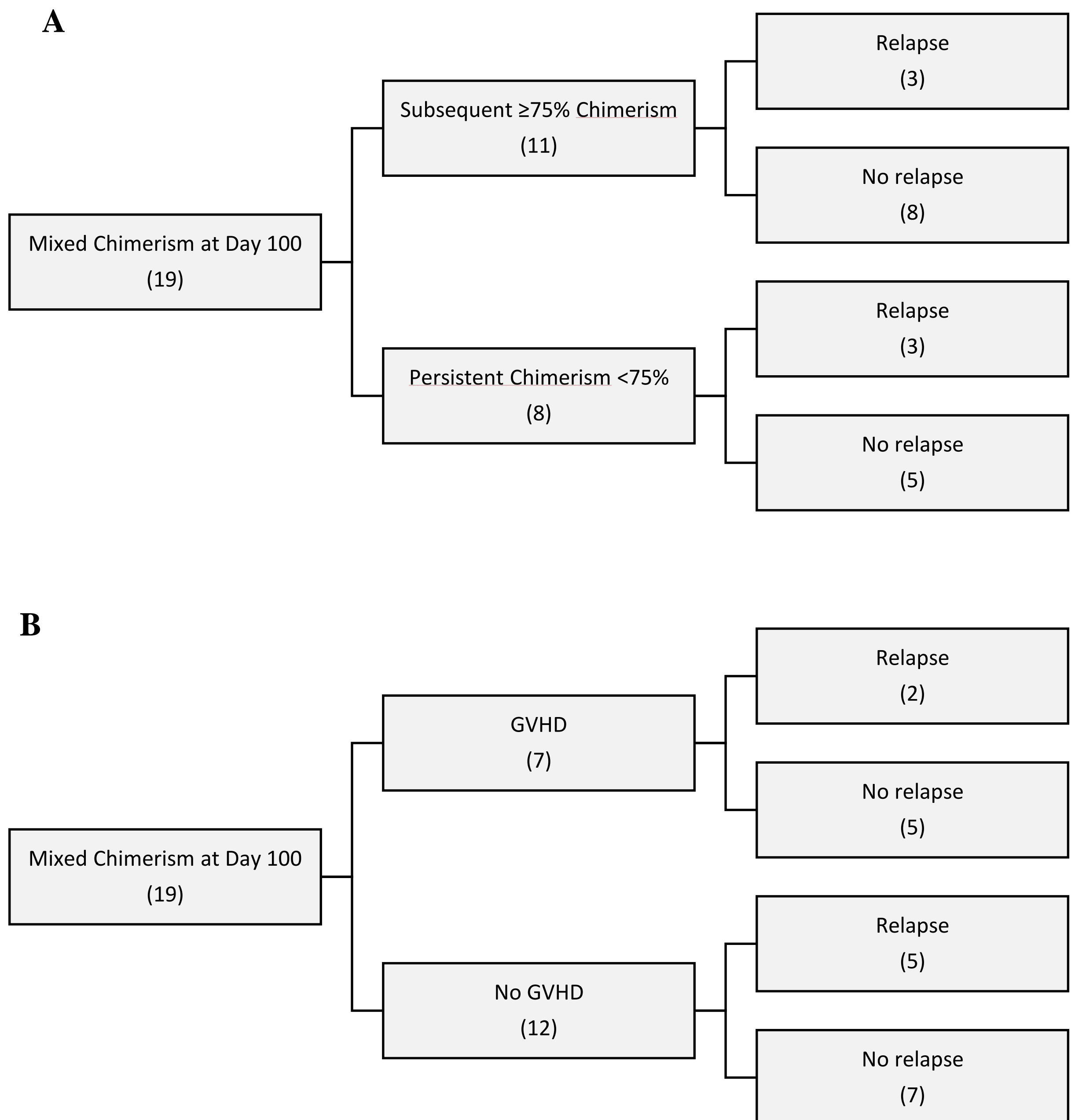


Figure 7.3 Outcomes following mixed chimerism: lymphoid neoplasms

Outcomes according to post-immunomodulation chimerism response (**A**) or development of subsequent GVHD (**B**). (Patient numbers in parentheses)



7.2.3 Discussion

The kinetics and quality of donor T-cell engraftment following RIC alloSCT for haematological malignancies play important roles in post-transplant outcomes including relapse and GVHD. In this cohort I first examined the impact of conditioning regimen intensity and T cell depletion on chimerism. The degree of donor T-cell engraftment at day 100 varied considerably according to conditioning regimen, with higher degrees of donor chimerism seen in the more intensive FluMel conditioning regimen compared with FluHDCy or FluLDCy. This observation is consistent with previous reports which have demonstrated a greater degree of mixed chimerism with less intensive conditioning regimens, and is thought to reflect insufficient host immune suppression resulting in a residual host-versus-graft effect that inhibits robust donor engraftment[135-137].

While there was no significant impact on T-cell chimerism with the use of *in-vivo* T-cell depletion in patients receiving either FluMel or FluHDCy, there was an increase in the incidence of secondary graft loss in patients who received the lowest intensity regimen, FluLDCy, in combination with T-cell depletion. This suggests that in very low intensity conditioning regimens where there is incomplete suppression of recipient immunity, donor T-cell depletion results in an inability of donor haematopoiesis to overcome residual recipient immunity[136].

T-cell chimerism was significantly associated with reduced relapse albeit at the cost of increased NRM secondary to increased chronic GVHD. In these analyses, I analysed chimerism as a continuous variable, and hence demonstrated that there was an incremental and linear increase in risk of relapse and NRM with decreasing donor

chimerism at day 100. These observations are broadly consistent with previous reports in RIC alloSCT[127], [138].

Finally, I examined the impact of immunomodulation by means of rapid immunosuppression withdrawal, and in some cases the use of DLI in patients with low mixed chimerism at day 100. These interventions were able to improve T-cell chimerism to greater than 75% in 60% of patients, although this ability was dependent on the baseline chimerism being at least 50%. A similar observation was noted in a prospective study of prophylactic DLI for mixed chimerism following RIC alloSCT in which improvement in donor chimerism following DLI was exclusively observed in patients with pre-DLI chimerism of at least 40%[139]. In contradistinction, patients with less than 20% donor chimerism universally developed graft loss despite DLI[139].

In this cohort, one third of patients developed GVHD following immunomodulation. The onset of GVHD was universally accompanied by an increase in donor chimerism to >75%. This study was inadequately powered to examine the impact of improvement of chimerism or the development of GVHD with regards to reduced relapse or improvement in survival. In addition, I acknowledge variability in the rate of immunosuppression withdrawal and intensity of DLI which may have impacted upon chimerism outcomes following immunomodulation. Nevertheless, I observed a small proportion of patients able to maintain long-term disease remission despite persistent low mixed chimerism. Notably, all of these patients had low or intermediate DRI, suggesting that the aggressiveness of the underlying malignancy is an important factor in determining the likelihood of long-term remission despite a state of low mixed T-cell chimerism.

In conclusion, this retrospective study demonstrates that T-cell chimerism following RIC alloSCT is dependent on conditioning intensity and that the combined use of T-cell depletion with very low intensity conditioning regimens is associated with a greater risk of secondary graft loss. There is limited efficacy of immunomodulation in improving mixed T-cell chimerism values less than 50%. Despite the progressive increase in relapse with decreasing day 100 chimerism, a subgroup of patients with low or intermediate DRI can maintain long-term remission despite a state of low mixed T-cell chimerism.

7.3 Part 2. The prognostic limitations of donor T-cell chimerism after myeloablative allogeneic stem cell transplantation for acute myeloid leukaemia and myelodysplastic syndromes

7.3.1 Patients and Methods

Adult patients (≥18 years) who underwent myeloablative conditioning followed by peripheral blood or bone marrow alloSCT from a matched sibling or unrelated donor for AML or MDS at the Royal Melbourne Hospital (RMH) between 2000 and 2016 were included in this retrospective analysis. Patients who relapsed or died before day 100 post-alloSCT were excluded. This study was approved by the RMH institutional human research ethics committee. The myeloablative conditioning regimens utilized over this period included cyclophosphamide 120mg/kg in combination with either intravenous busulphan 12.8mg/kg (BuCy) or total body irradiation (TBI) 12Gy (CyTBI). The choice of conditioning regimen was not protocolized and was according to physician discretion. Graft versus host disease (GVHD) prophylaxis consisted of cyclosporin 3mg/kg i.v commencing day -1, with transition to an equivalent oral dose upon recommencement of oral intake, and short course methotrexate 15mg/m^2 day +1 followed by 10mg/m^2 days 3, 6, 11. AlloSCT recipients from sibling donors also received prednisolone 0.5mg/kg/d days 14-34 decreasing to 0.25mg/kg/d days 35-48, while transplants from unrelated donors received T-cell depletion with antithymocyte globulin 4mg/kg pre-alloSCT in divided doses (Thymoglobulin, Genzyme Corporation, Cambridge, MA). Cyclosporin taper commenced from day +100 or earlier depending on physician discretion due to perceived risk of relapse or toxicity.

Chimerism analysis

Donor/recipient chimerism analysis was performed routinely on day 100 post-alloSCT on PB samples collected in ethylenediaminetetraacetic acid. Recipient samples were separated into CD3-positive (T-cell) and CD3-negative (non-T cell) fractions using immunomagnetic cell separation with density gradient centrifugation (RosetteSep, StemCell Technologies, VIC). Polymerase chain reaction (PCR) was performed using oligonucleotide primers specific for short tandem repeats which were identified pre-transplant to be able to distinguish donor from recipient DNA (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA). Products were analyzed using an Applied Biosystems 3730 DNA Sequencer, and chimerism results were expressed as the percentage of donor-specific DNA present.

Endpoints and statistical analysis

The primary objective of the study was to evaluate the sensitivity and specificity of day 100 donor T-cell chimerism (D100chim) as a predictor of relapse free survival at 6 months (RFS6) and 12 months (RFS12) post-alloSCT, treated as binary outcomes. Relapse of AML or MDS was defined as >5% blasts or unequivocal morphologic dysplasia not attributable to other causes on bone marrow aspirate and trephine biopsy morphology. Logistic regression was used to investigate the relationships between RFS6 and RFS12, and D100chim, age, sex, disease risk index (DRI), cytogenetics, graft type, donor relation and use T-cell depletion. D100chim was investigated as both a continuous variable and using pre-defined thresholds of 55%, 65%, 75%, 85% and 95%. DRI was separated into two categories of low/intermediate or high/very high, as previously defined[134]. Cytogenetics risk categories were defined according to the refined Medical Research Council (MRC) classification. Variables found to be

statistically significant in univariate analyses were included in a multivariate model. . Significance level was set at $\alpha<0.05$. Statistical analysis was performed using R analysis software (Comprehensive R Archive Network Project).

7.3.2 Results

A total of 103 patients were included in the analysis (Table 7.4). Of the patients with AML, 72 (80%) were in complete remission at the time of alloSCT, while 11 patients (12.2%) underwent transplantation in early morphologic relapse. Twelve patients with MDS (92.3%) had not received any disease-modifying therapy prior to alloSCT, and one patient had received the hypomethylating agent azacitidine without response. Of the 49 patients (54.4%) who received transplants from unrelated donors, 5 patients were mismatched at a single HLA class II loci (DR or DQ), while the remainder were 10/10 matches. 38 (42.2%) patients received glucocorticosteroids for GVHD prophylaxis in addition to cyclosporine and short-course methotrexate. The median duration of follow-up was 48.0 months (interquartile range [IQR] 29.1-76.0 months). Overall survival at 6 and 12 months post-alloSCT were 97.1% (95% CI 91.2-98.5%) and 91.0% (95% CI 83.4-95.2%) respectively. Relapse-free survival at 6 and 12 months were 85.4% (95% CI 77.0-91.0%) and 73.2% (95% CI 63.4-80.8%). The cumulative incidence of relapse, with non-relapse mortality (NRM) as a competing risk at 6 and 12 months post-alloSCT was 13.6% (95% CI 7.8-21.0%) and 23.8% (95% CI 16.0-32.5%) respectively. The cumulative incidence of extensive chronic GVHD at 6 and 12 months was 36.9% (95% CI 27.6-46.2%) and 45.7% (35.8-55.0%) respectively.

Table 7.4 Patient and treatment characteristics

Characteristic	Value
Age (median years, range)	43 (18-60)
Sex	
Male	52
Female	51
Disease	
AML	90
MDS	13
Cytogenetic group	
Favourable	10
Intermediate	72
Adverse	21
Disease Risk Index	
Low/Intermediate	80
High/Very High	23
Stem cell source	
Peripheral blood	80
Bone marrow	23
Donor type	
Sibling	54
Unrelated	49
T-cell depletion	
Yes	45
No	58
Conditioning regimen	
CyTBI	18
BuCy	85

Associations between D100chim and RFS6 and RFS12

The median D100chim was 96% (IQR 89-100%), and complete D100chim (defined as ≥95%) was achieved by 62.2% of patients. Patients who received T-cell depletion paradoxically had a higher median D100chim compared to patients who did not (100% vs 91.5%), although this was confounded by the observation that a greater number of patients who did not receive T-cell depletion had bone marrow grafts (34.5% vs 6.8%). On univariate analysis, covariates significantly associated with RFS6 included D100chim as a continuous variable as well as D100chim as a binary variable dichotomized by pre-specified thresholds of 65%, 75% and 85% (Table 7.5). Disease risk index was also significantly associated with RFS6 (OR 3.94, 95% CI 1.22-12.60, P=0.020). Cytogenetic classification, donor relation, stem cell source or administration of T-cell depleting antibodies were not significantly associated with RFS6. In a multivariate model including DRI and D100chim, DRI (odds ratio [OR] 3.616, 95% CI 1.03-12.58, P=0.04) and D100chim (OR 1.06, 95% CI 1.02-1.11, P=0.006) remained significantly associated with RFS6.

Ninety-seven patients had complete 12 month follow-up and were included in the analysis of associations with RFS12. At 12 months, D100chim as a continuous variable, as well as D100chim at thresholds of 65%, 75%, 85% were significantly associated with RFS12. However, only the continuous variable remained significantly associated with RFS12 (OR 0.95, 95% CI 0.92-0.99, P=0.01) in a multivariable model also including DRI. There was only moderate correlation between D100chim (i.e. CD3-positive T-cell) and CD3-negative chimerism, (Spearman correlation coefficient R= 0.46). There was also no significant association between CD3-negative chimerism and RFS6 or RFS12, suggesting that D100chim was not merely a surrogate for myeloid chimerism.

Table 7.5 Univariate analysis for associations with RFS6 and RFS12

	RFS6		RFS12	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***
Age	0.99 (0.95-1.04)	0.76	1.00 (0.97-1.05)	0.85
DRI				
Low/Intermediate	Ref[1]		Ref	
High/Very high	3.94 (1.22-12.60)	0.02	2.55 (0.91-7.05)	0.07
Cytogenetics				
Favourable	Ref		Ref	
Intermediate	1.62 (0.26-31.45)	0.66	3.20 (0.54-61.34)	0.29
Adverse	1.50 (0.16-32.74)	0.74	2.29 (0.28-48.93)	0.49
Stem cell source				
Peripheral blood	Ref		Ref	
Bone marrow	1.94 (0.55-6.23)	0.27	1.02 (0.33-2.87)	0.97
Donor type				
Sibling	Ref		Ref	
Unrelated	0.50 (0.15-1.53)	0.24	0.79 (0.31-1.96)	0.61
T-cell depletion	0.84 (0.26-2.53)	0.76	1.35 (0.54-3.41)	0.52
D100chim				
Continuous[2]	1.06 (1.02-1.11)	<0.01	1.05 (1.01-1.09)	<0.01
≤65%	10.30 (2.03-58.49)	<0.01	8.75 (1.74-64.41)	0.01
≤75%	6.83 (1.71-27.10)	<0.01	6.64 (1.80-27.66)	<0.01
≤85%	6.13 (1.83-20.65)	<0.01	4.50 (1.50-13.86)	<0.01
≤95%	2.76 (0.90-9.48)	0.09	1.52 (0.61-3.83)	0.37

[1]Reference value; [2]D100chim as a continuous variable decreasing from 100%.

Sensitivity and specificity of D100chim as a predictor of RFS6 and RFS12

I analyzed the performance of D100chim as a predictor of RFS6 and RFS12 by generating receiver-operating characteristic (ROC) curves and calculating the sensitivity and specificity of D100chim at different pre-determined threshold values. The area under the ROC curve (AUC) for D100chim as a predictor of RFS6 was 0.68 (Figure 7.4). Using a D100chim threshold of 65%, the specificity for RFS6 was 96.6%, however sensitivity was poor at 26.7%. In this study cohort, this equated to a negative predictive value (NPV) of 88.5% and positive predictive value (PPV) of 57.1%. Changing the threshold for D100chim to 75% or 85% modestly improved the sensitivity of D100chim for RFS6; however this was at the expense of specificity (Table 7.6). In this cohort, PPV decreased when the D100chim was increased from 65% to 75% or 85%. The AUC for D100chim as a predictor of RFS12 was 0.62, which was lower than that observed for RFS6. In keeping with this, D100chim at thresholds of 65%, 75% and 85% demonstrated poorer sensitivity for RFS12 compared to RFS6.

Given that DRI and D100chim were independently associated with RFS6 in the multivariable model, I investigated the utility of combining both variables for the prediction of RFS6. The AUC of the multivariable regression model was 0.77, demonstrating an improved ability to predict RFS6 compared to D100chim alone. Accordingly, I devised a scoring algorithm where high/very high DRI scored 1 point, and D100chim less than 65% scored 1 point. The specificity of a total score of 1 or greater for RFS6 was 79.5% and sensitivity 66.7%. Using an alternative D100chim threshold of 75% improved sensitivity at the expense of specificity. There was no further benefit in sensitivity or specificity of using a D100chim threshold of 85%.

Figure 7.4 ROC curve of D100chim as a predictor of RFS6

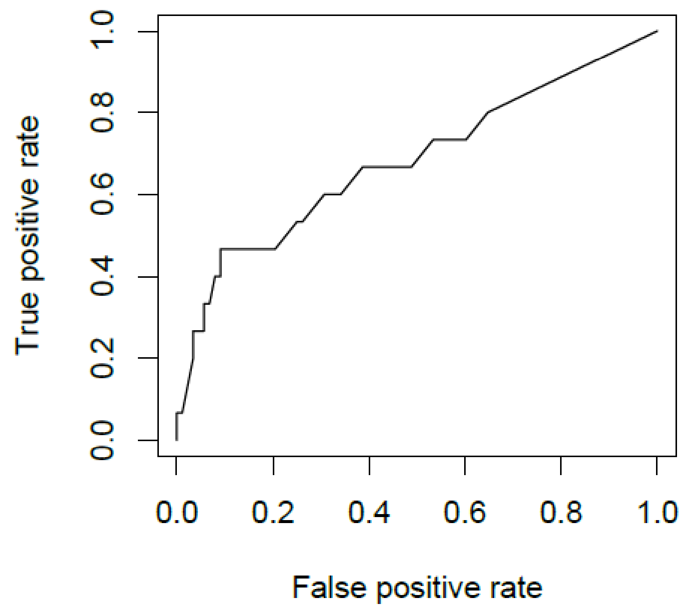


Table 7.6 Sensitivity, specificity, PPV and NPV of D100chim for RFS6 and RFS12

RFS6	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
D100chim ≤65%	26.7	96.5	57.1	88.5
≤75%	33.3	93.2	45.5	89.1
≤85%	46.7	87.5	38.9	90.6
DRI + D100chim				
≤65%	66.7	79.5	35.7	93.3
≤75%	73.3	78.4	36.7	94.5
≤85%	73.3	73.9	32.4	94.2
RFS12				
D100chim ≤65%	20.0	97.5	71.4	77.8
≤75%	28.0	94.4	63.6	79.1
≤85%	36.0	88.9	52.9	80.0
DRI + D100chim				
≤65%	52.0	80.6	48.1	82.9
≤75%	60.0	51.7	51.7	85.3
≤85%	60.0	76.4	46.9	84.6

Kinetics of T-cell chimerism up to day 100

T-cell engraftment following alloSCT is a dynamic process that may be reflected in the change in T-cell chimerism over time, particularly in the first few months following donor cell infusion. Of the 103 patients in the day 100 chimerism cohort, there were 80 patients in whom T-cell chimerism was examined between day 30 to day 60 post-alloSCT in addition to D100chim. The characteristics of this subset of patients did not differ significantly from the original cohort with respect to age, sex, disease type, disease risk index, donor relation or use of T-cell depletion. The median T-cell chimerism between days 30-60 was 93.5% (IQR 85-98.3%). In 4 patients (5%), T-cell chimerism increased by 10% or greater from day 30-60 to day 100 post-alloSCT, and in another 4 patients T-cell chimerism decreased by 10% or greater over the same time period. There was an association between patients who had a falling chimerism (by 10% or greater) and RFS6 (OR 18.8, 95% CI 2.2-400.1, $P = 0.01$). The sensitivity of a decrease in T-cell chimerism by 10% or greater for RFS6 was 21.4% and specificity was 98.5%. The PPV of this dynamic measure of T-cell chimerism was 75%; however this was hampered by a high rate of false negative predictions (78.6%).

7.3.3 Discussion

The ability to accurately predict early relapse after alloSCT is an area of significant clinical need, given the poor prognosis of established relapse, and the potential for pre-emptive immunological manipulation to mitigate relapse risk. Donor T-cell chimerism is particularly attractive as a candidate biomarker given its ability to be performed on peripheral blood samples and relatively widespread availability to the extent that it is routinely performed post-alloSCT. In addition, from a mechanistic point of view donor T-cell chimerism reflects the degree of donor T-cell engraftment and by extension may

be used to infer the availability of donor T-cells to exert a graft-versus-leukemia effect. Several groups have described an association between donor T-cell chimerism and relapse risk post-alloSCT, however the interpretation of these reports have been hampered by patient and disease heterogeneity[127, 138]. In the largest homogeneous cohort of AML/MDS patients reported to date with respect to post-transplant T-cell chimerism, Lee and co-workers reported an association between day 100 T-cell chimerism and relapse following myeloablative alloSCT[126]. In patients in CR1 or CR2 at the time of alloSCT, donor T-cell chimerism ≤85% was significantly and independently associated with the cumulative incidence of relapse at 3 years post-alloSCT, with hazard ratio 2.4 ($p=0.02$). In this study, I likewise demonstrate that D100chim is significantly associated with RFS6 and RFS12, both as a continuous variable as well as using thresholds of between 65% and 85%.

However, the utility of D100chim as a predictive biomarker of relapse post-alloSCT is inadequately characterized by measures of association such as odds ratios in logistic regression models. For a biomarker to accurately predict prognosis, the distributions of the biomarker in patients that have disease/relapse or not must be sufficiently separated to allow dichotomous classification[121]. This frequently, although indirectly, corresponds to a strength of association rarely observed in logistic regression models[140]. Therein lies the considerable difference between statistical methodology for investigation of classification rather than association. Thus, rather than measures of association, the performance of a diagnostic or predictive tool is best described by the AUC of a receiver-operating-characteristic curve, or when using a binary classification tool, the sensitivity and specificity. I demonstrate that the AUC of D100chim as a predictor of RFS6 was 0.68, where only values of greater than 0.7 would be considered a reasonably

predictive tool. When using D100chim as a binary classification tool using a threshold of 65%, this demonstrated high specificity (96.6%) for RFS6 but poor sensitivity (26.7%). Increasing the D100chim threshold to 75% or 85% only modestly improved sensitivity, at the expense of specificity. In this patient cohort, the high specificity but low sensitivity of D100chim corresponded to a high NPV but poor PPV. In practical terms, 43% of patients with D100chim of less than 65% will be incorrectly classified as being at risk of relapse (false positive predictions). This suggests that patients with a low D100chim may not universally require potent immunomodulatory therapy, such as donor lymphocyte infusions, which are associated with a high rate of GVHD toxicity.

In clinical practice, physicians often use a combination of biomarkers which increase the power of prediction compared to one marker alone. Disease risk index has been validated in several cohorts as being independently associated with OS and RFS after alloSCT[134, 141]. When I combined DRI and D100chim, sensitivity was improved however in this cohort it did not translate to an improved PPV, due to the presence of an increased number of false positive predictions.

The strength of this analysis is the use of a homogenous patient cohort of AML/MDS and myeloablative conditioning. In addition, this analysis of the sensitivity and specificity of D100chim is unique. This was facilitated by the selection of primary outcomes as dichotomous outcome variables. Furthermore, the choice of RFS6 and RFS12 as the primary endpoints of the analysis recognized that the prognostic impact of D100chim is likely to be time-dependent; that is, the predictive ability of D100chim for relapse is likely to decrease over time. This was confirmed by the observation that sensitivity of D100chim for predicting RFS6 was greater than RFS12. I hypothesize that

extending the duration of minimum follow-up (e.g. RFS24 or RFS36) would reveal a similar pattern. This study had a relatively modest sample size which likely contributed to the lack of association observed between cytogenetic risk group and RFS, however I doubt that the analysis of D100chim would be significantly altered in a larger cohort as I was able to replicate the association between D100chim and RFS as previously reported in the literature. This cohort also demonstrated some heterogeneity with regards to disease risk and donor source, however it is unlikely that these factors would have significantly impacted on the results.

Biomarkers predictive of relapse post-alloSCT are desperately required to identify patients who may benefit from post-transplant immunological strategies to prevent relapse. Other than donor T-cell chimerism, donor/recipient chimerism in CD34-expressing cells (CD34 chimerism) and minimal residual disease (MRD) are other candidate biomarkers that have being described as being associated with relapse in patients with AML and other haematological malignancies[142-144]. CD34 chimerism has been reported to a highly sensitive predictor of relapse of AML post-alloSCT (sensitivity > 80%) and is likely to be more sensitive than T-cell chimerism[145]. For the moment, I encourage caution when using D100chim as a predictor of early relapse and mortality after myeloablative alloSCT in AML and MDS, as it demonstrates a high specificity but lacks sensitivity which in this cohort corresponded to a poor PPV and a high rate of false positive predictions. Clinicians should thus tread carefully when using D100chim to guide post-transplant immunological therapy such as donor lymphocyte infusions which are associated with substantial rates of GVHD.

8 Clinical determinants of T-cell receptor diversity after allogeneic haematopoietic stem cell transplantation

8.1 Introduction

T-cell reconstitution after alloSCT is critical for protection against infection and to mediate the GVT effect against haematological malignancies including AML[146]. The immediate peri-transplant period is characterised by profound immunodeficiency as a consequence of pre-transplant conditioning therapy with gradual reconstitution of T-cell immunity over time in a dynamic non-linear fashion. In addition to time post-transplant, T-cell reconstitution post-alloSCT is also significantly impacted by endogenous factors including donor and recipient age reflecting the potential for thymic recovery post-transplant and exogenous factors including T-cell depleting strategies, immunosuppressive medications and the prohibitive effects of GVHD and infection[27, 30, 31].

Effective T-cell immunity is also contingent on a diverse T-cell receptor (TCR) repertoire able to recognise antigens specific to infectious pathogens and leukaemia cells. The TCR is comprised of α and β subunits in 95% of T-cells, encoded by *TRA* and *TRB* loci respectively; somatic recombination of variable (V) and joining (J) gene segments in the *TRA* locus and V, J and diversity (D) gene segments in *TRB* during T-cell development generates a vast array of different gene sequences translating to a multitude of different TCRs. The early T-cell repertoire post-alloSCT is oligoclonal, consistent with peripheral expansion of a fixed pool of donor-derived memory T-cells[147]. Gradual recovery of the naïve T-cell pool facilitates recovery of TCR diversity which may begin to approach that of healthy persons by 12 months after T-cell replete

alloSCT[148, 149]. However, clinical events such as infection and GVHD may be expected to adversely impact recovery of the TCR repertoire. Furthermore, the impact of an oligoclonal TCR repertoire on the potency of the T-cell mediated GVL and leukaemia control remains unclear. Previous work has suggested that TCR restriction may increase the risk of AML relapse post-alloSCT in a GVHD-dependent manner, however this analysis did not control for important pre-transplant characteristics including use of T-cell depletion and donor or recipient age[150]. The objectives of this study were to interrogate the patterns of TCR repertoire reconstitution post-alloSCT, to investigate clinical determinants of TCR diversity at day 100 post-alloSCT and to investigate the impact of TCR diversity on risk of early AML relapse after alloSCT.

8.2 Methods

8.2.1 Patient Selection

To minimise the impact of confounding transplant-related factors on any relationship between TCR diversity and AML relapse, a retrospective matched cohort design was adopted whereby patients who relapsed between day 60 and day 180 post-transplant were matched using a propensity score matching algorithm to patients who did not relapse post-transplant. Variables identified as potential confounders and hence included in the matching algorithm included recipient age, donor type, donor age, use of *in-vivo* T-cell depletion as part of GVHD prophylaxis, conditioning regimen intensity, AML cytogenetics and time from transplant to peripheral blood sampling for TCR repertoire analysis. Twenty-nine patients who underwent matched sibling or unrelated donor alloSCT at Royal Melbourne Hospital were included in this cohort comprising 16 patients with AML relapse at day 100 to 180 post-alloSCT and 13 control patients who did not relapse post-alloSCT. All patients were required to have T-cell chimerism of at

least 50% at day 100 in order to ensure that TCR repertoire was predominantly of donor origin. Patient and treatment characteristics are summarised in Table 8.1. All patients received unmanipulated peripheral blood or bone marrow stem cells. *In-vivo* T-cell depletion with anti-thymocyte globulin was administered to all patients receiving unrelated donor stem cells as per institutional practice. Graft-versus host disease (GVHD) prophylaxis consisted of cyclosporin commencing day -1 and short course methotrexate. Surveillance for cytomegalovirus (CMV) viremia in peripheral blood (plasma) was monitored twice weekly using polymerase chain reaction (PCR) and pre-emptive therapy with intravenous ganciclovir or oral valganciclovir was commenced in patients with plasma viral load of 400 copies/mL or greater. Acute (aGVHD) and chronic GVHD (cGVHD) were diagnosed clinically with confirmation by histology where feasible and staging was according to established criteria[151, 152]. The study protocol was reviewed and approved by the institutional human research ethics committee.

Table 8.1 Patient characteristics

Characteristic	Value
Recipient age median, years (range)	45 (20-62)
Cytogenetic risk	
Intermediate	19
Adverse	10
Conditioning intensity	
Myeloablative	18
Reduced intensity	11
Conditioning regimen	
Busulfan/Cyclophosphamide	14
Fludarabine/Melphalan	8
Fludarabine/Cyclophosphamide	3
Other	4
Stem cell source	
Peripheral blood	28
Bone marrow	1
Donor	
Sibling	20
Unrelated	9
Donor age median, years (range)	42 (15-69)
Anti-thymocyte globulin	9
CMV recipient/donor serostatus	
Negative/Negative	8
Negative/Positive	2
Positive/Negative	9
Positive/Positive	10
T-cell chimerism, median at day 100 (range)	89 (59-100)

8.2.2 Peripheral blood samples and TCRβ sequencing

Archival DNA from peripheral blood CD3+ cells remaining post clinical testing from all patients as close to day 100 post-alloSCT as possible were retrieved from the institutional biobank (hereafter referred to as 'early' time point). Eleven patients had additional DNA samples available between 1 and 2 years post-alloSCT for evaluation of the change in TCR diversity over time ('late' time point). At the time of collection, peripheral blood was collected in EDTA and CD3+ T-cells were isolated using immunomagnetic cell separation with density gradient centrifugation (RosetteSep; StemCell Technologies, Vancouver, CA). DNA was isolated using the QIAamp DSP Mini Kit and purified using the MagAttract DNA M48 automated platform (Qiagen, Hilden, Germany).

TCRβ sequencing was performed using LymphoTrack TRB (Invivoscribe, San Diego, CA) as per manufacturer's instructions. Sequence assembly from FASTQs, annotation and error correction was performed by MiXCR[153]. Secondary analysis including diversity assessment, VDJ family usage analysis, repertoire overlap analysis and dynamics of clonotype frequencies over time were all performed by VDJtools (version 1.1.9), an open-source analysis tool designed for immune repertoire sequencing[154]. Repertoire cluster analysis was performed also within the VDJtools analysis software which is based on the hierarchical clustering method using Euclidean distance and the hclust function in R. Identification of VDJ sequence specificities was performed by comparison with a curated database of TCR sequences with known specificities with a focus on those specific for infectious pathogens (VDJdb)[155].

8.2.3 Statistical Analysis

TCR diversity was quantified using the inverse Simpson's diversity index ($1/D_S$) as previously described. Briefly, the range of potential values of $1/D_S$ ranges from 1 to ∞, whereby a higher index represents a more diverse TCR repertoire. $1/D_S$ values are expressed as median values with interquartile ranges (IQR). The degree of overlap in TCR clonotype frequency distributions between paired patient samples were quantified using the Jensen-Shannon Divergence (JSD), which has been described in detail previously and used for TCR analyses[156, 157]. The Mann-Whitney U test for non-parametric variables was used for comparisons between unpaired groups; paired comparisons were performed using the Wilcoxon matched-pairs signed rank test. Overall survival was calculated using the Kaplan-Meier estimate, with the log-rank test used for comparisons between groups. The cumulative incidence of cGVHD was determined taking into account competing risks of relapse and death prior to cGVHD. $P<0.05$ was considered statistically significant.

8.3 Results

TCRβ sequencing of the entire cohort of 29 patients was performed with a mean of 454516 sequence reads per patient. Median time from transplant for the early post-alloSCT timepoint was 99 days (IQR 91-102). Median TCR repertoire diversity ($1/D_S$) early post-transplant was 104.3 (IQR 46.5-398.4). Analysis of V-J recombination patterns in individual patients identified broad usage of V and J genes (Figure 8.1). Twenty-seven (93%) patients had at least one dominant clonotype (defined as a frequency of greater than 1% of all clones) and seventeen patients (59%) had at least one highly dominant clonotype (clonal frequency greater than 5%). There was significant heterogeneity in TCRβ repertoire composition among patients with at least

one shared HLA class I allele. There were 3 recipients who received 10/10 HLA-matched alloSCT from donors with one HLA-A*02:01 allele (sample identifiers UPN12, UPN11, UPN14); out of a total clonotype count ranging between 4145 to 16520 clones per patient, between 0.47% and 1.86% of TCRβ clonotypes were shared by all patients (Figure 8.2).

Figure 8.1 TCR repertoire post-alloSCT

TCR V and J segment usage (**A, B**) in 2 representative patients with each arc representing different V and J segments and the thickness of the connecting ribbon corresponding to clonotype frequency. The accompanying spectratype of complementarity determining region 3 (CDR3) lengths (**C, D**) with highlights of the top 10 clone sequences by frequency.

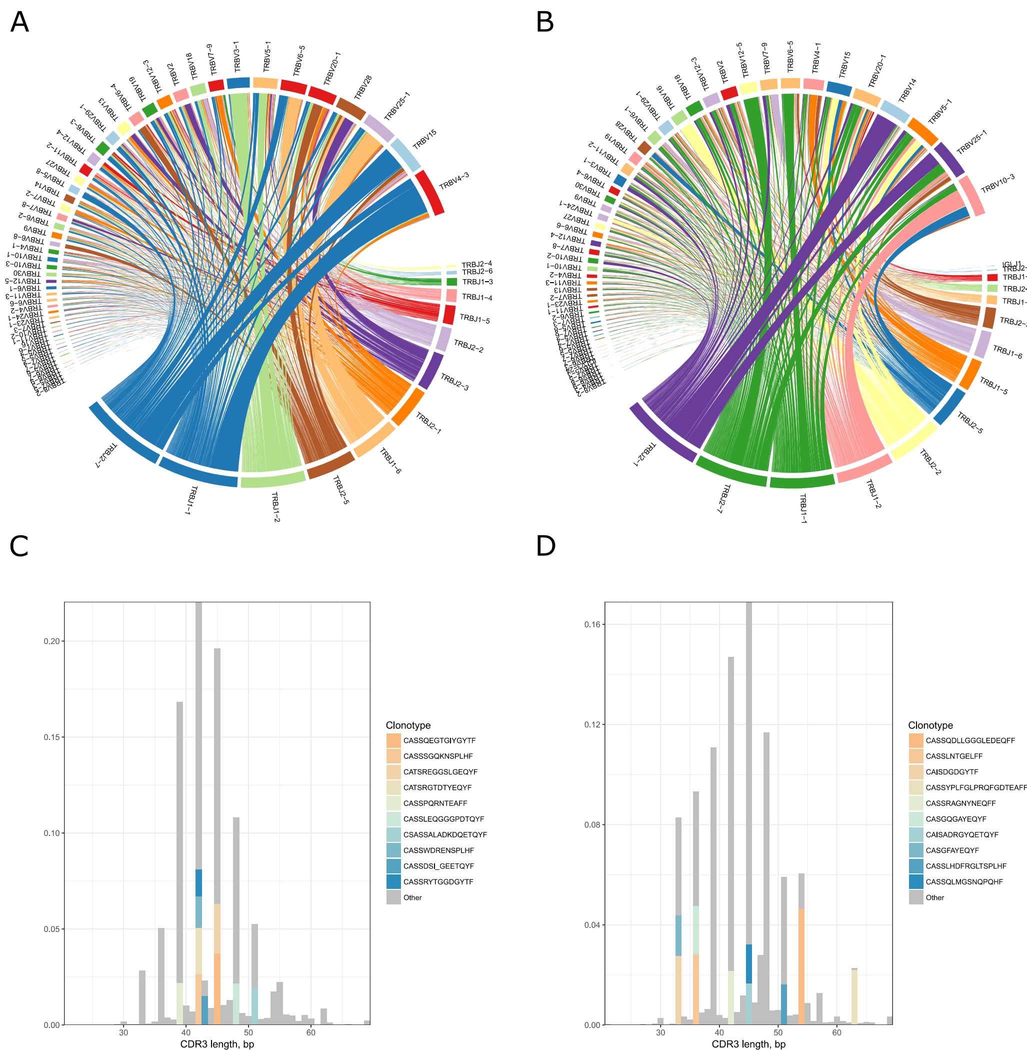
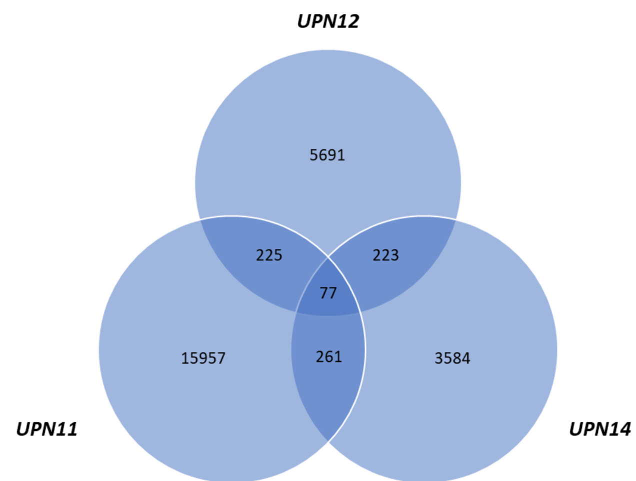


Figure 8.2 Venn diagram of overlapping TCR clonotypes among 3 patients with HLA-A*02:01 alleles early post-transplant.



8.3.1 Clinical determinants of early TCR diversity

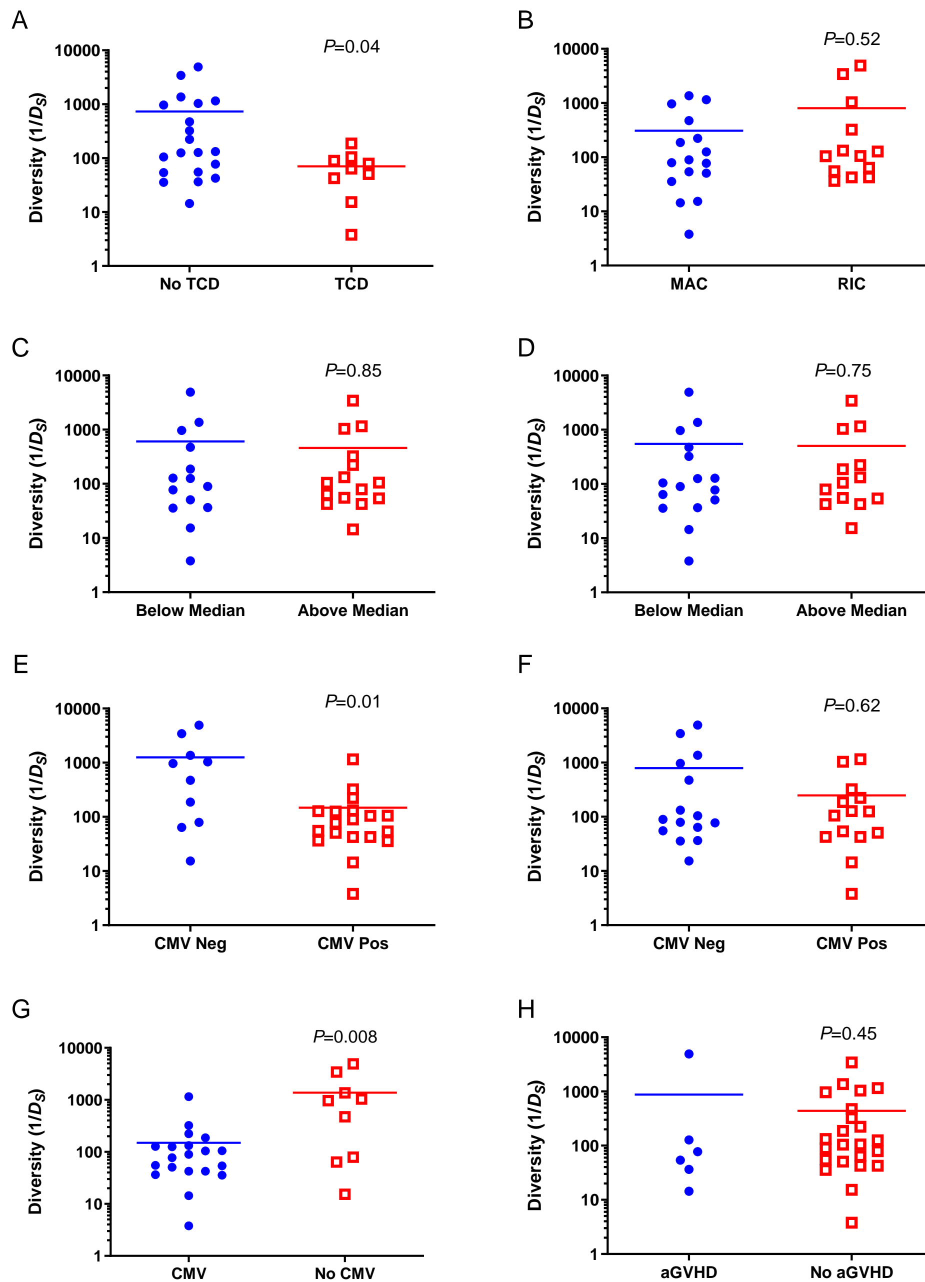
Pre-transplant characteristics were investigated for associations with early TCR diversity (Figure 8.3A-H). TCR diversity was significantly greater in patients who received T-cell replete transplants from matched sibling donors compared with T-cell depleted transplants from unrelated donors (siblings 130.1 [IQR 54-1017] vs unrelated donors 64 [IQR 28.9-96.9]; P=0.04) (Figure 8.3A). Early TCR diversity was significantly reduced in recipients who were CMV seropositive prior to transplant compared with seronegative patients (77.5 [IQR 42.4-127.5] vs 718.8 [IQR 75.5-1884]; P=0.01) (Figure 8.3E). There was no significant difference in median diversity according to donor CMV serostatus (donor seronegative 89.5 [IQR 55-964.4] vs seropositive 115.2 [IQR 42.5-248.2]; P=0.62). Early TCR diversity was not significantly different in subgroups stratified by conditioning regimen intensity (myeloablative versus reduced intensity), donor age and recipient age.

Similarly, clinical events including CMV viremia and acute GVHD prior to day 100 post-alloSCT were investigated for associations with early TCR diversity. Twenty patients (69%) developed CMV viremia prior to day 100 post-alloSCT (median onset 34 days post-alloSCT). Of these, 19 patients (95%) were CMV seropositive prior to alloSCT; one patient was CMV seronegative and had a transplant from a seropositive donor. Early TCR diversity was significantly reduced in patients with CMV viremia within the first 100 days post-alloSCT (83.5 [IQR 42.4-131.5] vs 964.4 [IQR 71.7-2399]; P=0.02) (Figure 8.3G). Six patients (20.7%) developed acute GVHD of any grade at a median of 48 days post-transplant, including 1 patient with grade 4 gastrointestinal acute GVHD at 35 days post-alloSCT. There was no significant difference in TCR diversity at day 100 in patients who had prior acute GVHD compared

to those who did not (patients with acute GVHD 65.6 [IQR 10-1327 vs no acute GVHD 104.9 [IQR 50.6-473.2]; *P*=0.45).

Figure 8.3 Impact of pre- and post-transplant variables on early TCR diversity

Impact of A) use of T-cell depleting antibodies, B) conditioning intensity, C) recipient age, D) donor age, E) recipient pre-transplant CMV IgG serological status, F) donor CMV IgG serostatus, G) CMV viremia before day 100 and H) presence of acute GVHD before day 100 post-alloSCT. TCD, T-cell depletion; MAC, myeloablative conditioning; RIC, reduced intensity conditioning.



8.3.2 Impact of early TCR diversity on relapse, chronic GVHD and survival

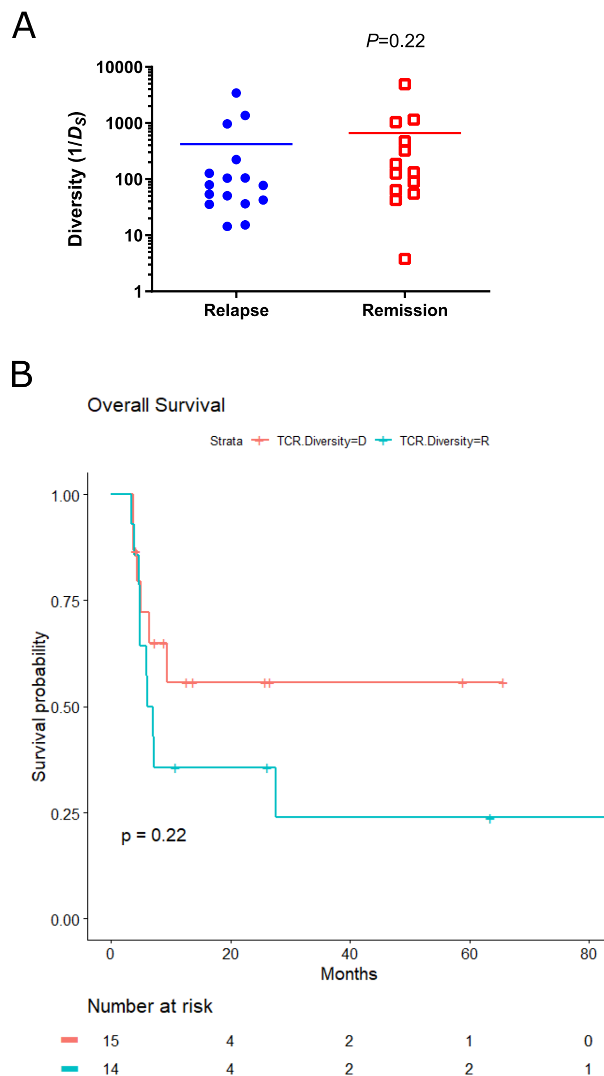
Sixteen patients developed AML relapse at a median of 98.5 days post-alloSCT (IQR 85-105.3 days). Peripheral blood samples were obtained from all relapse patients either prior to or at diagnosis of AML relapse and analysed for TCR diversity. Comparison was made with the 13 patients who did not relapse post-alloSCT. Importantly, TCR diversity was assessed at comparable times between the relapse (median 92 days post-alloSCT) and non-relapse (median 101 days post-alloSCT) cohorts thereby eliminating the effect of time post-transplant as a potential confounder. In addition, pre-transplant variables including recipient and donor age, conditioning intensity, graft type, donor type and use of antithymocyte globulin as GVHD prophylaxis were similar between relapse and non-relapse patients demonstrating effectiveness of the propensity score matching process (Table 8.2). There was no significant difference in early TCR diversity between patients who relapsed compared with those who did not (78.4 [IQR 38-799.2] vs 132.8 [IQR 59.5-753.6]; P=0.22), suggesting that a restricted TCR repertoire early post-transplant is not a mechanism of AML relapse (Figure 8.4A).

Table 8.2 Baseline characteristics of relapse (cases) and non-relapse (controls) patients

Characteristic	**Relapse Cases (*N*=16)**	**Non-Relapse Controls (*N*=13)**
Age, median years (range)	41 (21-62)	45 (20-58)
Cytogenetic Risk		
Intermediate	12 (75%)	7 (54%)
Adverse	4 (25%)	6 (46%)
Conditioning Intensity		
MAC	10 (63%)	8 (62%)
RIC	6 (38%)	5 (38)
Graft Source		
PB	16 (100%)	12 (92%)
BM	0	1 (8%)
Donor Source		
Sibling	11 (69%)	9 (69%)
UD	5 (31%)	4 (31%)
Donor Age, median years (range)	43.5 (15-69)	42 (22-63)
Anti-thymocyte globulin	5 (31%)	4 (31%)

Figure 8.4 Impact of early TCR diversity on relapse and survival

Early TCR diversity among patients with AML relapse compared with those in remission at approximately day 100 post-transplant (**A**) demonstrating no significant difference in TCR diversity between the two groups. Relapse and remission patients were matched for time post-transplant and key transplant variables including recipient age, donor type, donor age, use of in-vivo T-cell depletion as part of GVHD prophylaxis and conditioning regimen intensity. **B**) Overall survival among patients with diverse (D) or restricted (R) early TCR repertoires.



The cumulative incidence of chronic GVHD (cGVHD) of any grade in the entire cohort at 12 months post-alloSCT was 25.7% (95%CI 10.9-43.5). Patients were stratified into groups according to early TCR diversity values above (diverse) or below (restricted) the median. There was no significant difference in cGVHD incidence between diverse (12 month cGVHD incidence 37.1%, 95% CI 11.8-63.2%) and restricted groups (14.3%, 95% CI 1.8-39.1; *P*=0.36). Overall survival for the overall cohort at 12 months post-alloSCT was 46.1% (95% CI 27.4-64.8%). There was no significant difference in OS between patients with diverse (12 month OS 55.7%, 95% CI 28.6-82.9%) or restricted (35.7%, 95% CI 10.6-60.8%) early TCR repertoires defined as above or below the median value (*P*=0.22) (Figure 8.4B).

8.3.3 Dynamics of TCR diversity over time after alloSCT

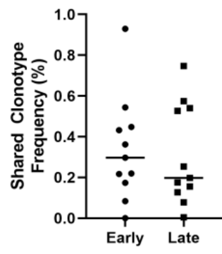
Eleven patients had serial samples analysed at early (day 100) and late (between 1-2 years post-transplant) timepoints. All patients remained free of leukemia relapse between these two timepoints. Four of the 7 patients who had CMV reactivation prior to day 100 had recurrence of CMV viremia after day 100; none of the patients who were free of CMV viremia pre-day 100 developed later viremia. The median cumulative frequency of shared clonotypes was 29.7% at the early time point (IQR 17.4-44.8%) and 19.8% (IQR 12.7-54.0%) late post-transplant indicating an overall high degree of clonotype repertoire similarity between two time points (Figure 8.5A). An alternative measure of overall similarity between two frequency distributions is the Jensen-Shannon Divergence (JSD) which is a quantitative representation of the overall degree of overlap normalised to a scale of 0 to 1, where 0 represents the samples being identical in TCRβ clonotype repertoires and 1 representing no overlap[156-158]. The median JSD between early and late time points in paired patient samples was 0.06 (IQR

0.01-0.16) once again demonstrating a high degree of repertoire overlap (Figure 8.5B). The dynamics of individual shared clonotypes between early and late timepoints was also assessed. Scatterplots of overlapping clonotypes demonstrate that in 10 out of 11 patients there was a positive linear correlation in shared clonotype frequency between the two times, with Pearson correlation coefficient r^2 values between 0.163 and 0.559 (Figures 8.5C, D). One patient demonstrated a particularly poor correlation due very few shared clonotypes being present between the two timepoints (Figure 8.5F). Of the 10 patients with multiple shared clonotypes between early and late time points, 4 patients demonstrated increases in shared clonotype frequency over time and the remaining 6 patients had a decrease in shared clonotype frequency.

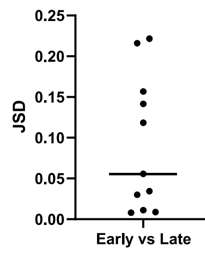
Figure 8.5 Paired comparison of early and late TCR repertoires in patients who remained in remission post-alloSCT

Overlap between paired samples assessed by Jensen-Shannon Divervence (JSD) (**A**) and frequency of shared clonotypes (**B**). Scatterplot of overlapping clonotype frequencies and linear regression of correlation between early and late timepoints in 2 representative patients demonstrating either strong linear correlation with multiple shared clonotypes present at both timepoints in one patient (**C**) and few overlapping clonotypes with poor correlation between timepoints in the second patient (**D**). Scatterplot axes represent log10 clonotype frequency. Histograms on the horizontal and vertical axes depict total (grey) and overlapping clonotype frequency distributions. Stacked clonotype frequency plots from the same 2 patients demonstrating dynamic changes overtime in the frequency of clonotypes (**E, F**). Coloured bars represent the top 20 shared clones, dark grey bar represents the remainder of shared clonotypes, light grey bar represents non-overlapping clones.

A



B



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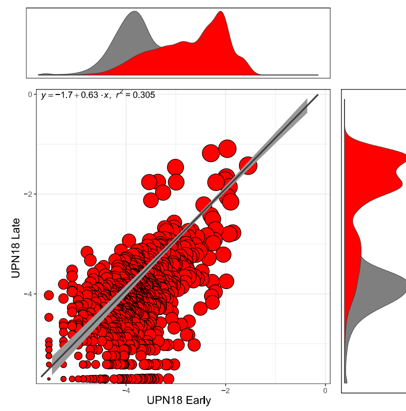
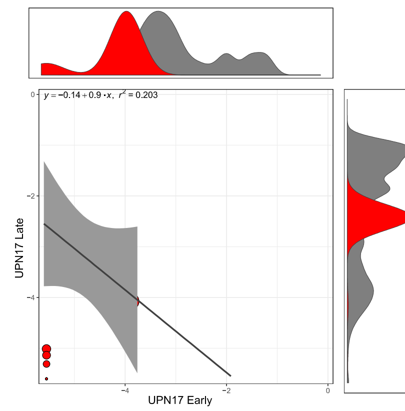
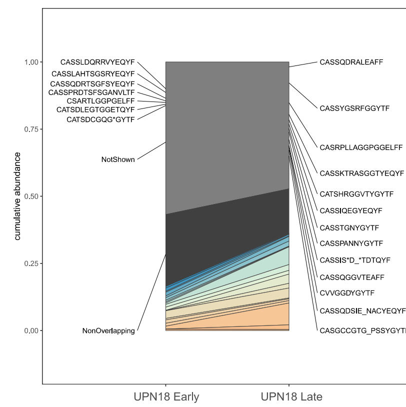


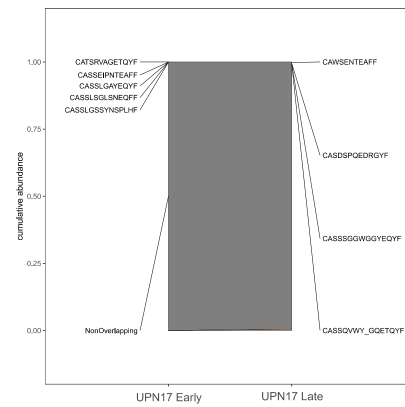
D



E



F



There was no significant difference in TCR diversity between early and late timepoints (median diversity 187.6 [IQR 64-1034] vs 61.1 [IQR 32-3551]; *P*=0.83) (Figure 8.6A). Patients with early CMV viremia (prior to day 100) continued to have a significantly reduced TCR diversity late post-transplant compared with patients who did not have early CMV reactivation (33.2 [IQR 27.3-61.4] vs 3868 [IQR 1421-4565]; *P*=0.006), indicating that early CMV viremia had a persistent effect on post-transplant T-cell recovery extending to 1-2 years post-transplant (Figure 8.6B). Additionally, patients with early CMV viremia had a significant decrease in TCR diversity between early and late timepoints (132.8 [IQR 55-323.6] vs 33.2 [IQR 27.3-61.4]; *P*=0.05). There was a non-significant trend towards an increase in TCR diversity between early and late timepoints in patients without early CMV reactivation (753.6 [IQR 166.3-3952] vs 3868 [IQR 1421-4565]; *P*=0.38). When looking at individual patient trends, 6 out of 7 patients with CMV reactivation had a decrease in TCR diversity between early and late timepoints; in contrast, 3 out of 4 patients without CMV reactivation had an increase in TCR diversity from early to late post-alloSCT (Figure 8.7).

Figure 8.6 Changes in TCR diversity according to post-transplant CMV viremia

TCR diversity at early and late post-transplant timepoints (**A**) and according to the presence or absence of early CMV reactivation (**B**).

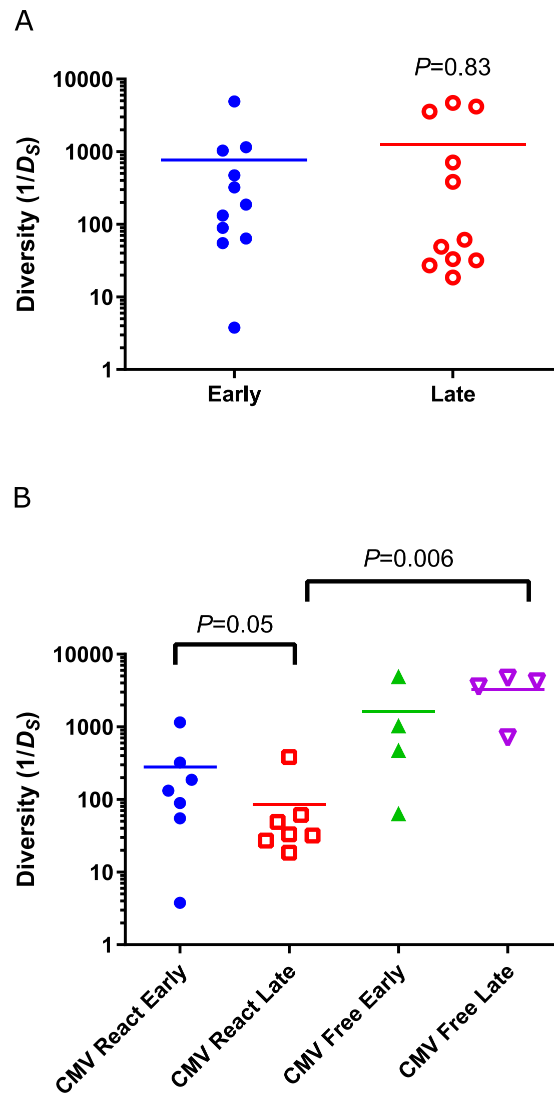
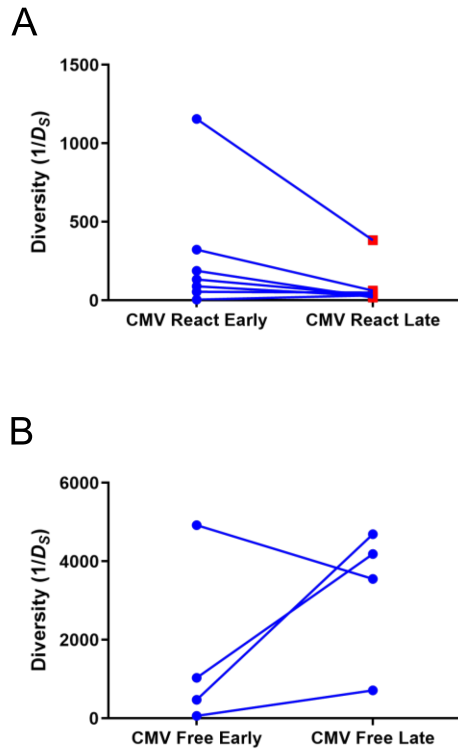


Figure 8.7 Individual patient changes in TCR diversity according to CMV viremia

Individual patient changes in TCR diversity in patients with (**A**) and without (**B**) post-transplant CMV reactivation



8.3.4 Early CMV infection induces a distinct TCRβ repertoire late post-transplant

Given that early CMV infection impaired TCR diversity early and late post-transplant, we investigated the impact of early CMV infection on the TCR repertoire at both timepoints to identify if the post-CMV repertoire was dominated by CMV-specific clones. Hierarchical clustering of TCRβ repertoires of the 29 patients early post-transplant demonstrated that early CMV infection did not appear to result in a distinct clonotype repertoire. However, at the late post-transplant time point, there was considerable clustering of TCRβ repertoires among patients with early CMV infection, suggesting that early CMV infection was associated with a distinct TCRβ repertoire (Figure 8.8). To identify if this distinct TCRβ repertoire was dominated by an abundance of CMV-specific clonotypes, we investigated the frequency of CMV-specific clones using a curated database of TCR specificity (VDJdb), acknowledging the limitation of this technique to public CMV-specific clonotypes with an inability to account for private patient-specific clonotypes. TCR Vβ clonotypes specific for CMV p65, p50 or IE1 were identified in all 11 patients late post-transplant, albeit at relatively low frequency (median 0.07% [IQR 0.03-0.08%]). There was no significant difference in frequency of CMV-specific clonotypes between patients who had CMV viremia prior to day 100 or not (Figure 8.9).

Figure 8.8 Hierarchical clustering of TCR clonotype repertoires late post-transplant

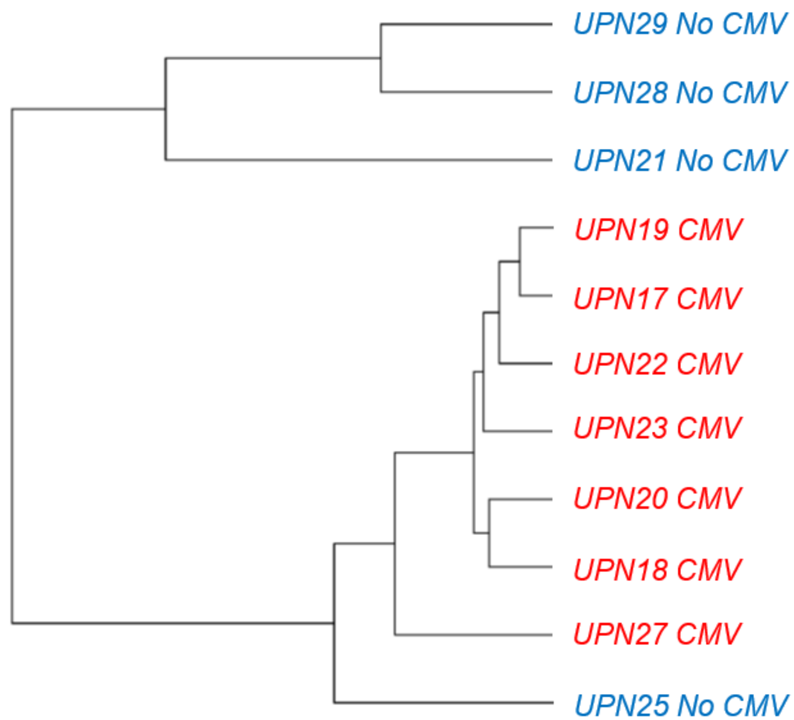
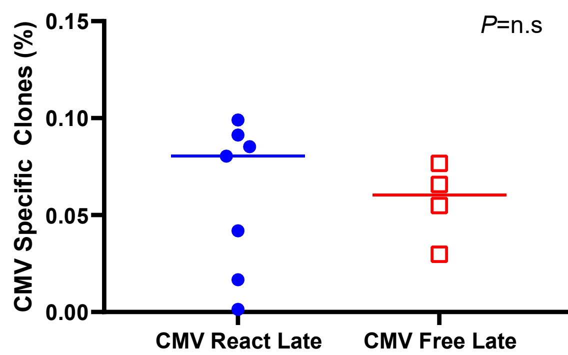


Figure 8.9 Frequency of CMV specific TCR clonotypes late post-transplant in patients with and without prior CMV reactivation



8.4 Discussion

In this paper we have performed an in-depth analysis of the dynamics of TCR repertoire reconstitution post-transplant and clinical determinants of TCR diversity post-alloSCT. A strength of this analysis was the longitudinal evaluation of TCR diversity over time, ranging from early (circa day 100) post-transplant to later time points (between 1 and 2 years post-transplant). Using this sequential analysis we observed that the TCR repertoire was oligoclonal early post-transplant and that there were dynamic changes in individual TCR clonotype frequency over time.

We identified that the most significant factors that impact TCR diversity at day 100 post-alloSCT are use of T-cell depletion and/or donor type, recipient CMV serostatus and post-transplant CMV viremia. Similar to our results, Van Heijst *et al.* previously reported that TCR repertoire at 6 months post-alloSCT was significantly more restricted in patients receiving T cell depletion[28]. All but one patient in our cohort received a peripheral blood stem cell graft, and therefore we were unable to examine the impact of graft source (bone marrow or peripheral blood) on TCR repertoire recovery. While previous reports have described differences in TCR reconstitution between peripheral blood and double umbilical cord transplants in adult patients, the similarity in naïve and memory T-cell content between granulocyte colony stimulating factor (G-CSF) mobilised peripheral blood grafts and unstimulated bone marrow suggests that there may not be a significant difference in early TCR reconstitution between these two graft types[28, 159].

Importantly, our results describe that post-transplant CMV viremia prior to day 100 was associated with impaired TCR reconstitution and diversity that persisted for up to 2

years post-transplant. We observed that there was divergence of TCR repertoire recovery over time dependent on early CMV viremia; there was a decrease in TCR diversity between early and late timepoints in patients with early CMV viremia whereas patients without CMV viremia demonstrated either stable or improved diversity over time. Furthermore, early CMV infection was associated with a distinct TCR repertoire compared with patients without CMV infection. The observations in this cohort of the impact of recipient CMV seropositivity and early CMV viremia on post-transplant TCR diversity mirrors work previously published[160, 161]. However, despite previous work, it remained unclear as to the impact of early CMV viremia on late post-transplant TCR diversity, as opposed to CMV viremia merely having a contemporaneous impact on repertoire restriction. The work presented herein clarifies that early CMV viremia (that is, within the first 100 days post-alloSCT), can have a persistent impact on both early and late post-transplant TCR diversity. These observations of the long-term adverse impacts of CMV viremia on post-transplant T-cell reconstitution mirror longitudinal analyses in healthy ageing. CMV seropositivity is a key driver of T-cell immunosenescence with ageing with an accumulation of senescent terminal effector memory T-cells specific for CMV and with restricted TCR repertoire in CMV seropositive elderly persons[162-164]. The median age of our cohort was 45 years and we did not observe any impact of recipient or donor age on TCR diversity, thereby excluding the possibility that ageing *per se* contributed to TCR restriction. Rather, it appears that CMV viremia may result in an accelerated deterioration in TCR diversity that is typically seen in significantly older individuals.

Despite the distinct clustering of TCRβ repertoires according to early CMV viremia, we did not observe a significant difference in CMV-specific clonotypes between patients

with or without CMV viremia prior to day 100. While this may on one hand suggest that the impact of CMV viremia on the TCR repertoire is independent of expansion of CMV-specific clonotypes, we cannot exclude the possibility that the public library of VDJ antigen specificities used may have been incomplete. Correlation of our observation with alternative methods to quantify CMV-specific T-cells is required for example using CMV-specific tetramers[165]. Indeed, Link et al. observed that while there were some shared CMV-specific TCRα clonotypes between patients, others remained private, thereby supporting the concept that public libraries of virus-specific TCR clonotypes may not adequately characterise the breadth of possible responses between patients[166].

Impairment of TCR repertoire recovery post-transplant has been suggested to have several clinical implications including the risk of post-transplant cancer relapse. In our analysis, we did not identify any impact of TCR diversity on early relapse of AML. The strength of our analysis with regards to relapse outcomes is that a well matched cohort design was used in which relapse cases were matched to controls for key variables that may impact TCR diversity in particular time post-transplant and use of T-cell depletion. In addition, we did not identify any impact of TCR diversity on the incidence of chronic GVHD or overall survival, although the small sample size may have limited our ability to detect differences in these outcomes which may also require a longer period of follow-up or analysis of TCR diversity at even later timepoints.

A limitation of this analysis is the small patient cohort and inherent heterogeneity in patient and transplant characteristics including use of *in-vivo* T-cell depletion and donor type. As a consequence, analyses were univariate in nature without sufficient power to

examine interactions in a multivariate model. Analysis of a larger cohort to strengthen the current findings and to allow more comprehensive analyses of the impact of TCR on survival is required.

In conclusion, we have demonstrated that there are dynamic changes in TCR repertoire composition over time with changes in individual clonotype abundance between day 100 and 2 years post-transplant. T-cell depletion and CMV viremia post-transplant were significantly associated with restriction of the early TCR repertoire post-alloSCT. Furthermore, early CMV reactivation post-transplant has persistent and deleterious effects on TCR repertoire reconstitution by limiting recovery of clonotype diversity. The potential clinical impact of this observation requires further examination, particularly amid new and effective antiviral or cellular therapy approaches to prevent CMV viremia post-transplant[167, 168].

9 MicroLEN: Micro-dose lenalidomide as maintenance therapy post-alloSCT for patients with acute myeloid leukaemia or myelodysplastic syndromes at high risk of relapse

9.1 Introduction

AlloSCT is an established therapy for patients with AML and MDS, however disease relapse remains a major cause of post-transplant mortality. In patients with high risk AML defined either by adverse risk cytogenetic or molecular features, relapse occurs in up to 50% of patients (unpublished data, RMH institutional transplant database). In approximately 43% of patients relapse occurs within 6 months of transplantation[169]. The prognosis of patients with relapsed AML post-alloSCT is poor; large registry-based cohort studies have repeatedly demonstrated that overall survival following relapse ranges between 10-30% at 2 years, with key prognostic indicators being time elapsed from transplant to relapse, disease burden at relapse and GVHD prior to relapse (presence of GVHD conferred greater risk of death post-relapse)[2, 169, 170]. There is therefore a need to identify patients who are at greatest risk of relapse, initiate novel therapies to attempt to prevent relapse in high risk individuals, and finally to improve outcomes for patients with established disease relapse.

Maintenance strategies post-alloSCT have been and continue to be explored to mitigate the risk of relapse in patients with high risk AML or MDS. These approaches may be broadly categorised into hypomethylating agents, targeted small-molecule inhibitors and immunomodulatory agents including lenalidomide, DLI or checkpoint inhibitors[171]. Although a thorough discussion of the merits of each maintenance strategy is outside the scope of this introduction, several common themes are emerging. Firstly, the unique

post-transplant combination of frequent cytopenias due to fragile engraftment, infection risk in the context of the expected kinetics of immune reconstitution, and importantly the risk of GVHD mean that the evaluation of maintenance strategies post-transplant need to be carefully considered in this specific population, and that therapies that may be well tolerated pre-transplant are often less so post-transplant. Secondly, and most interestingly, established therapies against AML and MDS that are employed post-transplant may have unexpected but beneficial immunological effects. For example, azacitidine when used as maintenance post-alloSCT was demonstrated to enhance CD8+ T-cell responses to several AML-associated antigens while simultaneously increasing the frequency of peripheral blood regulatory T-cells (Tregs) which may reduce the risk of GVHD[172]. Likewise, the Fms-like tyrosine kinase 3 (FLT3) inhibitor sorafenib increases IL-15 production by AML blasts that potentiates the CD8+ T-cell GVL effect post-alloSCT[173]. Both of these examples highlight strategies by which the CD8+ T-cell GVL may be augmented post-transplant.

Lenalidomide is an immunomodulatory agent which may potentiate GVL responses post-transplant. Lenalidomide has wide-ranging immunological effects including T- and NK-cell activation, shift of CD4+ T-cell responses to favour a Th1 phenotype, inhibition of regulatory T cells (Tregs) and enhancement of interleukin-2 (IL-2) and interferon gamma (IFNγ) production. Several of these pleiotropic effects of lenalidomide were revealed to be secondary to the interaction between lenalidomide and cereblon, the substrate receptor of the cullin ring E3 ubiquitin ligase complex, first identified to be the molecular target of thalidomide[104, 174]. Lenalidomide, following binding to cereblon, induces the recruitment of transcriptional repressors Ikaros and Aiolos to the E3 ubiquitin ligase complex and promotes their degradation. Aiolos is a

transcriptional repressor of IL-2; therefore, lenalidomide-induced degradation of Aiolos results in increased transcription of IL-2 to result in many of the T-cell stimulatory effects observed with lenalidomide treatment[104, 105].

The strategy of post-alloSCT maintenance therapy with lenalidomide has been explored in small phase II studies in patients with AML/MDS and myeloma. Sockel *et al.* conducted a trial of lenalidomide 10mg daily days 1-21 in patients with AML or high risk MDS with del(5q) following alloSCT, 3 of whom from HLA-mismatched unrelated donors[73]. Lenalidomide was commenced at a median 2.5 months post allograft. This study was ceased prematurely due to a high incidence of grade 3-4 acute GVHD (60% of patients, within 2 cycles of lenalidomide). Similarly, a high incidence of acute GVHD was also reported in the HOVON76 study, a phase II trial of lenalidomide maintenance after tandem autologous followed by non-myeloablative alloSCT for patients with newly diagnosed myeloma[74]. Fifty-three percent of patients developed grade 2-4 acute GVHD or extensive chronic GVHD, with median onset 18 days after commencement of lenalidomide. These studies demonstrate that lenalidomide can induce potent allogeneic responses post-transplant; however they also highlight the difficulty in extracting an augmented GVT response from the simultaneous promotion of GVHD. While it is clear that lenalidomide at a dose of 10mg daily as an early post-transplant maintenance strategy is not tolerable, it remains unknown if smaller doses in carefully selected patients may have a more acceptable safety profile post-alloSCT whilst still augmenting GVT responses in patients with high risk malignancies. In this context, I conducted microLEN, an investigator-initiated phase I dose-escalation clinical trial to determine the maximum tolerated dose of lenalidomide when used as a

maintenance strategy in combination with early immunosuppression taper in patients with high-risk AML and MDS.

9.2 Clinical trial design

MicroLEN was conducted at the Royal Melbourne Hospital and Peter MacCallum Cancer Centre commencing 4 April 2016 and remains open to patient recruitment. The primary objective is to determine the maximum tolerated dose (MTD) of lenalidomide when used in combination with early immunosuppression taper in patients with high-risk AML and MDS post-alloSCT. Secondary and exploratory objectives are to assess overall survival (OS), relapse, non-relapse mortality (NRM), incidence of acute and chronic GVHD and to assess the kinetics of NK and T-cell reconstitution post-alloSCT.

9.2.1 Participant enrolment

Participants were screened for participation in the study at any time after the decision to undergo alloSCT prior to day 40 post-alloSCT. Patients underwent a bone marrow biopsy and clinical assessment between days 25 to 40 post-alloSCT, and patients with evidence of responsive disease (defined as complete morphologic remission on bone marrow biopsy or at minimum a partial response) and without grade 2-4 acute GVHD were enrolled in the study and continued with planned immunosuppression taper and lenalidomide. This study has a recruitment target of up to 20 patients which takes into account 5 dose levels.

Inclusion criteria

Inclusion in the study required patients to meet one of the following criteria:

- High risk AML defined as any of:

- Not in complete remission (CR) at time of alloHCT
- Adverse risk cytogenetics at any stage of disease
- FLT3-ITD mutation
- Prior induction failure
- Evidence of pre-transplant minimal residual disease either by cytogenetics or by flow cytometry. If flow cytometry is the selected method used, MRD must be greater than 0.1%.
- In second complete remission if duration of first complete remission was ≤6months.
- Transformation from myeloid neoplasm at any stage

OR

- High risk MDS defined as any of:
  - Adverse cytogenetics
  - Over 10% blasts in blood or marrow aspirate

In addition, patients must meet all the following criteria prior to inclusion in the study:

- Age 18 years or older
- No prior exposure to lenalidomide
- Alkaline phosphatase and transaminases ≤ 2 x ULN
- Creatinine clearance ≥ 30 ml/min (calculated by Cockcroft-Gault formula
- Females of childbearing potential must use an effective method of contraception or practice absolute abstinence for 4 weeks prior to lenalidomide therapy, during treatment and 4 weeks after treatment discontinuation

- Male patients must use contraception during lenalidomide treatment and for 1 week after completion of treatment
- ECOG performance status 0-2
- Life expectancy > 6 months
- Patient must provide written informed consent
- Patients must agree not to share their medication and return unused supplies

Commencement of study treatment between day 25-40 post-alloSCT will be dependent on the following criteria:

- Responsive disease (morphologic complete or partial response) based on bone marrow biopsy performed between days 25 and 40 post-alloSCT
- Absence of grade 2-4 acute GVHD

Exclusion criteria

Patients meeting any of the following criteria were excluded from this study:

- Grade 2-4 aGVHD
- Relapsed or progressive disease on screening bone marrow biopsy
- Active second malignancy currently requiring treatment
- Known hypersensitivity with anaphylactic reaction to lenalidomide
- Class III or IV cardiac disease defined by the NYHA.
- Severe or debilitating pulmonary disease.
- Severe or debilitating central nervous system disease or cerebral dysfunction.
- Active bacterial, viral or fungal infection

- Human Immuno-deficiency Virus (HIV) infection.
- Any coexisting medical or psychological condition that would preclude participation in the required study procedures.
- Female patients who are both lactating and breast-feeding or have a positive serum pregnancy test during the screening period or a positive pregnancy test on day 1 before first dose of study drug

9.2.2 Study treatment

Study treatment comprised of ciclosporin taper in combination with lenalidomide, both commencing between day 40-45 post-alloSCT.

Ciclosporin taper

The protocol of ciclosporin taper was designed so that ciclosporin would cease over a period of 9 weeks, corresponding to approximately 100 days post-alloSCT. The schema of ciclosporin taper is described in Table 9.1 and was dependent on the dose of ciclosporin at day 40 post-alloSCT.

Lenalidomide treatment

Lenalidomide treatment commenced day 40-45 post-alloSCT and continued for up to 12 cycles with each cycle length of 4 weeks (therefore, maximum of 48 weeks of lenalidomide treatment). Each patient commenced lenalidomide at a specified dose level, with no intra-patient dose escalation permitted. The dose of lenalidomide in each cohort is specified in Table 9.2. The dose levels chosen reflect uncertainty as to whether there was any safe dose of lenalidomide when used early post-transplant. To the authors' knowledge, this is the first phase I dose-finding study of lenalidomide in this

context, and hence the first dose level comprised a particularly low dose of lenalidomide. In addition, the highest dose level of 10mg every second day was selected in recognition of previous reports describing high rates of acute GVHD (50-60%) with lenalidomide at a dose of 10mg/d[73, 74].

Table 9.1 Ciclosporin tapering schedule

Starting Ciclosporin dose (mg bd)	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
100	100	75	75	75	50	50	25	25	0
125	100	100	75	75	50	50	25	25	0
150	125	125	100	100	75	75	50	25	0
175	150	150	125	125	100	75	50	25	0
200	175	175	150	125	100	75	50	25	0
225	200	175	150	125	100	75	50	25	0
250	225	200	175	150	125	100	50	25	0
275	225	200	175	150	125	100	50	25	0
300	250	200	175	150	125	100	50	25	0

Table 9.2 Lenalidomide dose cohorts

Dose cohort	Lenalidomide dose
1	2.5mg once per week
2	2.5mg twice per week
3	5mg twice per week
4	5mg alternate daily
5	10mg alternate daily

9.2.3 Determination of cohort size and dose escalation parameters

This study was designed to have a cohort size of 4 patients. The first 2 patients were enrolled at each dose level and observed for DLT for 2 cycles of lenalidomide treatment. Subsequent patients were only enrolled into that dose cohort if none or 1 patient developed a DLT. This cautious approach was taken to avoid rapid accrual of patients into dose cohorts that may have eventually be deemed overly toxic. Dose escalation to the next pre-defined level was permitted when fewer than 2 patients developed a DLT. The maximum tolerated dose (MTD) is defined as the highest dose at which fewer than 2 out of 4 patients develop a DLT.

The rationale for this study design, in contrast to the classical 3+3 dose escalation strategy, was to take into account the 'expected' rate of acute GVHD and other sequelae defining a DLT that occurs in the context of alloSCT even in the absence of accelerated immunosuppression withdrawal or lenalidomide therapy. Reported rates of grade 3-4 acute GVHD with ciclosporin and methotrexate as prophylaxis in HLA-matched sibling transplants and the addition of anti-thymocyte globulin for HLA-matched unrelated donor transplants are between 10-20%.[133, 175] Taking this into account, the anticipated rate of DLT in patients undergoing alloSCT outside of this trial was estimated to be between 20-30%. A conventional 3+3 study design tolerates a DLT rate of 16.7%, with a higher event rate triggering study termination. In the post-alloSCT setting, such a study design would prematurely trigger study termination even with an event rate that is not significantly greater than that expected in patients not receiving study treatment. In contrast, a 4+4 design, as adopted in this study, tolerates an event rate of up to 25% which was deemed suitable to simultaneously meet the needs to patient safety without compromising the ability of the study hypothesis to be investigated.

9.2.4 Definition of dose limiting toxicity

Dose limiting toxicity (DLT) was defined as any of the following adverse events considered at least possibly related to lenalidomide that occur from the time lenalidomide commencement until 120 days after the first dose lenalidomide:

- Grade 3-4 acute GVHD
- Moderate or severe chronic GVHD
- Grade 3-4 neutropenia, unresponsive to G-CSF
- Grade 3-4 thrombocytopenia
- Grade 3-4 infection at any site
- New venous thromboembolism
- Grade 3-4 non-haematologic toxicity requiring dose interruption except anaemia
- 3 or more episodes of dose interruptions for any reason

9.2.5 Dose escalation for lack of efficacy

The protocol requires that at least 4 patients receive a minimum of 2 cycles of lenalidomide, with fewer than 2 patients experiencing a DLT, before permitting dose escalation to the subsequent dose level. However, disease relapse is a competing risk for the development of a DLT because trial participation for a particular patient will also cease at the time of disease relapse post-allograft. Given the high-risk cohort specifically included in this trial, occurrence of relapse will thus interfere with dose escalation, meaning that the trial may become 'blocked' at a low and likely sub-therapeutic dose if several relapses were to occur. This occurred at the first dose level, where 3 out of the first 4 patients ceased study treatment during the second cycle of lenalidomide due to leukemia relapse. None of these patients had experienced a DLT.

Therefore, the protocol was amended to allow dose escalation for the instance of lack of efficacy to avoid ‘roadblocks’ in the trial at likely subtherapeutic doses of lenalidomide if several relapses were to occur, while preserving the requirement for safety and determination of the MTD. The protocol revision stipulated that once a minimum of 4 patients have been commenced on lenalidomide at a given dose level, and ≥50% of patients experience disease relapse within 2 cycles of lenalidomide, and <2 patients (i.e.. 0 or 1 patient) at that dose level have experienced a DLT, dose escalation to the next level will be permitted.

9.2.6 Management of graft versus host disease while on study

The occurrence of Grade 1 aGVHD did not require drug cessation. Immunosuppression withdrawal was halted until resolution of aGVHD or until aGVHD has remained stable (grade 1) for at least 2 weeks, whichever occurred first. Management of aGVHD was according to local guidelines.

Grade 2 aGVHD occurring in a trial participant after commencing lenalidomide, required doses of lenalidomide to be withheld until improvement of GvHD symptoms to grade 1 or less for a duration of at least 2 weeks. Immunosuppression withdrawal was halted until improvement in aGVHD to grade 1 or less for at least 2 weeks. Following improvement in aGVHD to grade 1 or less for 2 weeks, immunosuppression withdrawal and lenalidomide administration resumed.

The occurrence of grade 3-4 aGVHD in a trial participant after commencing lenalidomide was an end point and further lenalidomide was not administered.

Likewise, moderate to severe chronic GVHD was an end point and lenalidomide treatment was ceased.

9.3 Clinical trial results

9.3.1 Patient recruitment

Since opening to recruitment on the 4 April 2016 until 4 July 2019, 16 patients were recruited into the study. One patient was in the acute phase of her transplant and was yet to commence lenalidomide. One patient was enrolled on the study prior to transplant, however did not proceed to transplant due to identification of a non-haematological malignancy during their pre-transplant assessment. Three patients did not commence lenalidomide post-alloSCT as they did not meet inclusion criteria at re-assessment between days 25-40, due to multi-organ failure (1 patient), sepsis and liver GVHD (1) and severe acute kidney injury (1). The remaining 11 patients commenced ciclosporin taper and lenalidomide therapy as per study protocol.

Over the same period of time that the study has been open to recruitment, an additional 22 patients were screened for eligibility for microLEN but did not proceed to enrolment. Fourteen patients met one or more exclusion criteria, the most common of which were patients not proceeding to allograft due to refractory disease (3 patients), active infection (2), and acute GVHD (2). Six patients declined the invitation to participate in the study, most frequently due to concerns about toxicity and the addition of an investigational approach to an already high-risk allograft procedure. A further 2 patients did not participate due to physician preference, namely to use a FLT3 inhibitor as maintenance post-alloSCT (1) and concern about lenalidomide toxicity (1). Overall, of

all patients invited to participate in the study, 30% (11 out of 37) commenced study treatment with lenalidomide.

9.3.2 Patient baseline characteristics

The characteristics of the 11 patients who commenced post-transplant lenalidomide are described in Table 9.3.

Table 9.3 MicroLEN baseline characteristics

Characteristic	Value
Age, median (range)	48 (18-62)
Sex, M/F	7/4
Cytogenetic group	
Intermediate	6
Adverse	5
Disease status pre-transplant	
CR	9
PR	2
MRD status pre-transplant	
Positive	6
Negative	3
Transplant donor source	
Sibling	7
UD	4
Conditioning intensity	
MAC	6
RIC/NMA	5
Anti-thymocyte globulin	5
HCT-CI	
<3	5
≥3	6
Median peripheral blood counts prior to commencement of lenalidomide	
Haemoglobin, g/L (range)	98 (80-117)
White cells, $x10^9$/L (range)	4.9 (1.7-17.5)
Neutrophils, $x10^9$/L (range)	3.1 (0.8-14.9)
Platelets, $x10^9$/L (range)	95 (32-180)

CR, complete remission; PR, partial remission; MRD, measurable residual disease; UD, unrelated donor; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; NMA, non-myeloablative; HCT-CI, haematopoietic cell transplant comorbidity index.

All 11 patients were transplanted for AML. As required for inclusion into the study, all patients had at least one high risk feature, including adverse risk cytogenetics (5), prior relapse with short duration of first remission (2), therapy-related AML (1), refractoriness to previous chemotherapy (3) or adverse-risk molecular mutations including 3 patients with FLT3 internal tandem duplications (ITD). Measurable residual disease (MRD) assessments in bone marrow aspirates were available in 9 patients, 8 of which were performed by flow cytometry and 1 patient with MRD assessed by NPM1 quantitative PCR. Six patients were MRD positive pre-transplant; two of these patients remained MRD positive at day 30 post-transplant, prior to commencement of immunosuppression withdrawal and lenalidomide therapy. One patient with morphologic persistence of disease pre-transplant was in CR by day 30 with persistent MRD, and one patient who was MRD negative pre-transplant had detectable MRD at day 30. All patients received peripheral blood stem cell grafts. The 6 patients who received myeloablative conditioning (MAC) had busulfan and cyclophosphamide. Of the 5 patients who had reduced-intensity (RIC) or non-myeloablative conditioning (NMA), 4 had fludarabine and melphalan and 1 patient had fludarabine and cyclophosphamide. The 4 patients who received transplants from unrelated donors had anti-thymocyte globulin (Thymoglobulin 4.5mg/kg) as part of their GVHD prophylaxis regimen. Additionally, one patient who received stem cells from a single antigen mismatched sibling donor also received anti-thymocyte globulin. Prior to commencement of lenalidomide, all patients had cytopenia in at least one lineage. Eleven patients had grade 1-2 anaemia, 7 patients had grade 1-2 thrombocytopenia and 2 patients had grade 3 neutropenia.

9.3.3 Ciclosporin withdrawal

Ciclosporin withdrawal commenced as per protocol at a median of 45 days post-alloSCT (range 40-57 days). Six patients had progressive dose reductions of ciclosporin strictly at the proscribed intervals in the study protocol. Four patients had delays in ciclosporin dose reductions due to 2 patients with acute GVHD, 1 patient with diarrhoea later biopsy demonstrated not to be GVHD, and 1 patient with pancytopenia and concern for immune-mediated graft rejection. One patient had ciclosporin dose reduction accelerated due to EBV-driven post-transplant lymphoproliferative disease. Overall, six patients had ciclosporin ceased while on study at a median of 98 days post-alloSCT (range 52-124 days).

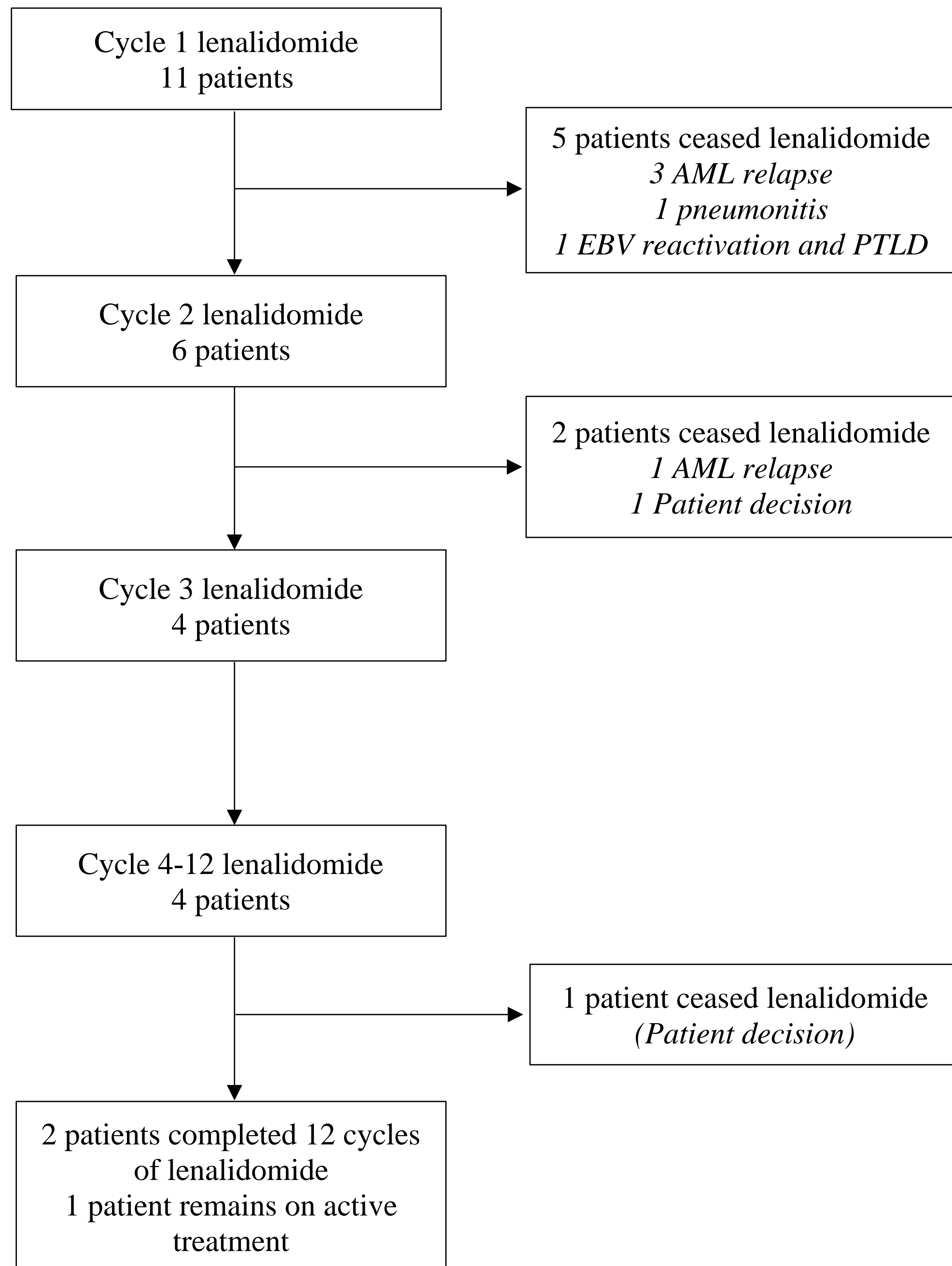
9.3.4 Lenalidomide therapy

Lenalidomide was commenced at a median of 45 days post-alloSCT (range 40-57 days). As of 4 July 2019, 4 patients had received lenalidomide at each of dose levels 1 (2.5mg weekly) and 2 (2.5mg twice per week), and 3 patients at dose level 3 (5mg twice per week) at which the study remains open to recruitment (Figure 9.1). To date, the maximum tolerated dose (MTD) of lenalidomide has not been reached.

The median number of lenalidomide cycles completed was 2 (range 1-12). Lenalidomide was ceased prematurely in 8 patients. Of the 4 patients in dose level 1, 3 patients relapsed during the second cycle of lenalidomide therefore requiring treatment cessation. The fourth patient in dose level 1 developed grade 3 pneumonitis at day 64 post-alloSCT possibly related to lenalidomide. Due to 3 out of the first 4 patients developing relapse which precluded completion of the first 2 cycles of lenalidomide, and none of these patients developing toxicity at least possibly related to lenalidomide,

progression to dose level 2 was permitted following a protocol amendment which allowed dose escalation for lack of efficacy in the absence of significant toxicity. In dose level 2, 3 patients completed at least 2 cycles of lenalidomide without a DLT. One patient ceased lenalidomide after cycle 2 due to relapse. Another 2 patients ceased lenalidomide due to patients' decision to end study treatment. One patient developed a DLT at dose level 2, due to EBV reactivation and post-transplant lymphoproliferative disease (grade 4) considered not related to lenalidomide treatment. Three patients have received lenalidomide at dose level 3, of which 2 patients have completed 12 cycles of lenalidomide, and 1 patient remains on lenalidomide therapy (completed 4 cycles to date).

Figure 9.1 Schematic flowchart of lenalidomide treatment



9.3.5 Graft versus host disease

Two patients developed acute GVHD. One patient developed acute pneumonitis as an atypical manifestation of acute GVHD 64 days post-alloSCT (22 days after commencement of lenalidomide at dose level 1, 2.5mg once per week) which was treated and resolved with oral prednisolone. Due to the degree of hypoxia and hospitalisation required, this was classified as grade 3 acute GVHD and considered a DLT. A second patient developed oral GVHD 66 days post-alloSCT (21 days after lenalidomide commenced) which resolved with topical corticosteroids. Two patients developed mild chronic GVHD, both of whom received lenalidomide at dose level 3 (5mg twice per week). One of these patients developed mild oral and liver GVHD at 181 days post-alloSCT (138 days post-lenalidomide) that responded to topical corticosteroids and ciclosporin mouth rinse, and the other patient developed mild oral GVHD 218 days post-alloSCT (173 days post-lenalidomide) that resolved spontaneously without therapy. There were no cases of moderate or severe chronic GVHD involving any organ.

9.3.6 Adverse events

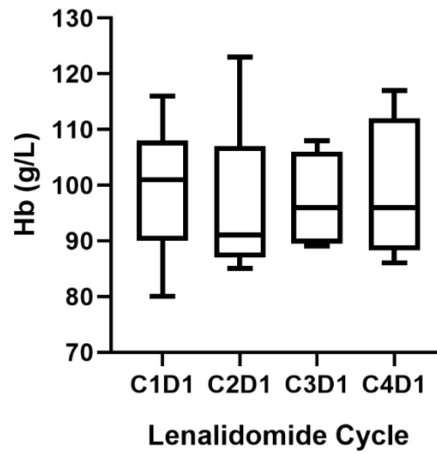
There were 41 adverse events of any grade considered at least possibly related to lenalidomide treatment; all 11 patients had at least one adverse event. The most common adverse events were haematological cytopenias. Four patients developed either new or worsening of baseline anaemia (maximum grade 3). Four patients developed thrombocytopenia including one patient with grade 4 thrombocytopenia. Seven patients developed neutropenia including 5 patients with grade 4 neutropenia, for which lenalidomide was withheld for all 3 patients. Haematological toxicity occurred at all dose levels of lenalidomide. The trend of peripheral blood counts for patients on day 1

of cycles 1 to 4 are depicted in Figure 9.2a-c. There was no significant difference haemoglobin or platelet counts from day 1 cycle 1 and the median of day 1 cycles 2-4 (haemoglobin: 101 vs 93g/L, P=0.66; platelet count 116 vs 114x10^9/L, P=0.54). However, there was a significant decrease in neutrophil count between day 1 cycle 1 and the median of subsequent cycles (2.3 vs 1.7x10^9/L, P=0.01).

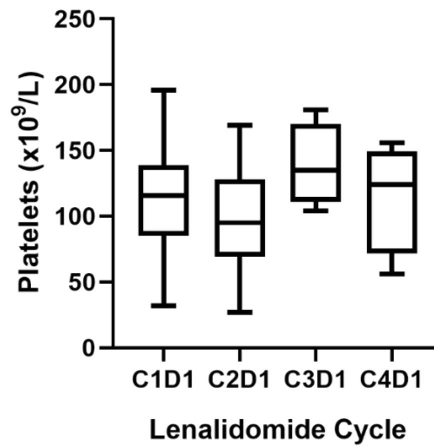
The most common non-haematological toxicities were infection and liver function test derangements. Three patients developed infection considered possibly related to lenalidomide, including 2 patients with pulmonary infections and 1 patient with a perioral viral exanthem. One patient developed EBV reactivation and post-transplant lymphoproliferative disease (PTLD) that was considered unrelated to lenalidomide therapy. This patient had undergone a second alloSCT for relapsed AML with reduced intensity conditioning and anti-thymocyte globulin. Ciclosporin was ceased and the patient received rituximab for treatment of PTLD. Although lenalidomide was considered not to have contributed to the initial EBV reactivation, lenalidomide treatment was withheld (investigator's decision) due to concern about potentiating immunosuppression in the context of significant PTLD. Three patients developed elevated liver transaminases including 1 patient with grade 3 elevated alanine aminotransferase. There were 8 severe adverse events (SAE), classified a such due to requirement for admission to hospital. Of these, one was considered possibly related to lenalidomide (grade 2 pneumonia). Lenalidomide was withheld during this episode which resolved with antibiotic therapy.

Figure 9.2 Peripheral blood counts on lenalidomide treatment

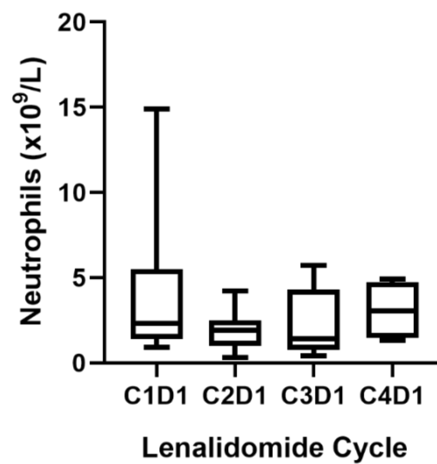
a) Haemoglobin



b) Platelet count



c) Neutrophil count

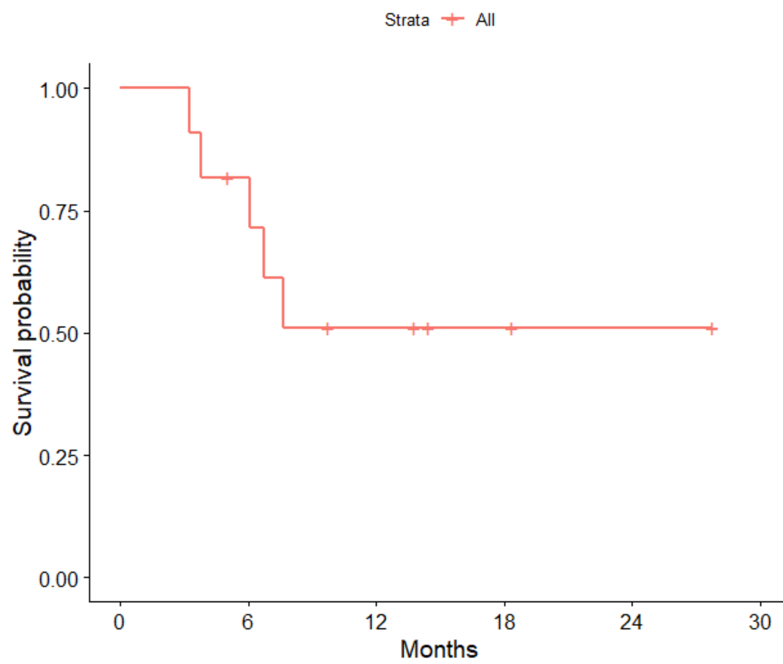


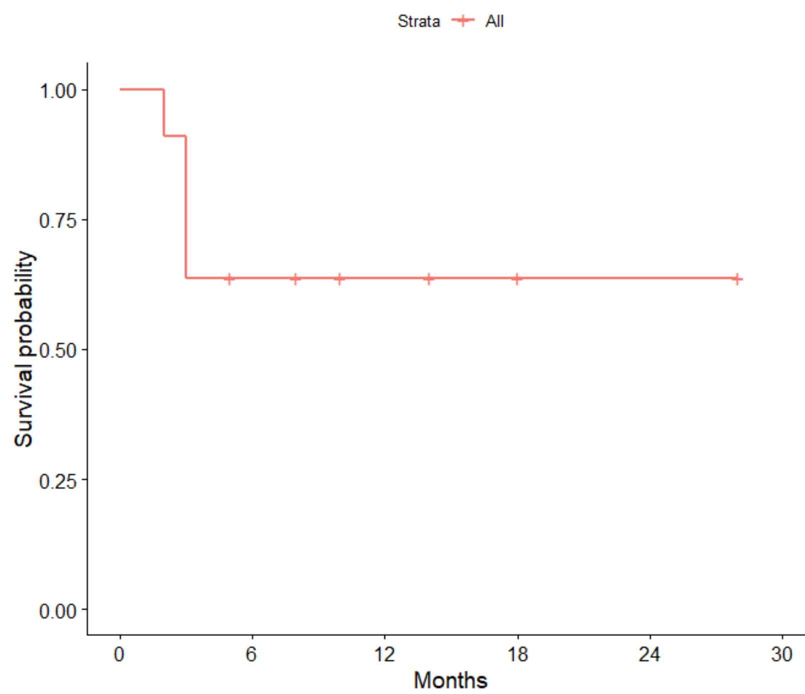
9.3.7 Leukaemia relapse and survival

Four patients relapsed with AML post-alloSCT at a median of 81 days post-transplant (range 72-89 days); 3 of these patients received 1 cycle of lenalidomide prior to relapse, and one patient relapsed after completing 2 cycles of lenalidomide. All of these patients had particularly adverse prognosis features including adverse risk cytogenetics, FLT-ITD positivity and either morphologic residual disease prior to transplant or measurable residual disease. Notably, three of these patients received lenalidomide at the lowest dose level, and one patient at the second dose level. There were no AML relapses at the third dose level.

Overall survival (OS) and relapse-free survival at 12 months from transplant were 51.1% (95% CI 27.9-93.6%) and 63.6% (40.1-99.5%) respectively (Figure 9.3). Median OS and RFS were not reached.

Figure 9.3 Survival outcomes post-alloSCT





9.4 Correlative immunophenotyping of T and NK-cell subsets with post-transplant lenalidomide therapy

Detailed immunophenotypic analysis of T-cell and NK-cell populations in peripheral blood were performed in study participants with comparison with both normal controls and patients undergoing alloSCT for AML and MDS who did not receive post-transplant lenalidomide therapy. In both study participants and control patients, samples were analysed at day 30 and day 100 post-transplant. At the time of writing, the paucity of patients who had completed 12 months of post-transplant lenalidomide precluded analysis of this timepoint.

9.4.1 T-cell analysis

CD8 T-cells

CD8 T-cells outnumbered CD4 T-cells at day 30 and day 100 post-transplant in both lenalidomide treated and control patients, consistent with previously reported kinetics of T-cell reconstitution post-transplant in which CD8 T-cell recovery precedes that of CD4 T-cells[146]. There was a statistically significant increase in CD8 T-cell proportion from day 30 to day 100 in lenalidomide treated patients (42.2% vs 53.5%, P=0.027), however at day 100 there was no difference in the proportion of CD8 T-cells between lenalidomide treated and untreated patients (53.8% vs 58.9%, P=0.99) (Figure 9.4).

At day 30 post-transplant, CD8 T-cells comprised predominantly CD45RA-CCR7- effector memory T-cells (Tem) and CD45RA+CCR7- terminally differentiated effector memory (TEMRA) cells in both lenalidomide (Tem 41.3%, TEMRA 42.7%) and control transplant patients (Tem 32.3%, TEMRA 49.2%). Accordingly, the proportion of CD45RA+CCR7+ naïve T cells (Tn) and CD45RA-CCR7+ central memory T cells

(Tcm) comprised the minority of CD8 T-cells in lenalidomide patients (Tn 7.2%, Tcm 0.7%) and transplant controls (Tn 14.6%, Tcm 2.2%). Lenalidomide treated patients had a significantly lower proportion of Tn at day 30 post-alloSCT (i.e. prior to lenalidomide therapy) compared with transplant controls (P=0.014); there was no significant difference in other T-cell memory subsets between the two cohorts. By day 100 post-transplant, there was a statistically significant decrease in Tn in lenalidomide patients (3.0% vs 7.2%, P=0.049) and a non-significant trend towards the same in transplant controls (2.2% vs 20.0%, P=0.13). There was no significant difference in Tn between lenalidomide patients and controls at day 100 (P=0.95). There were no significant changes in the proportion of Tcm, Tem or TEMRA between days 30 and 100 post-transplant in either lenalidomide or transplant control patients.

The expression of HLA-DR by T-cells was analysed as a marker of cellular activation. HLA-DR expression on CD8 T-cells was significantly increased at day 30 post-transplant on both lenalidomide treated (33.2%) and control transplant patients (14.5%) compared with normal non-transplant controls (3.8%; lenalidomide vs normal P<0.001, control transplant vs normal P=0.03) consistent with a state of heightened T-cell activation post-transplant (Figure 9.5). There was no significant change in HLA-DR expression from day 30 to day 100 post-alloSCT in both lenalidomide and control transplant patients. Lenalidomide treatment did not result in a significant difference in HLA-DR expression by day 100 in comparison with control transplant patients (lenalidomide 34.5% vs transplant controls 19.8%, P=0.84).

The expression of PD-1 and TIM3, among other coinhibitory checkpoint receptors, occurs following T-cell activation which if sustained can result in functional exhaustion.

The frequency of PD-1+ CD8 T-cells at day 30 post-alloSCT (prior to lenalidomide therapy) was significantly increased in the lenalidomide group compared with non-transplant normal controls (31.8% vs 5.3%; *P*=0.008)(Figure 9.5); there was likewise a numerical increase in PD-1 expressing CD8 T-cells in transplant controls compared with normal controls however this did not reach statistical significance, possibly due to the smaller cohort size (21.8% vs 5.3%; *P*=0.19). The frequency of PD-1+ CD8 T-cells remained elevated at day 100 post-alloSCT in both the lenalidomide and transplant control groups, however there was no significant impact of lenalidomide therapy on PD-1 expression (lenalidomide 27.0% vs transplant controls 27.1%; *P*=0.95). Similarly, the frequency of TIM3+ CD8 T-cells was increased at day 30 post-alloSCT in lenalidomide patients (*P*=0.02) and transplant controls (*P*=0.06) compared with normal controls, however there was also no significant impact of lenalidomide treatment on TIM3+ cells by day 100 post-alloSCT (lenalidomide patients 13.2% vs transplant controls 24.0%; *P*=0.14).

Figure 9.4 CD8 T-cell reconstitution post-alloSCT

HD, healthy donor; TP, transplant controls; LEN, lenalidomide treated patients

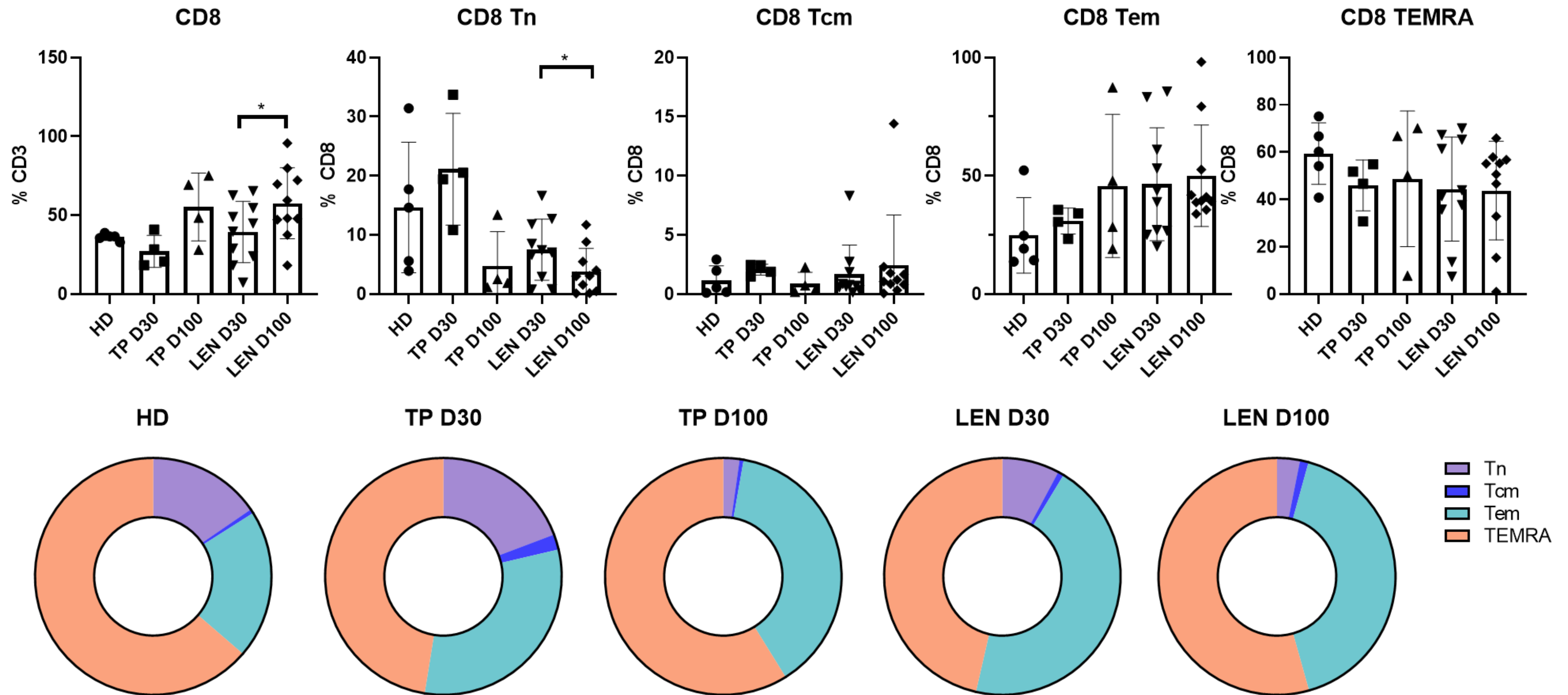
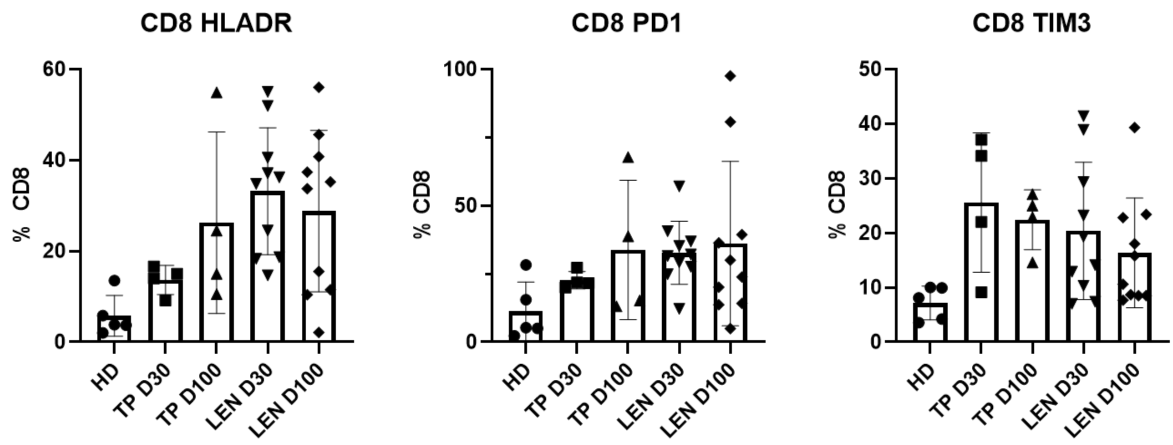


Figure 9.5 CD8 T cells: HLA-DR, PD1 and TIM3 expression



CD4 T-cells

The frequency of CD4 T-cells decreased in lenalidomide-treated patients from day 30 to day 100 (39.3% vs 33.9%; *P*=0.037) and a similar trend was noted in transplant controls (69.6% vs 27.4%; *P*=0.13) (Figure 9.6). The effector memory subset comprised the largest proportion of CD4 T-cells in both lenalidomide and transplant control patients, with a small but statistical increase in CD4 Tem between day 30 and day 100 in lenalidomide patients (48.8% vs 66.8%; *P*=0.049). Simultaneously, the proportion of CD4 Tn and TEMRA both decreased between days 30 and 100 post-alloSCT in the lenalidomide group (Tn: 16.2% vs 10.2%, *P*=0.006; TEMRA 4.6% vs 2.2%; *P*=0.002). Similar trends, although non-significant, were observed in control transplant patients. There was no significant difference in the proportion of Tn, Tem, Tcm or TEMRA between lenalidomide and transplant control patients at day 100 post-alloSCT.

Regulatory CD4 T-cells (Treg) have well-described roles in shaping innate and adaptive immune responses and suppression of autoreactivity via production of immunosuppressive cytokines including IL-10 and TGF-β and also induction of direct apoptosis of effector cells by Fas-Fas ligand interactions[53]. Diminution of Tregs post-alloSCT has been associated with acute and chronic GVHD, while simultaneously Tregs may be implicated in diminishing alloreactivity to promote malignancy relapse post-alloSCT[176, 177]. Administration of low dose IL-2 preferentially increased Tregs *in-vivo* with clinical responses observed in patients with steroid-refractory chronic GVHD[178]. Given that lenalidomide increases T-cell production of IL-2, the impact of post-transplant lenalidomide on Treg reconstitution was investigated, defined as CD3+CD4+CD25+CD127^{low}. Compared with normal controls, the frequency of Tregs was increased at day 30 post-transplant in the lenalidomide group (2.9% vs 1.5%;

P=0.037) and a similar trend was also observed in transplant controls (2.5% vs 1.5%; *P*=0.11) (Figure 9.7). The frequency of Tregs remained constant between day 30 and day 100 post-alloSCT in both lenalidomide and transplant control patients, and at day 100 there was no significant impact of lenalidomide treatment on Treg frequency (lenalidomide 3.3% vs transplant controls 2.7%; *P*=0.37).

Expression of HLA-DR, PD-1 and TIM3 on CD4 T-cells were increased in lenalidomide and transplant control patients compared with normal controls at both day 30 and day 100 post-transplant (Figure 9.8). However, there was no significant difference between lenalidomide patients and transplant controls at either timepoint, suggesting lack of impact of lenalidomide treatment on the expression of these activation and co-inhibitory markers.

Figure 9.6 CD4 T cell reconstitution

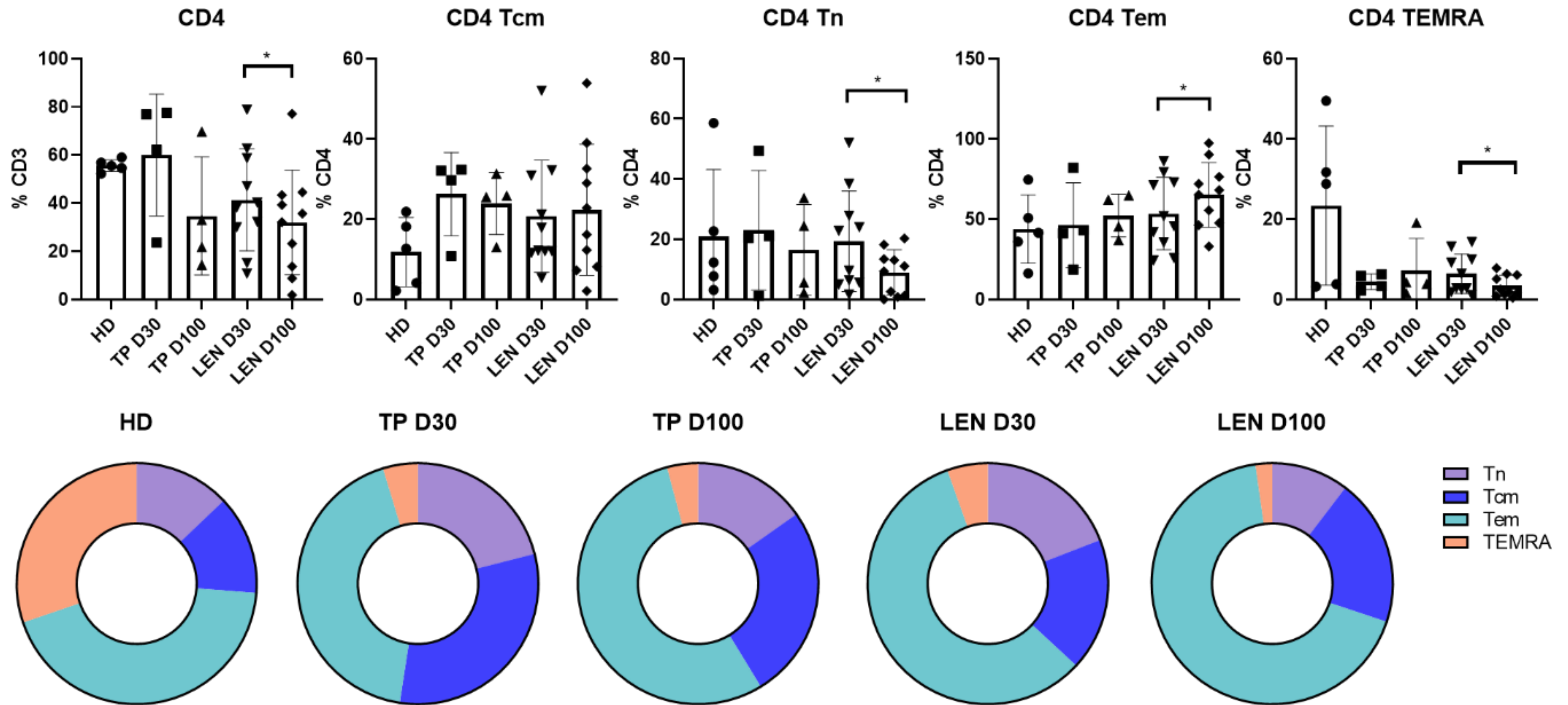


Figure 9.7 CD4 Treg reconstitution

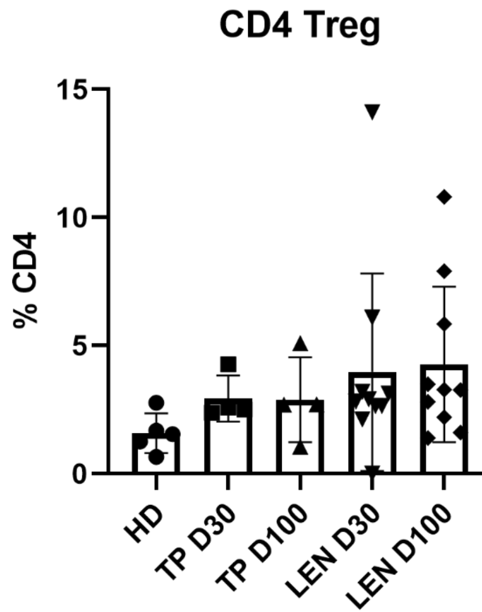
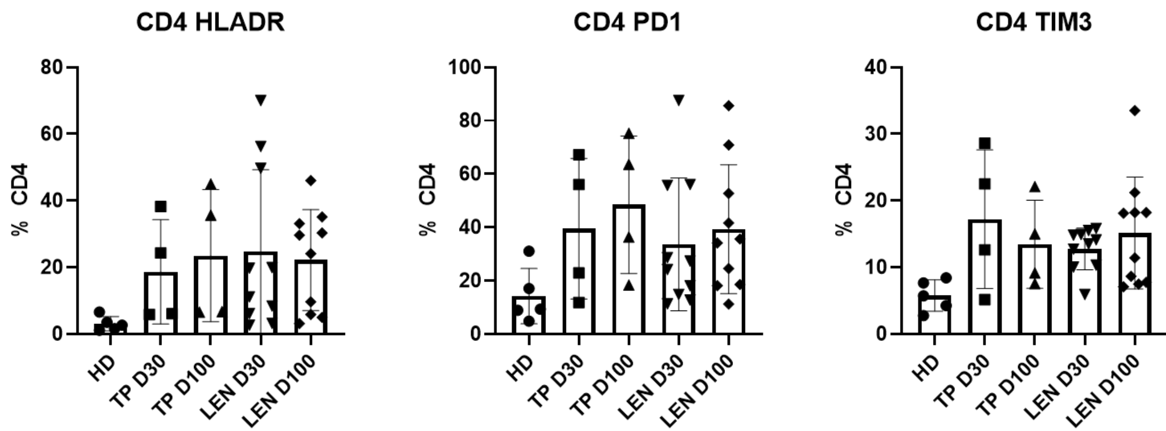


Figure 9.8 CD4 T-cells: HLA-DR, PD1 andTIM3 expression



9.4.2 NK-cells

NK-cells defined as CD3-CD56+ were significantly increased as a proportion of all lymphocytes at day 30 in both lenalidomide patients (22.2%) and transplant controls (24.9%) compared with normal controls (7.2%; lenalidomide vs normal *P*=0.0013; transplant controls vs normal *P*=0.032) (Figure 9.9), consistent with previous reports describing prompt numerical recovery of the NK cell compartment post-alloSCT[20]. By day 100, there was a decrease in the proportion of NK cells due to a corresponding increase in CD4 and CD8 T-cells in the lymphocyte compartment in both lenalidomide and transplant controls, so that NK cell frequency was similar to that of normal controls (day 100 lenalidomide 11.5% vs normal 7.2%; *P*=0.16; day 100 transplant controls 16.1% vs 7.2%; *P*=0.11). There was no significant difference in NK cell frequency at day 100 in lenalidomide patients or transplant controls (*P*=0.37).

Natural killer cells can be further subclassified into CD56dimCD16br NK cells which are functionally mature and demonstrate potent cytotoxic properties, and CD56brCD16dim NK cells which have less cytotoxic ability however are able to secrete cytokines including IFNγ. Lesser described NK cell subtypes include CD56brCD16br and CD56dimCD16dim. In healthy controls, the majority of NK cells were CD56dimCD16br (64.1%) with a lesser proportion being CD56brCD16dim (6.1%). In contrast, in both lenalidomide treated patients and transplant controls, the frequency of CD56brCD16dim NK cells exceeded that of the more functionally mature CD56dimCD16br subset at day 30 (CD56brCD16dim lenalidomide 30.0%, transplant controls 33.4%; CD56dimCD56br lenalidomide 19.1% transplant controls 17.5%) and day 100 post-transplant (CD56brCD16dim lenalidomide 27.5%, transplant controls 37.8%; CD56dimCD56br lenalidomide 28.0% transplant controls 22.8%).

Natural killer cell reactivity is determined by interaction between NK cell receptors and their cognate ligand on target cells and this interaction may deliver either an activating or inhibitory signal for NK cell function. The primary NK cell receptor is the killer immunoglobulin receptor (KIR) which interacts with MHC class I on target cells to deliver an inhibitory signal. Modulating the signal from KIR/MHC binding are other receptors including NK2GA, TIM3 and TIGIT which deliver inhibitory signals and NKG2D which provides an activating signal. The expression of NK cell receptors is dynamic and in part dependent on functional maturity. In addition, expression of NK cell receptor ligands is also variable, resulting in a highly context-dependent nature of NK cell reactivity against target cells such as leukaemia. In previous work, I and co-workers have demonstrated that the expression of NK receptor ligands on AML blasts significantly impact prognosis post-chemotherapy, thereby demonstrating that NK cell receptor and ligand interactions impact maintenance of remission in AML[123]. In the present microLEN study, I therefore investigated the impact of post-transplant lenalidomide on NK cell receptor expression.

At day 30 post-transplant, the frequency of KIR+ NK cells was significantly reduced in transplant controls compared with normal controls (39.6% vs 55.5%, *P*=0.032), with a similar trend also observed in the lenalidomide group (38.1% vs 55.5%, *P*=0.071) (Figure 9.10). On the contrary, the frequency of NKG2A+ NK cells was significantly greater at day 30 in both the lenalidomide group (57.7% vs 23.8%, *P*=0.013) and transplant controls (74.3% vs 23.8%, *P*=0.016) compared with normal controls. There was no significant difference in the expression of TIGIT, TIM3 or NKG2D between normal controls and day 30 post-transplant. Between day 30 and day 100 post-transplant, there was no significant change in the frequency of KIR, NKG2A, NKG2D,

TIM3 or TIGIT-expressing NK cells in either the lenalidomide or transplant control groups.

Figure 9.9 NK-cell reconstitution

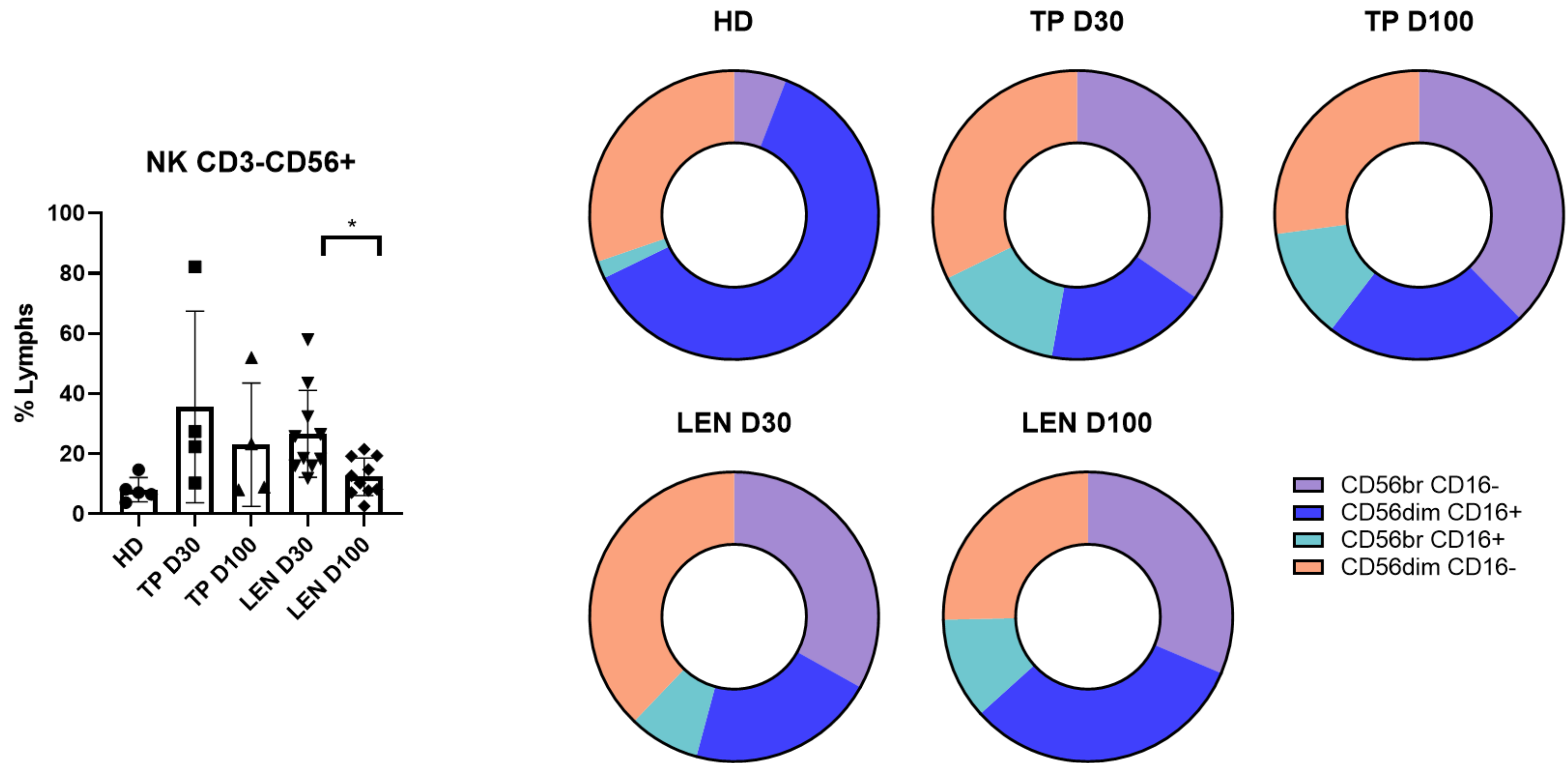
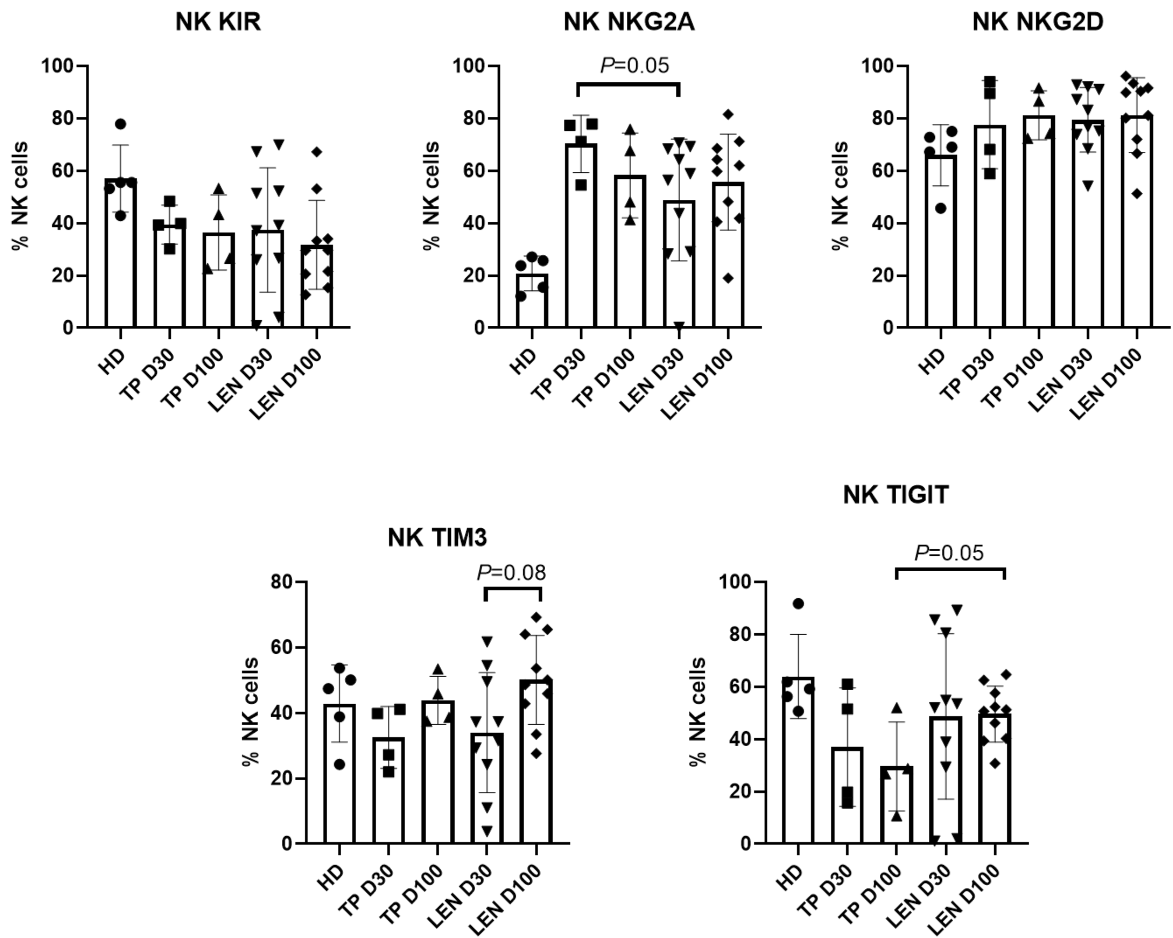


Figure 9.10 NK-cell receptor expression



9.5 Discussion

In this chapter, I have described preliminary clinical and correlative immunology findings from this phase I trial of lenalidomide in combination with early immunosuppression taper in patients with high risk AML and MDS. At the time of writing, the MTD of lenalidomide had not yet been reached and the trial remains open to patient accrual at the dose level of 5mg twice per week. At this interim stage of the study, several observations may be made with regards to both study design and preliminary results with a focus on safety.

The design of this study was notable for having a cohort size of 4 as opposed to the conventional 3+3 study design for phase I dose escalation studies. This design was used in order to accommodate an event rate (namely GVHD, cytopenias and infection) that would be expected for patients undergoing alloSCT in the absence of post-transplant immune manipulation with either early ciclosporin withdrawal or lenalidomide. As discussed in the methods section, the anticipated rate of such events which would be classified as DLTs was estimated to be 20-30% in a normal transplant population, hence making a 3+3 study design impractical for the high probability of resulting in premature study termination. This point highlights a specific design element that should be taken into account in post-transplant studies: that standard alloSCT procedures are high-risk in themselves and inherently prone to complication, therefore post-alloSCT maintenance studies should build-in a higher degree of tolerance to these expected adverse events than would be otherwise used in non-transplant trials. This is particularly the case in clinical trials that seek to add a therapeutic intervention early post-transplant, particularly within the first 3-6 months due to this period being the most prone to GVHD, infection and a fragile or dysfunctional graft. It is therefore not surprising that

many interventional studies avoid this period altogether. Given that the median time from transplant to relapse of AML is 5.5 months and that therapeutic interventions are most likely to be effective with low burden of disease, commencement of post-transplant therapies to prevent relapse are required in the early post-transplant period is needed, despite the practical difficulties of doing so[2]. The second and related issue for consideration in post-transplant interventional studies is the maximum tolerated event/DLT rate considered permissible for an intervention to be deemed as tolerable post-transplant. In the 4+4 study design adopted in this trial, a DLT rate of 50% was used to determine the MTD. Previous phase II studies using lenalidomide 10mg daily post-alloSCT were deemed excessively toxic with the incidence of grade 3-4 acute GVHD and other stopping criteria being between 60-70%[73, 74]. Although the classification of the limit of tolerability is somewhat arbitrary, a limit of tolerance set at less than 50% as was done in this study is potentially more clinically acceptable although results in a narrow therapeutic window which adds to the complexity of conducting post-transplant studies.

The lenalidomide dosing strategy for this study was cautious with a low starting dose of 2.5mg once per week (dose level 1). It is pertinent to note that in this study, lenalidomide was combined with early immunosuppression withdrawal, and hence the combined effect of these two strategies on the potential to provoke acute GVHD was unknown. This cautious approach therefore acknowledged the narrow therapeutic window to enhance immunological potency post-alloSCT. At the time of writing, the preliminary results of this study have demonstrated that lenalidomide at a dose of up of 2.5mg twice per week in combination with early ciclosporin taper is tolerable, with current recruitment ongoing at the 5mg twice per week cohort and the MTD of

lenalidomide as yet not being reached. At the dose levels completed, there have been 2 DLT's, with both cases being acute pneumonitis and occurring within 3 months post-alloSCT and within 2 months of commencement of lenalidomide. The precise aetiology of these two cases of pneumonitis are unknown with possibilities including either infective or inflammatory in nature. Pulmonary infections have been well described in patients receiving lenalidomide. In the phase 3 randomised study of lenalidomide 25mg daily in combination with either low or high-dose dexamethasone in patients with newly diagnosed myeloma, 12% of patients developed grade 3 or 4 infection or pneumonia with a significant difference in favour of the low dose dexamethasone group[179]. Similarly, in the phase 2 study of lenalidomide as monotherapy for patients with relapsed mantle cell lymphoma, 6% of patients developed grade 3 or 4 pneumonia[180]. A further 13% of patients developed upper respiratory tract infections although all of these were of grade 1 or 2 in severity. There have not been reports of pneumonitis occurring in patients receiving lenalidomide post-alloSCT. Pneumonitis and respiratory tract infections were not specifically noted in the phase 2 studies of lenalidomide post-alloSCT nor in the pilot study of lenalidomide in combination with ipilimumab post-transplant[73, 74, 181]. Pneumonitis has been described as a rare manifestation of acute GVHD following alloSCT and remains a diagnosis of exclusion[182]. It is possible that lenalidomide may increase the risk of this rare complication through a myriad of potential mechanisms including increased risk of infection provoking secondary pneumonitis, direct lung injury promoting local alloreactivity, or promoting immune tropism to the lung. The small number of patients in this study precludes more definitive conclusions about this observation and warrants further investigation in the final analysis of this trial.

The most common non-dose limiting toxicities were haematological cytopenias in particular worsening of baseline neutropenia. Eight patients (73%) developed grade 3 or 4 cytopenia in at least one lineage. Cytopenias occurred at all dose levels of lenalidomide without an apparent relationship between dose and degree of toxicity. Peripheral blood cytopenias are a well described adverse effect of lenalidomide, with grade 3 to 4 cytopaenias occurring in approximately 50% of patients receiving lenalidomide 25mg/d in non-transplant trials[179, 180, 183]. In HOVON-76 trial of lenalidomide 10mg/d post-alloSCT, grade 3 or 4 cytopenias were observed in 44% of patients[74]. Within the limitations of inter-study comparisons, there was a higher rate of cytopenias noted in our study. One contributing factor to this may have been the timing of lenalidomide commencement post-transplant, with myelosuppressive medications potentially having a greater effect on early marrow engraftment post-alloSCT. Consistent with this, in the present study lenalidomide was commenced significantly earlier (median day 45) compared with other phase 2 studies post-alloSCT (between 3 to 6 months post-transplant).

The incidence of acute GVHD in this study was 18% with one case of grade 3 acute GVHD. Importantly there were no cases of grade 3-4 acute gastrointestinal GVHD or cases of steroid refractory acute GVHD. The frequency of acute GVHD in this study is in keeping with registry data where acute GVHD (all grades) rates are approximately 30% in sibling transplants and 50% in unrelated donor transplants[184]. Likewise the observed incidence of chronic GVHD in this study (20% of patients who survived beyond day 100) is similar to expected rates of chronic GVHD in standard sibling and unrelated donor alloSCT[184]. This is somewhat surprising given the early reduction of immunosuppression incorporated into this study and suggests that even in the absence

of post-transplant lenalidomide, early immunosuppression taper is safe in patients with high-risk of disease relapse.

Overall and relapse free survival post-alloSCT in this study at 12 months was 51% and 64% respectively. Four deaths were due to leukaemia relapse and 1 death was secondary to transplant-related mortality (unrelated to lenalidomide) in a patient who developed EBV-induced post-transplant lymphoproliferative disease and cerebral haemorrhage. These figures are consistent with published data that in high risk leukaemia disease relapse remains the most common cause of death post-transplant over and above non-relapse mortality[185]. The cases of AML relapse following alloSCT all occurred within 3 months post-transplant with all patients having particularly adverse risk features. That 3 cases of relapse occurred in the first dose level and none to date at dose level 3 suggests that there may be a dose-response relationship of lenalidomide, however this observation is preliminary only and requires further evaluation after completion of accrual.

A strength of this study is the correlative immunology that was performed detailing T- and NK-cell reconstitution post-transplant. The analyses performed in this study demonstrated that there are significant differences in the frequency of T and NK-cell subsets between patients post-transplant and normal controls. Recapitulating previous descriptions of post-transplant immune reconstitution, patients post-transplant demonstrated a higher frequency of CD8 T-cells compared with CD4 T-cells resulting in an inversion of the CD4:8 ratio; there was a relative paucity of naïve T-cells in circulation with an increase in the frequency of Tem and TEMRA at both day 30 and 100 post-transplant likely reflecting peripheral expansion of donor-derived effector T-

cells upon allo-antigen stimulation and limited thymic generation of naïve T cells in an older patient population; T-cells demonstrated an activated phenotype with increased frequency of HLA-DR positive T-cells[20, 147]. Although not specifically examined here, the dependence on homeostatic expansion of adult-derived T-cell populations for early T-cell reconstitution likely accounts for the restricted TCR repertoire post-transplant as described in chapter 8 of this thesis. The expression of the inhibitory checkpoint receptors PD-1 and TIM-3 were also increased in CD4 and CD8 T-cells at day 30 and day 100 in transplant recipients compared with normal controls. Although T-cell expression of PD-1 and TIM-3 occur in the stepwise process of T-cell exhaustion following prolonged antigen stimulation, expression of these immune checkpoint receptors also occurs in the acute phase of T-cell activation[186]. In the post-transplant setting, work by Simonetta and co-workers demonstrated that despite increased frequency of PD-1+ T-cells early post-transplant, T-cells remained functionally competent with preserved cytotoxic potential[187]. In the present study, *in-vitro* functional analyses were not performed; however together with the increased T-cell expression of HLA-DR, our findings are consistent with an overall state of T-cell activation with associated PD-1 and TIM-3 upregulation in this context rather than one of T-cell exhaustion.

There were also significant differences in NK-cell phenotype between transplant patients and normal controls. Numerical recovery of NK-cells occurs rapidly following alloSCT and this is reflected in a relative increase in the frequency of NK-cells at both day 30 and day 100 as a proportion of lymphocytes. Post-transplant patients demonstrated an increase in the frequency of phenotypically immature CD56brightCD16dim NK cells with increased expression of the inhibitory receptor

NKG2A. Increased expression of NKG2A has been reported following haploidentical alloSCT and *in-vitro* impairs the NK-cell mediated GVT effect[26]. Therapeutic blockade of NKG2A restored NK-cell reactivity *in-vitro* and therefore may be a means of enhancing the NK-cell mediated GVT effect post-alloSCT.

A strength of this study is the comparison of immune reconstitution between lenalidomide treated patients with a contemporaneous transplant control group who did not receive post-transplant lenalidomide or early immunosuppression withdrawal. Immune reconstitution post-transplant is a highly dynamic process that is time-dependent; therefore between-group comparisons when evaluating the impact of a therapeutic intervention on immune reconstitution should take into account the time from transplant. At the dose cohorts completed to date, lenalidomide did not appear to significantly alter CD4, CD8 T-cell, Treg or NK-cell reconstitution by day 100 post-alloSCT. There was also no significant impact on T-cell activation or NK-receptor expression including NKG2A. It remains possible that either higher doses of lenalidomide, longer duration of treatment or longer follow-up (for example up to 1 year post-transplant) are required to observe any impact of post-transplant lenalidomide on immune reconstitution. At the time of writing, such analysis was not possible due to the paucity of patients who had completed 12 months of therapy. Further analysis upon completion of the entire cohort may be more informative.

Post-transplant maintenance strategies have been a focus of significant investigation in order to reduce the incidence of post-transplant AML relapse. Two such strategies, other than post-transplant lenalidomide, include hypomethylating agents and targeted small molecule inhibitors including FLT-3 inhibitors. Azacitidine as maintenance post-

alloSCT has been investigated in a phase 1 study post-alloSCT with the maximum tolerated dose identified to be 32mg/m^2 for 5 days when commenced 6 weeks post-alloSCT[188]. Although this dose was deemed to be tolerable, the primary toxicities were haematological cytopenias and 58% of patients completed only 1 or 2 cycles of azacitidine, findings that are mirrored in the present lenalidomide post-alloSCT study. In a phase 2 study, Craddock et al. used azacitidine 36mg/m^2 for 5 days commencing 42 days following reduced intensity alloSCT (fludarabine, melphalan and alemtuzumab) for high risk AML (RICAZA)[189]. In contrast to the previous phase 1 study, 43% of patients in the RICAZA study were able to complete 10 cycles of azacitidine. It is noteworthy that patients in the RICAZA study only commenced azacitidine upon stable engraftment defined as neutrophils greater than 1x10^9/L and platelets greater than 50x10^9/L (as opposed to a threshold of 15x10^9/L), suggesting that careful patient selection in particular those with robust engraftment is necessary prior to post-transplant azacitidine maintenance. Interestingly, patients who developed a CD8 T-cell response to leukaemia-specific antigens following azacitidine therapy had a reduced risk of relapse, suggesting that azacitidine may have a direct ability to enhance the GVT effect post-transplant[189]. More recently, a phase I study of the oral hypomethylating agent CC-486 as maintenance post-transplant demonstrated tolerability at doses up to 200mg/d for 14 days per cycle when started between days 42-84 post-alloSCT[190]. Alternatively, small molecule targeted inhibitors including FLT3 inhibitors have been investigated as maintenance post-transplant including several currently accruing phase 3 studies. The SORMAIN study was a placebo-controlled phase 3 study using sorafenib 400mg bd as post-transplant maintenance for patients with FLT3-ITD positive AML which demonstrated a significant reduction in relapse risk and improvement in RFS with sorafenib maintenance[191]. Similarly, the RADIUS study of post-transplant midostaurin

50mg bd commencing days 28-60 post-transplant with maintenance for 48 weeks demonstrated a reducing in relapse and improvement in RFS compared with standard of care, however no improvement in OS was observed[192]. Within this evolving landscape of post-transplant maintenance for high-risk AML, the utility of post-transplant lenalidomide is likely to be limited to a subset of patients without FLT3-ITD and for whom hypomethylating agents are not tolerable. This cohort may be restricted further in the future as other targeted therapies demonstrate efficacy in the post-transplant setting, including the potential for IDH1 and IDH2 inhibitors or venetoclax for NPM1-mutated AML, noting that these agents are yet to be investigated post-transplant[193].

In conclusion, the preliminary results of this phase 1 study demonstrate that the MTD of lenalidomide as maintenance post-alloSCT in combination with early immunosuppression taper has not been reached at a dose of up to 5mg twice per week. The most common toxicities were haematologic cytopenias and the rate of acute and chronic GVHD were low. This study remains open to patient accrual and further data is required to evaluate the MTD and expand upon correlative immunology analyses which as yet have not demonstrated significant impact of lenalidomide on post-transplant immune reconstitution.

10 NIVALLO: Nivolumab treatment for relapsed or residual haematological malignancies after allogeneic haematopoietic stem cell transplantation

10.1 Introduction

The current clinical armamentarium by which to treat relapsed haematological malignancies after alloSCT is limited and frequently unsuccessful. Fewer than 20% of patients with conditions other than chronic myelogenous leukaemia (CML) will obtain a response to DLI alone and the majority of these responses are not sustained[194]. Donor lymphocyte infusions also carry the risk of significant toxicity with graft-versus-host disease (GvHD) observed in up to 60% of patients. A second alloSCT achieves modest responses, however many patients are unable to proceed with a second alloSCT due to comorbidity or lack of donor availability.

New approaches to treat relapse after alloSCT are therefore required, based on an understanding of the potential mechanisms that facilitate escape from the graft-versus-leukemia effect. There is some evidence that the programmed cell death protein-1 (PD-1) and associated ligand (PD-L1) pathway may be implicated in disease relapse after alloSCT either through T-cell exhaustion (PD-1) or through leukaemia upregulation of PD-L1 allowing immunological escape. PD-1 is a negative costimulatory receptor of the immunoglobulin gene superfamily that can be expressed on the surface of T-cells, B-cells, NK-T cells, activated monocytes, and dendritic cells. The binding of PD-1 by its ligands PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273) results in phosphorylation of PD-1 on intracellular tyrosine residues within its immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). Protein phosphatases recruited to ITSM are then activated and inhibit

TCR signalling.[195] PD-L1 is expressed on many solid tumours including glioblastoma and melanoma among others. PD-L1 is also expressed on the malignant cells in a variety of haematologic malignancies, including Hodgkin lymphoma, primary mediastinal B-cell lymphoma, T-cell lymphomas, multiple myeloma, and acute leukaemias. In addition, PD-L1 expression by non-malignant cells in the tumour microenvironment also contribute to tumour evasion from the host immune response.[196]

In the post-alloSCT setting, Kong and colleagues performed phenotypic and functional studies on T-cells from patients with acute myeloid leukaemia after alloSCT.[50] The PD-1^{bright}TIM3+ CD8+ T-cell phenotype seen as a marker of T-cell exhaustion was more frequently observed in patients with AML relapse than in those in ongoing remission. These T-cells also demonstrated functional impairment (reduced intracellular TNF-α and IL-2 release following PMA/ionomycin and antiCD3/CD38 stimulation), consistent with the phenotype of T-cell exhaustion. Norde and colleagues demonstrated that PD-L1 expression was increased on leukaemia cells of two patients (one chronic myelogenous leukaemia in accelerated phase; one acute myeloid leukaemia) who relapsed after alloSCT, and that minor histocompatibility antigen (MiHA) specific CD8+ T-cells also expressed increased levels of PD-1.[49] *In-vitro* blockade of PD-1 augmented the proliferation of MiHA specific CD8+ T-cells. These findings support the hypothesis that PD-1/PD-L1 are a mechanism by which leukaemic cells are able to evade the immune response by inducing T-cell dysfunction or exhaustion, and that inhibition of PD-1 is able to restore T-cell proliferative capacity.

The greatest experience with the therapeutic use of checkpoint inhibitors is in the non-alloSCT setting. In a phase I study, 23 patients with relapsed refractory Hodgkin

lymphoma (HL) received nivolumab 3mg/kg every 2 weeks until complete response, tumour progression, or excessive toxicity.[197] Overall response rate (ORR) was 87% including 17% complete response. Adverse events were predominantly of grade 1 or 2. Notably, the incidence of grade 3 immune-related adverse events (irAE) such as colitis, pneumonitis or pancreatitis was less than 20%. An open-label phase I dose escalation study of the PD-1 inhibitor nivolumab in relapsed or refractory lymphoid malignancies included B-cell non-Hodgkin lymphoma (B-NHL; 29 patients), primary mediastinal B-cell lymphoma (PMBL; 2), T-cell NHL (T-NHL; 23) and multiple myeloma (MM; 27)[198]. Patients with prior alloSCT were excluded. Response rates varied according to malignancy type, with ORR 28% for B-NHL, 17% for T-NHL, however no responses were observed in MM. The incidence of irAE ranged from 4-7%.

Although acting via a different pathway, reports of CTLA-4 blockade post alloSCT support the concept that checkpoint blockade may be an effective means of enhancing GVM activity post alloSCT. Twenty-nine patients with relapsed or progressive haematological malignancies post alloSCT were treated with a single infusion of the CTLA-4 inhibitor ipilimumab in a phase 1 dose-escalation study.[199] Hodgkin lymphoma comprised 48% of cases, NHL/CLL 9%, and myeloid malignancies 12%. Responses were observed in 3 patients (2 HL, 1 B-NHL), and no patient developed GvHD of any grade. Immune-related adverse events were observed in 4 patients, including polyarthritis, hyperthyroidism, and pneumonitis in 2 patients.

Taken together, the current pre-clinical and clinical data support the concept that pharmacological blockade of co-inhibitory pathways may be able to augment the GVM effect to treat relapse post-alloSCT. To explore this hypothesis I conducted a

prospective clinical trial to evaluate the safety and efficacy of PD-1 blockade with nivolumab to treat relapsed haematological malignancies post-alloSCT (NIVALLO).

10.2 Clinical trial design

NIVALLO is a phase Ib open-label study of nivolumab, a fully human monoclonal IgG4 antibody targeting the PD-1 receptor on T-cells. It has been conducted at the Royal Melbourne Hospital and Peter MacCallum Cancer Centre commencing 4 April 2017 and remains open to accrual at both sites. The primary endpoint of the study is the cumulative incidence of GVHD at specified time points (8, 24 and 48 weeks) after the first dose of nivolumab. The secondary endpoint of the study is the overall response rate (ORR) at specified time points (8, 24 and 48 weeks) after the first dose of nivolumab.

10.2.1 Participant Enrolment

This study has a recruitment target of 14 patients with a potential to increase to 20 patients if required (after evaluation of the initial cohort) to further determine the efficacy (secondary) endpoint. At the time of writing, this study had enrolled 11 patients of which 9 had received at least 1 dose of nivolumab. The enrolment plan recognised that the intervention is intending to induce a response (anti-tumour effect) through the same mechanism as it might induce the AE of most concern (GVHD). Therefore the study recruitment plan was staggered so that once three patients had completed a minimum of two cycles and grade 2 or higher GVHD occurred in not more than one patient, the study opened three further enrolment slots. The initially enrolled patients and the three subsequently enrolled patients were included in an interim safety assessment. If any patients withdrew from study for any reason other than GVHD they were to be replaced as one of the six patients for the interim safety assessment.

The safety and data monitoring committee (SDMC) comprising the principal investigator, senior co-investigators and an independent haematologist with expertise in management of alloSCT patients convened after the initial 6 patients had received at least one dose of nivolumab on this study. Based on this experience as well as other local experience indicating that a lower dose of PD-1 inhibitor post-alloSCT may be able to produce potent GVT responses with less GVHD, the protocol was amended to reduce the starting dose of nivolumab. Review of further results by the SDMC was planned after treatment of a further 3 patients with at least one dose of nivolumab.

10.2.2 Inclusion Criteria

All the following criteria were required for inclusion in the study:

- Age>18 years
- Prior alloSCT for a haematological malignancy (all types of transplant irrespective of donor, conditioning regimen, or GVHD prophylaxis will be eligible)
- Confirmed relapse of haematological malignancy or persistent disease post alloSCT, documented by either tissue biopsy or peripheral blood/bone marrow morphology, flow cytometry, cytogenetics or molecular analysis (see below for definition of relapsed or persistent disease for specific diseases).
- Immunosuppression cessation for a minimum of 2 weeks
- Life expectancy >2 months
- ECOG performance status 0-2
- Screening laboratory values must meet the following criteria and should be obtained within 28 days prior to registration:

- ≥30% CD3+ T-cell donor chimerism
- Serum creatinine ≤1.5 x ULN or creatinine clearance (CrCl) ≥40mL/min using the Cockroft-Gault formula
- AST/ALT≤3 x ULN
- Total bilirubin ≤ 1.5 x ULN (except participants with Gilbert Syndrome, who must have total bilirubin < 50micromol/L).

- Women of childbearing potential (WOCBP), as defined in this protocol below, must use appropriate method(s) of contraception. WOCBP should use an adequate method to avoid pregnancy for 32 weeks (30 days plus the time required for nivolumab to undergo five half-lives) after the last dose of nivolumab
- Women of childbearing potential must have a negative serum or urine pregnancy test within 24 hours prior to the start of nivolumab
- Men who are sexually active with WOCBP must use any contraceptive method with a failure rate of less than 1% per year. Men receiving nivolumab and who are sexually active with WOCBP will be instructed to adhere to contraception for a period of 32 weeks after the last dose of nivolumab.
- Signed written informed consent

Relapse or residual disease for specific diseases was defined as follows:

- Acute leukaemia:
  - Relapse with between 5-30% blasts or reappearance of minimal residual disease either
    - untreated, or

        - no less than 4 weeks after salvage therapy consisting of chemotherapy alone, or
        - a minimum of 2 months after completion of DLI

- Myelodysplastic syndrome:
    - Relapse or residual disease defined as:
        - Evidence of previously observed or new cytogenetic abnormalities
        - Bone marrow blasts 5-20%
        - Evidence of morphologic dysplasia in two or more lineages not attributable to other causes including, but not limited to, medications, post-transplantation changes, infection or haematinic deficiencies
    - AND
    - Patients must be either untreated or no less than 4 weeks after salvage chemotherapy or a minimum of 2 months after completion of DLI
- Non-Hodgkin lymphoma, CLL, multiple myeloma, Hodgkin lymphoma, CML, myelofibrosis, other myeloproliferative neoplasms:
    - Relapse or persistent disease after alloSCT either
        - untreated, or
        - no less than 4 weeks after salvage therapy consisting of chemotherapy alone, or
        - a minimum of 2 months after completion of DLI

10.2.3 Exclusion Criteria

Any of the following were considered criteria for exclusion from the study:

- Current evidence of any grade of GVHD
- Prior history of grade 2 or higher acute GVHD
- Moderate chronic GVHD within the previous 6 months or any prior history of severe chronic GVHD
- Active, known or suspected autoimmune disease (excluding vitiligo, type 1 diabetes mellitus, residual hypothyroidism due to autoimmune condition only requiring hormone replacement, psoriasis not requiring systemic treatment, or conditions not expected to recur in the absence of an external trigger)
- Positive hepatitis B virus surface antigen
- Positive hepatitis C virus antibody
- Known human immunodeficiency virus infection

10.2.4 Study Treatment

Participants received nivolumab intravenously in 2 weekly intervals for a maximum duration of 48 weeks. The study was commenced using the nivolumab dose of 3mg/kg which was administered to the first 6 participants. Following review of these preliminary results by the SDMC, the study protocol was revised on the 28 May 2018. The revised dosing strategy took into account emerging observations that 1) lower doses of nivolumab may afford less GVHD but still provide a potent GVT effect; 2) in patients without early GVHD and who are yet to achieve responses, there may be scope to increase the dose of nivolumab to 3mg/kg.

Therefore, the revised dosing strategy (reflected in the revised Study Protocol version 1.6) reduced the initial dose of nivolumab for cycles 1 and 2 and subsequent dosing was based according to an assessment of response after these first two cycles:

- Cycle 1 and cycle 2
    - Nivolumab 1.5mg/kg
- Response assessment was performed after cycle 2 (but before cycle 3) to determine subsequent dosing:
    - If CR or PR and ≤grade 1 acute GVHD or ≤mild chronic GVHD, nivolumab was continued at 1.5mg/kg
    - If SD or no response and no GVHD, nivolumab dose was increased to 3mg/kg.
    - If SD or no response and grade 1 acute GVHD or mild chronic GVHD, nivolumab 1.5mg/kg was continued for the remainder of dosing
    - If grade 2 acute GVHD developed once the dose was been increased to 3mg/kg, further doses were withheld and nivolumab resumed at 1.5mg/kg when GVHD resolved
    - If grade 2 acute GVHD developed at the dose of 1.5mg/kg, further doses were withheld and nivolumab resumed at 0.75mg/kg when GVHD resolved
    - If grade 3 or greater acute GVHD or moderate/severe chronic GVHD, nivolumab was discontinued
- If a participant achieved a prior PR to nivolumab on study but developed progressive disease at a subsequent time point, with no evidence of GVHD, and remained at 1.5mg/kg, consideration was made to increase nivolumab to 3mg/kg for up to 4 doses prior to subsequent response assessment. If there was evidence of response (CR or PR) or stable disease at this subsequent time point with no evidence of GVHD, nivolumab was continued at 3mg/kg. If there was evidence of disease progression, study participation was ceased.

While the maximum duration of therapy was permitted to be 48 weeks, early cessation was permitted in patients achieving complete response, in recognition that further administration of nivolumab may provoke GVHD in patients already achieving maximal responses. Therefore, the following criteria were established for continuation of therapy following response assessment at cycles 4, 8, 12 and 24:

- Complete remission: nivolumab was continued for 2 ‘maintenance’ cycles then ceased; patient then entered the follow-up period.
- >50% tumour response: nivolumab continued until next response assessment timepoint, then re-assessment performed;
- Stable disease: nivolumab continued until next response assessment timepoint, then re-assessment performed;
- Progressive disease: nivolumab was ceased.

Re-initiation of study therapy for participants in follow-up period

Participants in the follow-up period with ongoing disease control (CR or PR) for a period of at least 6 months after the last dose of nivolumab were permitted to re-initiate study therapy upon confirmed disease progression. Participants re-initiating therapy were required to meet eligibility criteria at the time the study drug resumed and should not have experienced a SAE requiring discontinuation of study therapy. Participants that resumed therapy in this setting received study therapy for up to an additional 48 weeks, or until study cessation, whichever came first.

10.2.5 Management of Graft Versus Host Disease

Murine models have suggested that GVHD is in part mitigated by T-cell inactivation via the PD-1/PD-L1 pathway. As a result, PD-1 blockade may conceivably increase the occurrence of GVHD. The management of GVHD in this clinical trial was according to the institution's local policy, which included commencement of systemic corticosteroids at 1-2mg/kg for grade 2 or higher acute GVHD. Acute GVHD was graded according to the Glucksberg criteria and chronic GVHD according to the NIH consensus criteria[151, 152]. Wherever possible, histological confirmation of GVHD of affected organs was obtained.

Doses of nivolumab were withheld following the onset of GVHD according to the following criteria:

- Grade 1 acute GVHD: further doses of nivolumab were withheld until GVHD resolved then nivolumab resumed at the same dose
- Grade 2 acute GVHD: further doses of nivolumab were withheld. Consideration was made to resume at a lower dose and schedule if GVHD was responsive and resolved within 4 weeks and in the absence of tumour progression
- Grade 3-4 acute GVHD: nivolumab was ceased
- Participants who developed new onset moderate to severe chronic GVHD permanently ceased nivolumab.

10.2.6 Nivolumab Discontinuation Criteria

The following criteria were established for permanent discontinuation of nivolumab:

- Grade 3 or 4 acute GVHD or new onset moderate to severe chronic GVHD

- Any Grade 2 drug-related uveitis or eye pain or blurred vision that did not respond to topical therapy and did not improve to Grade 1 severity within the re-treatment period OR required systemic treatment
- Any Grade 3 non-skin, drug-related adverse event lasting > 7 days, with the following exceptions for drug-related laboratory abnormalities, uveitis, pneumonitis, bronchospasm, hypersensitivity reactions, and infusion reactions, and endocrinopathies:
- Any Grade 4 drug-related adverse event or laboratory abnormality, except for the following events which did not require discontinuation:
    - Isolated Grade 4 amylase or lipase abnormalities that were not associated with symptoms or clinical manifestations of pancreatitis and decreased to < Grade 4 within 1 week of onset.
    - Isolated Grade 4 electrolyte imbalances/abnormalities that were not associated with clinical sequelae and were corrected with supplementation/appropriate management within 72 hours of their onset
    - Grade 4 lymphopenia or leucopenia
    - Grade 4 drug-related endocrinopathy adverse events, such as adrenal insufficiency, ACTH deficiency, hyper- or hypothyroidism, or glucose intolerance, which resolved or were adequately controlled with physiologic hormone replacement (corticosteroids, thyroid hormones) or glucose-controlling agents, respectively, did not require discontinuation
- Any dosing interruption lasting > 6 weeks with the following exceptions:
    - Dosing delays or interruptions to allow for prolonged steroid tapers to manage drug-related adverse events were allowed.

- Dosing interruptions or delays lasting > 6 weeks that occur for non-drug-related reasons were allowed

- Any adverse event, laboratory abnormality, or intercurrent illness which presented a substantial clinical risk to the subject with continued nivolumab dosing required treatment cessation

10.2.7 Response Assessments

Prior to the amendment Protocol 1.6, the first response assessment was after 4 doses of nivolumab, with subsequent assessments after 8, 12 and the last dose of nivolumab. The first 6 participants therefore followed this assessment schema. Following amendment Protocol 1.6, an earlier response time-point was introduced to determine subsequent nivolumab dose; therefore, the first response assessment was performed after 2 cycles, with subsequent response time-points of 4, 8, 12 and the last dose of nivolumab. Response assessments were performed according to established criteria for each category of haematological malignancy[200-208].

10.3 Clinical trial results

10.3.1 Patient recruitment

NIVALLO was opened to patient recruitment on the 4 April 2017 at the Royal Melbourne Hospital and Peter MacCallum Cancer Centre. Up to the 20 June 2019, 11 patients with relapsed haematological malignancies after alloSCT provided written informed consent for inclusion into the study. Two of these participants failed the screening process for commencement of study treatment due to development of interstitial pneumonitis in one patient and acute GVHD in the second patient, both occurring within the screening period.

Over the same period of time that the study was been open to recruitment at this site, an additional 43 patients developed relapse of a haematological malignancy post-alloSCT but did not participate in the NIVALLO study. Twenty-nine patients (67%) were ineligible to participate in the study due degree of disease burden (26%), current or previous GVHD (30%), T-cell chimerism less than the required 30% donor origin (5%) or concurrent illnesses which precluded trial participation (5%). Fourteen patients (32%) met criteria for inclusion into the study however did not participate due to physician preference for alternative treatment for relapse, including chemotherapy alone or in combination with donor lymphocytes (14%), novel agents and targeted therapies (12%), DLI alone (2%), radiotherapy (2%) or an alternative clinical trial (2%).

10.3.2 Patient baseline characteristics

Nine patients received treatment with nivolumab on this study. Patient characteristics at baseline are described in Table 10.1. The most common indication for treatment was post-transplant relapse of acute myeloid leukaemia (AML). Of the 3 patients with AML relapse, 2 patients had isolated extramedullary AML. The third patient with AML had a poor prognosis subtype with a complex monosomal karyotype and three TP53 coding mutations. There were 2 patients with Hodgkin lymphoma included in this study, one of whom had prior exposure to a PD-1 inhibitor with no response pre-transplant. Eight out of the 9 patients had morphologic relapse post-transplant. One patient with T-cell acute lymphoblastic leukaemia had emergence of minimal residual disease (MRD) in the bone marrow detected by multiparameter flow cytometry without overt morphologic relapse. No patient had active acute or chronic GVHD at the time of study inclusion; 3 patients had a prior mild chronic GVHD which had completely resolved at the time of study

participation. None of the patients were on any systemic immunosuppression for the treatment of GVHD at the time of study inclusion. However, two patients (1 AML, 1 T-ALL) were receiving oral prednisolone for treatment of disease-related fever.

Table 10.1 NIVALLO baseline characteristics

Characteristic	**Value**
Age, median (range)	52 (24-66)
Sex, M/F	7/2
Haematological malignancy	
AML	3
ALL	2
HL	2
NHL	1
CLL	1
Transplant donor source	
Sibling	2
UD	6
Umbilical cord blood	1
Conditioning intensity	
MAC	5
RIC/NMA	4
Stem cell source	
PB	8
BM	0
Umbilical cord blood	1
T-cell depletion	5
Haematopoietic chimerism at relapse, median % (range)	

CD3+	100 (70-100)
CD3-	100 (48-100)
Months from alloSCT to relapse, median (range)	6 (1-49)
Months from alloSCT to nivolumab, median (range)	11 (4-75)

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukaemia; UD, unrelated donor; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; NMA, non-myeloablative; PB, peripheral blood; BM, bone marrow.

10.3.3 Nivolumab treatment

The median time from alloSCT to first dose of nivolumab was 6 months (range 1-49 months). Three patients commenced study treatment with nivolumab within 6 months of alloSCT. The median time from immunosuppression cessation to first dose of nivolumab was 6.7 months (range 0.5-69.8 months). A median of 4 doses of nivolumab were administered per patient (range 1-19 doses). One patient received an initial course of 6 doses of nivolumab and was re-treated with a further 13 doses for subsequent relapse as per study protocol. Seven patients commenced on nivolumab at a dose of 3mg/kg; 2 patients commenced at a dose of 1.5mg/kg following the study protocol amendment.

10.3.4 Adverse events

Adverse events including GVHD are described in Table 10.2. Eight patients developed at least 1 adverse event of any grade; 3 patients developed grade 3 or 4 adverse events. Three patients developed acute GVHD of any grade, including 2 patients who developed grades 3 to 4 acute GVHD. Out of these 3 patients, 2 had been commenced on nivolumab at 3mg/kg, and 1 patient commenced treatment at the reduced dose of 1.5mg/kg. The patients who developed acute GVHD did so rapidly following initiation of nivolumab. The two patients who developed grades 3-4 acute GVHD had onset 5 days and 13 days following the first dose of nivolumab. In both of these cases nivolumab was ceased after the first dose. Both patients had multi-organ acute GVHD requiring systemic immunosuppression. An additional patient developed grade 1 cutaneous acute GVHD and mild oral and ocular chronic GVHD following two doses of nivolumab. The three patients who developed GVHD commenced nivolumab a median of 4.8 months post-alloSCT (range 4.8-7.1 months). In contrast, patients who did not

develop GVHD commenced nivolumab a median of 33.4 months post-alloSCT (range 3.9-74.6) (Mann Whitney U test *P*=0.15). None of the patients who developed GVHD with nivolumab had a prior history of GVHD post-alloSCT. The three cases of GVHD are described in detail below due to their clinical significance.

Acute GVHD case 1

RMH007 was a 38 year old man with Hodgkin lymphoma refractory to multiple lines of treatment including ABVD chemotherapy, brentuximab, high dose chemotherapy with autologous stem cell support and 6 cycles of PD-1 blockade with pembrolizumab with no response. He achieved a second complete remission with prednisolone, etoposide and cyclophosphamide chemotherapy. This was consolidated with a reduced intensity conditioned matched sibling alloSCT. He subsequently relapsed post-alloSCT at day 123 with stage 4 disease including widespread lymph node involvement and extranodal involvement of the left scapula, ribs and sacrum. Immunosuppression was rapidly withdrawn. Peripheral blood chimerism demonstrated that 99% of T-cells were donor-derived. Study treatment with nivolumab 3mg/kg was commenced 145 days post-alloSCT (15 days following immunosuppression cessation). Five days following the first dose of nivolumab he developed acute onset large volume diarrhoea and hyperbilirubinemia consistent with acute gastrointestinal and hepatic GVHD (grade 3) which was confirmed on colon biopsy. Treatment of GVHD consisted of sequential initiation of methylprednisolone (2mg/kg), cyclosporin (3mg/kg) and etanercept (25mcg for four doses) with complete resolution of this episode of GVHD within 26 days. The patient subsequently developed several other complications including CMV reactivation, microangiopathic haemolytic anaemia secondary to ciclosporin, and recurrence of GVHD following cessation of ciclosporin. Despite re-initiation of

immunosuppression including methylprednisolone and ciclosporin, he developed an acute bowel perforation and died.

Acute GVHD case 2

RMH008 was a 57 year old man with mantle cell lymphoma who underwent a non-myeloablative (fludarabine and low-dose cyclophosphamide) 8/8 HLA-matched unrelated donor alloSCT. Post-transplant relapse occurred at day 56. Study treatment with nivolumab 3mg/kg was commenced 140 days post-alloSCT (54 days following immunosuppression cessation). He developed multi-organ acute GVHD (gastrointestinal, ocular, cutaneous and liver, maximum grade 3) with onset 13 days following nivolumab. Intravenous methylprednisolone and mycophenolate mofetil were commenced with rapid improvement of gastrointestinal and cutaneous manifestations but persistent liver and ocular GVHD. He was commenced on extracorporeal photopheresis (ECP) for treatment of GVHD with gradual improvement noted by 9 months following the first dose of nivolumab.

Following the first 2 cases of grade 3-4 acute GVHD, which occurred after a total of 6 patients had been treated with at least 1 dose of nivolumab, the study SDMC convened to assess the appropriateness of the study design. Acknowledging that although severe GVHD is undesirable, however a modicum of GVHD may be required to afford tumour responses, the study protocol was amended with the hypothesis that 1) lower doses of nivolumab may afford less GVHD but still provide a potent GVT effect; 2) in patients without early GVHD and who are yet to achieve responses, there may be scope to increase the dose of nivolumab to 3mg/kg. Therefore, the protocol was amended to reduce the starting dose of nivolumab to 1.5mg/kg with an assessment after cycle 2 to

determine dose escalation depending on tumour response as well as the occurrence of GVHD (refer to 'Study Treatment' above). The third case of acute GVHD occurred following commencement at the reduced dose of 1.5mg/kg.

Acute and chronic GVHD case 3

RMH010, a 52 year old man with T-cell ALL (T-ALL) underwent a sibling myeloablative alloSCT in first complete remission, and was in a minimal residual disease (MRD) negative CR at day 100 post-alloSCT. At day 186 post-alloSCT, repeat bone marrow biopsy demonstrated re-emergence of MRD detectable by flow cytometry. He commenced nivolumab treatment on post-transplant day 215 at a dose of 1.5mg/kg. Following 2 doses of nivolumab, he developed grade 1 cutaneous acute GVHD and mild oral and ocular chronic GVHD. A repeat bone marrow biopsy was performed at this time, which showed clearance of MRD. Due to the depth of response achieved as well as emerging GVHD, the study investigators elected to cease further nivolumab treatment. The cutaneous acute GVHD was treated with topical hydrocortisone to a complete response. His chronic GVHD symptoms have also been managed with topical agents (topical ocular lubricants); his symptoms persist at the time of last assessment.

Overall, three patients (33%) developed dose-limiting toxicities (DLT), including the aforementioned 2 cases of grade 3-4 acute GVHD. The third DLT occurred in a patient who developed grade 3 pneumonitis with onset 47 days after commencement of nivolumab. There were 11 severe adverse events (SAE) recorded on this study, all of which were considered thus due to requirement for hospital re-admission. Notable SAEs included both cases of acute GVHD, 2 cases of pneumonitis, and one patient who developed postural orthostatic tachycardia syndrome (POTS).

Table 10.2 NIVALLO adverse events

Adverse event	Number of events
Acute GVHD	
Grade 1-2	1
Grade 3-4	2
Chronic GVHD	
Mild	1
Other adverse events, grade 2 and considered possibly related to nivolumab	
Hypothyroidism	1
Dizziness	1
Dyspnoea	1
Pharyngeal mucositis	1
Fall	1
Pneumonitis	1
Fever	1
Headache	1
ALT increased	1
AST increased	1
Other adverse events, grade 3 or 4 of any relatedness to nivolumab	
Pulmonary embolism	1
Pneumonitis	1
Haemorrhage	1
GGT increased	1
Neutrophil count decreased	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase.

10.3.5 Clinical responses

All 9 patients were evaluable for disease response. The overall response rate (ORR) was 44%, comprising four patients who achieved complete responses (CR) to nivolumab treatment; there were no partial responses. Responses were observed at both 3mg/kg and 1.5mg/kg nivolumab doses. The disease subtypes of patients who achieved responses were Hodgkin lymphoma (2 patients), mantle cell lymphoma (1) and T-cell ALL (1). Of the patients with Hodgkin lymphoma, 1 patient had prior exposure and non-response to PD-1 blockade prior to alloSCT. This patient achieved CR following 1 dose of nivolumab, demonstrating that prior non-response to PD-1 inhibition pre-transplant may not correlate with post-transplant responses, likely due to the latter being in the context of a new donor immune system.

The median time to response was 38.5 days (range 26-112 days). In 3 patients, the best response was achieved at first disease assessment. One patient with mantle cell lymphoma achieved a partial response at first assessment; nivolumab treatment was ceased after the first dose due to grade 3 acute GVHD. Later disease reassessment at day 112 showed that the patient had achieved CR. The median duration of response was 5.6 months (range 1.7-8.2 months). Two patients relapsed 8 months after achieving CR. One of these patients with Hodgkin lymphoma who did not develop GVHD with nivolumab treatment was re-commenced on nivolumab and achieved a second CR. The second patient who developed relapse had grade 3 GVHD following nivolumab therapy and therefore was not eligible for re-treatment. One patient died in CR due to GVHD.

Of the 4 patients who achieved CR, 3 patients (75%) developed GVHD. The one patient who achieved CR without GVHD had Hodgkin lymphoma with a low burden of disease

and was considerably later post-transplant (55 months) compared with the 3 patients who developed CR with GVHD (median 4.8months). Five patients did not have demonstrable responses to nivolumab treatment. This included all 3 patients with AML, 1 patient with large cell transformation of CLL and 1 patient with B-ALL. There were no cases of GVHD in patients who did not respond to nivolumab.

10.4 Peripheral blood T-cell immunophenotype and function

Detailed characterisation of peripheral blood T-cell memory subsets, cellular activation and functional studies of *in-vitro* cytokine production were performed to interrogate the impact of nivolumab treatment post-alloSCT. Comparison was made to normal patient controls.

10.4.1 CD8 T-cells

Trial participants had a significantly greater proportion of CD8 T-cells prior to nivolumab treatment compared with normal controls (64.2% [IQR 48-65.6%] vs 27.6% [IQR 15.1-37.5%]; *P*=0.004) (Figure 10.1A). Trial participants also had a significantly greater proportion of CD8 Tem (49.1% [IQR 35.5-61.9%] vs 26.7% [IQR 18.2-35.0%]; *P*=0.02) and a lower proportion of Tn (10.1% [IQR 1.5-16.5%] vs 33.4% [23.4-49.4%]; *P*=0.006) at baseline compared with normal controls (Figure 10.1B). There were no significant differences between trial participants and normal controls with respect to the proportion of Tcm or TEMRA. T-cell expression of CD38 and HLA-DR is representative of cellular activation. The frequency of CD8 T-cells that expressed CD38 or HLA-DR was greater among trial participants compared with healthy donors (CD38: 2.2% [IQR 1.8-6.1%] vs 0.8% [IQR 0.6-0.9%] *P*=0.002; HLA-DR: 7.6% [IQR 3.4-11.1%] vs 0.7% [0.5-1.4%] *P*=0.008) (Figure 10.1C, D). The increase in HLA-DR+

CD8 T-cells in trial participants was observed in all naïve and memory subsets; the increase in CD38+ CD8 T-cells was observed in Tem and TEMRA subsets but not Tn or Tcm. There was a significantly greater frequency of TIM3+CD8+ T-cells in trial participants compared with normal controls (15.8% [IQR 11.5-25.7%] vs 5.0% [IQR 4.0-7.7%]; P=0.002), however there was no difference in PD-1 expression between the two groups (18.6% [IQR 9.6-39.3%] vs 12.1% [8.6-16.6%]; P=0.24) (Figure 10.1J, K).

Serial peripheral blood samples were obtained following treatment with nivolumab to allow analysis of the immunological impact of therapy. CD8 T-cell PD-1 receptor binding by nivolumab was near complete with minimal residual unbound PD-1. Nivolumab treatment did not significantly impact the proportion of naïve and memory T-cells or expression of CD38, HLA-DR or TIM3 over time (Figure 10.2).

Figure 10.1 CD8 T-cell naïve and memory subsets at baseline and following treatment with nivolumab

Comparisons between study participants and normal controls (HD). * denotes P<0.05.

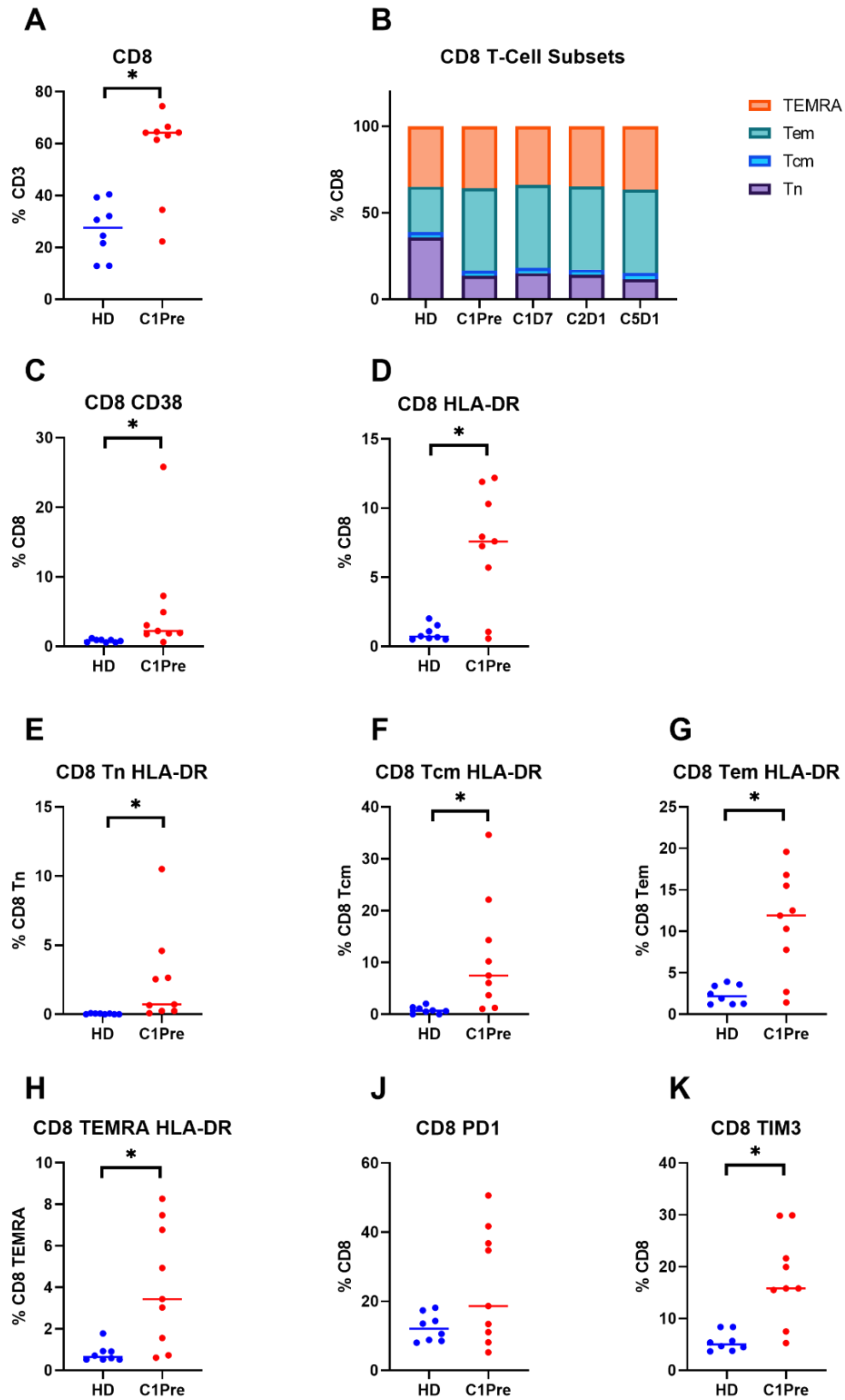
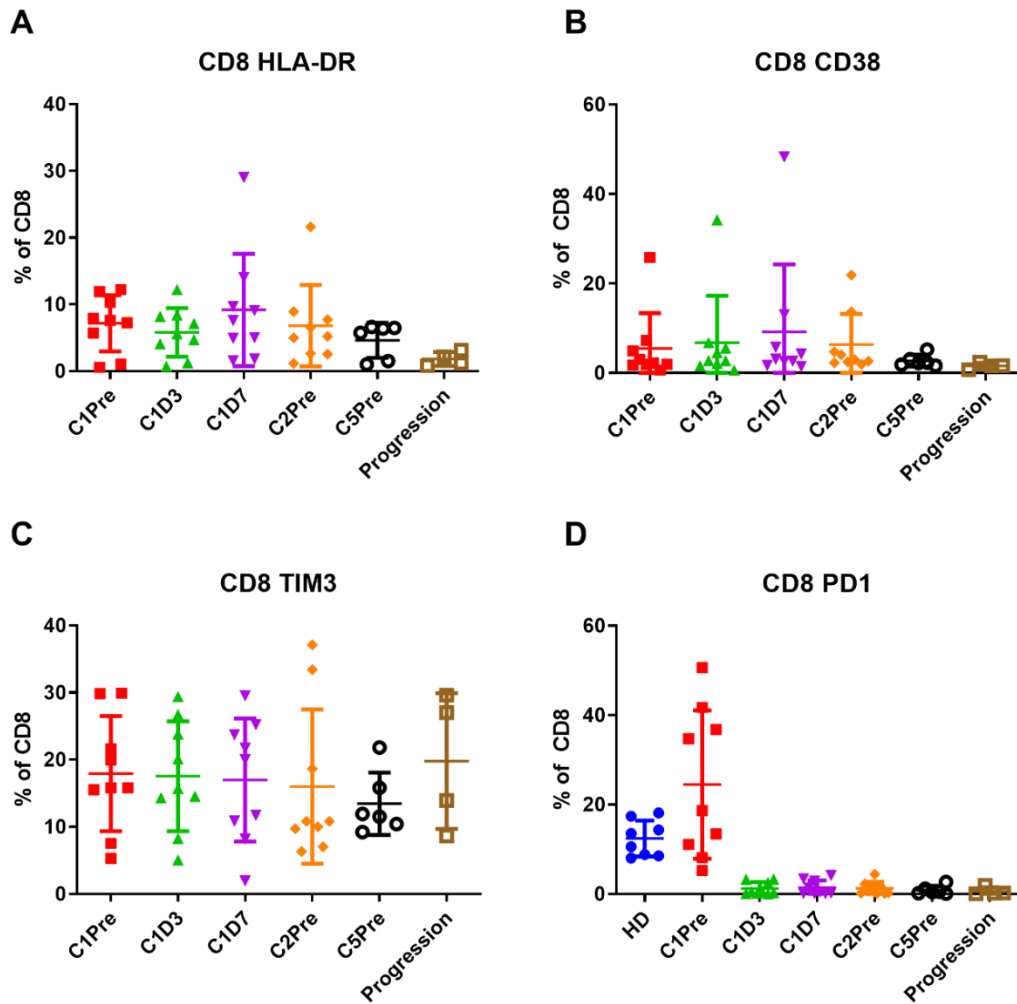


Figure 10.2 Changes in CD8 T-cell activation and inhibitory receptor expression following nivolumab treatment

There was no significant difference in (**A**) HLA-DR, (**B**) CD38 or (**C**) TIM-3 expression following treatment with nivolumab. (**D**) PD-1 receptor occupancy was virtually complete following nivolumab treatment.



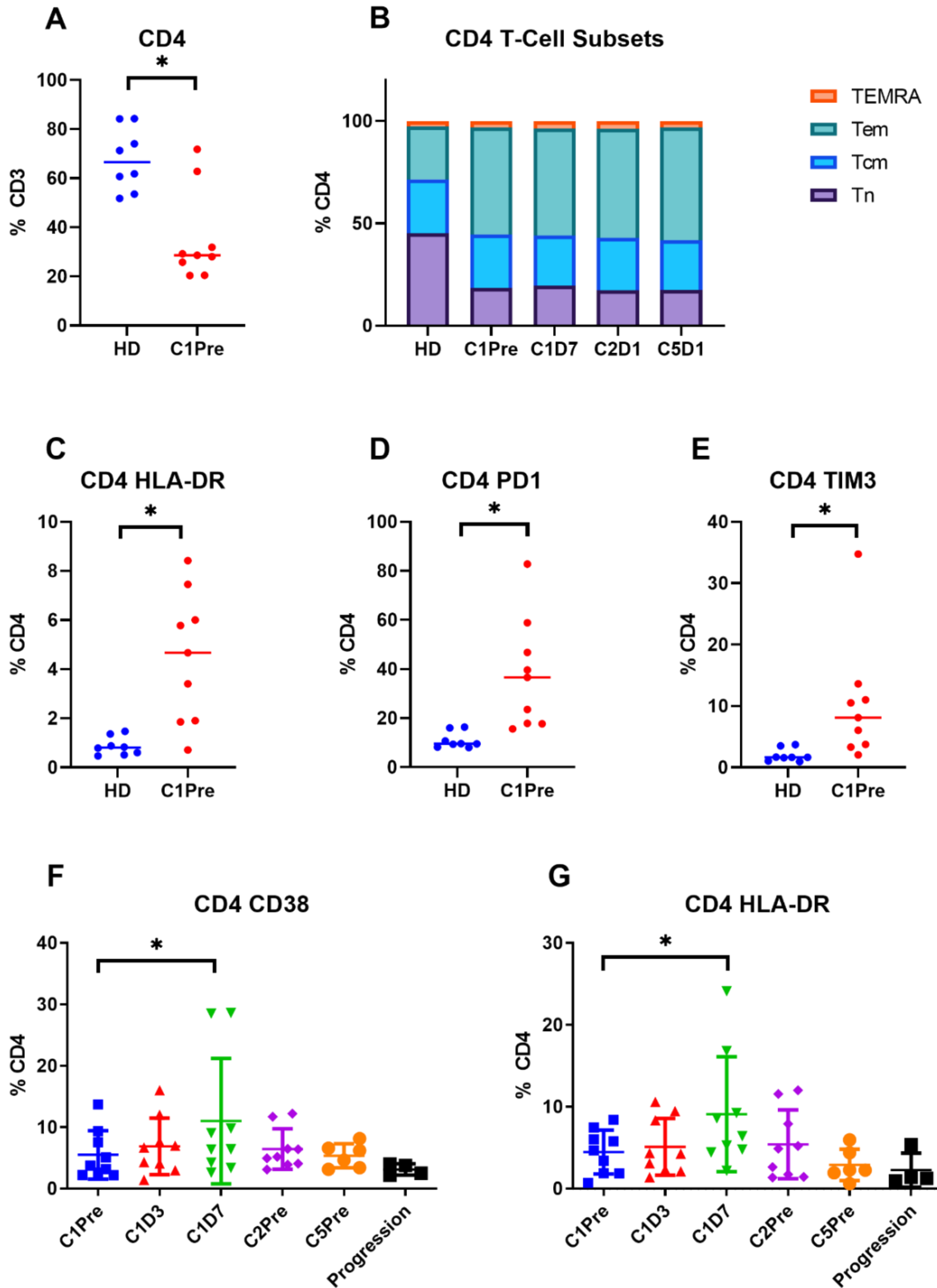
10.4.2 CD4 T-cells

CD4 T-cells were significantly reduced as a proportion of the total T-cell pool in trial participants compared with normal controls (28.6% [IQR 23.1-47.4%] vs 66.6% [IQR 55.3-81.7%]; P=0.008), consistent with the normal post-transplant pattern of prolonged CD4 T-cell lymphopenia (Figure 10.3A). Similar to that observed in the CD8 T-cell compartment, trial participants had a reduced proportion of Tn (13.1% [IQR 7.7-30.9%] vs 46.1% [35.4-54.6%]; P=0.001) and an increased frequency of Tem (52.6% [IQR 32.4-67.4%] vs 26.4% [IQR 21.2-28.8%]; P=0.02) compared with normal controls (Figure 10.3B). At baseline, trial participants had a greater frequency of HLA-DR+ CD4 T-cells (4.7% [IQR 1.9-6.7%] vs 0.8% [IQR 0.5-1.2%]; P=0.002) and also increased expression of PD-1 (36.6% [17.8-52.9%] vs 9.6% [8.5-14.7%]; P<0.001) and TIM3 (8.1% [3.5-12.3%] vs 1.7% [1.2-3.1%]; P<0.001) compared with controls (Figure 10.3C-E).

Treatment with nivolumab was accompanied by an increase in HLA-DR and CD38 expression among CD4+ T-cells by day 7 that returned to baseline by cycle 2, suggesting that CD4 T-cell activation by nivolumab may be cyclical in nature (Figure 10.3F, G). CD4 T-cell naïve and memory subset frequencies remained unchanged following nivolumab treatment.

Figure 10.3 CD4 T-cell subsets and activation at baseline and follow nivolumab treatment

Comparisons between baseline and normal controls (**A-E**) and dynamic changes in CD4 T-cell activation following treatment with nivolumab (**F, G**).



10.4.3 Intracellular Cytokine Production

CD8 T-cells from trial participants at baseline demonstrated increased IFNγ production following *in-vitro* stimulation compared with normal controls (CD8: 1.8% [IQR 1.3-3.1%] vs 0.9% [IQR 0.4-1.2%], *P*=0.02), however there was no difference in IL2 or TNF production by CD8 T-cells between the two groups (Figure 10.4A-C). CD4 T-cells from trial participants at baseline had greater IFNγ (1.6% [IQR 1.1-2.9%] vs 0.4% [IQR 0.2-0.5%]; *P*=0.003 and IL2 (2.6% [IQR 1.9-5.5%] vs 1.4% [IQR 0.8-1.5%]; *P*=0.02) production compared with controls, suggesting that although CD4 T-cells from trial participants had increased surface expression of PD1 and TIM3 they remained functionally intact (Figure 10.4D-F).

Paradoxically, cytokine production by CD4 T-cells decreased in serial patient samples taken following treatment with nivolumab. Production of IFNγ by CD4 T-cells was reduced by day 7 following nivolumab treatment compared with baseline (1.1% [IQR 0.5-2.5%[vs 1.6% [IQR 1.1-2.9%]; *P*=0.008) as was IL2 production (2.0% [IQR 1.2-3.4%] vs 2.6% [IQR 1.9-5.5%]; *P*=0.02) (Figure 10.5). There was no significant change in TNF production by CD4 T-cells over time. Similarly, there was a non-significant trend towards decreased IFNγ production by CD8 T-cells by cycle 1 day 7 of nivolumab treatment compared with baseline (1.1% [IQR 0.6-2.4%] vs 1.8% [IQR 1.3-3.1%]; *P*=0.06). The cause for this unexpected finding remains to be clarified, however one possible explanation is that treatment with nivolumab *in-vivo* resulted in T-cell activation and cytokine release which then resulted in a gradual loss of ability of T-cells to respond to subsequent re-stimulation *in-vitro*.

Figure 10.4 CD8 and CD4 T-cell cytokine production in study participants at baseline compared with normal controls

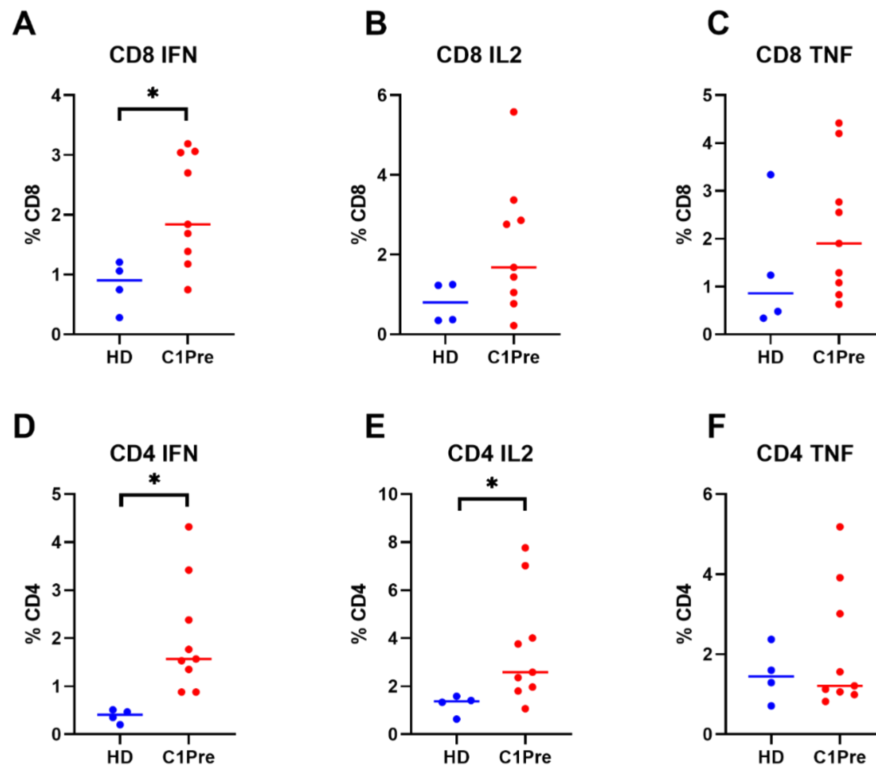
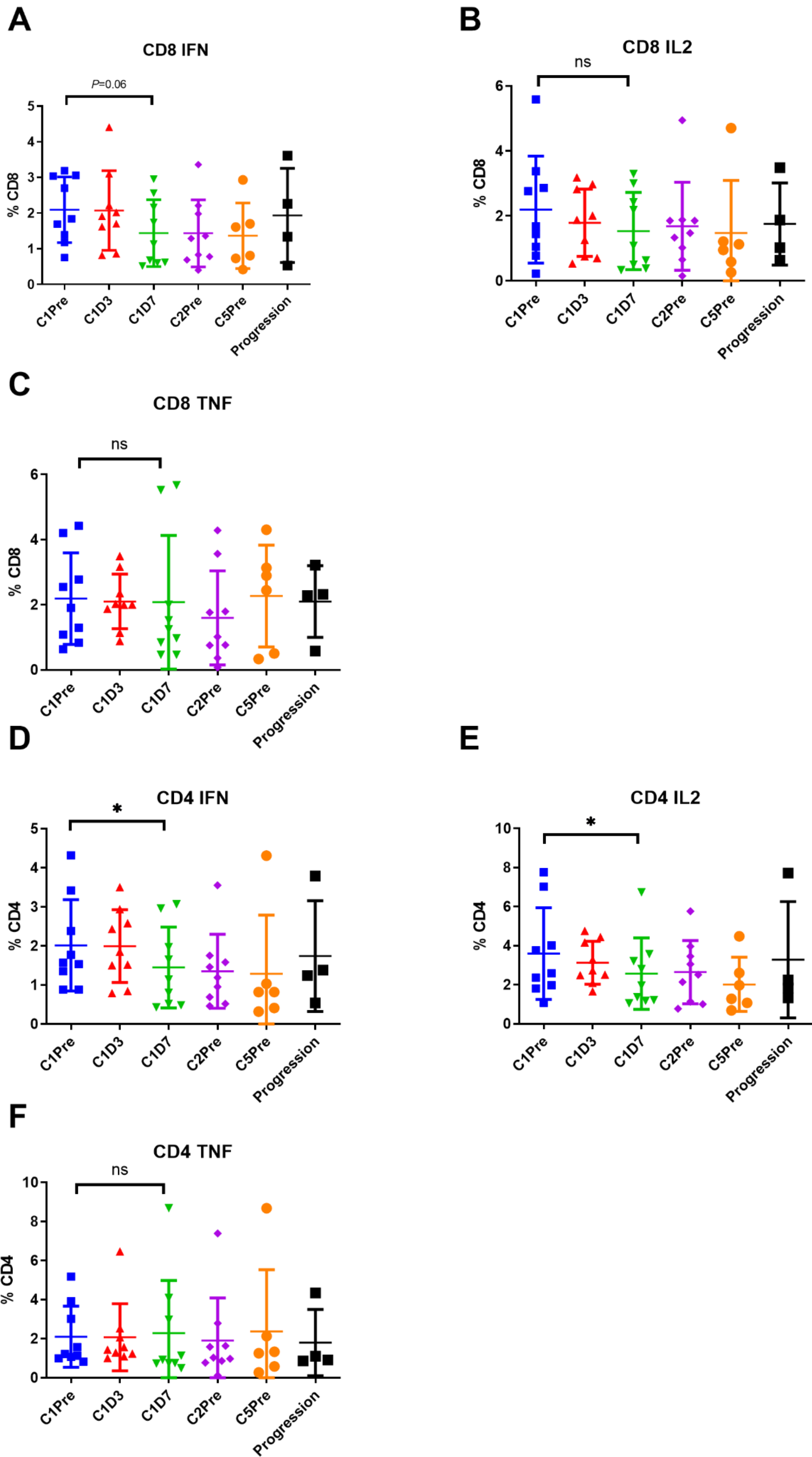


Figure 10.5 T-cell cytokine production following nivolumab treatment.

Intracellular cytokine staining following in-vitro CD3/CD28 bead stimulation demonstrated a non-significant trend towards decreased IFN secretion by CD8 T-cells at C1D7 compared with baseline (**A**) but no significant differences in CD8 T-cell IL2 (**B**) or TNF (**C**) production over time. There were statistically significant decreases in CD4 T-cell IFN (**D**) and IL2 (**E**) production over time and no change in CD4 T-cell production of TNF (F) following nivolumab treatment.

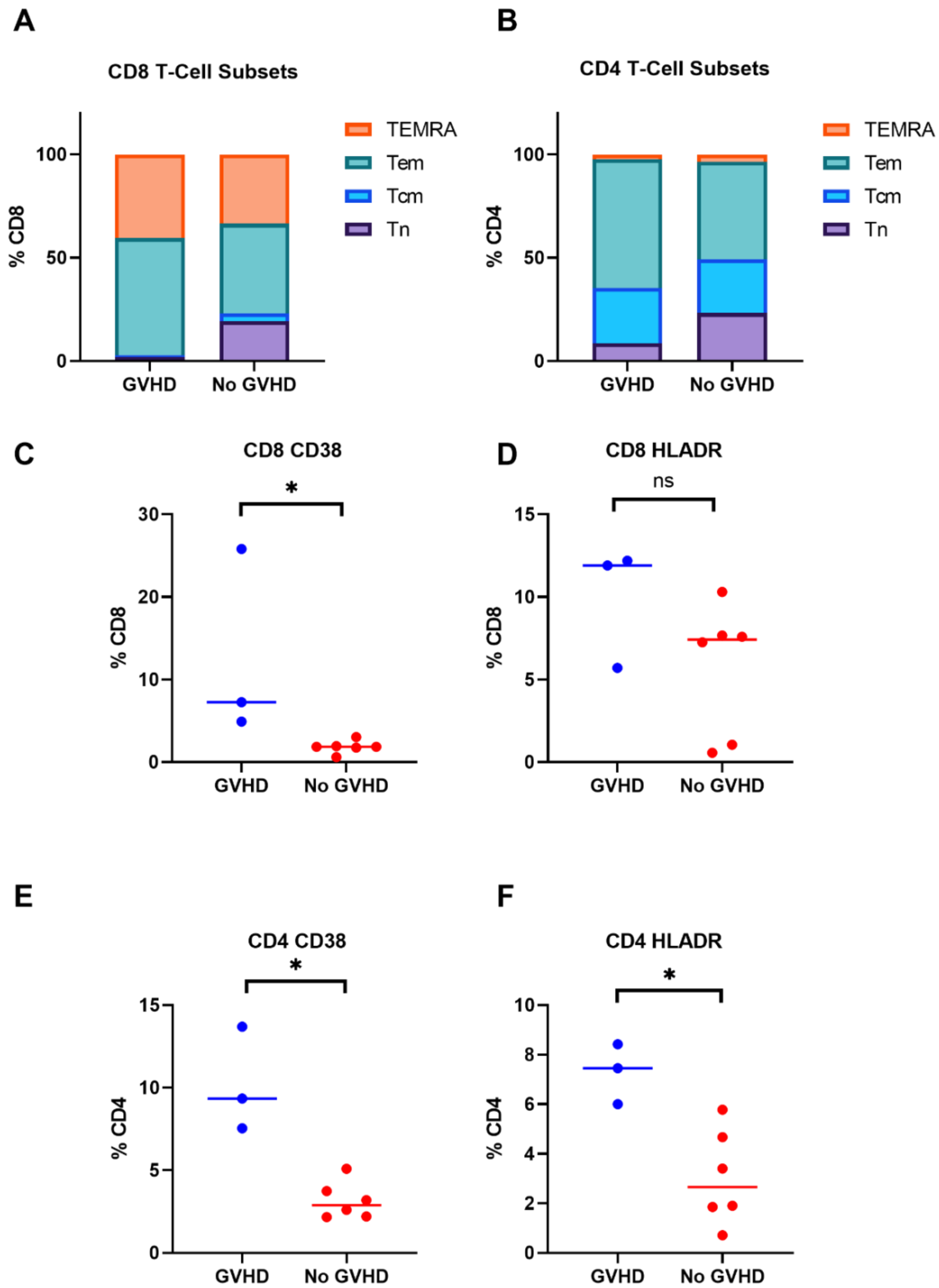

A
CD8 IFN
P=0.06
% CD8
C1Pre
C1D3
C1D7
C2Pre
C5Pre
Progression
B
CD8 IL2
ns
% CD8
C
CD8 TNF
ns
% CD8
D
CD4 IFN
*
% CD4
E
CD4 IL2
*
% CD4
F
CD4 TNF
ns
% CD4

10.4.4 Baseline predictors of GVHD and response

Given that GVHD is a major concern with post-transplant checkpoint inhibitor therapy, immunological biomarkers at baseline prior to nivolumab treatment that can identify those patients at greatest risk of GVHD may be able to guide therapy. Likewise, baseline predictors of response may also allow nivolumab treatment to be used in a patient-specific manner. There were no significant differences in the proportion of naïve or memory CD8 or CD4 T-cell subsets in baseline samples between participants who subsequently developed GVHD following nivolumab treatment compared to whose who did not (Figure 10.6A, B). Patients who developed GVHD had a greater proportion of CD38+ CD8 T-cells at baseline (7.3% [IQR 4.9-25.8%] vs [1.9% [IQR 1.5-2.2%]; P=0.03) and also a greater proportion of CD38+ CD4+ T-cells (9.4% [IQR 7.5-13.7%] vs 2.9% [IQR 2.2-4.1%]; P=0.02) and HLA-DR+ CD4 T-cells (7.5% [IQR 6.0-8.4%] vs 2.7% [IQR 1.6-4.9%]; P=0.02) compared to patients who did not develop GVHD (Figure 10.6C-F). There were no significant differences in CD4 or CD8 T-cell production of IL2, TNF or IFNγ between patients who developed GVHD compared to those who did not. There were also no significant differences in T-cell phenotype or cytokine production between responders and non-responders to nivolumab treatment.

Figure 10.6 Immunophenotypic predictors of subsequent GVHD following nivolumab treatment.

Interrogation of CD8 and CD4 T-cell subsets (**A, B**) and expression of CD38 and HLA-DR (**C-F**) at baseline in patients who subsequently developed GVHD compared to those who did not develop GVHD.



10.4.5 CD8 T-cell gene expression

Gene expression by CD8 T-cells prior to and following nivolumab treatment was examined with particular attention to 13 pre-specified key mRNA transcripts and a larger exploratory cohort of 594 genes. Nivolumab treatment resulted in a decrease in TNF (baseline log2 mRNA expression 6.0±1.6 vs C1D7 4.9±0.6; *P*= 0.05) and IFNG (7.6±0.4 vs 7.1±0.4; *P*=0.05) mRNA expression by day 7 that persisted until the end of treatment (Figure 10.7), consistent with the previously observed decrease in cytokine production following *in-vitro* stimulation in flow cytometry experiments. There were no significant changes in expression of other genes over time.

T-cell immunophenotyping described in this work previously identified that patients who subsequently developed GVHD had an activated T-cell phenotype at baseline. Consistent with this, patients who developed GVHD had significantly greater baseline expression of GZMA (11.6±0.04 vs 8.4±0.3; *P*<0.001), GZMB (9.9±0.1 vs 7.2±0.4; *P*=0.003) and PRF1 (12.3±0.08 vs 9.8±0.3; *P*<0.001) compared to patients who would remain free of GVHD (Figure 10.8A-C), further lending support to the concept that some patients may have been primed to develop GVHD following nivolumab therapy due to having a greater degree of T-cell activation and cytotoxic potential. Patients who subsequently developed GVHD also had increased expression of CD45RO (9.6±0.7 vs 7.9±0.3; *P*=0.02), CTLA4 (4.3±0.1 vs 3.1±0.2; *P*=0.006) and LAG3 (8.5±0.3 vs 6.7±0.4; *P*=0.02) compared to patients who did not develop GVHD, however there was no difference in PDCD1 or HAVCR2 expression at baseline (Figure 10.8D-J). There were no significant differences in baseline TNF, IFNG expression between patients with or without GVHD also consistent with flow cytometry results. Furthermore, there were no significantly differentially expressed genes among the expanded exploratory gene

set, noting that the small sample size and the large number of genes analysed with subsequent B-Y adjustment for multiple comparisons may have limited the statistical power to identify such differences. Despite the lack of statistically significant differences in this expanded gene set, there were trends towards differential expression of several genes that may be further explored in a larger cohort of patients (Figure 10.9). These included upregulation of SLAMF7, IL10RA, CX3CR1 in patients who developed GVHD compared to those who did not.

Figure 10.7 CD8 T-cell TNF and IFNG gene expression at baseline and following nivolumab treatment

Comparison of paired samples between baseline and C1D7 (n=8) (**A, B**) and baseline and end of treatment (EOT; n=10) (**C, D**).

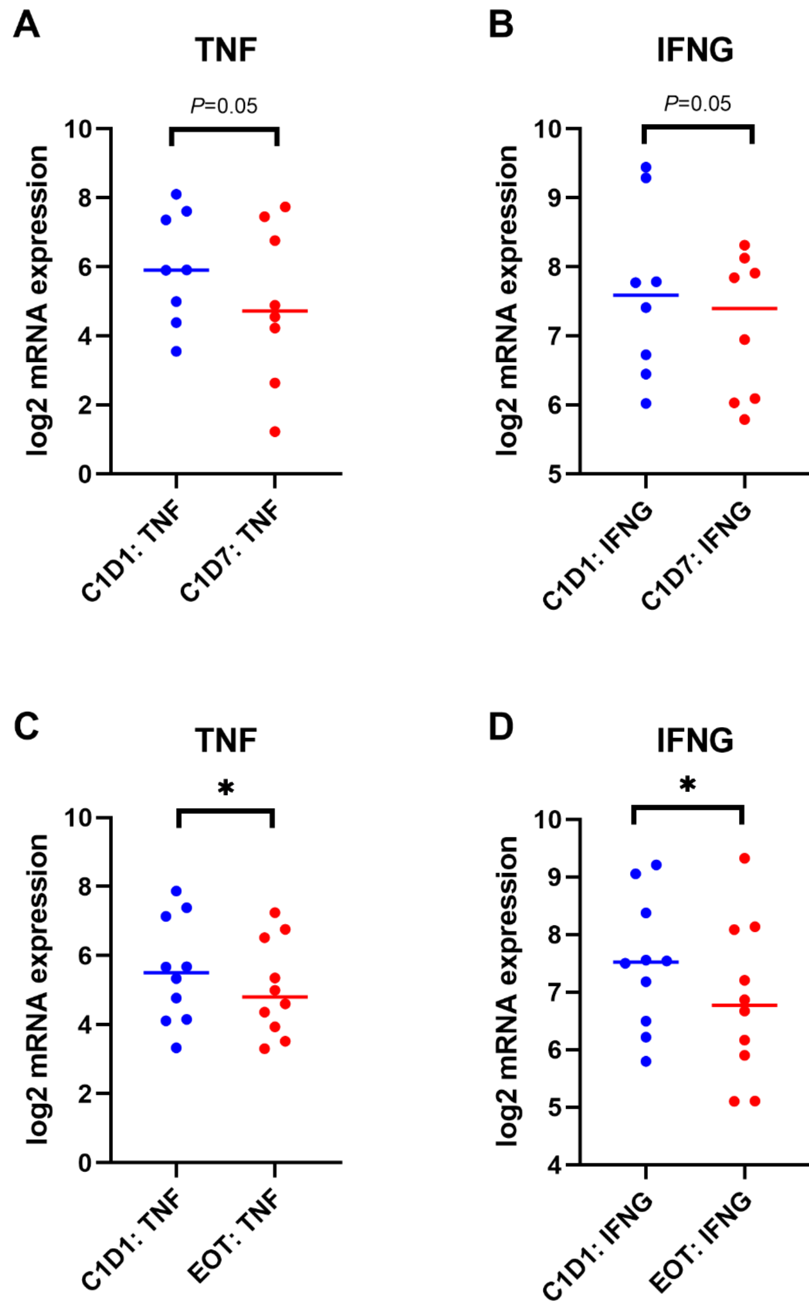


Figure 10.8 Gene expression predictors of subsequent GVHD following nivolumab treatment

CD8 T-cell gene expression at baseline in patients who subsequently developed GVHD compared to those who did not.

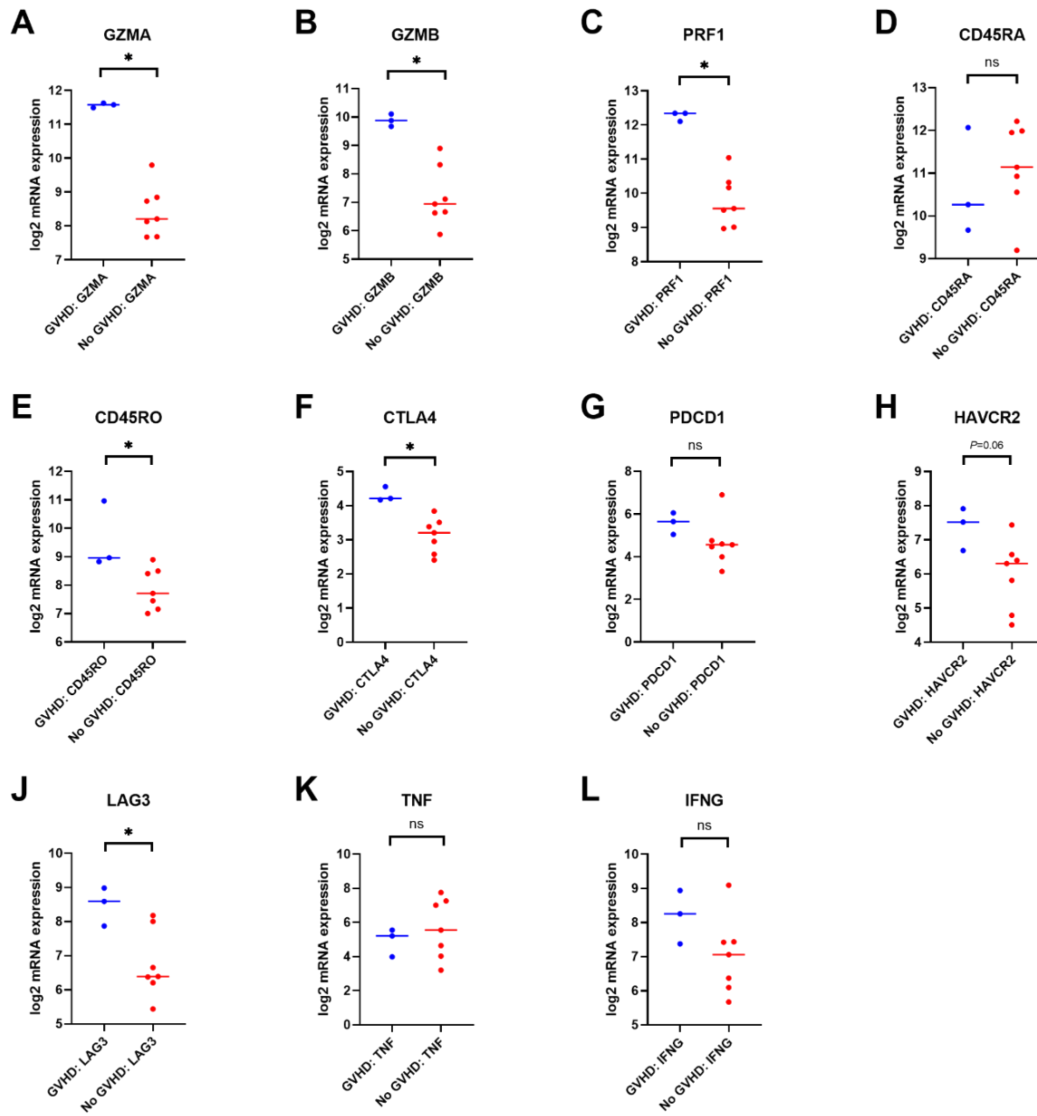


Figure 10.9 Differentially expressed genes in the extended gene target set between patients with or without subsequent GVHD

10.5 Discussion

Immune checkpoint inhibitors have been touted as a potential strategy to treat relapse of haematological malignancies post-alloSCT based on evidence of dysregulated T-cell co-stimulation and upregulation of PD-L1 by tumour cells at the time of post-transplant relapse[209, 210]. In this context, NIVALLO is a sentinel clinical trial to evaluate the safety and efficacy of PD-1 inhibition with nivolumab post-alloSCT. The preliminary results of this trial presented herein demonstrate that this is a highly potent strategy that can produce complete responses in refractory haematological malignancies albeit with a considerable risk of GVHD.

The incidence of GVHD observed in this study was 33% including 2 patients with severe acute GVHD of which 1 patient died of complications relating to GVHD. Both cases of severe acute GVHD were steroid refractory and required multiple immunosuppressive agents for either temporary or complete resolution. Several key themes arose from the cases of GVHD observed in this study. Firstly, all 3 cases of GVHD occurred rapidly following the first dose of nivolumab, typically within 2 weeks. A similar observation was noted in a retrospective series of 20 patients who received nivolumab for relapsed Hodgkin lymphoma after alloSCT where 30% of patients developed GVHD all within 1 week of the first infusion[81]. Secondly, all 3 patients who developed GVHD in our study received nivolumab within 7 months post-transplant. This was considerably shorter than the median time from alloSCT to nivolumab treatment in patients who did not develop GVHD (33.4 months) and suggests that the duration of time from alloSCT to the nivolumab therapy may be an important risk factor for the development of GVHD. The precise mechanism of this association remains unclear, however it does not appear to be related to either

differences in naïve and memory T-cell subsets and the changes in these over time post-transplant. A potential mechanism which deserves further exploration is the role of regulatory T-cells (Tregs) in limiting PD-1 induced post-transplant GVHD given that Tregs are known to be important in induction of immune tolerance after alloSCT and their reconstitution is partly dependent on time post-transplant[211].

Given that GVHD is a major concern with post-transplant checkpoint inhibitor therapy, immunological biomarkers to identify those patients at greatest risk of GVHD may be able to guide therapy. In this cohort, patients who subsequently developed GVHD demonstrated a significantly greater degree of T-cell activation at baseline as evidenced by CD38 and HLA-DR expression and increased granzyme and perforin mRNA expression. This suggests that even prior to nivolumab therapy, a subset of patients may already be primed to develop subsequent GVHD. This important finding may therefore allow the use of checkpoint inhibitors post-transplant to be personalised to patients who are least likely to develop significant toxicity.

Interestingly, the expression of PD-1 or other co-inhibitory receptors by T-cells did not correlate with either response nor the incidence of GVHD. Indeed, although PD-1 and TIM-3 expression was increased in post-allograft patients compared with normal control patients, T-cells from patients post-transplant remained functionally able to produce cytokines following *in-vitro* stimulation, suggesting that in the post-transplant setting expression of co-inhibitory receptors *per se* is not a reliable indicator of functional exhaustion. These findings are consistent with recent reports describing the dynamics of co-inhibitory receptor expression by T-cells post-transplant, wherein PD-1 expression can remain upregulated for prolonged periods of time post-alloSCT even in

the absence of functional exhaustion as T-cells retained normal expression of granzyme and perforin[187]. Taken together with the previous findings of T-cell activation correlating with GVHD risk, this suggests that the clinical impact of checkpoint blockade post-transplant is independent of the level of PD-1 expression by T-cells but rather on their functional ability following receptor binding.

The overall response rate to nivolumab in this study was 44%, all of which were complete responses. These occurred rapidly following nivolumab treatment, with 3 patients achieving complete remissions after a single dose of nivolumab. Responses were observed in a range of haematological malignancies including those typically associated with high response rates to PD-1 inhibitors such as Hodgkin lymphoma, as well as those not classically associated with responses to checkpoint blockade including mantle cell lymphoma and T-ALL[198, 212]. A particularly interesting case in point is one patient with Hodgkin lymphoma that was refractory to a PD-1 inhibitor prior to alloSCT who achieved a complete response to nivolumab after transplant, suggesting that prior lack of responsiveness to checkpoint blockade before alloSCT may not predict response after transplantation. Responses to nivolumab treatment were accompanied by GVHD in 3 out of 4 patients, demonstrating the difficulty in separating the beneficial GVT effect from GVHD. A similar observation was reported by Davids *et al*. in a phase 1/1b study of CTLA-4 inhibition with ipilimumab after alloSCT in which responses were all associated with GVHD[79]. Indeed, there are similarities in both the rate of GVHD or immune-related adverse events (33% in NIVALLO and 35% following ipilimumab) and response (NIVALLO 44%, ipilimumab 32%) in the present study using nivolumab compared with the ipilimumab study, suggesting that similar clinical and

immunological outcomes may be achieved regardless of the manner of checkpoint blockade used post-alloSCT[79].

It remains to be determined if lower doses of nivolumab may be able to achieve a balance between safety and efficacy post-alloSCT. Following the study protocol amendment to reduce the initial starting dose of nivolumab to 1.5mg/kg, one further case of acute and chronic GVHD was observed albeit of mild severity and the patient responded promptly to topical therapies alone. Early case reports suggest that lower doses of PD-1 inhibitors remain highly potent in stimulating alloimmune responses post-transplant. Minson *et al*. reported 3 patients with relapsed Hodgkin lymphoma post-alloSCT, all of whom had current GVHD at the time of administration of pembrolizumab[213]. Dose-reduced pembrolizumab (ranging from 25% to 50% of standard dosing) achieved partial or complete responses in all patients albeit accompanied by worsening GVHD. These cases are peculiar for all patients having current GVHD at the time of pembrolizumab treatment and the impact of such dose-reductions in PD-1 inhibitors post-alloSCT in patients without concomitant GVHD remains to be examined. Of note, the ipilimumab experience wherein no responses were observed at the initial lower dose of 3mg/kg while some patients still developed GVHD at this dose, may provide an insight that a lower dose of checkpoint inhibitor therapy that is able to provide robust responses without significant GVHD may not exist. Further experience with the 1.5mg/kg initial dose of nivolumab adopted in this study moving forward will provide further insights into this.

In summary, the preliminary results of this sentinel study demonstrate that nivolumab post-alloSCT is able to achieve highly potent alloimmune responses and can induce

clinical remission in highly refractory haematological malignancies albeit with a significant risk of GVHD. Importantly, an activated T-cell immunophenotype and upregulation of cytolytic granule gene expression identifies patients primed to develop GVHD following nivolumab therapy. The use of these immunological biomarkers may be able to identify patients at lower risk of toxicity and hence guide post-alloSCT checkpoint inhibitor therapy in a patient-specific manner.

11 Conclusion

The prognosis of patients who develop relapse of haematological malignancies after alloSCT is poor and strategies are required to identify patients who are greatest risk of relapse through the use of robust biomarkers and novel strategies are needed to prevent relapse or effectively treat relapse of disease once it has occurred. In this thesis, I have explored the prognostic utility and limitations of T-cell chimerism as a predictive biomarker of relapse, the use of lenalidomide as a maintenance strategy post-alloSCT, and investigated nivolumab as a novel strategy to treat relapse post-alloSCT.

The utility of T-cell chimerism as a predictive biomarker of relapse is limited by its poor statistical sensitivity. In the cohort of patients with AML and MDS with what many would consider to be a standard rate of relapse at 12 months post-alloSCT (25%), T-cell chimerism at thresholds between 65% to 85% demonstrated overall poor negative and positive predictive values. This demonstrates that caution is required when using T-cell chimerism alone to assess the requirement for adjunctive immunomodulation including donor lymphocyte infusions. Moreover, there was a cohort of patients with low and intermediate risk disease (DRI) that maintained long-term remission despite persistent mixed chimerism, demonstrating the flaws in using T-cell chimerism alone to guide therapy. An alternative or adjunctive diagnostic tool is measurable residual disease (MRD), which through improved technologies including deep sequencing through digital droplet PCR or error-corrected sequencing results in highly sensitive assays that are also likely to be more predictive of relapse in the pre- or post-alloSCT settings[214, 215]. One possible future direction of exploration is the utility of MRD in combination with T-cell chimerism and whether these two methodologies, which largely reflect two different but parallel processes with MRD reflective of tumour

persistence and T-cell chimerism a marker of post-transplant immunology, may be complementary.

Immunomodulatory strategies to enhance the allogeneic GVT effect to prevent or treat relapse post-alloSCT remain a challenging albeit powerful tool due to a very narrow therapeutic window of opportunity. The example of post-transplant lenalidomide is a case in point with previous work demonstrating that lenalidomide at a dose of 10mg daily resulted in an unacceptable rate of GVHD. The microLEN study has to date demonstrated that lenalidomide doses of up to 5mg twice per week are safe and tolerable. Completion of recruitment of this study of this study and expansion into a phase 2 trial will determine if lenalidomide may be added to the expanding armamentarium of strategies to prevent post-transplant relapse.

Finally, I have described the potency of nivolumab as a strategy to treat post-transplant relapse, albeit at a significant risk of GVHD. Patient selection may be important in identifying those at lower risk of GVHD, including longer time post-alloSCT and T-cell phenotype and granzyme or perforin expression. The preliminary experience from the NIVALLO study demonstrates once again the difficulty in separating the beneficial GVL effect from GVHD, and strategies to identify the precise mechanisms of disease relapse and therapies that target these evasion mechanisms while simultaneously identifying patients at lowest risk of toxicity may guide selection of the most appropriate therapies in a patient-specific manner.

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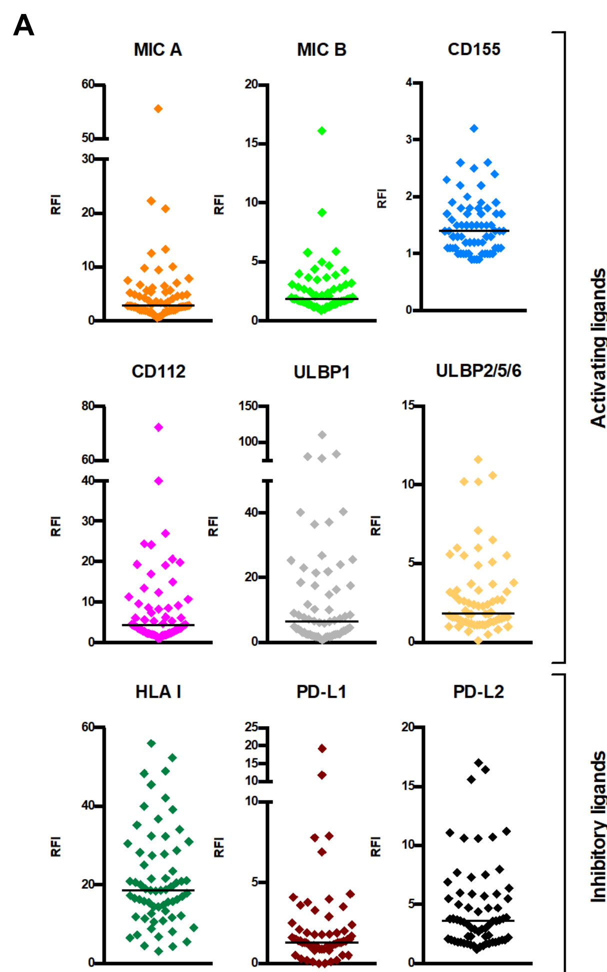
13 Appendices

13.1 NK receptor ligand expression by AML blasts impacts prognosis after chemotherapy.

Figure 13.1 Analysis of AML blasts from 66 patients undergoing remission induction chemotherapy

Expression of activating and inhibitory NK receptor ligands were examined by flow cytometry. AML blasts displayed heterogeneous expression of NK receptor ligands (**A**). An overall activating pattern of NK receptor ligands was associated with improved overall survival and reduced incidence of relapse (**B**; 0=inhibitory pattern, 1=activating pattern)[123].



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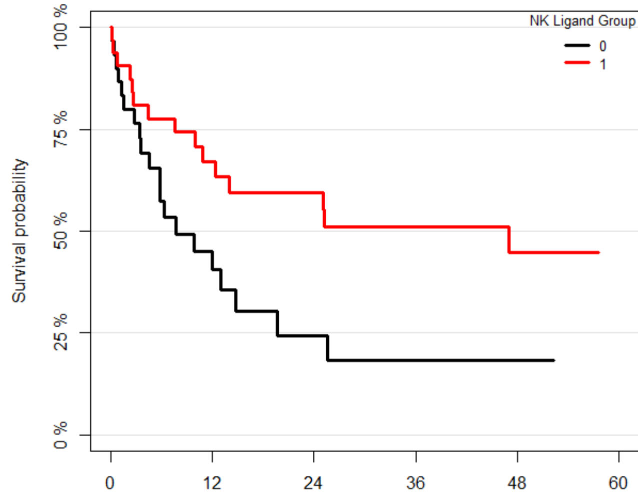

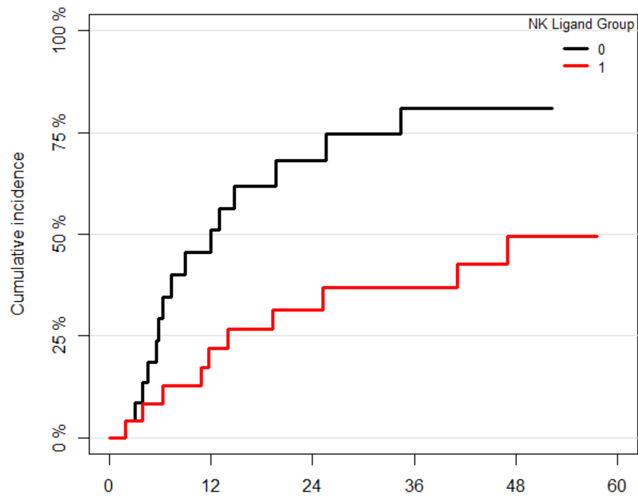

13.2 NIVALLO Case Descriptions

RMH001

RMH001 was a 26 year old man with multiply relapsed Hodgkin lymphoma. He initially achieved a complete metabolic response following chemotherapy (Adriamycin, bleomycin, vinblastine and dacarbazine; ABVD), however relapsed within 6 months of completing chemotherapy. He underwent salvage chemotherapy with ifosfamide, carboplatin and etoposide (ICE) which was consolidated with high-dose chemotherapy and an autologous stem cell transplant to which he achieved a complete response. His second relapse occurred within 2 months of his autologous transplant with widespread nodal disease. He then received radiotherapy (total nodal and mantle field) and achieved a complete response. His third relapse occurred 6 months later with small volume nodal disease, within the previous field of radiotherapy. Following 15 months of observation for slowly progressive but asymptomatic disease, he then received a reduced-intensity double umbilical cord allogeneic stem cell transplant with fludarabine, cyclophosphamide and total body irradiation conditioning. A complete metabolic response was achieved post-alloSCT. He developed mild oral and cutaneous chronic GVHD which resolved with corticosteroids. Four years following alloSCT, he relapsed with a left inguinal mass which was histologically confirmed as relapsed Hodgkin lymphoma. He was asymptomatic and therefore observed with repeat imaging. On further nodal progression, he was enrolled in the NIVALLO clinical trial and received his first dose of nivolumab 6 months after his latest relapse, and 55 months following his alloSCT. He had complete donor chimerism in peripheral blood at the time of study commencement. There was no active GVHD at the time of enrolment. He received nivolumab as per the approved study protocol at the time at a dose of 3mg/kg. A complete metabolic response was achieved at the first response assessment timepoint

after 4 doses of nivolumab. He then received 2 further doses and then ceased treatment as per study protocol. A further relapse occurred 6 months after ceasing nivolumab on surveillance PET imaging, with small volume nodal disease in previously involved sites post-alloSCT. He then re-commenced nivolumab and achieved a complete response after a further 4 doses. He received a further 8 doses of nivolumab before deciding to cease further treatment due to social circumstances. He did not develop acute or chronic GVHD during or following nivolumab treatment. He developed grade 3 community acquired pneumonia during his second course of treatment which was thought not related to study treatment. He remains in ongoing remission 4 months after ceasing treatment.

RMH002

RMH002 was a 53 year old man with extramedullary AML (right maxillary chloroma) without evidence of marrow involvement. Cytogenetics performed on the tissue biopsy revealed complex cytogenetics. He received induction chemotherapy with cytarabine and idarubicin to which he achieved a complete remission, and this was followed by one cycle of consolidation chemotherapy. He relapsed one year later with leukemia cutis and received 30Gy radiotherapy. This was followed by a myeloablative alloSCT from an 8/8 HLA-matched unrelated donor with peripheral blood stem cells, complicated by mild oral chronic GVHD that resolved with topical corticosteroids. He developed post-transplant relapse in the right testicle 2 years after alloSCT, which was treated with high dose methotrexate to complete response. A further relapse occurred in the left testicle for which he underwent an orchidectomy. Five years after alloSCT he developed a further relapse with multiple subcutaneous nodules, extensive nodal involvement, and a soft tissue mass in the right orbit. Biopsy of a subcutaneous nodule confirmed the

diagnosis of relapsed extramedullary AML. There was no evidence of bone marrow involvement. Nivolumab was commenced on study, 75 months following alloSCT and 35 days following the diagnosis of relapse. He received 4 doses of nivolumab, after which repeat PET scan demonstrated disease progression with new subcutaneous lesions, and study treatment was ceased due to lack of response. Notable adverse events included new onset subclinical hypothyroidism (grade 2) and an isolated increase in serum amylase (grade 1) without clinical or radiological evidence of pancreatitis, both of which were possibly related to nivolumab treatment. He did not develop acute or chronic GVHD.

RMH003

RMH003 was a 52 year old woman with CLL who received first-line treatment with fludarabine, cyclophosphamide and rituximab with subsequent transformation to diffuse large B cell lymphoma 7 years following diagnosis. She received chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) to which she achieved complete remission. This was consolidated with an 8/8 HLA-matched unrelated donor alloSCT with cyclophosphamide and total body irradiation as pre-transplant conditioning. She developed an early post-transplant relapse with the high-grade lymphoma at day 75 despite complete donor chimerism. She received rapid immunosuppression withdrawal in combination with 4 doses of rituximab to which she achieved a complete response. She did not develop acute or chronic GVHD. She then developed a further relapse 46 months post-alloSCT with numerous cervical, submandibular and axillary lymph nodes, and extra-nodal involvement of the nasopharyngeal soft tissue, bilateral femora and humeri. Histology of an excised lymph node demonstrated effacement with CLL however with a high Ki67 proliferation index

consistent with an aggressive relapse despite the absence of definite features of large cell transformation. She commenced study treatment and received 8 doses of nivolumab, after which there was no evidence of response by CT criteria, although several nodal sites demonstrated reduced FDG avidity. Due to lack of response, she ceased further treatment with nivolumab. She did not develop post-nivolumab GVHD or any other adverse events of grade 2 or higher.

RMH004

RMH004 was a 43 year old man with myelodysplastic syndrome (MDS) with secondary AML with adverse prognosis features including a complex monosomal karyotype and three TP53 coding region mutations. Induction chemotherapy achieved complete morphologic remission from AML but persistent dysplasia consistent with the underlying MDS and persistent measurable residual disease (MRD) was also able to be identified. He proceeded to alloSCT from an HLA-matched unrelated donor with non-myeloablative conditioning (fludarabine and cyclophosphamide) due to severe hepatic steatosis. GVHD prophylaxis comprised of *in-vivo* T-cell depletion with thymoglobulin in combination with ciclosporin and short course methotrexate. Bone marrow biopsy at day 30 demonstrated complete morphologic remission. Relapse of AML occurred at 97 days post-alloSCT with 23% bone marrow blasts, with karyotypic and molecular features consistent with re-emergence of a pre-existing leukaemic clone. Immunosuppression was ceased without any ensuing GVHD. T-cell chimerism at relapse was 69% donor origin. Due to leukemia-associated pyrexia, he was commenced on dexamethasone 4mg daily. Study treatment with nivolumab was commenced 120 days post-alloSCT (28 days after cessation of immunosuppression). Twenty-five days following the first dose of nivolumab, he developed postural dizziness and tachycardia

secondary to newly acquired postural orthostatic tachycardia syndrome (POTS) requiring hospitalisation. He was subsequently admitted on a second occasion due to persistent symptoms despite therapy (fludrocortisone and propranolol) complicated by a fall and acquired a subdural haemorrhage and humerus fracture, both of which were managed conservatively. There was no other evidence of autonomic or peripheral nervous system dysfunction. Due to there being no previous reports of POTS following checkpoint inhibitor therapy, the association between this patient's symptoms and study treatment was unclear, and he continued study treatment. Disease re-assessment after 4 doses of nivolumab demonstrated reduction in blast count to 12%. However, this response was transient as after 8 doses of nivolumab, repeat bone marrow biopsy demonstrated an increase in blast percentage to 17% and treatment was discontinued. Following nivolumab treatment, he did not develop classical features of acute or chronic GVHD and there was a progressive decrease in his T-cell and myeloid chimerism.

RMH007

RMH007 was a 38 year old man with Hodgkin lymphoma refractory to multiple lines of treatment including ABVD chemotherapy, brentuximab and high dose chemotherapy with autologous stem cell support. A complete remission was achieved following 30Gy mantle-field radiotherapy. He then developed lymphoma relapse, which was treated with 6 cycles of PD-1 blockade with pembrolizumab with no response. He achieved a second complete remission with prednisolone, etoposide and cyclophosphamide chemotherapy. This was consolidated with a reduced intensity (fludarabine and melphalan) conditioned matched sibling alloSCT. At day 46 post-transplant PET scan demonstrated ongoing complete metabolic response. He subsequently relapsed post-alloSCT at day 123 with stage 4 disease including widespread lymph node involvement

and extranodal involvement of the left scapula, ribs and sacrum. Immunosuppression was rapidly withdrawn. Peripheral blood chimerism demonstrated that 99% of T-cells were donor-derived. Study treatment with nivolumab 3mg/kg was commenced 145 days post-alloSCT (15 days following immunosuppression cessation). Five days following the first dose of nivolumab he developed acute onset large volume diarrhoea and hyperbilirubinemia consistent with acute gastrointestinal and hepatic GVHD (grade 3) which was confirmed on colon biopsy. Treatment of GVHD consisted of sequential initiation of methylprednisolone (2mg/kg), cyclosporin (3mg/kg) and etanercept (25mcg for four doses) with complete resolution of this episode of GVHD within 26 days. Hodgkin lymphoma response assessment was performed 28 days after the dose of nivolumab, which demonstrated complete metabolic remission. The patient subsequently developed several other complications including CMV reactivation, microangiopathic haemolytic anaemia secondary to ciclosporin, and recurrence of GVHD following cessation of ciclosporin.

RMH008

RMH008 was a 57 year old man with mantle cell lymphoma with high risk features including a high mantle-cell international prognostic index (MIPI). He underwent induction chemotherapy with rituximab and the hyper-CVAD regimen to which he achieved complete remission, and this was consolidated with busulfan and melphalan with autologous stem cell support. He relapsed 2 years later and achieved partial response to salvage chemotherapy, followed by complete remission following treatment with the Bcl2-inhibitor venetoclax. He subsequently relapsed 3 years later and received the BTK-inhibitor ibrutinib to which he achieved a complete nodal response but had persistent bone marrow lymphoma. He then underwent a non-myeloablative

(fludarabine and low-dose cyclophosphamide) 8/8 HLA-matched unrelated donor alloSCT. By day 56, there was progressive bone marrow involvement, and immunosuppression was rapidly withdrawn and ceased by day 86 post-alloSCT. Re-staging at day 121 post-alloSCT demonstrated persistent marrow involvement with recurrence of widespread nodal disease. Study treatment with nivolumab 3mg/kg was commenced 140 days post-alloSCT (54 days following immunosuppression cessation). He developed multi-organ acute GVHD (gastrointestinal, ocular, cutaneous and liver, maximum grade 3) with onset 13 days following nivolumab. Intravenous methylprednisolone and mycophenolate mofetil were commenced with rapid improvement of gastrointestinal and cutaneous manifestations but persistent liver and ocular GVHD. He was commenced on extracorporeal photopheresis (ECP) for treatment of GVHD with gradual improvement noted by 9 months following the first dose of nivolumab. Response assessment was performed 21 days following nivolumab treatment which demonstrated complete metabolic response on PET with minimal residual disease evident in the bone marrow. Subsequent repeat bone marrow assessment on day 91 demonstrated complete remission. Duration of complete remission was 245 days, following which he developed relapse which was treated with the BTK inhibitor ibrutinib.

RMH009

RMH009 was a 66 year old man with B-cell acute lymphoblastic leukaemia (B-ALL), Philadelphia chromosome negative, who underwent a myeloablative unrelated donor alloSCT in second complete remission (CR2) following response to salvage treatment with blinatumomab. He did not develop post-transplant GVHD. He developed post-transplant relapse of B-ALL in extramedullary sites (lymph nodes, cutaneous nodule,

pericardial disease) without bone marrow involvement 9 months post-alloSCT which was treated with blinatumomab which achieved a partial response, followed by vincristine, doxorubicin and dexamethasone to which he achieved a complete response. A second post-transplant relapse occurred at 19 months post-alloSCT in the same extramedullary sites as previous. He commenced study treatment with nivolumab (3mg/kg) 1 month later and received a total of 4 doses. Following the fourth dose of nivolumab, he developed a lower respiratory tract infection and grade 3 pneumonitis, possibly related in part to nivolumab treatment and requiring hospitalisation and treatment with systemic corticosteroids. Nivolumab was ceased due to the pneumonitis. A repeat PET scan showed a reduction in metabolic activity of known sites of disease, however this did not meet criteria for response (Deauville score 5).

RMH010

RMH010, a 52 year old man with T-cell ALL (T-ALL) underwent a sibling myeloablative alloSCT in first complete remission, and was in a minimal residual disease (MRD) negative CR at day 100 post-alloSCT. At day 186 post-alloSCT, repeat bone marrow biopsy demonstrated re-emergence of MRD detectable by flow cytometry. He commenced nivolumab treatment on post-transplant day 215 at a dose of 1.5mg/kg, in accordance with a study protocol amendment. Following 2 doses of nivolumab, he developed grade 1 cutaneous acute GVHD and mild oral and ocular chronic GVHD. A repeat bone marrow biopsy was performed at this time, which showed clearance of MRD. Due to the depth of response achieved as well as emerging GVHD, the study investigators elected to cease further nivolumab treatment. The cutaneous acute GVHD was treated with topical hydrocortisone to a complete response. His chronic GVHD

symptoms have also been managed with topical agents (topical ocular lubricants); his symptoms persist at the time of last assessment.

RMH011

RMH011 developed extramedullary relapse of AML 11 months following a myeloablative unrelated donor alloSCT performed in first complete remission. Extramedullary sites of AML relapse included one cutaneous nodule as well as widespread non-bulky lymph node involvement. There was no evidence of leukaemia in the bone marrow or cerebral spinal fluid. Donor chimerism in both CD3-positive and negative cell fractions in the peripheral blood were 100%. She commenced nivolumab treatment on study initially at 1.5mg/kg for the first two doses, which was subsequently increased to 3mg/kg for doses 3 and 4 as per study protocol. Repeat PET imaging and bone marrow biopsy following 4 doses of nivolumab demonstrate progressive nodal disease, and morphologic evidence of leukaemia in the bone marrow, and therefore study treatment was terminated. She did not develop demonstrable GVHD.

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