Description of a Novel MHC Class II Restricted Allogeneic TCR T Cell Therapy for Acute Myeloid Leukaemia

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Green Templeton College
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A thesis submitted for the degree of Doctor of Philosophy

Trinity 2023

Abstract

Acute myeloid leukaemia (AML) is an aggressive blood cancer that can be treated with allogeneic haematopoietic stem cell transplantation (allo-HSCT). Although allo-HSCT reduces the chance of relapse because of the Graft-versus-leukaemia (GvL) effect, it is also associated with the Graft-versus-Host Disease (GvHD). This and other treatment or disease related complications mean that only around half of transplanted AML patients survive at 3-years[1]. To harness the GvL effect, on-going research is trying to identify novel GvL-specific allogeneic peptide antigens presented by the major histocompatability complex (MHC), called minor histocompatability antigens (miHA). However, it remains unclear which miHAs mediate a successful GvL effect and what GvL-specific T cell functions are associated with it. This work describes a novel HLA-DP-restricted *PADI4* miHA response identified as part of a previous allogeneic antigen screen in a cohort of AML patients with long-term remission post-transplant. Antigen-specific allogeneic T cells were successfully isolated and their T cell receptor (TCR) transferred to third-party primary T cells. PADI4 TCR T cell activation was seen to cancer cell lines, where it could also exert a growth inhibitory effect. Although this work did not show evidence of direct T cell activation to primary leukaemia samples, leukaemia-derived antigen was successfully presented by primary monocyte-derived dendritic cells. *PADI4* miHA expression is haematopoietically restricted, suggesting no reactivity to the GvHD target tissues. However, PADI4 TCR T cell activation, without target cell killing, was seen to healthy monocytes. Overall, this data supports the idea that the *PADI4* miHA response could exert an anti-leukaemic or a broader anti-haematopoietic effect. This work provides an important starting point to further investigate the PADI4 TCR for its anti-leukaemic effect and the importance of the *PADI4* miHA response in other AML patients. It also highlights the role of $CD4^+$ MHC class II-restricted T cell responses in GvL. **Word count: 45,957**

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Acknowledgements

Firstly, I want to I thank my supervisors Paresh Vyas, Persephone Borrow, Ronjon Chakraverty and Andrew McMichael, as well as my *DPhil* funding body, Cancer Research UK (CRUK). I am thankful to the CRUK Oxford Centre and its Team, especially Mark Middleton and Michael Youdell. In addition, I am grateful to receive financial support from the Vilnius Lyceum Alumni Association to continue my BMBCh-DPhil studies. I thank my college, Green Templeton, and especially my college advisor Sue Ziebland.

I thank the patients, who teach us, and allow the use of their samples for cancer research.

I am grateful for my colleagues, friends and students, who have taught me and helped me - Connor Sweeney, Maria Barbanti, Bing Tseu, Sven Turkalj, Dave Grainger, Gina Sangha, Kinam Gupta, Susann Rahmig, Asger Jakobsen, Batchimeg Usukhbayar, Vivien Ho, Gaurav Agarwal, Anne Colas and other members, transient and constitutive, of the Vyas and Porcher research groups.

I thank the research support available at the Weatherall Institute of Molecular Medicine (WIMM) and Oxford, especially the Flow Cytometry Facility, expertly run by Paul Sopp, Kevin Clark, Sally-Ann Clark and Craig Waugh, the Genome Engineering Facility (Philip Hublitz), the Virus Screening Facility (Ryan Beveridge) and the Sequencing Facility (Timothy Rostron and John Frankland). I also thank Margarida Rei and Mai Vuong, for their brilliant help with primary T cell transductions.

In light of this milestone, I want to express my gratitude for the supervisors of my first research project - Rachel Exley, Rhian Ford, Guangyu Liu (George) and Christoph Tang (Sir William Dunn School of Pathology). Your invested time and passion made me discover my affinity for research.

I thank Somerville College and the Oxford Medical School, who are supportive of combined BMBCh-DPhil studies, especially Catherine Swales, Daniel Anthony and Damian Tyler.

I thank my family, Inga, Marius and Ben, as well as my friends. You are my PADI4!

Abstract

Acute myeloid leukaemia (AML) is an aggressive blood cancer that can be treated with allogeneic haematopoietic stem cell transplantation (allo-HSCT). Although allo-HSCT reduces the chance of relapse because of the Graft-versus-leukaemia (GvL) effect, it is also associated with the Graft-versus-Host Disease (GvHD). This and other treatment or disease related complications mean that only around half of transplanted AML patients survive at 3-years[1]. To harness the GvL effect, on-going research is trying to identify novel GvL-specific allogeneic peptide antigens presented by the major histocompatability complex (MHC), called minor histocompatability antigens (miHA). However, it remains unclear which miHAs mediate a successful GvL effect and what GvL-specific T cell functions are associated with it. This work describes a novel HLA-DP-restricted *PADI4* miHA response identified as part of a previous allogeneic antigen screen in a cohort of AML patients with long-term remission post-transplant. Antigen-specific allogeneic T cells were successfully isolated and their T cell receptor (TCR) transferred to third-party primary T cells. PADI4 TCR T cell activation was seen to cancer cell lines, where it could also exert a growth inhibitory effect. Although this work did not show evidence of direct T cell activation to primary leukaemia samples, leukaemia-derived antigen was successfully presented by primary monocyte-derived dendritic cells. *PADI4* miHA expression is haematopoietically restricted, suggesting no reactivity to the GvHD target tissues. However, PADI4 TCR T cell activation, without target cell killing, was seen to healthy monocytes. Overall, this data supports the idea that the *PADI4* miHA response could exert an anti-leukaemic or a broader anti-haematopoietic effect. This work provides an important starting point to further investigate the PADI4 TCR for its anti-leukaemic effect and the importance of the *PADI4* miHA response in other AML patients. It also highlights the role of $CD4^+$ MHC class II-restricted T cell responses in GvL. **Word count: 45,957**

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List of Abbreviations

2-HG 2-hydroxyglutarate

ALL . . . . . Acute lymphoblastic leukaemia

Allo-HSCT . . Allogeneic haematopoietic stem cell transplant

AML . . . . . Acute myeloid leukaemia

APC . . . . . Antigen-presenting cell

AraC . . . . . Cytosine arabinoside

ASH . . . . . American Society of Hematology

ATG . . . . . Anti-thymocyte globulin

BLCL . . . . . B-lymphoblastoid cell line

BM . . . . . Bone marrow

BSA . . . . . Bovine serum albumin

BU . . . . . Busulfan

CB . . . . . Cord blood

CBF . . . . . Core binding factor

CLL . . . . . Chronic lymphocytic leukaemia

CLP . . . . . Common lympoid progenitor

CML . . . . . Chronic myeloid leukaemia

CMP . . . . . Common myeloid progenitor

CMV . . . . . Cytomegalovirus

CNI . . . . . Calcineurin inhibitor

CR . . . . . Complete remission

CRi . . . . . Complete remission with incomplete count recovery

CSA . . . . . Cyclosporine

CY . . . . . Cyclophosphamide

DAMP . . . . . Damage associated molecular pattern

DC . . . . . Dendritic cell

DLI . . . . . Donor lymphocyte infusions

DVT . . . . . Deep vein thrombosis

EBV . . . . . Epstein-Barr virus

EBV-LCL . . . EBV-transformed lymphoblastoid cell line

ELN . . . . . European LeukaemiaNet

EMA . . . . . European Medicines Agency

ESMO . . . . . European Society for Medical Oncology

E:T . . . . . Effector-to-target

FACS . . . . . Fluorescence-activated cell sorting

FBS . . . . . Fetal bovine serum

FLU . . . . . Fludarabine

G-CSF . . . . . Granulocyte colony-stimulating factor

GFP . . . . . Green fluorescent protein

GI . . . . . Gastrointestinal tract

GM-CSF . . . . Granulocyte-macrophage colony-stimulating factor

GMP . . . . . Granulocyte-monocyte progenitor

GO . . . . . Gemtuzumab ozogamicin

gRNA . . . . . Guide RNA

GvHD . . . . . Graft-versus-host disease

GvL . . . . . Graft-versus-leukaemia

HDR . . . . . Homology directed repair

Het . . . . . Heterozygous

Hom . . . . . Homozygous

HLA . . . . . Human leukocyte antigen

HMA . . . . . Hypomethylating agent

HSC . . . . . Haematopoietic stem cell

HSPC . . . . . Haematopoietic stem and progenitor cells

HSCT . . . . . Haematopoietic stem cell transplant

ICD . . . . . Immunogenic cell death

IDAC . . . . . Intermediate-dose cytarabine

IDH . . . . . Isocitrate dehydrogenase

iPSC . . . . . Inducible pluripotent stem cell

IFNγ . . . . . Interferonγ

IS . . . . . Immunosuppression

ITD . . . . . Internal tandem duplication

KI . . . . . Knock-in

KIR . . . . . Killer-Cell Immunoglobulin-like Receptor

KO . . . . . Knock-out

LAA . . . . . Leukaemia-associated antigen

LAIP . . . . . Leukaemia-associated immunophenotype

LEL . . . . . Low-expression loci (in reference to HLA)

LMPP . . . . . Lymphoid-primed multipotent progenitor

LPS . . . . . Lipopolysaccharide

MAC . . . . . Myeloablative conditioning

MACS . . . . . Magnetic-activated cell sorting

MDS . . . . . Myelodysplastic syndrome

MEP . . . . . Megakaryocytic-erythroid progenitor

MHC . . . . . Major histocompatability complex

miHA . . . . . Minor histocompatability antigen

MIP-1α . . . . Macrophage inflammatory protein 1α

MIP-1β . . . . Macrophage inflammatory protein 1β

MLP . . . . . Multi-lymphoid progenitor

MMF . . . . . Mycophenolate mofetil

MOI . . . . . Multiplicity of infection

MPP . . . . . Multipotent progenitor

MRD . . . . . Minimal residual disease

mTCRβ . . . . murine T cell receptor β chain

MTX . . . . . Methotrexate

NCBI . . . . . National Center for Biotechnology Information

NCCN . . . . . National Cancer Centers Network

NCI . . . . . National Cancer Institute

NeoAg . . . . . Neoantigen

NET . . . . . Neutrophil extracellular trap

NFAT . . . . . Nuclear factor of activated T cell

NGS . . . . . Next generation sequencing

NHSBT . . . . National Health Service Blood& Transplant

NIH . . . . . National Institutes of Health

NMA . . . . . Non-myeloablative

NPM1 . . . . . Nucleophosmin 1

NRM . . . . . Non-relapse mortality

PADI4 . . . . . Peptidyl arginine deiminase 4

PADI4-Var . . Refers to PADI4 with variant SNP genotype (recognised by PADI4-TCR)

PADI4-WT . . Refers to PADI4 with wild-type SNP genotype (not recognised by PADI4-TCR)

PAMP . . . . . Pathogen associated molecular pattern

PB . . . . . Peripheral blood

PBMC . . . . . Peripheral blood mononuclear cells

PCR . . . . . Polymerase chain reaction

PHA . . . . . Phytohaemagglutinin

PIRCHE . . . . Predicted Indirectly ReCognizable HLA Epitope

pMHC . . . . . peptide-MHC complex

PR3 . . . . . Proteinase 3

PRAME . . . . PReferentially expressed Antigen in MElanoma

PTCy . . . . . Post-transplant cyclophosphamide

qPCR . . . . . Quantitative PCR

RA . . . . . Rheumatoid arthritis

RIC . . . . . Reduced intensity conditioning

RT . . . . . Room temperature

SEER . . . . . Surveillance, Epidemiology, and End Results Program (National Cancer Institute)

SNP . . . . . Single nucleotide polymorphism

ssODN . . . . . single stranded oligodeoxynucleotide

TAA . . . . . Tumour-associated antigen

TAC . . . . . Tacrolimus

TBI . . . . . Total body irradiation

TCR . . . . . T cell receptor

Th1 . . . . . Type 1 T helper cell

Th2 . . . . . Type 2 T helper cell

Th17 . . . . . Type 17 T helper cell

TPM . . . . . Transcripts per million

UMAP . . . . . Uniform Manifold Approximation and projection

UTR . . . . . Untranslated region

Var . . . . . Variant genotype

WB . . . . . Western blot

WT . . . . . Wild type genotype

WT1 . . . . . Wilms tumour 1

Chapter 1

Introduction

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1.1 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia (AML) is a blood cancer characterised by the aberrant expansion of immature myeloid progenitors. Patients with AML can progress from asymptomatic to critically-ill within weeks. The disease originates in the bone marrow (BM), where the malignant blasts displace normal haematopoiesis. Hence, AML patients typically present with symptoms of BM failure, like shortness of breath and fatigue (anaemia), easy bruising and bleeding (thrombocytopaenia) as well as recurrent

infections (neutropaenia). Alongside, abnormal myeloblasts can be seen in both BM and peripheral blood (PB).

AML diagnosis is made based on complimentary clinical, morphological, molecular and cytogenetic findings. First of all, complete blood counts highlight the cytopaenias. Additionally, PB and BM smears are used to quantify presence of undifferentiated blasts. Typically, the lower diagnostic threshold for AML is at 10-20% frequency of immature myeloblasts in either PB or BM, depending on the genetic abnormalities identified in parallel[9]. Indeed, a complete AML diagnosis is accompanied by karyotyping and targeted next generation sequencing (NGS), which not only classifies the AML, but also informs on its appropriate treatment and likely prognosis.

Under the umbrella term of AML, there is a wide variety of cytogenetic and molecular AML subtypes as well as distinct blast morphologies as seen under the microscope. AML can range from undifferentiated myeloblast to myelomonocytic, monocytic, erythroid or megakaryoblastic. Although in all cases the cancer originates from an undifferentiated cell of the myeloid lineage, the different subtypes reflect different haematopoietic stem and progenitor states. These morphological and simple cytochemical differences led to the development of the French-American-British (FAB) morphological AML classification in the 1970s[10].

Additionally, AML is commonly characterised using multiparameter flow cytometry, where it shows wide heterogeneity. It can express both haematopoietic stem and progenitor cell (CD34 and CD117) as well as myeloid (CD33, CD11b), monocytic (CD14), megakaryocytic (CD41) and erythroid (CD235a) cell markers. Interestingly, typical markers of non-myeloid lineages can also be aberrantly expressed in AML blasts, such as CD56[11] (natural killer cell marker), CD7[12] (T cell marker) and CD19[13] (B cell marker). In turn, the knowledge on the diversity as well as heterogeneity of the AML cell surfaceome has proven useful in the clinical monitoring of the disease. In some cases, leukaemia-associated immunophenotype (LAIP) can be characterised at the time of diagnosis and this knowledge may be subsequently used to accurately track low-level disease post-treatment, called minimal residual disease (MRD) monitoring.

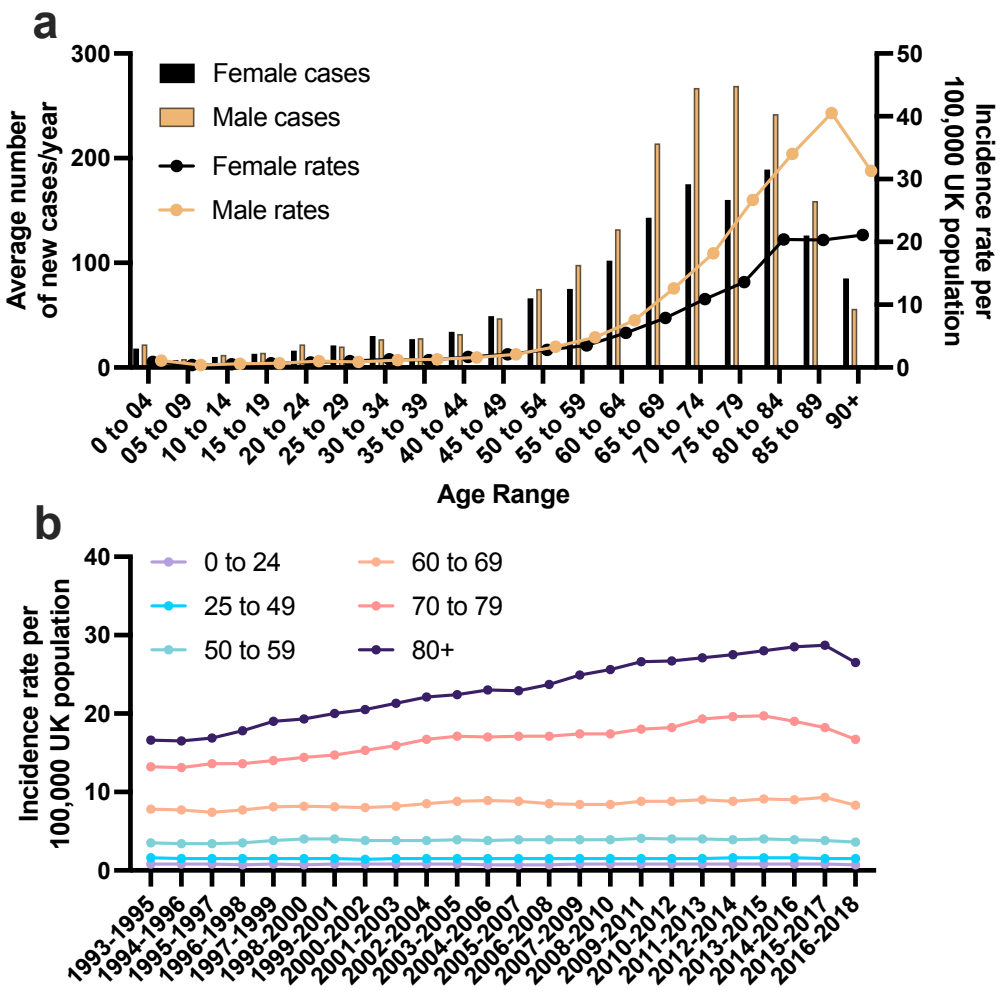

Figure 1.1: AML diagnosis incidence by age and time in the United Kingdom. a Average number of new cases of AML each year for different age ranges, stratified by sex. **b** AML diagnosis incidence rate over time, stratified by age. Based on a graphic created by Cancer Research UK[14].

Alternatively, MRD monitoring may be performed with NGS to detect low level leukaemia mutations (NGS MRD)[9].

Demographics and burden

Of all adult acute leukaemias, AML is the most common[15]. Between 2016 and 2018, an average of 3,089 new cases of AML were diagnosed each year in the UK, which makes up less than 1% of total cancer diagnoses[14]. However, the incidence of AML is closely related to increasing age and is slightly more prevalent amongst males. For example, data from Cancer Research UK shows that whilst incidence rates for 60-64

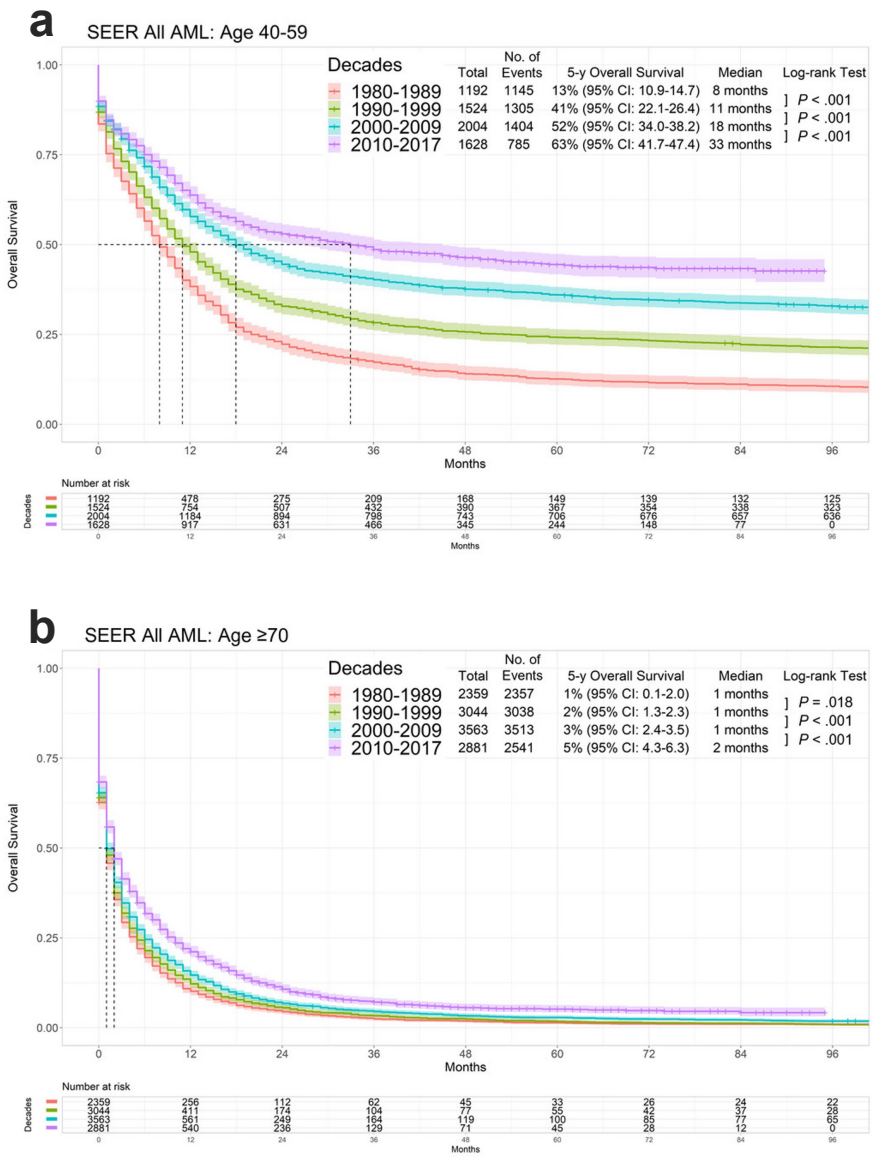

Figure 1.2: Kaplan-Meier survival estimates for all types of AML from Sasaki et al.[2] analysis of NCI SEER data. **a** and **b** show survival for all types of AML over time for age groups 40-59 and 70+, respectively. Figure adapted from Sasaki et al.[2].

year olds are 5.5 female and 7.5 male cases per 100,000 UK population, in 85-89 year olds the incidence rises to 20.3 and 40.5, respectively (Fig. 1.1a). In addition, the older age groups saw an increase in the AML incidence rates since the 1990s, by 27% for 70-79 year olds and by 60% for 80+ year olds (Fig. 1.1b)[14]. Although the reason for this increase is unclear, it may reflect an improvement in AML diagnosis and record keeping over time, rather than a true increase in population incidence[16].

Moreover, survival with AML is closely age-related as well. National Cancer Institute

Surveillance, Epidemiology, and End Results Program (SEER) data on *de novo* AML survival between years 2000 and 2017 showed patient age groups of 40-59 and 70+ to have stark differences in survival. The younger group had a 27% 4-week mortality and 40% 5-year survival, whilst the older group had 45-50% and less than 5%, respectively[2]. The age group separation in AML survival has been further exacerbated over the last few decades, where the survival of younger age groups has progressively increased but the survival of 70+ age group has virtually remained unchanged (Fig. 1.2)[2].

AML classification and risk stratification

Initial stratification of AML subtypes occurred by morphological observation supplemented with cytochemical tests, called the French-American-British (FAB) classification[10]. Introduced in the 1970s, this classification largely relies on the expertise of the observer and thus is somewhat subjective. Nevertheless, it is still used today, to indicate the level of AML blast maturation in PB/BM smears. Soon after the introduction of FAB classification, a wider availability of antigen-specific antibodies allowed studies to compare the immunophenotypes of different AML FAB subtypes. This highlighted that the morphological AML classification correlates with a corresponding immunophenotype only somewhat and a substantial inter- as well as intra-patient immunophenotypic variability exists[17]. Similar association between FAB classification and specific cytogenetic abnormalities was found as well[18].

Therefore, it became apparent that the significant morphological, immunophenotypic and cytogenetic variability requires careful integration to accurately stratify AML. Currently, the widely used World Health Organisation (WHO) AML classification defines different AML subtypes based on the morphological and cytogenetic characteristics as well as specific mutations, identified by using targeted NGS. Furthermore, it integrates the important clinical context of AML, chiefly by accounting for therapy-related AML, AML developed from previous myelodysplastic and myeloproliferative disorders and Down's syndrome related AML. This comprehensive characterisation allows efficient risk stratification and facilitates research on the effect of different disease characteristics

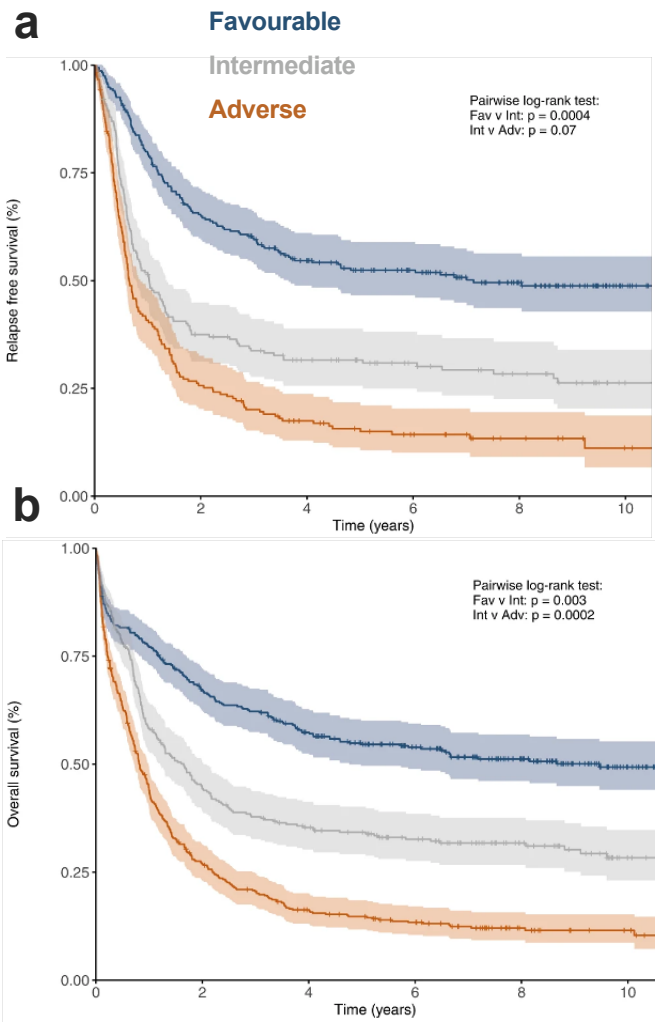

Figure 1.3: Patient outcomes according to ELN 2022 risk classification from Rausch et al.[3] analysis. a and **b** show relapse-free survival and overall survival of 1118 newly diagnosed AML patients between ages 18 and 86, stratified by ELN 2022 risk group. Study used patients treated with cytarabine-based induction between years 1999 and 2012. Figure adapted from Rausch et al.[3].

(*e.g.* gene mutations) or treatments on risk/outcomes in the context of different AML subtypes[19].

In terms of clinical practice, efficient AML classification allows disease treatment and patient prognosis to be tailored to a specific AML subtype. Currently, there are two prevalent risk classifications allowing to do this, the European LeukaemiaNet (ELN) guidelines[9] and the National Cancer Centers Network (NCCN) guidelines[20]. In both cases, AML is stratified into favourable/better, intermediate and poor/adverse

risk at the time of diagnosis. These groups stratify well according to overall survival and relapse free survival (Fig. 1.3)[3].

Treatment methods

While AML was considered an incurable disease in the 1960s, improvements in diagnosis, disease classification, drugs, allogeneic haematopoietic stem cell transplantation (allo-HSCT) and treatment regimens have changed this[21]. In order to establish a consistent standard-of-care, three different bodies have published treatment guidelines for adult AML: European Society for Medical Oncology (ESMO)[22], NCCN[23] and American Society of Hematology (ASH)[24]. The following text is focused on the treatment options available to Europe (ESMO guidelines).

After the initial diagnosis and work-up, treatment decision is taken together with the patient. It accounts both for the disease risk classification as well as for patient fitness to undergo different chemotherapy and allo-HSCT regimens[22]. However, given the fast progression of the disease, treatment must be started as soon as possible. In cases where acute promyelocytic leukaemia (APL) is suspected, all-trans-retinoic-acid (ATRA) with arsenic trioxide (ATO) are given, which allows to specifically target the promyelocytic leukemia-retinoic acid receptor alpha fusion oncoprotein (PML-RARA) and induce leukaemia blast differentiation. This targeted approach has transformed the prognosis for patients with APL[22, 25].

For cases other than APL, it is first important to decide whether the patient is more suitable for a standard induction and consolidation chemotherapy or a non-intensive treatment regimen. In practice, there are many different treatment variations, and many more are currently in development. For the sake of brevity, below outlines the main principles and most established/common treatments. The standard induction regimen involves a 7-day cytarabine and 3-day daunorubicin infusion, commonly referred to as the 7+3 regimen. More recently, this was improved by complementing with targeted therapeutics, such as gemtuzumab ozogamicin (GO) and midostaurin, additional anti-neoplastic agents, such as cladribine or fludarabine (in adverse risk AMLs only), or replacement with CPX-351 (Vyxeos). GO is an anti-CD33 (myeloid marker) antibody

conjugate with a cytotoxic drug calicheamicin. It is approved for use with 7+3 induction regimen for leukaemias with 30% or more blasts expressing CD33. It is currently recommended for treatment of favourable risk leukaemias with core binding factor (CBF) gene rearrangements, where it was shown to be the most effective[22, 26, 27], but can also be indicated for intermediate risk AML[9]. Meanwhile, midostaurin is a specific FLT3 receptor inhibitor, recommended for use in patients with activating FLT3 mutations[22]. Finally, Vyxeos is a liposomal formulation of daunorubicin and cytarabine, which improves drug bioavailability and selective uptake by leukaemia blasts. It is recommended for use in therapy or myelodysplasia-related AMLs, where it was shown to improve overall survival by around 3.5 months when compared to the standard 7+3 regimen[28].

The aim of the induction regimen is a complete remission (CR; defined as 5% or less blasts in BM) or a complete remission with incomplete count recovery (CRi; *i.e.* residual thrombocytopaenia/neutropaenia). Subsequently, different consolidation regimens may be given, commonly either intermediate-dose cytarabine (IDAC) or Vyxeos (only if it was used for induction beforehand). Additionally, IDAC can be supplemented with GO or midostaurin. Finally, an important role in consolidation of intermediate or adverse risk AMLs is played by the allo-HSCT (discussed below)[22].

Lastly, for those patients with AML who are deemed not eligible for the standard induction regimen at the time of the diagnosis, non-intensive treatments may be chosen. Typically, this involves hypomethylating agents (HMA; azacitidine/decitabine) or low-dose cytarabine (LDAC) with or without venetoclax (inhibitor of anti-apoptotic protein Bcl-2). This offers a more tolerable regimen than standard induction chemotherapy. In 2023, the European Medicines Agency (EMA) approved the use of ivosidenib, a small-molecule inhibitor used for IDH1-mutant AML. The mutation is known to change the activity of the gene protein product isocitrate dehydrogenase, resulting in the production of oncometabolite 2-hydroxyglutarate (2-HG). In those not eligible for the standard induction, ivosidenib with azacitidine showed the median overall survival of 24 months compared to just under 8 months for azacitidine alone[29]. However, as such, HMA/LDAC based treatments do not offer a cure and should be continued either

until intolerance or disease improvement. If the latter occurs, this could be used as an opportunity to consider allo-HSCT (discussed below)[22].

Outlook and unmet need

To summarise, AML is a heterogenous disease occurring mostly in those over the age of 60. Its study and stratification by morphology, immunophenotype, cytogenetics, specific gene mutations and important clinical considerations allows to better understand which factors are important in predicting disease prognosis and thus the requirement for different treatment regimens.

Concurrently, the refinement of chemotherapies and the development of novel cancer-specific cytotoxic or differentiating treatments (ATRA, ATO, Vyxeos, GO, midostaurin, ivosidenib) allowed to gradually improve patient survival. However, youngest patient groups have benefited from care improvements over time the most (Fig. 1.2). The reason for this is likely determined by several factors, including the fact that older patients are more likely to have adverse risk disease[30, 31], myelodysplasia or therapy-related secondary AML[32] and poorer overall health[33, 34]. Moreover, some studies report that less than half of older (65+) patients diagnosed with AML proceed to receive chemotherapy[35, 36].

This strongly indicates that the highest unmet need is for those with the higher risk disease and/or those who are older. Indeed, the 5-year survival for patients diagnosed with ELN adverse risk AML is just around 20% in all ages (Fig. 1.3). One way to potentially address this is by studying one of the most important factors that changed between the 1960s (when AML was considered universally incurable) and today to allow a reduction in the risk of relapse for adverse risk patients - allo-HSCT.

1.2 Bone Marrow Transplantation for AML

Human haematopoietic stem cell transplantation (HSCT) was first reported in 1957 and was initially motivated by the prospect of discovering a cure for radiation injury-induced bone marrow failure[37]. However, initial HSCT trials in patients with malignant

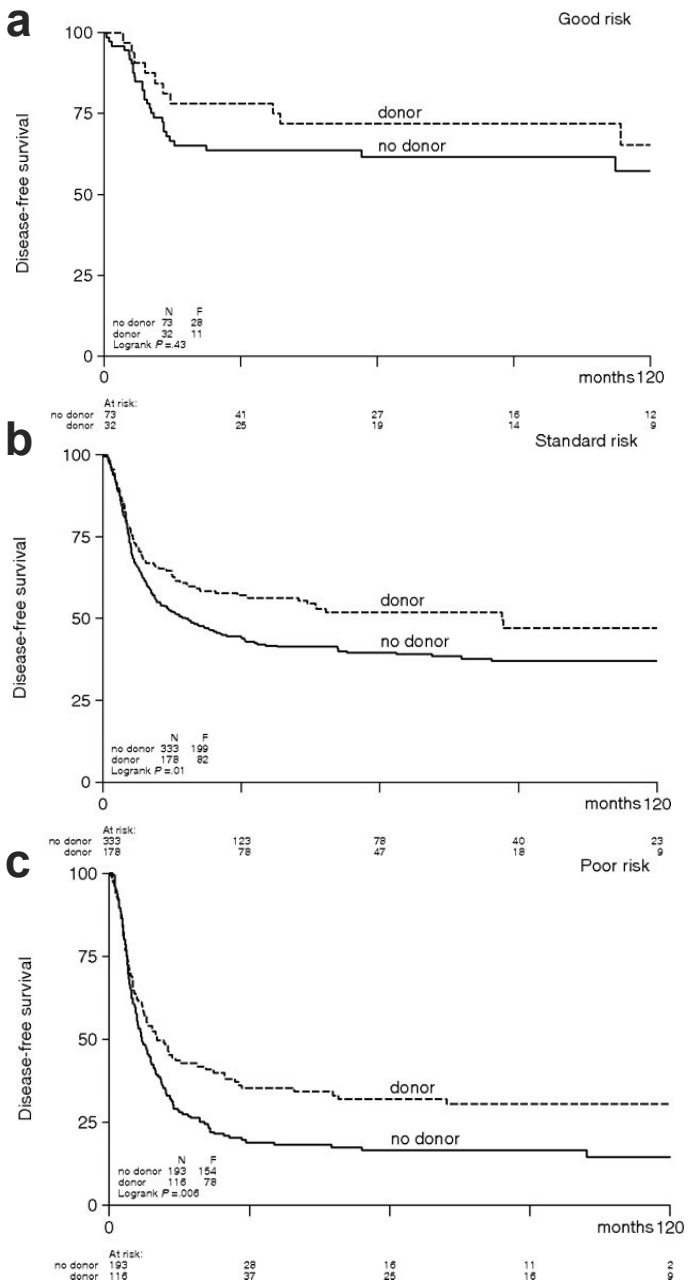

Figure 1.4: Relapse-free survival in AML patients according to risk stratification and allo-HSCT availability (HOVON-SAKK study[4]). **a**, **b** and **c** show good, standard and poor risk AML patients from HOVON-SAKK study. Donor arm received allo-HSCT. Figure from Cornelissen *et al.*[4])

and non-malignant diseases reported a 100% failure rate, related to graft failure, infections, recurrence of malignant disease and graft-versus-host disease (GvHD)[38].

Retrospectively, the initial failure of adopting HSCT into clinical practice is not surprising - the significance of many important factors for the success of transplantation was not established, including the relevance of human leukocyte antigen (HLA) matching, adequate conditioning regimens and immunosupression (discussed below)[39]. With key advancements in these areas, as well as the establishment of specialised patient care, initial successes in human HSCT were reported from late 1960s onwards[39]. The first patients successfully treated with HSCT were suffering from both malignant as well as non-malignant (*e.g.* aplastic anaemia) diseases. For haematologic cancers, HSCT, whether autologous or allogeneic, was proposed to have a dual benefit: 1) rescue from bone marrow failure after treatment with high dose chemotherapy and/or radiotherapy; 2) a graft-versus-leukaemia (GvL) effect, where the HSCT graft can target and eradicate remaining cancer[40, 41].

Indications for HSCT

Today, autologous and allogeneic HSCT is performed in several different treatment scenarios for AML. Most commonly, allo-HSCT is used as a consolidation treatment for intermediate and high risk AMLs after the initial induction chemotherapy regimen and during the first clinical remission (CR1). This guidance is based on the risk of relapse after consolidation with chemotherapy/auto-HSCT versus allo-HSCT for AMLs of different risk classifications. For example, the rate of relapse for patients with very poor risk AML is more than 90% after chemotherapy/auto-HSCT consolidation. With allo-HSCT, this is reduced to 40-50%. Meanwhile, for patients with good risk prediction, relapse after chemotherapy/auto-HSCT is 35-40%, whereas with allo-HSCT it is 15-20%[42]. These rates are reflected by the HOVON-SAKK study, which compared AML patients who were able to receive an HLA-matched allo-HSCT to those who were not (Fig. 1.4)[4]. Overall, the significant relapse-free survival and overall survival benefit for intermediate and poor risk, but not good risk AMLs was shown with a meta-analysis by Koreth and colleagues[43].

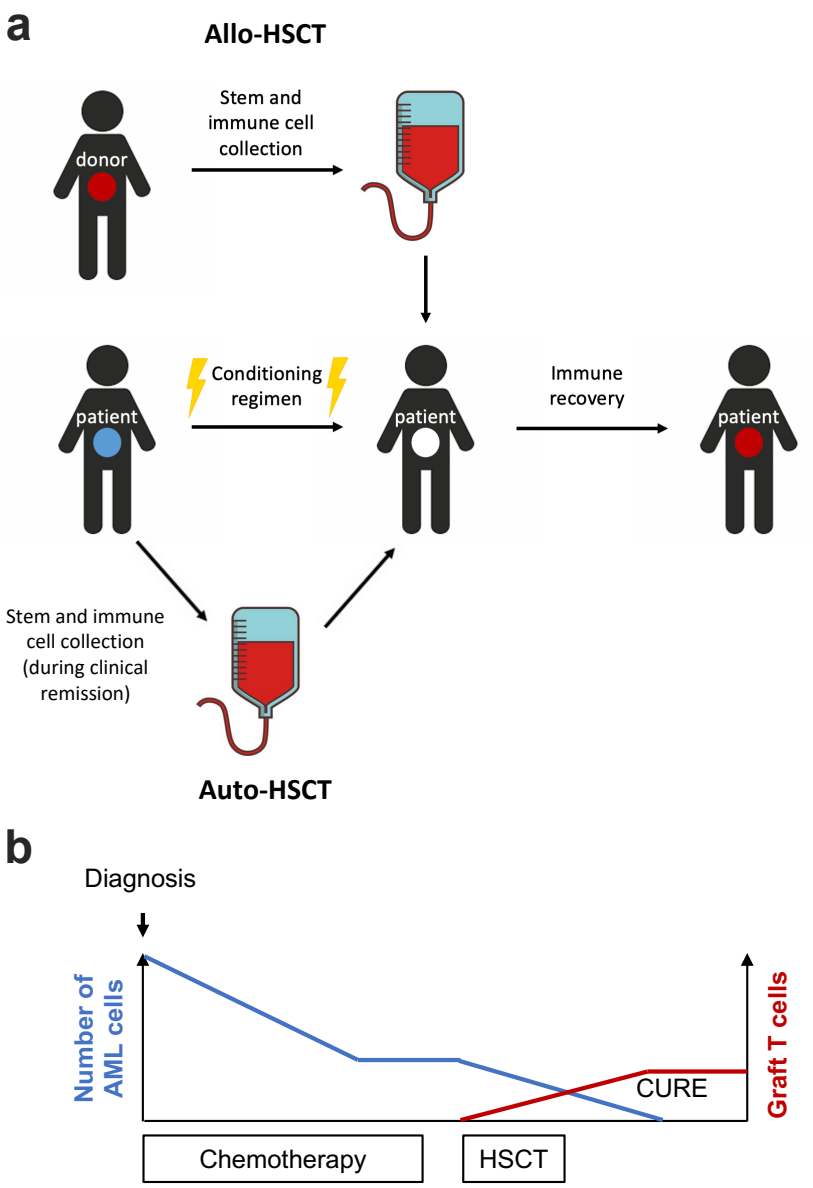

Figure 1.5: Schematic overview of allogeneic and autologous HSCT for AML. a shows the major steps required for bone marrow transplantation, including haematopoietic stem cell collection (either from a donor or the patient, during the clinical remission), conditioning regimen and transplant infusion. Circles represent the engrafted haematopoietic stem cell origin (blue - patient; red - donor). **b** shows an ideal treatment course for someone diagnosed with intermediate or adverse risk AML, starting with diagnosis, bulk disease burden reduction by chemotherapy (induction ± consolidation) and cancer-specific allogeneic T cell expansion following allo-HSCT, which exerts the GvL effect.

While generally not recommended for favourable risk disease, allo-HSCT may be indicated in cases where after induction and consolidation patients still have low level disease, termed as being minimal residual disease positive (MRD+). Furthermore, allo-HSCT is recommended in those who are primary refractory (do not achieve CR or CRi after up to two cycles of induction chemotherapy) or relapse after the initial treatment course[22].

Although not as common (see below), auto-HSCT also has its indications in the treatment for AML. For those with favourable risk disease, it may be used as a superior alternative for chemotherapy-based consolidation[44]. Similarly, in intermediate and adverse risk AML patients, were allo-HSCT is unavailable or inappropriate, auto-HSCT may be considered as well, due to better overall survival than treatment with chemotherapy alone[45, 46]. Indeed, one study has shown that for intermediate risk AML patients in CR1, the auto-HSCT arm had similar overall survival as the allo-HSCT arm, although the relapse-free survival in the auto-HSCT group was lower[46].

Modern transplantation

The schematic representation of the HSCT process is shown in Figure 1.5a. Whether for autologous or allogeneic HSCT, the patient first undergoes induction chemotherapy with the intention to enter CR. Whether further chemotherapeutic consolidation occurs is patient-dependent and is often determined by the availability of the allo-HSCT donor[22]. If the route of auto-HSCT is chosen, haematopoietic stem cells are collected during CR and cryopreserved for later reinfusion. Subsequently, patients undergo a conditioning regimen (discussed later), which is followed by the HSCT infusion. In proceeding weeks, months and years it is hoped that the transplant successfully reconstitutes all the haematopoietic and immune compartments[47], establishing a life-long chimerism. Importantly, as this occurs during allo-HSCT, it is hoped that the engrafting T cells are able to exert a GvL effect, thus curing the patient from AML (Fig. 1.5b; discussed below).

To minimise the risk of graft failure, GvHD, disease relapse and infections, the modern transplantation procedure is a complex, multi-step process. Decision to proceed with transplantation is taken after a careful consideration of the potential risks and

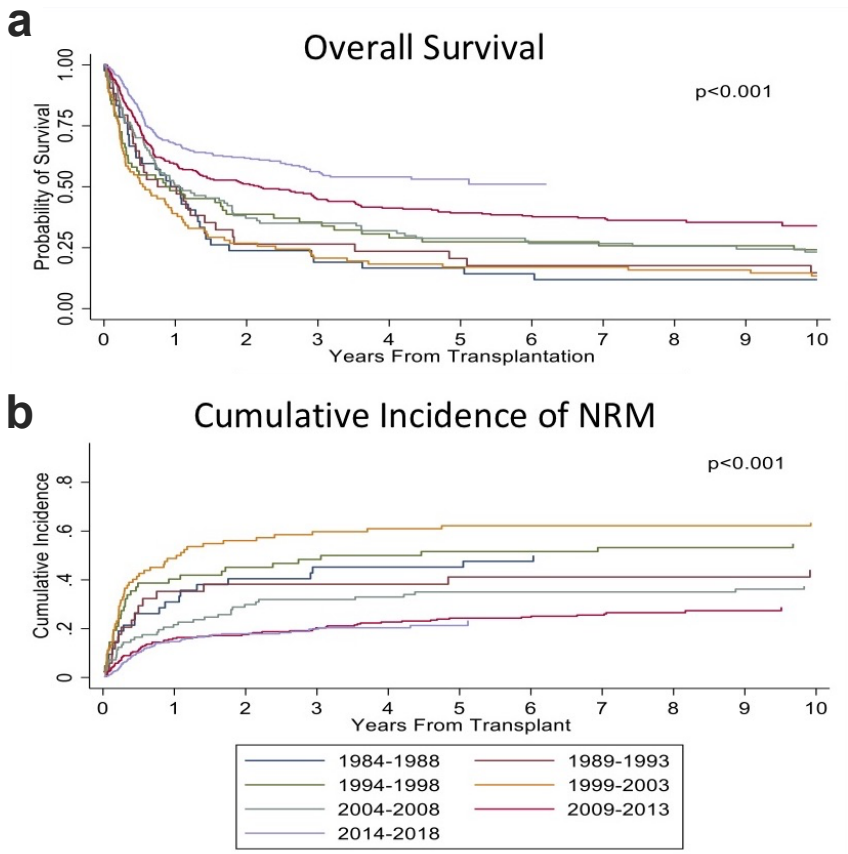

Figure 1.6: Overall survival and non-relapse mortality for AML and myelodysplastic syndrome (MDS) patients treated with allo-HSCT[5]. **a** and **b** show overall survival and non-relapse mortality, respectively, for 900 (705 of which were AML) patients treated with allo-HSCT, stratified by year of transplantation. P-value indicates the significance of difference in survival/mortality between groups. Figure adapted from Sigmund *et al.*[5]

benefits. Furthermore, the patient's wishes, comorbidities, frailty and available support at home must all be considered as part of the final decision. To help predict the non-relapse mortality after allo-HSCT, a haematopoietic cell transplantation-comorbidity index (HCT-CI) was developed, which considers multiple clinical factors and produces a single predictive numerical score[48]. Moreover, this can be used as a guiding factor when deciding on the most suitable conditioning regimen[22].

Having said that, transplant over the age of 75 is virtually non-existent, as indicated by the 2019 transplantation data from the United States of America[49]. This is in stark contrast with the peak age of AML diagnosis being 85-89 years old (Fig. 1.1a) as well as the knowledge that the older age groups are more likely to suffer from higher risk disease,

where patients benefit from transplantation the most[30–32]. Reassuringly, non-relapse mortality and overall survival after transplantation has been steadily improving over time(Fig. 1.6)[5]. Therefore, one hopes that the field of transplantation continues to push for further refinements that make the treatment less intensive and risky, whilst preserving, or even enhancing, its potential to cure AML.

Today, AML is the most common indication for allo-HSCT, making up over a third of total allo-HSCTs in Europe and the U.S.[1, 50]. From the year 2000, a steady increase in allo-HSCT for AML treatment in the U.S. has been primarily driven by an increase in transplant recipients who are 40+ years old, including those who are 65+. Meanwhile, although auto-HSCT can be indicated as an alternative to chemotherapy or allo-HSCT consolidation, it is virtually non-existent as an AML treatment in practice[1, 50].

Transplant source

In terms of the allogeneic transplant type, different donor sources as well as levels of matching are considered. For the former, haematopoietic stem cells may be retrieved directly from the BM, cord blood (CB) or PB. While $CD34^+$ (hameatopoietic stem and progenitor cells; HSPC) are naturally found at higher concentrations in BM and CB, this is not the case for PB. Therefore, PB allo-HSCT donors must be pre-treated with granulocyte colony-stimulating factor (G-CSF), such as filgrastim or lenograstim, for several days before apheresis[39]. G-CSF allows stem cell mobilisation and migration into the bloodstream through modification of important cell adhesion interactions[51].

Importantly, different $CD34^+$ cell sources have been associated with different benefits and risks to the recipient. Mobilised PB grafts are associated with faster engraftment, lower graft failure and may have slightly lower rates of disease relapse. However, they may also have somewhat higher rates of both acute and chronic GvHD[52–57]. Meanwhile, CB grafts have highly limited number of stem cells, due to the original size of the donation, but the level of HLA matching for CB transplant recipients is not as stringent[58]. These considerations are important in the context of other factors, such as donor age, disease and its level of progression; for someone with non-malignant disease, BM graft is generally preferred, as GvHD is the main concern. However, for

AML, preservation of the GvL effect is particularly important, which is intricately linked with the GvHD occurrence (see below)[39]. Having said that, mobilised PB is by far the most common source, making up ∼85% of all allo-HSCTs in the U.S.[1].

Transplant matching

When considering donor selection, HLA matching is by far the most important and practically stringent criterion. For BM or PB sources, matching is typically sought for both alleles of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQ, more commonly referred to as a 10/10 match. Where possible, HLA-DPB1 may also be matched (11/12 or even 12/12 match), as matching for HLA-DPB1 may reduce risks for acute GvHD[59, 60] and graft rejection[61, 62], but is also associated with leukaemia relapse[63, 64]. In cases where this is not possible, 9/10 or 8/8 matches are considered (where HLA-DQ is mismatched or not considered)[39]. Where 8/8 match is not available as well, one could consider related donor haploidentical transplantation, where each of the 6 HLA loci are matched in one allele. The probability of finding such a donor is high, since both parents of the recipient are nearly guaranteed to be a match, whilst any sibling is predicted to be a match ∼50% of the time. However, haploidentical HSCT requires special care to mitigate graft rejection and GvHD (see below). As an alternative, lower than 8/8 unrelated donor matches may also be considered, although this is associated with higher recipient mortality[65]. Meanwhile, CB transplants are only typically matched at class I alleles, with 5/6 or 6/6 matches[58], but their use has been largely superseded by the advent of haploidentical transplantation[39].

However, it is important to note that HLA matching is not necessarily straightforward. For most patients, the chance of finding an unrelated adult 8/8 donor is highly dependent on their ethnic group, and varying from 16% to 75%[66]. Importantly, not all HLA, and not all allelic mismatches, appear equally important. It is recognised that some HLA have generally lower expression (HLA-DRB3/4/5, HLA-DQ and HLA-DP) and are thus referred to as low expression loci (LEL). This explains why the predominant allo-HSCT matching for unrelated donors is 8/8, excluding all LEL HLAs[67]. Nevertheless, this is not to say that these loci are not important, and indeed a combination mismatch

for different LEL is associated with increased mortality[68]. Moreover, the concept of permissive HLA mismatches has emerged, where mismatches for certain HLA alleles are more tolerable than for others[39]. For example, HLA-DPB1 alleles can be grouped according to similar T cell epitopes (TCE). HLA-DPB1 mismatches that are permissive (same TCE group) are associated with decreased transplant-related mortality[69–72]. Overall, current research is only now beginning to decode the intricate rules of permissive and beneficial HLA mismatches. It is hoped that in the future, this will refine the treatment and widen allo-HSCT accessibility.

Beyond HLA-matching of the transplant, other donor matching factors are routinely considered as well, provided there is a choice between different suitably matched HLA donors. Firstly, age is regarded as the second most important characteristic in a donor, with preference of a younger donor than the recipient. This is based on a retrospective study finding that those who received a transplantation from a 10-year younger donor had 3% higher 2-year survival post-transplant[73]. Secondly, donor matching for latent cytomegalovirus (CMV) infection using a serological test is also important. A negative status for both the donor and the recipient is associated with the best outcomes, while CMV status mismatching in either direction may be worse than matching a positive recipient to a positive donor[74–76]. Lastly, matching for sex and ABO blood group may be considered, while the influence of latter on transplant outcomes appears to have decreased over time[73, 77–79].

Conditioning

Once a suitable allo-HSCT donor is identified, preparation for the transplantation includes conditioning, which is used to reduce the bulk of the disease (Fig. 1.5b), abolish host haematopoiesis (myeloablation) and host immune responses (lymphodepletion). In turn, this minimizes the chances of graft rejection and disease re-occurrence. Today, there is a wide variety of conditioning regimens and they must be chosen upon careful consideration of the disease, its progression, the level of donor HLA matching, graft source (PB/BM/CB) and comorbidities.

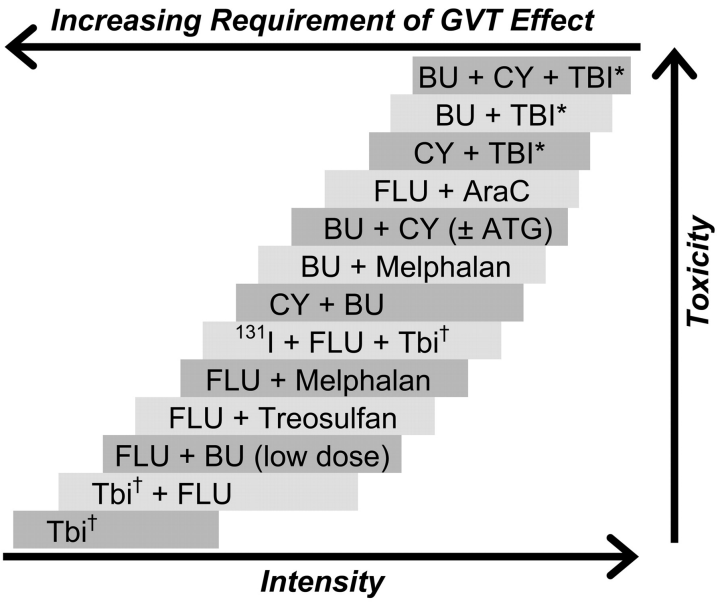

Figure 1.7: The continuum of HSCT conditioning regimen intensities from Deeg and Sandmaier[6] GvT: graft versus tumour, BU: busulfan, CY: cyclophosphamide, TBI: total body irradiation, FLU: fludarabine, AraC: cytosine arabinoside, ATG: antithymocyte glubulin, ^{131}I: anti-CD45 antibody conjugate with ^{131}I. Asterisk denotes high-dose and cross denotes low-dose TBI. Diagram from Deeg and Sandmaier[6]

Generally, regimens vary in their intensity. Most intense, myeloablative conditioning (MAC), is considered to produce lasting cytopaenias and such damage to the host haematopoiesis that the transplantation is absolutely necessary for recovery. One step lower is reduced intensity conditioning (RIC), which generally has lower doses of the same drugs used in MAC. Therefore, it can result in prolonged, but variable cytopaenias, which should require transplantation to aid the recovery. Lastly, non-myeloablative (NMA) conditioning is least intensive, can cause minimal cytopaenias and does not require HSCT for the haematopoietic recovery[80, 81]. However, it is important to note that different MAC, RIC and NMA conditioning regimens form a continuum of progressive intensity (*i.e.* myeloablation & lymphodepletion) (Fig. 1.7).

The HSCT conditioning regimen intensity is intricately linked with treatment toxicity and the subsequent reliance on the GvL effect. The most intense conditioning regimens can reduce tumour burden the most, whilst also posing the highest risk of toxicity and vice versa. As a result, the less one is reliant on chemotherapy and/or radiotherapy to reduce the tumour burden, the more it is necessary that the allo-HSCT produces a meaningful

GvL effect (Fig. 1.7). Initially, conditioning regimens were exclusively myeloablative, such as high-dose TBI or a combination of high-dose TBI with chemotherapeutic agents such as cyclophosphamide (CY). Although high-dose TBI regimens reduced the risk or relapse, they also caused significant side effects, such as pulmonary, gastrointestinal, kidney and liver toxicities, infertility and secondary malignancies[82]. To address this, the TBI+CY regimen was compared to Busulfan(BU)+CY in several studies, to show comparable survival and relapse rates[83–86].

With the discovery of the GvL effect (see below) came the rationale for development of lower intensity RIC and NMA conditioning regimens, which are more reliant on the GvL to eradicate the disease. Importantly, this allowed the recruitment of older patients for allo-HSCT. The seminal study by Slavin *et al.*[87] reported a RIC regimen with Fludarabine (FLU), BU and anti-thymocyte globulin (ATG), but since then, various different RIC and NMA conditioning regimens were reported (Fig. 1.7)[39, 82]. Importantly, a retrospective study by Shimoni *et al.* has shown that AML patients treated with MAC and RIC had similar leukaemia-free and GvHD/relapse-free 10-year survival, however, MAC had lower relapse rate and higher non-relapse mortality[88]. The inception of RIC/NMA regimens was transformative - today over half of conditioning regimens for AML are RIC/NMA, a change which likely contributed to the increase of allo-HSCT for recipients with malignant diseases over the age of 65[1].

Immunosuppression

Integral to allo-HSCT is the prophylactic immunosuppression (IS) to curb acute and chronic GvHD incidence. It is expected that 30-50% of allo-HSCT patients will experience acute GvHD, while 15% will have severe acute GvHD. In turn, acute GvHD is the main risk factor for chronic GvHD[39]. Acute GvHD involves three main sites - skin, liver and gastrointestinal tract (GI) and its severity can be graded from I (lowest) to IV (most severe). In turn, chronic GvHD may involve these sites as well as others, such as hair, nails, lungs, muscles, joints and genitalia. Chronic GvHD is defined as occurring after day 100 from allo-HSCT and can too be graded, into mild, moderate and severe.

Conditioning regimen, source of transplant (PB versus BM) and level of HLA matching are important risk predictors for GvHD. As discussed before, PB transplants as well as reduced HLA matching are directly associated with increased GvHD incidence. Meanwhile, increased intensity of the conditioning regimen, *i.e.* MAC versus RIC, is also associated with the increased incidence of GvHD[89–91]. Therefore, prophylactic IS regimens are different dependent on individual patients.

The most common combinations of prophylactic IS for MAC regimens are calcineurin inhibitors (CNI) (cyclosporine(CSA)/tacrolimus(TAC)) with methotrexate (MTX), which were shown to reduce GvHD incidence in prospective clinical trials in the 1980s and 1990s[92–94]. Whilst MTX is an antifolate agent, CNI specifically inhibits a Calcium-dependent phosphatase which is essential for the activation of the nuclear factor of activated T cell (NFAT). In turn, this inhibits T cell activation and effector functions[39]. CNI can also be used in combination with mycophenolate mofetil (MMF), which is a common IS prophylaxis for RIC[39]. Unfortunately, there is a lack of good quality clinical studies comparing MTX versus MMF in the aforementioned combination IS regimens. A Cochrane systematic review suggests no significant difference in GvHD incidence, overall survival, non-relapse mortality and incidence of relapse. However, there is evidence to suggest a more suitable safety profile with MMF[95].

For haploidentical allo-HSCT, where HLA disparity is higher than for the typical matched sibling or matched unrelated donors, IS regimen requires appropriate adjustment to minimise the risks of GvHD. Thus emerged the PTCy (post-transplant cyclophosphamide) regimen, which combines high-dose cyclophosphamide with TAC and MMF. With the use of PTCy, the progression-free survival and GvHD incidence between haploidentical and matched allo-HSCTs appears similar[96].

Finally, IS prophylaxis may be supplemented with targeted anti-T cell or anti-lymphocyte treatments, anti-thymocyte globulin (ATG) and anti-CD52 antibody (Alemtuzumab/Campath/Lemtrada), respectively. Several prospective clinical trials have shown the efficacy of ATG in reducing GvHD incidence[97–100]. However evidence on Alemtuzumab is lacking, despite its popularity in the U.K.[39].

Post-transplant monitoring and intervention

One final consideration on modern transplantation that is discussed here is on the importance of continuous post-transplant monitoring and early intervention. The level of HSCT engraftment, referred to as chimerism, is monitored periodically post-transplant using amplification and detection of variable genomic regions, such as repetitive DNA (tandem repeats) and single nucleotide polymorphisms (SNPs)[39]. With the advent of lower intensity conditioning, this measurement became especially important as RIC and NMA conditioning can result in varied and mixed chimerism immediately post-allo-HSCT[82].

Importantly, falling donor chimerism may be a sign of impeding graft rejection or malignant disease relapse. This is because the increased number of recipient cells could be either due to leukaemic blasts or the recovering recipient haematopoiesis, or both. However, even if it is the latter, host haematopoietic cells may interfere with the allogeneic GvL effect[101]. Therefore, chimerism analysis can and should be supplemented with cytometry or NGS-based MRD monitoring. In cases where mixed chimerism raises significant concerns, it can be addressed by cessation of post-transplant immunosuppression, infusions of donor lymphocytes or additional pharmacological therapy[39].

Outlook and unmet need

Having considered the complex landscape of modern day allo-HSCT, it is clear that numerous factors contributed to the improved treatment accessibility and outcomes since human HSCT was first introduced. Practice improvements focused on mitigating three major allo-HSCT complications - graft failure, GvHD and disease relapse. Novel conditioning regimens, post-transplant immunosuppression, donor matching and careful post-transplant monitoring were only few amongst many factors that likely contributed to the steady increase in the survival of allo-HSCT patients over time (Fig. 1.6a). Importantly, improved safety and tolerability of allo-HSCT allowed wider patient recruitment, improving access to those over the age of 65[1].

Despite this, allo-HSCT remains an intensive treatment route. Conditioning and immunosuppression regimens can cause considerable side effects. Moreover, as a

result, patients are immune-deficient and susceptible to a variety of different infections. Furthermore, despite the prophylaxis to mitigate the risk of GvHD, both acute and chronic GvHD can occur in around 30-60% of patients. Worryingly, in those who require systemic intervention to curb their GvHD, the first line treatment (steroids) improves the disease in only up to a half of patients[39], necessitating alternative treatment methods.

Hence, the 3-year survival in patients with intermediate and adverse risk AML who receive any type (matched, mismatched or haploidentical, related or unrelated) allo-HSCT during the CR1 is between 49 and 57% (2010-2020 U.S. CIBMTR data)[1]. Unfortunately, we know that virtually all of these patients are 75 or younger[49], which means that a substantial fraction of AML diagnoses are never treated with HSCT (peak age of diagnosis is 85-89; Fig. 1.1a). This means that whatever improvements were made to make allo-HSCT more accessible, they did not outweigh the risks of treatment for the oldest patient groups. However, one important aspect of the allo-HSCT was not discussed yet. Knowing that two mechanisms were proposed by which allo-HSCT can cure AML, namely high-dose chemotherapy and GvL, what do we know about the existence and the underlying biology of the latter?

1.3 Graft-versus-Leukaemia (GvL)

The first empirical description of the GvL effect came in the late 1970s and early 1980s, when a group in Seattle showed that acute leukaemia patients were more likely to remain in remission post-transplant if they also experienced grade II-IV GvHD (involvement of skin and liver/GI)[7] (Fig. 1.8a). Soon after, with the advent of acute versus chronic GvHD classification, the same group reported higher remission rates in acute leukaemia patients who experienced chronic or chronic and acute GvHD[8] (Fig. 1.8b). In the proceeding years, numerous studies reported on the existence and nature of the GvL effect through several different associations of cancer remission with transplant type, level of GvHD, conditioning type and donor lymphocyte infusions. Furthermore, cancer relapse post-transplant was reported to show distinct mechanisms of immune evasion. These lines of evidence made the GvL effect ubiquitously accepted. Today, allo-HSCT is considered to be the world's most established as well as oldest immunotherapy.

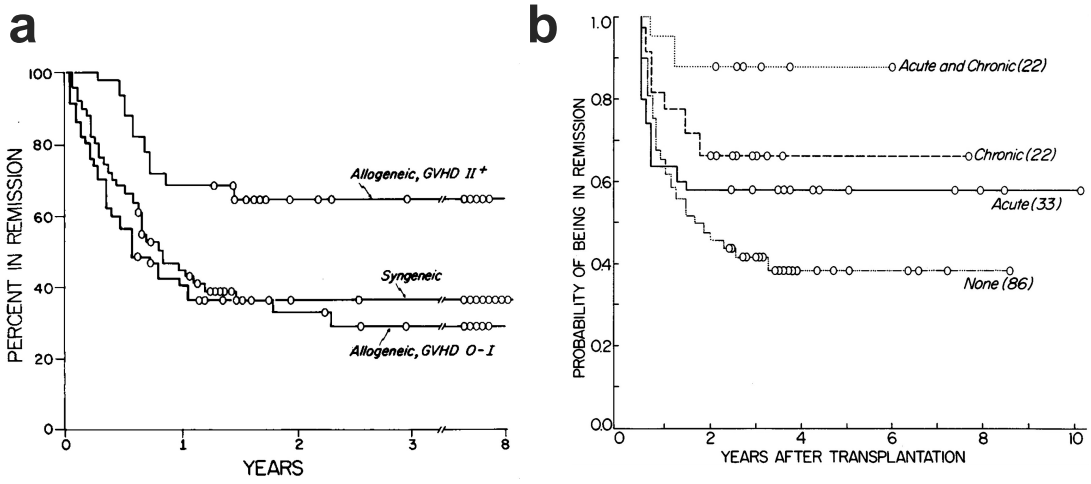

Figure 1.8: Post-transplant remission is higher in acute leukaemia patients with GvHD (Weiden *et al.*[7, 8]) **a** and **b** show percentage of acute leukaemia patients in remission after HSCT, stratified by severity or type of GvHD, respectively. 'Syngeneic', refers to patients who received allo-HSCT from monozygotic twins. All other transplant groups were HLA-matched sibling allo-HSCTs. Figures **a** and **b** are from Weiden *et al.* 1979[7] and Weiden *et al.* 1981[8], respectively.

Evidence for the GvL effect

GvHD and GvL correlation

With further association studies of the GvL effect with the GvHD, two important concepts emerged. For one, GvL and GvHD are closely related, which means that the overall patient survival is balanced between the risk of relapse and the risk of GvHD. This was elegantly shown in 1989 with a retrospective analysis of 1,200 patients transplanted with unmodified HLA-matched sibling transplants. While the probability of relapse was greatly reduced in those who had either acute or chronic GvHD, or both, the overall survival was similar between those who never had GvHD and those who had both the acute and chronic GvHD. Instead, highest survival was seen in those who had either chronic or acute GvHD, but not both. Furthermore, comparing patients in different stages of chronic myeloid leukaemia (CML), namely in chronic and in accelerated or blast phase, showed opposite effects for those who had GvHD. Whilst GvHD decreased overall survival in those with chronic CML, it improved survival as well as decreased relapse incidence in those with accelerated or blast phase CML[102].

Similar trends were shown for a cohort of ∼1000 AML cases treated with HLA-matched sibling allo-HSCT in CR1, where relative risk of relapse was reduced with GvHD, especially if both acute and chronic types co-occurred. However, this benefit was countered by the overall treatment failure (a composite measure of relapse and death), where AML patients who had both acute and chronic GvHD were 1.51 times more likely to experience it compared to the no GvHD group[103]. A more recent retrospective study on nearly 1,900 AML patients confirmed this[104]. Additionally, it compared overall survival, relapse incidence and non-relapse mortality in patients with different severities of GvHD. While those with any grade GvHD were less likely to experience relapse, only those with lowest grade GvHD had higher overall survival. This was explained by increased non-relapse mortality for those with grade II-IV GvHD. Finally, a smaller prospective study with 93 AML patients confirmed that absence of chronic GvHD is associated with increased relapse rate and decreased overall survival[105].

Are GvHD and GvL two sides of the same coin? The second concept that emerged from the GvHD and the GvL effect association studies is that GvL may occur in isolation. Horowitz *et al.* study showed that AML patients who received a syngeneic transplant (from a monozygotic twin) had a relative relapse risk of 2.58 compared to those who received an HLA-matched sibling allo-HSCT and experienced no GvHD[103]. This suggested that some GvL persists, even in the absence of clinically measurable GvHD. Of note, the seminal study by Weiden *et al.*[7] did not observe this effect (Fig. 1.8a). However, the Weiden *et al.* cohort was considerably smaller. Hence, a subsequent, larger cohort study by the same Seattle team was able to show a significantly higher relapse rate in patients who received syngeneic transplants[106]. More recently, a retrospective analysis of nearly 50,000 patients treated with allo-HSCT for different haematological malignancies showed that an increasing severity or extent of acute and chronic GvHD correlate well with reduced relapse incidence of ALL and CML, but this correlation is not as strong for AML[107]. This further suggested that GvL and GvHD have distinct mechanisms.

Association with allogeneic transplant type

However, allo-HSCT type, level of matching and constitution may vary considerably. Given that allogeneic, but not syngeneic transplants were associated with the GvL effect, one would expect that increasing levels of genetic mismatch between the donor and the patient would augment it. However, within the context of HLA-matched transplants, studies report conflicting results on the difference in relapse incidence between sibling and unrelated transplantations[108, 109]. Meanwhile, transplant HLA mismatches (7/8 or 6/8) are not associated with decreased risk of relapse either, despite increasing non-relapse mortality[65]. Similarly, PB-derived grafts may have higher GvHD incidence as compared to BM[39, 52–57] but the relation between PB grafts and relapse incidence is varied, with some studies reporting a positive association[52, 54] and others none[53, 55–57].

But perhaps the more convincing allo-HSCT variable associated with the GvL effect is allogeneic T cell depletion. It can be performed both *ex vivo* and *in vivo*, with the intention to minimise the risk of GvHD. *Ex vivo* depleted HLA-matched sibling BM grafts for the treatment of AML were retrospectively associated with increased graft failure, decreased acute and chronic GvHD as well as increased disease relapse incidence[103, 110]. Notably, a more recent, smaller prospective study by Pasquini *et al.* employed *ex vivo* $CD34^+$ cell selection as a way of T cell depletion in HLA-matched sibling PB grafts. It showed lower chronic GvHD rates and no difference in graft failure or AML relapse[111]. Although the reasons for these conflicting findings are unclear, it is important to note that the graft source in these studies was different (PB versus BM) and any differences in the level of T cell depletion are unknown. Importantly, unmodified PB grafts have approximately 10 times higher numbers of T cells than BM grafts[39]. Nevertheless, Pasquini *et al.* study suggests that the GvL effect may be preserved in T cell depleted PB allo-HSCT, despite reduction in GvHD.

Association with RIC

With the discovery of the GvL effect, the popularity for MAC decreased, making RIC the more common allo-HSCT conditioning regimen today[1]. Knowing that RIC is less

capable at eliminating disease (the myeloablative effect is regarded as less pronounced or even reversible)[80, 81], sustained remission is theoretically more dependent on the GvL effect. Most studies comparing MAC and RIC regimens for allo-HSCT in AML patients are retrospective. Taken together, there is a consensus for lower non-relapse mortality and higher relapse rates in RIC[112].

In addition, one prospective randomised study investigated RIC versus MAC in 272 patients with AML and myelodysplastic syndorme (MDS). The overall survival at 18 months post-transplant was somewhat higher in MAC (by 9.7%) but was not statistically significant. Meanwhile, non-relapse mortality and grade II-IV GvHD were higher in MAC, whilst relapse lower (13.5%) than in RIC (48.3%)[113]. Therefore, it is clear that relapse is more likely with RIC, while GvHD and non-relapse mortality are more prevalent in MAC. However, RIC increased transplant accessibility for those who would not be fit to undergo MAC. Despite the higher relapse rate, allo-HSCT with RIC still offers an opportunity to reduce the risk of relapse.

To further indicate that the reduced risk of relapse after RIC and allo-HSCT is because of the GvL effect, rather than less frequent cure rate with RIC versus MAC alone, a large retrospective analysis of nearly 50,000 patients treated with allo-HSCT has shown that the hazard ratio for relapse is not significantly different between AML patients with different grades of GvHD that received MAC or RIC. *I.e.* decreasing risk of relapse was associated with increasing GvHD and this correlation was unaffected by MAC versus RIC. In contrast, if MAC itself was associated with higher cure rates, relapse risk would correlate less with increasing GvHD than in RIC[107].

DLIs promote GvL

In addition, discovery of the GvL effect prompted the use of donor lymphocyte infusions (DLIs). Given the importance of allogeneic T cells for reduced relapse incidence in BM allo-HSCTs (see above), it was proposed that post-transplant infusion of donor lymphocytes may improve patient remission rates. This was initially introduced in 1989, when a small cohort of mixed haematologic cancer patients (AML, ALL, CML and lymphoma) was treated with pre-emptive DLI infusions immediately post-transplant (day

1 through 5). Perhaps unsurprisingly, this study proved unsuccessful - those who received the DLIs saw highest rates of GvHD and non-relapse mortality, with no difference in relapse incidence[102]. Since, it was found that several factors determine DLI risk versus benefit - disease type and stage as well as the timing of DLI infusion. Later studies reported significant improvements in relapse incidence for CML patients who relapsed post-transplant[114, 115]. Importantly, DLI infusions were carried out months to years after initial allo-HSCT. In a prospective study involving 66 transplanted and subsequently relapsed CML patients, 67% achieved molecular remission. Meanwhile, those who relapsed with accelerated/blast phase disease or soon after allo-HSCT were less likely to benefit from DLI[115, 116].

However, CML was found to be the most sensitive haematological malignancy to DLIs. In AML, response to DLI after post-transplant relapse is considerably lower. In one study, while response rate for CML was 60%, for AML it was only 15.4%[116]. However, a larger retrospective study showed that those who receive DLI to treat post-transplant AML relapse are more than twice likely to survive at 2 years than those who do not (21% versus 9%)[117]. Similarly to previous findings with CML, response to DLI was also associated with later relapse post-allo-HSCT (5 months or longer), lower disease burden at relapse and favourable AML cytogenetics[117]. Hence, even though AML has a considerably lower response rate to DLI, it can still significantly improve survival in those who relapse after transplant. But the reason for AML having a lower response rate to DLI is unclear. It may be that the proliferative rate of the disease is too high, overwhelming the GvL effect[118]. This is in line with lower observed DLI benefit in those who have advanced phase CML[115].

Given the benefit of DLI in the treatment of post-transplant relapse, pre-emptive or prophylactic DLIs are being explored as well[118, 119]. Whilst the latter is used to reduce the risk of relapse, the former is performed either at MRD^+ status or at reduced donor chimerism. In a mixed AML and ALL patient cohort, preemptive DLI at MRD^+ status was shown to improve 3-year survival (MRD^+ without DLI 26% and MRD^+ with DLI 80%) and reduce disease relapse (MRD^+ without DLI 63% and MRD^+ with DLI 6%)[120]. Meanwhile, in a matched-pair retrospective analysis of acute leukaemias (AML and

ALL), prophylactic DLI was found to be advantageous only for high-risk AML, where it improved 5-year survival (69.8% versus 40.2%), reduced relapse incidence (30.5% versus 46%)and even the non-relapse mortality rate (7.4% versus 18.6%)[121]. To summarise, under favourable circumstances, DLI can be a powerful tool to augment the GvL effect.

Immune evasion mechanisms post-transplant

Finally, the GvL effect can be inferred from the changes that occur in relapsed leukaemic blasts post-transplant. A seminal study published in 2009 and lead by a team in Italy, had reported that of 17 patients that relapsed post-haploidentical allo-HSCT, five showed acquired loss of the entire mismatched HLA locus[122]. Furthermore, mismatched HLA loss was reported in a patient who received multiple partially HLA-matched unrelated transplants and relapsed. Loss was seen for both the mismatched HLA-C and HLA-DP alleles[123].

Interestingly, HLA loss was seen outside of the HLA mismatched context too. A study of 12 relapsed patients who received matched unrelated donor transplants found two patients with HLA loss, one with class I HLA focal deletions and the other with a loss of heterozygosity in class I and class II HLA. This was despite a complete matching in class I and a partial matching in the class II HLA loci (10/10)[124]. It is important to note that for the studies described so far, leukaemia relapse was 'late', *i.e.* 100 or more days since the relevant transplantation. This further suggests that a meaningful GvL effect has had the time to occur. Overall, these findings imply that the GvL effect may operate through recognition of both major HLA (differences in HLA alleles) as well as minor HLA (differences in peptides presented on matched HLA) mismatches. Given that both class I and class II HLA loss had been observed, the GvL effect may be mediated through both.

Importantly, some immune evasion mechanisms appear solely at the transcriptional and protein level. Another seminal study, this time from a team in the U.S., has reported that out of 15 AML patients who relapsed post-transplant, none showed genetic mutations associated with antigen presentation. Instead, among seven relapsed patients who were RNA sequenced before and after relapse, significant reduction in expression of both class I and class II HLA as well as associated genes was seen at

relapse. The control group of AML relapse patients treated with chemotherapy only did not show this trend. Further, reduction in HLA expression was confirmed at a protein level using cytometry. Interestingly, for a few patient samples, transcriptional HLA class II loss could be averted by short-term *in vitro* culture with interferon-γ (IFNγ)[125].

Another study by Toffalori *et al.*[126] confirmed that some AML samples show transcriptional HLA class II loss post-transplant. In addition, it has reported that some samples also show a reduction in T cell co-stimulatory and adhesion molecules and an increase in T cell co-inhibitory molecules, at both transcriptional and protein level. These changes were mutually exclusive from transcriptional HLA class II loss, suggesting the two may be distinct immune evasion mechanisms. Therefore, study of post-transplant AML relapse samples strongly indicates that allo-HSCT may impose a selective pressure for the cancer to evade allogeneic T cell and HLA-mediated, but not necessarily HLA-mismatched, immune responses.

The GvL Effector Cells

Evidence presented so far suggests that the GvL effect is mediated by allogeneic T cells. For one, some studies have shown that the incidence of AML relapse increased with *ex vivo* T cell depleted BM allo-HSCT[103, 110]. Furthermore, at post-transplant relapse, AML blasts were shown to acquire immune evasion mechanisms consistent with allogeneic T cell recognition, namely loss of both class I and class II HLA at genomic level, transcriptional downregulation of HLA class II or change in the balance of T cell costimulatory versus coinhibitory surface proteins (see above). Given that mixed HLA class I and class II loss has been shown, the immune selection may occur through both $CD8^+$ and $CD4^+$ T cells.

However, T cells are not the only immune cells capable of recognising HLA. Natural Killer (NK) cells are innate lymphoid cells that express Killer-Cell Immunoglobulin-like Receptors (KIR). Different KIRs have the capacity to activate or inhibit the NK cell expressing them, and the balance of these signals ultimately determines if the cell kills its target[127]. NK cells can express different KIR inhibitory receptors for different class I HLA allelic groups. Therefore, in the context of allo-HSCT, a mismatched self receptor

(inhibitory KIR) may not recognise HLA class I alleles present on recipient cells, leading to a cytotoxicity response in the Graft-versus-Host (GvH) direction[128]. Given that the 'missing self' hypothesis relies on ubiquitously expressed HLA class I proteins[129], which are typically matched in sibling and unrelated donor matched transplants, the NK alloreactive response is most relevant in haploidentical or mismatched transplants. Indeed, it was shown that in AML patients transplanted with haploidentical allo-HSCT, alloreactive NK cells could be identified and isolated in cases where KIR ligand in the recipient was missing (*i.e.* a specific HLA class I allelic group). *In vitro* experiments showed that these NK cells could mount a cytotoxic response against the healthy recipient cells and the leukaemia blasts[130]. Furthermore, a retrospective study of AML patients transplanted with either NK-alloreactive or NK-non-alloreactive haploidentical allo-HSCT showed that the NK-alloreactive group was associated with significantly lower relapse incidence in patients transplanted at CR (3% versus 47%) [131].

Interestingly, alloreactive NK clones are detected only in the first few months after transplant and thereafter engrafted donor-derived NK cells become non-reactive to the mismatched HLA alleles[130, 131]. High NK cell chimerism is associated with decreased relapse incidence early post transplant (at day 14), but has no association with incidence of acute GvHD. On the opposite, high early T cell chimerism is associated with acute GvHD and has no association with disease relapse[132]. However, another study reports that successful immune reconstitution, as measured by $CD4^+$ T cell chimerism, is associated with significantly lower disease relapse and non-relapse disease mortality in children who received allo-HSCT for myeloid leukaemia. NK cell chimerism was not as good a predictor as the $CD4^+$ chimerism[133]. Therefore, it remains unclear how engraftment dynamics correlate with different types of transplantation (haploidentical versus matched), alloreactive T cell and NK cell responses, and patient outcomes. Different transplantation, treatment and disease contexts may influence what cells, and when, exert a successful GvL effect. In turn, greater harmonisation between different transplantation centres regarding standardised immune subset tracking platforms post-transplant could inform us on any potential risk factors for relapse as well as transplant-related complications[39, 134].

1.4 Anti-Cancer T Cell Therapies

Despite a considerable body of research on the GvL effect, little can be done to harness it today. The GvL effect can only be inferred retrospectively, and no markers exist to measure it in real time or accurately predict its occurrence. Furthermore, little is known about the leukaemia antigens that mediate an effective or even curative GvL effect. Given the importance of alloreactive T cells in achieving AML remission (see above), one way to attempt closing this knowledge gap is by studying T cell responses and alloreactive T cell antigens in AML patients and pre-clinical models. In turn this could provide several therapeutic applications: 1) Refined HSCT donor matching to maximise the chance of curative GvL; 2) Real time post-transplant GvL effect measurement; 3) Delivery of antigen-specific immune therapies that augment/elicit the GvL effect and do not cause/promote GvHD. The third application could be especially useful for those cases where allo-HSCT is not possible - due to an absence of a suitable donor or patient's comorbidities and frailty. Therefore, this section focuses on the research efforts made so far to discover novel antigen-specific T cell responses against AML, especially within the HSCT context.

Putative antigen targets

There is a wide variety of HLA-restricted antigens that conventional $\alpha\beta$ T cell receptor (TCR) carrying allogeneic T cells may recognise on leukaemia blasts (Fig. 1.9). For one, cancer-specific mutations, such as gene fusions, insertions, deletions and non-synonymous amino acid substitutions, produce mutant peptide sequences (neoantigens or NeoAg) to which neither autologous nor allogeneic T cells would have been tolerised during thymic selection. These mutations can offer most specific cancer recognition, sparing all healthy tissues. Furthermore, NeoAgs are often produced by cancer-enabling mutations, such as those in proto-oncogenes, which could mean that antigen loss as an escape mechanism may be less likely to occur due to cancer's dependence on the mutation. However, it is important to note several drawbacks in NeoAg targeting. It

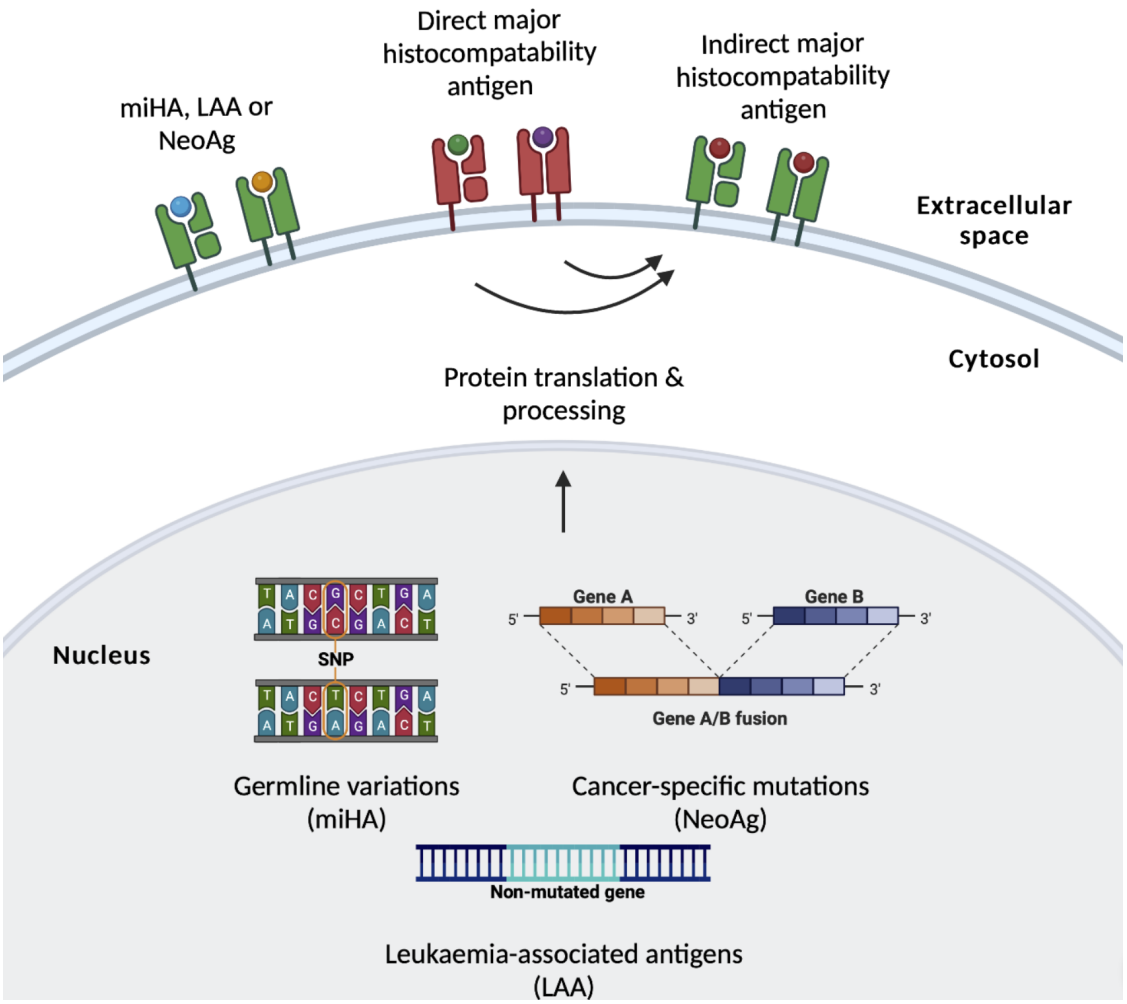

Figure 1.9: The variety of putative T cell receptor (TCR)-mediated AML targets within the allo-HSCT context. Antigens may be derived from germline variations (minor histocompatability antigens or miHA) or cancer-specific mutations (neoantigens or NeoAg), which result in amino acid sequence changes. Alternatively, leukaemia-associated antigens (LAA) are derived from non-polymorphic and non-mutated genes with higher expression in leukaemia than in normal tissues. Resultant peptides can be presented on either matched HLA (green) or mismatched HLA (red). Allogeneic T cells may be HLA-matched and may recognise miHA, LAA or NeoAg. Alternatively, allogeneic T cells may recognise mismatched HLA instead (direct major histocompatability antigen). Finally, mismatched HLA may also be processed to peptides and presented on matched HLA, resulting in allogeneic T cell recognition of indirect major histocompatability antigens. One or two transmembrane domains on the HLA proteins signify class I and class II HLA, respectively.

is known that cancers have considerable clonal diversity, including AML[135], which means that NeoAg$^-$ cancer cells could be selected for with a NeoAg-specific T cell therapy. Moreover, only some cancer mutations in AML are recurrent, limiting the choice of putative NeoAg targets for therapeutic development[136]. Lastly, AML is known to have a low mutational burden. A publication by the Cancer Genome Atlas Research Network showed that AML has an average of 13 mutations across the whole genome, with only $\sim$5 of those being recurrent[136]. Indeed, compared to other cancers, AML has one of the lowest mutational burdens[137].

An alternative type of HLA-restricted targets is minor histocompatability antigens (miHA), which are present in the allo-HSCT context only. MiHAs are produced by germline variations, such as single nucleotide polymorphisms (SNPs), that lead to amino acid changes (Fig. 1.9). As opposed to major histocompatability antigens (HLA), miHAs could be found in any protein-encoding gene that is not HLA. A typical human genome has about 10,000-12,000 such sites, depending on the ancestry[138]. Therefore, it is expected that an allo-HSCT donor will have thousands of miHA mismatches when compared to the recipient, and allogeneic T cells could potentially recognise them post-transplant. However, miHAs also have some drawbacks. First, they are encoded in the germline, meaning that all cells, healthy or malignant, have the required genotype to express them. Because of this, studies discovering novel miHA T cell responses in AML may focus on polymorphisms in haematopoietically-restricted genes. Second, it is not expected that miHA themselves are necessary for cancer survival, although the genes encoding them may be. As mentioned before, cancer cell dependence on the antigen in question may be therapeutically beneficial.

A third type of HLA-restricted targets is leukaemia-associated antigens (LAA). These are produced by neither polymorphic nor mutated protein-encoding genes, but rather are aberrantly expressed or overexpressed in leukaemia (Fig. 1.9). For example, it could be cancer testis antigens, which are normally expressed in germline cells only, however, the cancer cells are known to re-express it. Alternatively, it could be any gene which is expressed at a higher level in leukaemia cells than in healthy tissues, including healthy haematopoietic cells. Since these antigens are non-polymorphic and

non-mutated it is expected that the central tolerance mechanism eliminates any T cells possessing high-affinity LAA-specific TCRs. Therefore, it is generally agreed that tumour-associated antigens (TAA) elicit low-affinity T cell responses[139].

HLA mismatches may serve as GvL target antigens as well. Practically, we know such is the case because leukaemia blasts can lose mismatched class I and class II HLA post-transplant (see above, Section 1.3). However, the level of HLA matching is related to non-relapse mortality, graft rejection and GvHD occurrence (see above, Section 1.2), limiting the use of HLA mismatches to exert a GvL-specific effect. Notably, mismatched HLA recognition is not always direct; the allogeneic HLA complexes may be digested to peptides and presented on matched HLA, leading to indirect major histocompatability antigen presentation (Fig. 1.9). Their presentation on matched HLA can be predicted using the Predicted Indirectly ReCognizable HLA Epitope (PIRCHE) algorithm[140].

Finally, an important layer of antigen restriction is provided by the presenting HLA complex, which is necessary for the $\alpha\beta$ TCR-mediated recognition. It is known that different HLA alleles have different repertoires of presented peptides, determined by the peptide binding affinity to HLA. As a result, multiple different HLA binding prediction algorithms have been developed, although their accuracy is variable and generally lower for class II alleles[141]. In any case, putative antigen peptide binding to the desired HLA allele must always be functionally tested. Furthermore, HLA restriction means that the usefulness of a GvL-mediating TCR will be limited to those patients with the required allele. Hence, it is common to investigate only the antigens presented on common HLA alleles, such as HLA-A*02:01[142].

Importantly, AML is known to express HLA class II[125], which may offer a unique opportunity for leukaemia-specific and TCR-mediated targeting. Under steady-state conditions, HLA class II expression is restricted to professional antigen presenting cells (APC). Hence, after transplant, antigen presentation by the HLA class II may offer an opportunity to restrict allogeneic T cell responses to the recipient's haematopoiesis as well as leukaemia. As the transplant engrafts, toxicity to the healthy haematopoiesis would be self-limiting. Meanwhile, the other healthy tissues would not be expected to present the antigen, even if it is expressed, due to the absence of HLA class II machinery.

Antigen-specific T cell responses against AML

Neoantigen-specific

Despite the low mutational burden, some AML mutations were investigated for their ability to produce immunogenic neoantigens. Mutated nucleophosmin 1 ($NPM1^{mut}$) is a recurrent mutation found in just under a third of *de novo* AMLs[136].It is included in the ELN 2022 risk classification guidelines, where by itself it confers a favourible risk, but in combination with FLT3 mutation or adverse risk cytogenetics it is classed as intermediate or adverse risk, respectively[9]. In one study, putative $NPM1^{mut}$ neoantigen peptides were investigated for binding to HLA class I and II alleles, using bioinformatical prediction algorithms. The resultant peptides were screened *in vitro* against healthy volunteer and $NPM1^{mut}$ AML patient T lymphocytes. Both groups showed activation to $NPM1^{mut}$ peptides, and highest levels of T cell activation were associated with peptides that could activate both $CD4^+$ and $CD8^+$ T cells[143]. In an alternative approach, van der Lee *et al.* used $NPM1^{mut}$ patient AML samples to identify putative immunogenic $NPM1^{mut}$ peptides from mass spectrometry of immunoprecipitated HLA class I molecules. The identified $NPM1^{mut}$ HLA-A*02:01-binding peptide was used to raise antigen specific clones from healthy volunteer PBMC donations. Next, the antigen-specific TCR was identified and retrovirally transferred to third party T cells. The transgenic TCR therapy was shown to specifically lyse $NPM1^{mut}$ AML samples *in vitro* as well as decrease cancer cell line burden in an immunodeficient mouse xenograft model[144].

Another mutation proposed to produce a neoantigen response is in FLT3. Internal tandem duplications (ITD), a type of FLT3 mutation, are common in AML too[136]. A case report of an AML patient with the FLT3-ITD mutation has investigated putative FLT3 mutant peptides with high predicted binding affinity to the patient's own HLA. This identified an HLA-A*01:01-restricted peptide, which elicited patient T lymphocyte activation *ex vivo*. Antigen-specific lymphocytes could recognise target cell line K562 as well as patient's AML blasts[145].

Furthermore, fusion oncogenes can produce neoantigens as well. Core-binding factor (CBF) leukaemia can be characterised by the CBF-MYH11 fusion, which is associated with favourable risk disease[9]. A study by Biernacki *et al.* has shown that putative

CBF-MYH11 fusion neoantigen peptides can be predicted boinformatically. One peptide, presented by HLA-B*40:01, was used to raise an antigen-specific T cell response from healthy volunteer PBMCs. CBF-MYH11 fusion-specific T cells could recognise and kill both cancer cell lines and CBF-MYH11^{+} AML samples. Furthermore, CBF-MYH11 fusion-specific T cells could control primary AML progression in an immunodeficient mouse xenograft model[146].

While the aforementioned responses were largely isolated from the naive T cell subset of healthy voluneer PBMCs, there is some evidence that neoantigen-specific T cell responses can be found in AML patients, both in an autologous as well as an allogeneic setting. In one study, 25 NPM1^{mut} AML patients were investigated for *ex vivo* CD8^{+} T cell responses against NPM1^{mut} peptides to show that those with the antigen-specific cytotoxic T lymphocytes had better overall survival[147]. Furthermore, a case study by Hofmann *et al.* of an AML patient with MRD relapse post-transplant showed that DLI-induced molecular remission coincided with appearance of CD8^{+} T cell responses against several leukaemia-specific antigens, including NPM1^{mut} peptide[148]. Therefore, there is some suggestion that neoantigen specific responses can naturally occur in AML patients. However, further, more comprehensive study is required to assess what role allo-HSCT and DLIs have in eliciting or promoting neoantigen-specific T cell responses.

LAA-specific

Leukaemia-associated antigens, such as Wilms tumour 1 (WT1), proteinase 3 (PR3), survivin as well as cancer-testis antigen PReferentially expressed Antigen in MElanoma (PRAME), have been investigated for their significance in the GvL effect. WT1 gene encodes for a transcription factor that was shown to be overexpressed in leukaemia cells as compared to healthy haematopoietic progenitor cells[149]. Furthermore, it is lowly expressed in other healthy tissues, limiting its potential toxicity[150]. Multiple pilot in-human studies have studied the potential efficacy of WT1-specific T cells[150, 151]. Chapuis *et al.* study raised HLA-A*02:01-restricted WT1 peptide-specific T cell clones from allo-HSCT donor PBMCs. Subsequently, the expanded clones were infused into 11 relapsed or high-risk acute leukaemia patients. Of the cohort, two patients showed a

reduction in disease burden or remission after MRD^+ status, indicating that the therapy exerted an anti-leukaemic effect. Further three high-risk patients maintained disease remission, and did not require further therapy[150]. In a later study, 12 adverse risk AML patients were prophylactically infused with WT1-specific, donor-derived T cells at day $\sim$28 post-allo-HSCT. Subsequent infusions were given in cases where WT1-specific T cell persistence was poor. All patients remained disease-free (median follow-up of 44 months), while a comparative AML patient cohort had a relapse-free survival of 54%[152].

Multiple clinical case studies have identified LAA-specific T cells in patients with AML and other leukaemias. PRAME-specific T cells were isolated from a post-transplant AML patient using HLA-A*02:01 tetramers conjugated to the PRAME peptide. After *in vitro* expansion, these cells were shown to recognise a variety of cancer cell lines as well as primary AMLs. Concurrently, they had minimal toxicity to healthy tissues, with some on-target off-tumour activation seen to dendritic and kidney epithelial cells[153]. Furthermore, in a previously mentioned case study by Hofmann *et al.* antigen-specific T cell responses were found to PRAME, PR3, survivin as well as WT1. Importantly, appearance of these responses coincided with molecular remission post-DLI, and were absent pre-DLI[148]. In yet another case study, this time of therapy-related MDS relapse post-transplant, showed that following the treatment with DLI and azacitidine, the patient developed WT1-specific T cells. Furthermore, their frequency anticorrelated with WT1-specific mRNA, suggesting anti-leukaemic effect[154].

Phosphopeptides, a recently discovered new type of LAA, are also associated with the GvL effect. Cobbold *et al.* have hypothesised that leukaemia may be distinguished from normal cells by altered protein phosphorylation, which could be presented by the HLA class I machinery. Indeed, their analysis of phophopeptides presented by selected HLA-A and HLA-B alleles showed that leukaemia cells have an altered phosphopeptide library. Phosphopeptide-specific T cell clones could be identified and expanded from healthy volunteers, to show their cytotoxic capacity against both chronic lymphocytic leukaemia (CLL) and AML. Furthermore, the study has shown that the phosphopeptide-specific T cells are decreased in patients with AML or CLL when compared to healthy controls.

However, post-transplant, this was shown to improve, suggesting that allo-HSCT may restore anti-leukaemic phosphopeptide-specific responses[155].

Finally, most recent efforts to harness LAA for targeted therapy development have focused on using multiple LAA-specific T cell responses in combination. These approaches use donor derived PBMC to preferentially expand LAA-specific T cells using peptide-pulsed monocyte-derived dendritic cells (MDDCs) as the APC. In a phase I clinical trial, 25 post-transplant high-risk or relapsed AML or MDS patients were infused with donor-derived multiple leukaemia-antigen specific T cells (mLSTs; raised against WT1, PRAME, NY-ESO-1 and survivin). This was well tolerated, as reflected by 12% and 16% of patients developing *de novo*/worsening acute and chronic GvHD, respectively. Of those, only one patient necessitated steroid treatment to curb the GvHD. Where mLSTs were infused prophylactically, 11 out of 17 patients have not relapsed (study did not reach median leukaemia-free survival at a median follow-up of 1.9 years). For patients who had relapsed and refractory disease, in 2 out of 7 cases mLST infusion produced a partial or a complete response, demonstrating anti-leukaemic effect[156]. Additional two phase I clinical trials for mLST use in AML and/or acute lymphoblastic leukaemia (ALL) have shown similarly encouraging results[157, 158].

miHA-specific

Given the number of protein-encoding polymorphic sites across the human genome is in the range of thousands[138], perhaps it is not surprising that over a hundred of different miHAs have been discovered[151, 159]. However, these are alloreactive responses. Therefore, the potency of each miHA-specific T cell response to exert the GvL effect and not cause/accelerate GvHD must be assessed. For reference, the number of known haematopoietically-restricted miHAs are just around 10[151]. But, the discovery and characterisation of novel miHAs is just one aspect of the ongoing research. To fully understand the role of miHAs in exerting an effective GvL effect, we must also learn how the quantity and magnitude of miHA responses correlate with clinical outcome, whether certain miHA responses are immunodominant, and how to correctly design clinical intervention strategies that exploit known miHA responses.

Perhaps the most studied miHA response so far is that against the HA-1 antigen. It is produced by a non-synonymous SNP within the *HMHA1* gene[160] and the resultant peptide with the variant histidine amino acid in position 3 (VLHDDLLEA) is presented on the HLA-A*02:01 allele[159]. The *HMHA1* gene is expressed in both healthy and malignant haematopoietic cells, but has little expression in non-haematopoietic tissues at steady state or inflammatory conditions[161]. Furthermore, HA-1 specific cytotoxic lymphocytes show minimal to no reaction against human skin sections with the restricting HLA-A*02:01 and HA-1 genotype[162].

Importantly, HA-1 specific T cells can be found in patients treated with HSCT and DLIs[163–165]. One case study reported on three patients with CML or multiple myeloma (MM), who relapsed after transplant and subsequently responded to DLI. Of all isolated and expanded cytotoxic T cell clones, between 3-29% of them were HA-1 specific. Furthermore, these cells were shown to exert a cytotoxic or a cytostatic effect on peptide pulsed target cells and patient's own leukaemia, as well as healthy haematopoietic cells[163]. In another case report of a patient with ALL, who relapsed post-transplant and was successfully treated with DLI to induce cytogenetic and molecular remission, seven cytotoxic T cell clones were isolated and expanded using patient-derived dendritic cells. All were shown to be HA-1 specific and could kill patient-derived, but not donor-derived cells. Furthermore, lysis was shown for a peptide-pulsed cancer cell line as well as different primary lymphoblastic and myeloid leukaemia samples[164].

Based on the encouraging pre-clinical and observational findings, HA-1 is being explored therapeutically. One phase I/II clinical trial explored the use of donor-derived MDDCs loaded with several different miHA peptides, including HA-1. MDDCs were infused together with the DLI in 9 high-risk MM patients who previously relapsed after allo-HSCT and had a history of DLIs. The injections were well tolerated. Importantly, an increase in miHA-specific $CD8^+$ T cells was seen after the MDDC injection[166]. Another ongoing phase I clinical trial is testing the safety and feasibility of a genetically-modified HA-1-specific TCR T cell therapy[167].

However, the study and manipulation of the HA-1 miHA response is possible in only about $\sim$10-15%[159] of the patients due to the requirement for the HLA-A*02:01

allele and the HA-1 variant genotype as well as an HLA-matched donor with the HA-1 non-variant genotype. Therefore, new miHAs need to be discovered and tested across the whole population range. For this, multiple different experimental strategies are available. Broadly, some start with the identification of alloreactive T cell lymphocytes that specifically recognise an unknown cancer-derived antigen, whilst others start with putative antigen prediction, for which a specific T cell response is later sought for or artificially raised. These strategies are sometimes referred to as forward and reverse immunology methods, respectively. For example, one study used a forward immunology approach to identify 10 novel miHA from two patients, one with MDS and the other with CML, treated with DLIs. Activated $CD8^+$ T cells were isolated and expanded, to later identify clones which showed specificity to patient-derived, but not donor-derived Epstein Barr virus (EBV) transformed lymphoblastoid cell lines (EBV-LCL). Subsequently, using a large panel of different EBV-LCLs, T cell clone reactivity was related to the specific EBV-LCL germline genotype, an approach called whole genome association scanning[168].

The benefit of the alternative, reverse immunology, approach, is that it permits the researchers to refine what HLA alleles and putative miHAs they want to focus on from the start. In turn, this allows to eventually develop a repertoire of miHA responses that can cover the entire population[169–171]. One study focused on alleles HLA-A*02:01 and HLA-B*44:03, which together can cover around 55% of the population with European American descent. Using genomic analysis and mass spectrometry of HLA-bound peptides from 13 B-lymphoblastoid cell lines, 39 putative miHAs were identified with optimal features of preferential haematopoietic expression and common encoding germline variant[169].

Importantly, novel miHA discovery from a clinically relevant post-transplant context can reveal how the GvL specific effect is different from the GvL effect with GvHD, at the level of the alloreactive T cell repertoire diversity, specificity and phenotype. van Bergen *et al.* carefully selected 11 patients with CML, MDS or AML who received allo-HSCT and subsequent DLI to treat mixed chimerism, disease relapse or MRD^+ status. Some patients (5) experienced GvHD which necessitated systemic immunosuppressive treatment, whilst others did not, but all patients showed a response to the DLI in terms

of disease remission or conversion to full chimerism. Post-DLI samples were used to isolate activated T cells (HLA-DR$^+$) and expand them as separate clones. Interestingly, overall T cell clone reactivity to the patient-derived EBV-LCL lines was higher in the GvL with GvHD patient group. In addition, this group had a higher diversity of isolated T cell specificities - while the GvL only group had clones specific to 19 different miHA, the GvL with GvHD group had 48. However, the clones isolated from the GvL only group were less likely to become activated by fibroblasts. Therefore, this study suggests that the GvL-specific effect is distinguished from a mixed GvL and GvHD effect by its lower antigenic diversity and frequency of antigen-specific responses as well as skew of existing responses away from non-haematopoietic targets[172].

Another study suggested the importance of HLA class II-restricted miHA responses, as these were readily isolated using activation marker HLA-DR from four patients treated with CD4$^+$ cell purified DLI. These patients experienced no GvHD, but did convert to full donor chimerism after the DLI. Meanwhile, the isolated CD4$^+$ miHA-specific T cell clones showed lysis of healthy haematopoietic and malignant cells, with minimal reactivity against fibroblasts[173].

The discovery of miHAs associated with better clinical outcomes may allow to match patients with allo-HSCT donors based on the specific germline variant mismatches. Therefore, some large population studies aimed to assess if certain already known miHAs are retrospectively associated with better clinical outcomes. One multicenter study investigated over 800 patient-donor pairs to see if a panel of 20 previously known germline mismatches are related to treatment success. It found that mismatching for haematopoietically restricted miHA correlated with lower relapse rates, higher overall and relapse-free survival only within the patient cohort that experienced GvHD[174]. Another similar study of 327 patient-donor pairs found that one or more autosomal miHA mismatches within the context of related transplant were associated with higher relapse-free survival[175]. Furthermore, when the frequency of miHA-specific T cells was investigated using tetramer staining, some miHA-specific responses were more common across patients, despite the fact that all those investigated had the required

germline mismatches and the restricting HLA alleles. This result may indicate potential immunodominance in the GvL effect.

Most recently, the largest analysis so far, of 2249 patient-donor pairs, did not restrict its analysis to specific, already identified miHAs. Instead, all putative miHAs were computationally predicted from a large SNP typing array. Unfortunatelly, it did not find any single miHA associated with improved disease control or survival. Instead, greater than population median of predicted HLA class I miHA mismatches were associated with increased GvHD mortality[176]. Taken together, this may indicate that a few miHA responses may play a role in mediating the GvL effect, rather than the sheer quantity of germline disparities. In the future, it will be interesting to further relate not only the genetic mismatches (*i.e.* number of putative miHA) with the outcome, but also the quantity, specificity, magnitude and diversity of miHA-specific T cell responses, as the non-genetic factors may play a crucial role in the development of an effective GvL response, without the deleterious GvHD. Therefore, it remains crucial to identify further miHA responses within the clinically relevant contexts.

Finally, an alternative way to improve patient outcomes is by artificially enhancing or creating leukaemia-specific miHA responses in patients. Some early phase feasibility and safety studies have already been conducted or are in progress[177]. One study stimulated post-transplant lymphocytes *in vitro* with pre-transplant recipient cells and isolated $CD8^+$ T cells with the specificity to recipient, but not donor haematopoietic or recipient skin cells. Subsequently, expanded clones were re-infused into 7 different patients with post-transplant MDS/AML relapse. Unfortunately, this study reported pulmonary toxicity in 3 patients, which correlated with miHA expression in the lung[178]. Another study isolated CMV, EBV, LAA and miHA specific T cells from donor PBMC using multimers and expanded them *in vitro* before re-infusion. This approach aimed to prophylactically reduce the risk of relapse and viral infections. Of 24 post-transplant patients, none developed GvHD, and five showed disease progression, which coincided with non-persistence of LAA/miHA specific T cells[179]. Therefore, antigen-specific miHA T cell therapies may be feasible for further efficacy studies.

CAR T cell strategies for AML

Although the GvL effect is mediated by the TCR-driven alloreactive responses, the recent success of chimeric antigen receptors (CAR) suggests an artificial way to augment the GvL effect in the clinic. CAR receptors are synthetic transmembrane proteins which have an antigen-specific extracellular domain, typically derived from a single chain variable fragment of an antibody, a hinge and a transmembrane domain conjugated to the intracellular signalling domain. Typically, the latter has an activation component derived from CD3ζ and a costimulatory component derived from CD28 or CD137. However, this varies between CAR generations and constructs[180–182].

Recently, multiple CAR T cell therapies against the CD19 and the B cell maturation antigen (BCMA) were approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) after successful clinical trials for acute lymphoblastic leukaemia, mantle cell lymphoma, multiple myeloma and diffuse large B cell lymphoma[183–188]. These CAR T cell approaches have important advantages; they target a surface antigen that is not presented by the HLA and the antigen itself is neither mutated nor polymorphic. This means that all patients with $CD19^+$ or $BCMA^+$ malignancies may be eligible for CAR T therapy. However, it can also cause healthy B cell aplasia, which is a sign of CAR T cell persistence *in vivo*[189].

To translate this into the myeloid malignancies, including AML, a suitable surface antigen is required. Ideally, it should be cancer-specific and spare healthy myeloid cells, but also have uniform expression on cancer cells to avoid antigen loss and evasion. This presents a difficult balance, requiring novel targeting strategies. For example, both CD33 and CD123 antigens are expressed on healthy haematopoietic cells, but they are more frequently co-expressed and overexpressed in AML blasts. This suggests that novel Boolean "AND" gate CAR constructs might be promising[181]. Overall, multiple putative CAR targets are being explored for AML, including CD33, CD123, CLL-1, TIM-3, CD70, CD93 and others[181]. Some were already investigated for their safety and feasibility in phase I clinical trials[190–193]. However, the search for a suitable and effective CAR-based AML targeting strategy continues.

Roles of CD4$^+$ T cells in cancer immunity

Research discussed so far primarily focuses on the isolation and use of HLA class I, especially HLA-A*02:01-restricted, responses. These are mediated by the CD8$^+$ T cells, whose proposed role in cancer control is to exert a direct cytotoxic effect. Hence, historically, the putative roles of CD4$^+$ HLA class II-restricted T cells in cancer control and the GvL effect have been under-investigated. Given that the AML blasts both express HLA class II machinery as well as show its downregulation or loss at post-transplant relapse (see above), it is important to assess what role CD4$^+$ T cells may have in mediating the GvL effect.

CD4$^+$ can acquire a variety of phenotypes upon initial antigen encounter. This is determined by the inflammatory environment in which the T cell is primed as well as the strength of the TCR activation signal. There are multiple names to describe different CD4$^+$ T cell phenotypes, and the naming system adopted here is derived from a model proposed by Cannons, Lu, and Schwartzberg[194]. Briefly, T cells can acquire different effector programmes, such as Th1, Th2 and Th17. This determines what roles the primed T cell will fulfill, *e.g.* a Th1 effector (Teff1) cell is expected to release IFNγ upon activation and help recruit macrophages to the sites of inflammation. In addition, a CD4$^+$ T cell can also be a follicular helper cell (Tfh) or a germinal center follicular helper cell (GC Tfh). Teff, Tfh and GC Tfh cells are distinguished by their transcriptional programmes as well as sites of residence and functions[195]. However, this still does not capture the full phenotypic variety of different CD4$^+$ T cells. Some, in absence of appropriate pro-inflammatory signals, can become anti-inflammatory cells, called Treg cells. Others, can become cytotoxic CD4$^+$ T cells[196, 197].

Therefore, CD4$^+$ T cells have the capacity to carry out a diverse array of functions, from direct killing of targets, to recruitment and regulation of innate immune subsets and help for B cell responses[198]. Recent research on autologous immune responses against different cancers have suggested that antigen-specific anti-cancer CD4$^+$ T cell responses correlate with an overall anti-cancer immune response, patient survival and improved function of other immune subsets, such as B cells, CD8$^+$ T cells and myeloid cells (macrophages, dendritic cells (DC))[199–202]. In a phase I clinical trial

for patients with melanoma, Ott *et al.* describe a peptide vaccination approach directed against cancer neoantigens. To their suprise, most of the vaccine-induced responses were $CD4^+$[199]. In a recent, seminal paper Melenhorst *et al.* describe two long-term CLL survivors, who participated in a phase I clinical trial for CD19 CAR T cell therapy and subsequently experienced a decade-long remission. Both patients had CAR T cell persistence and years-long B cell aplasia. Interestingly, in both cases longest persisting CAR T cells were predominantly $CD4^+$. When these cells were isolated, co-culture with target cells showed that the $CD4^+$ CAR T cells could degranulate ($CD107^+$) as well as upregulate Granzyme B and Perforin, consistent with cytotoxic functions[200]. Another study sequenced primary melanoma tumour samples from four patients and identified neoantigen-specific $CD4^+$ T cells from the PD-1^{high} subset. Transcriptionally, antigen-specific T cells were characterised by their Tfh phenotype ($CXCL13^+$). In a larger melanoma patient cohort, presence of $CXCL13^+$ $CD4^+$ T cells correlated with $CD8^+$ T cell, B cell and myeloid cell recruitment into the tumour[202].

While correlative clinical evidence suggests that $CD4^+$ T cells may play a role in an anti-cancer immune response, this is more directly supported by pre-clinical cancer models. For one, $CD4^+$ T cells have been suggested to act synergistically with $CD8^+$ T cells. In an immunodeficient mouse model of a B cell malignancy, injection of both $CD4^+$ and $CD8^+$ CD19 CAR T cells resulted in the longest survival when compared to either of the T cell types injected alone[203]. This finding was confirmed in a more recent study which used an immunocompetent mouse B cell malignancy model and injected $CD4^+$ versus $CD8^+$ CD19 CAR T cells. The study also found that while $CD8^+$ CAR T cells were better at direct cancer cell killing, $CD4^+$ CAR T cells were better at recruiting NK cells, Natural killer T (NKT) cells, other $CD4^+$ T cells and myeloid cells. Furthermore, $CD4^+$ CAR T cells were better at promoting granzyme B and MHC class II expression in $CD8^+$ T cells and monocytes, respectively[204]. Taken together, this suggests that while the anti-cancer specific $CD8^+$ T cells are more conventionally cytotoxic, the $CD4^+$ T cells may be better at promoting a broader immune response and bridging the gap between adaptive and innate immune responses.

Moreover, $CD4^+$ activity has been suggested to promote B cell function in a lung adenocarcinoma (LUAD) model. Cui *et al.*[205] have produced a murine LUAD cancer cell line with expression of three model antigens for $CD8^+$ T, $CD4^+$ T and B cells. Using an immunocompetent mouse model, the group has carefully dissected that all relevant cell types ($CD4^+$, Tfh, $CD8^+$ and B cells) are important for optimal tumour growth control. Furthermore, Tfh cells promoted B cell and $CD8^+$ T cell function. To confirm that these findings may be applicable in human cancer, the researchers have also analysed a gene expression dataset from 478 LUAD cases, uncovering that patient survival is higher in those groups which express germinal center B cell and Tfh cell gene signatures[205]. Synergy between B cell and $CD4^+$ T cell anti-cancer responses was also found melanoma patients treated with an experimental 6 melanoma helper (*i.e.* HLA class II-restricted) peptides (6MHP) vaccine. Of all treated patients, those with both antibody and $CD4^+$ T cell responses to the vaccine had the highest survival probability[206]. Therefore, $CD4^+$ T cell - B cell communication axis may play a role in effective anti-cancer immune responses, and should be further investigated.

In addition, some pre-clinical evidence suggests that $CD4^+$ T cells play a role in promoting myeloid compartment anti-cancer function. A research group from Norway has reported on a murine multiple myeloma model which expresses a known MHC class II-restricted peptide. Transfer of antigen-specific $CD4^+$ T cells could improve mouse survival and the researchers showed that this depended on *in vivo* macrophage recruitment and activation. In turn, macrophages released short-lived nitric oxide, which exerted an anti-cancer effect[207–209]. A similar method of cancer killing was shown by DCs, which are activated by Th1 cells through IFNγ release[210]. The anti-cancer benefit of the myeloid compartment recruitment by the $CD4^+$ T cells was recently shown by Kruse *et al.*[211]. In mouse melanoma models, IFNγ-mediated recruitment and activation of macrophages allowed killing of MHC class II negative and IFNγ unresponsive cancer cells.

To summarise, $CD4^+$-based T cell responses could have many putative roles in cancer control, including, but not limited to, recruitment and activation of macrophages, DCs, B cells as well as cytotoxic $CD8^+$ T cells. In the context of AML, $CD4^+$ T cell responses are largely under-investigated, however, frequent loss of the HLA class II

machinery in leukaemia at post-transplant relapse suggests that the allogeneic $CD4^+$ T cells can have a direct cytotoxic effect.

HLA Class I and II presentation pathways

Another crucial difference between $CD4^+$ and $CD8^+$ T cells is their dependence on different antigen-presentation machineries, MHC class II and class I, respectively. There are many differences between them, including where the presented peptide is sourced from, how it is processed, delivered into the relevant peptide-loading organelle, loaded onto the MHC, and the MHC trafficked onto the cell surface. These processes are discussed in detail by multiple research reviews[212–214], and will not be described here.

However, for the purposes of this work, it is important to note three key aspects that distinguish the MHC class II machinery from the class I. For one, while MHC class I expression is seen in nearly every cell of the body, MHC class II expression is typically reserved to haematopoietic tissues and, as a resulta also frequently characterises AML blasts (see above).

Second, while the MHC class I complexes are comprised of the polymorphic chains (HLA-A, HLA-B or HLA-C) in a heterodimer with the non-polymorphic β2-microglobulin, MHC class II complexes form heterodimers with polymorphic α and polymorphic β chains (*e.g.* HLA-DPA1 with HLA-DPB1). Moreover, in the MHC class I, one protein forms the peptide binding groove, while in the MHC class II, both of the polymorphic α and β chains form the peptide binding groove. As a consequence, peptide binding to the MHC class II machinery is determined by its affinity to the polymorphic heterodimer. Importantly, considerable linkage disequilibrium exists in the MHC locus, which means that different α and β of the same MHC complex, or even genes between different MHC complexes, are inherited non-randomly (*e.g.* the HLA-DPB1*04:01 allele is invariably inherited with the HLA-DPA1*01:03 allele)[39, 215, 216].

Finally, the two classes differ by their typical peptide source. MHC class I is known to present intracellular (self) peptides derived from the proteasomal degradation pathway. Meanwhile, MHC class II antigens are typically derived from the outside of the cell (non-self) and are degraded into peptides for presentation by the endolysosomal system.

However, some 20-30% of peptides presented on the MHC class II are derived from the intracellular compartment. This is because the process of autophagy, *i.e.* engulfing of one's self macromolecules and organelles for degradation, is associated with the endolysosomal system - autophagosomes fuse with the endolysosomal compartments to form autophagolysosomes. Therefore, degraded peptides from self-proteins enter the compartment where MHC class II loading occurs[212]. This explains how leukaemia blasts with HLA class II expression could present self-peptides. Alternatively, some reports suggest that leukaemia has some phagocytic activity as well[217], which could mean that leukaemia cells may uptake and present non-self peptides from other malignant or nearby healthy cells.

1.5 Previous Study of GvL in AML Patients

Screen for identification of alloreactive T cell responses

Previous study in our group by Connor Sweeney (*DPhil* 2021) has focused on identifying novel miHA responses in 12 AML patients who were successfully treated with allo-HSCT. To maximise the chance of finding miHA responses relevant for the GvL effect, patients were carefully chosen based on clinical characteristics, which make the long-term disease remission more likely due to an effective GvL response rather than chemotherapy alone (Fig. 1.10a). Studied patients had intermediate or high risk AML, relapsed at least once pre-allo-HSCT or were MRD^+ before going into transplant. Most patients received reduced intensity conditioning (10/12). One (OX866) relapsed after transplant and subsequently went into remission following chemotherapy and DLIs. Two further patients received DLIs to treat reduced donor $CD3^+$ chimerism. Importantly, all 12 patients experienced long-term disease remission of at least 4 years at the time of writing (follow-up range of 4-10 years). Most experienced no or mild acute or chronic GvHD. Two patients experienced moderate/severe chronic GvHD and one had preceding grade 3 acute GvHD.

To identify putative miHAs, patient and donor protein-encoding genomes were compared and further filtering steps were applied (Fig. 1.10b). First, whole exome sequencing was performed on donor DNA, patient healthy haematopoietic and malignant cells to

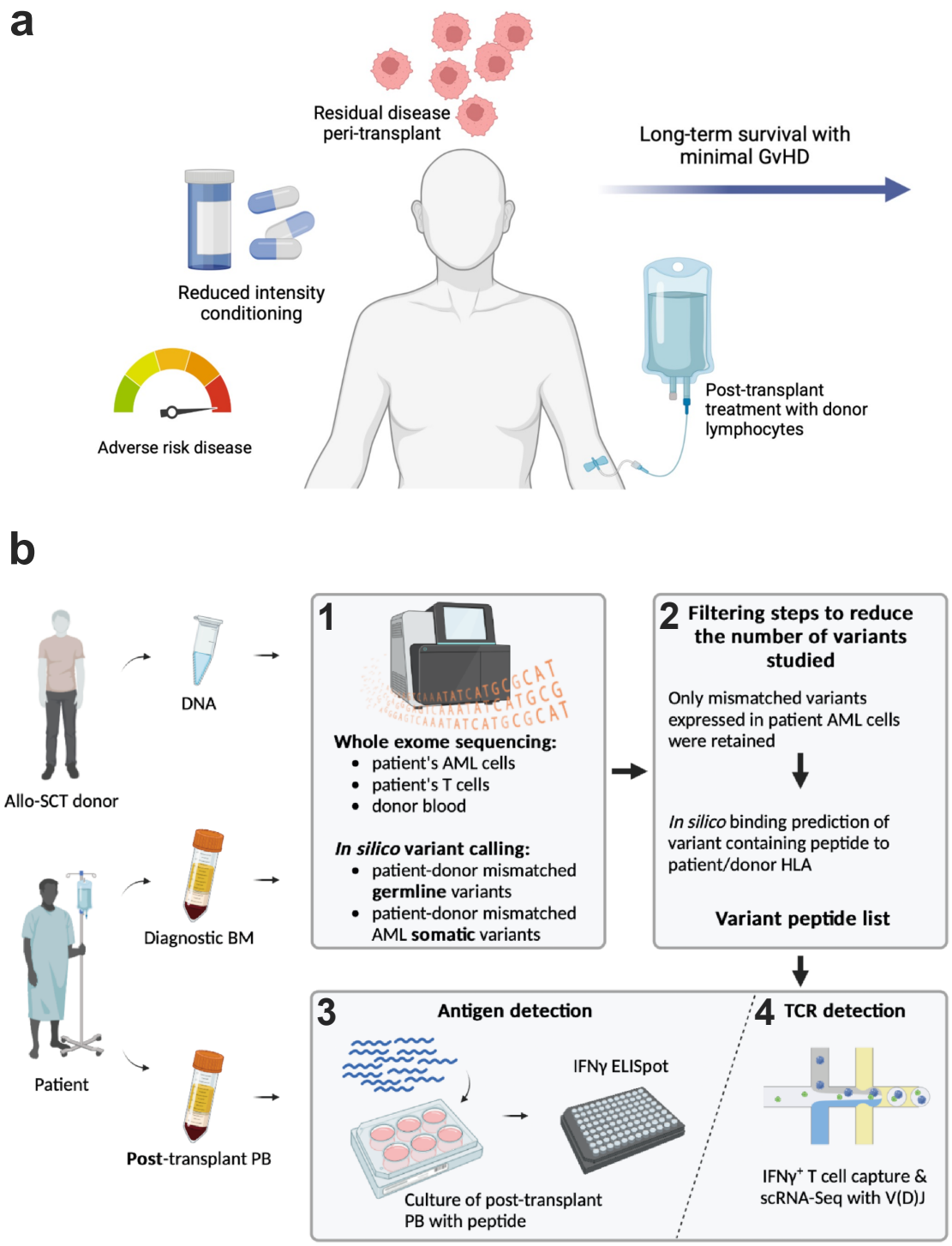

Figure 1.10: Patient selection criteria and the pipeline for unbiased miHA response detection (Connor Sweeney *DPhil* 2021). a Summarises some of the selection criteria for the patient cohort. **b** A graphical representation of the putative miHA screening platform developed by Connor Sweeney. Part of this figure is taken from the ASH Annual Meeting 2022 abstract[218].

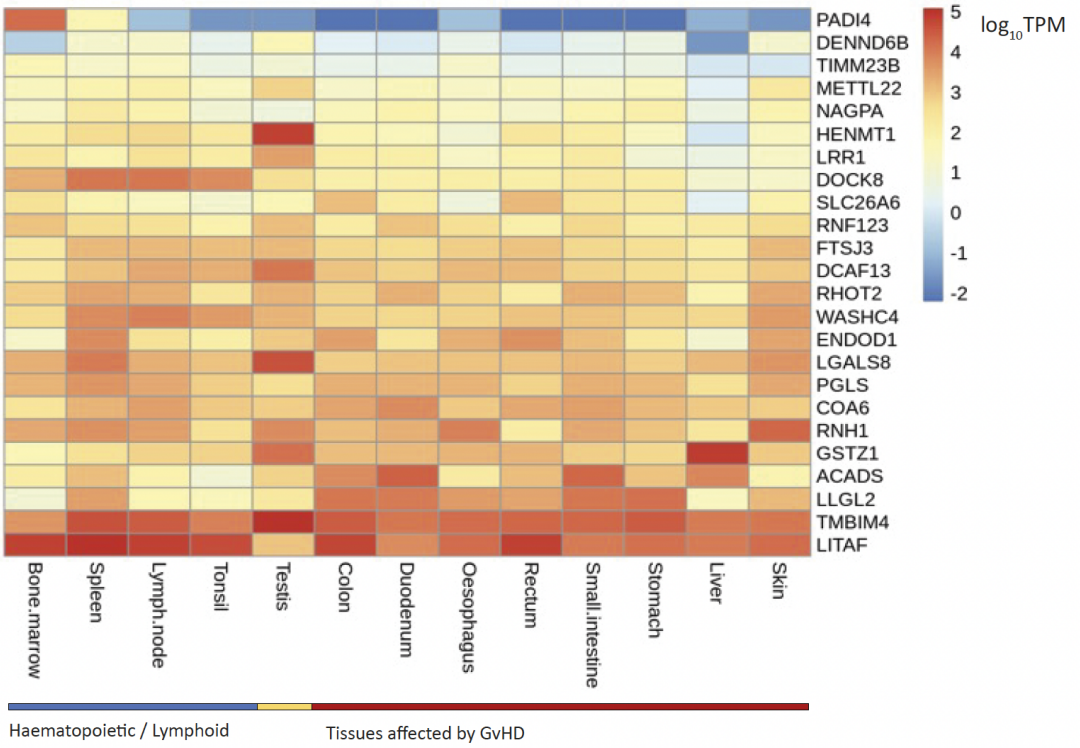

Figure 1.11: Healthy tissue expression pattern of the 22 discovered miHA responses (Connor Sweeney *DPhil* 2021). A heatmap showing gene expression across healthy tissue types from The Human Protein Atlas database[219, 220]. Each row is a different gene encoding a novel identified miHA. Note, that *TIMM23B* and *TMBIM4* are not part of the 22 identified miHA. This figure is taken from Connor Sweeney's *DPhil* thesis.

identify protein-encoding non-synonymous germline as well as somatic mismatches. Most of them were germline (98.7%). On average, 4281 mismatches were identified per patient-donor pair. The putative miHA list was narrowed by filtering for mismatches that were expressed in AML blasts by bulk RNA sequencing (expression of equal or more than 1 count per million (CPM)), to an average of 28.7% from the starting number (an average of 1211 mismatches per patient-donor pair). The list of expressed putative miHA was further filtered with *in silico* HLA-binding prediction using NetMHCpan 4.0 and NetMHCIIpan 3.0. Only predicted high affinity binders to patient's own HLA alleles were retained for further investigation (an average of 483 peptides per patient-donor pair).

This yielded a list of ∼5,300 peptides (putative miHA) for functional screening (range of ∼200-900 peptides per patient-donor pair) (Fig. 1.10b, sub-sections 3 & 4). For this, patient post-transplant PB samples (taken ∼2-7 years post-transplant) were cultured in

presence of peptide pools, to allow expansion of any low frequency antigen-specific T cell clones. Subsequently, T cell activation to pools was screened using IFNγ ELISpot, and any responses to the individual peptide pools were deconvoluted by IFNγ ELISpot with individual peptides. This way, 22 antigen-specific T cell responses were identified across all 12 patient-donor pairs. All responses were to peptides produced by non-synonymous SNPs mismatched between the patient and the donor. Interestingly, 16/22 responses were shown to be CD4$^+$ T cell mediated. Lastly, putative antigen-specific TCRs were identified by single cell RNA sequencing with V(D)J transcript enrichment of peptide-activated IFNγ^+ T cells (discussed in Chapter 3).

Population frequency of the newly discovered miHA-producing alleles varies between less than 1% and 72% in the Caucasian population (median frequency of 46%). Healthy tissue expression pattern of the miHA-encoding genes is also widely variable (Fig. 1.11). Of most interest is the miHA produced by the *PADI4* gene, which appeared highly haematopoietically restricted. The *PADI4* miHA is produced by a variant SNP allele with a population frequency of 6.04% (gnomAD v3.1.2 database).

Initial description of the *PADI4* miHA T cell response

Figure 1.12 summarises the initial experiments done by Connor Sweeney to characterise the *PADI4* miHA response. It was isolated from patient OX866 at 6 years post-transplant and the response to *PADI4* miHA overlapping peptides 21 & 22 was the only positive result from all of the screened peptides for that patient (Fig. 1.12a). A subsequent activation experiment confirmed that this was a true allogeneic response, as the T cells did not activate in presence of the reference (donor) *PADI4* peptide sequence (Fig. 1.12b). Furthermore, flow cytometry analysis showed that the activation occurred in the CD4$^+$ T cell subset (IFNγ^+; data not shown). To determine which HLA complex presents the antigen, an IFNγ ELISpot with different HLA blocking antibodies was perfomed. It confirmed that this response is HLA class II, and specifically HLA-DP, restricted (Fig. 1.12c). Meanwhile, the patient is homozygous for HLA-DPB1*04:01, and the donor was HLA-DP matched. This HLA-DPB1 allele is the most common amongst Northern Europeans (40.9%)[215].

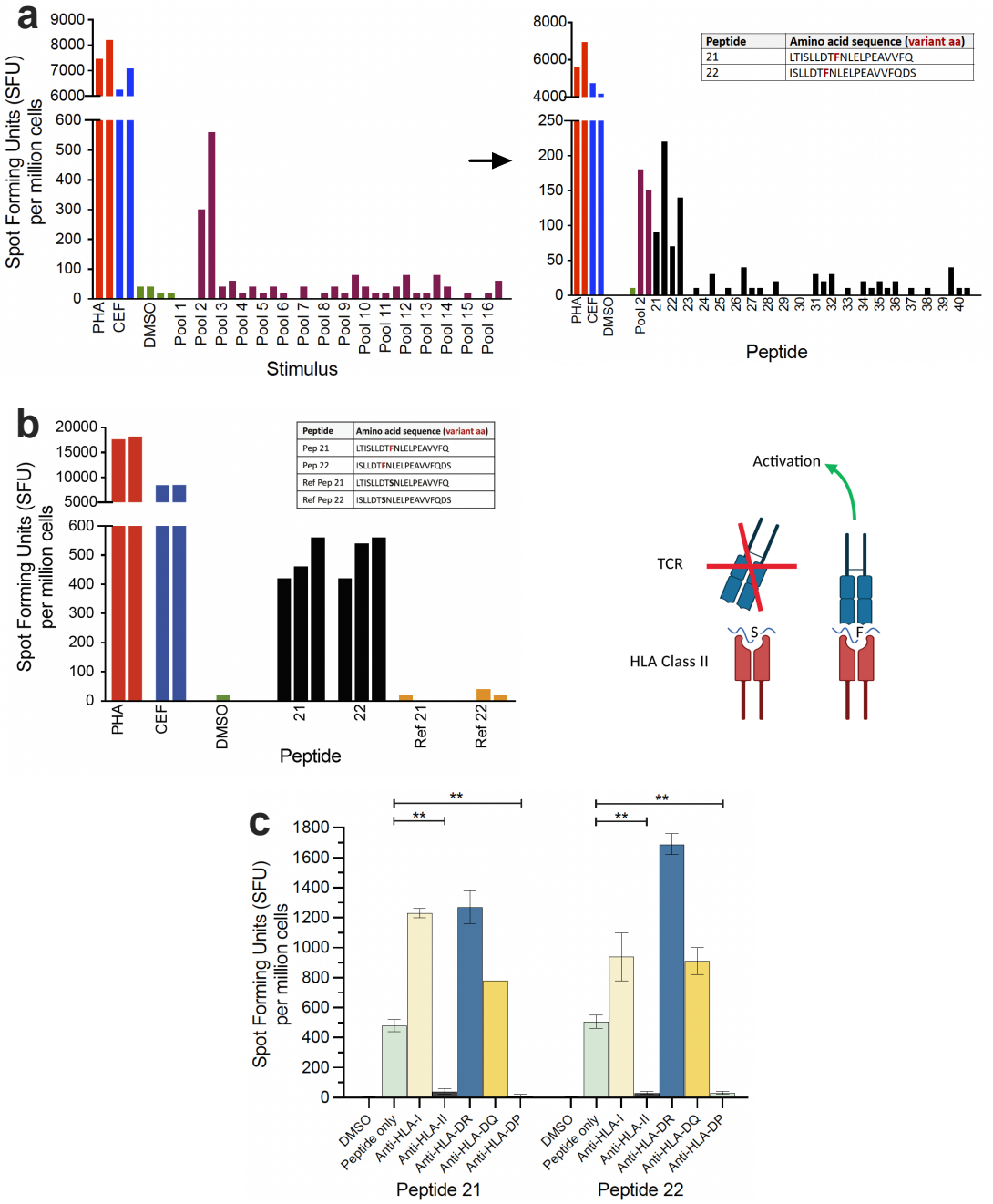

Figure 1.12: Characterisation of the *PADI4* miHA response from the post-transplant sample of the patient OX866 (Connor Sweeney *DPhil* 2021). PB sample (taken 6 years post-transplant) was cultured with peptide pools (in **a**) or individual peptides 21 & 22 (in **b** & **c**) prior to activation read out. **a** Shows IFNγ ELISpot on T lymphocytes incubated with different peptide pools of predicted miHA (left). When the response to the pool 2 was identified, the assay was repeated with individual peptides of the pool 2. This identified a response to the overlapping peptides 21 & 22, which are produced by an SNP within the *PADI4* gene. **b** Shows a repeat of the IFNγ ELISpot with the reference (donor) SNP allele (left). The adjacent diagram (right) illustrates the specificity of the discovered response. **c** Shows a repeat of the IFNγ ELISpot with different HLA blocking antibodies. **a** and **b** show technical replicates as individual bars adjacent to each other. Error bars in **c** show standard deviation between technical replicates. These figures are taken from Connor Sweeney's *DPhil* thesis.

1.6 PADI4 Functions

PADI4 is a Ca^{2+}-dependent enzyme[221] that catalyses arginine conversion to citrulline (citrullination or deimination). It was first described in a human leukaemia cell line HL-60, where its expression can be induced by treatmeant with agents to promote differentiation into monocyte or granulocyte lineages[222]. Since, PADI4 has been shown to citrullinate both histones[223–225] as well as other proteins[226, 227] involved in gene regulation. It has been implicated in gene repression[224, 225, 228–230] as well as activation[227, 231].

The proposed gene regulation roles of PADI4 are wide-ranging. It can citrullinate arginines on histones H3 and H4, thereby preventing arginine methylation[224, 225]. Alternatively, PADI4 can even convert histone methylarginine to citrulline (demethylimination)[224, 225], thus shutting off gene expression facilitated by arginine methylation. This was proposed to be a part of a normal cyclic gene expression regulation in response to oestrogen signalling[228]. A similar mechanism was proposed to regulate p53 response gene regulation as well[229, 230, 232], although some reports suggest PADI4 promotes p53-induced growth suppression and apoptosis instead[233, 234]. Moreover, hypoxia-inducible HIF-1α and HIF-2α were shown to induce expression of and then directly associate with PADI4 to facilitate histone citrullination and gene expression in hypoxia-responsive elements of breast cancer cell line models[223]. Lastly, *PADI4* expression was shown in both induced pluripotent stem cells as well as in early stage mouse embryos. Its expression was shown to be under control of pluripotency factor Oct4. Downregulation or enzymatic inhibition of PADI4 disrupted both pluripotent reprogramming and mouse embryo development with concominant decreases in expression of *Nanog* and *Tcl1*[235]. Thus, literature suggests that many of the proposed functions of PADI4 are not directly linked to haematopoietic cell identity and rather appear fundamental to cell function regardless of its origin.

Nevertheless, PADI4 has been implicated in both normal and pathologic functions of haematopoietic cells. Firstly, leukaemia cell line HL-60 differentiation into granulocytic

lineage by all-trans retinoic acid (ATRA) was shown to increase PADI4 expression[222, 236]. This finding is in line with public RNA sequencing data of healthy neutrophils and their immediate precursors (Chapter 5). Indeed, it was shown that ATRA-induced PADI4 binds to *SOX4* promoter and demethyliminates histone residues, repressing gene expression. In turn, depletion of SOX4 promotes expression of Pu.1, a known haematopoietic factor that promotes differentiation to granulocytic lineage[236]. Hence, this would suggest that PADI4 promotes normal granulocytic differentiation.

Conversely, PADI4 was implicated in the regulation of haematopoietic stem cell quiescence. It was shown that in LSK cells (mouse haematopoietic stem cells defined as Lineage$^-$ Sca-1$^+$ c-kit$^+$) PADI4 binds to regions upstream of *c-myc* and represses its expression. In turn, in PADI4^{KO} mice, c-myc is higher and the LSK compartment is expanded. Similarly to the extra-haematopoietic PADI4 functions discussed above, the proposed likely mechanism of *c-myc* repression in the LSK cells is by blocking gene-activating histone arginine methylation with citrullination[237].

But perhaps the most studied role of PADI4 is its involvement in neutrophil extracellular trap (NET) formation and the diverse sequelae of this process to the function of the immune system. Histone arginine conversion to citrulline results in a loss of a positive charge, leading to chromatin decompaction. Neutrophils were described to undergo histone hypercitrullination in response to inflammatory stimuli such as LPS, bacterial peptides, TNFα, licotechoic acid and H_2O_2. This process is closely associated with nuclear membrane breakdown and chromatin release into the extracellular space (NETosis). There, loose chromatin, decorated with antimicrobial proteins, acts as a trap for bacterial pathogens[238, 239]. Using PADI4^{KO} mice, it was shown that neutrophil PADI4 is essential for histone citrullination and the structural changes of chromatin during NETosis. Importantly, PADI4^{KO} neutrophils were unable to release NETs and thus had impaired killing capacity of *Shigella flexneri*[240].

The importance of PADI4-mediated NETosis in the immune system can be reflected by the different pathologies in which this mechanism is implicated. Rheumatoid arthritis (RA) is an autoimmune condition of the joints characterised by presence of antibodies against citrullinated autoantigens. NETosis and PADI4 are thought to play a major role

in RA pathogenesis[241]. Certain SNP variants of *PADI4* are associated with increased susceptibility to RA and higher *PADI4* mRNA transcript stability[242]. PADI4 is found in the joint-infiltrating haematopoietic cells as well as extracellular deposits[243, 244]. Furthermore, a proportion of RA patients even develop anti-PADI4 autoantibodies[245].

PADI4-mediated NETosis is also implicated in the development of deep vein thrombosis (DVT). Using a mouse model, it was shown that the thrombi are rich in citrullinated extracellular histone and neutrophils. Furthermore, it was shown that the rate of DVT is significantly reduced in PADI4^{KO} mice as compared to the wild-type. To confirm that this effect is determined by haematopoietic cells, PADI4^{KO} BM engrafted wild-type chimera mice had similarly reduced occurrence of DVT[246].

Finally, PADI4 has been studied in a wide range of cancer models. Although it is proposed to have haematopoietic restriction in healthy tissues (Chapter 5), its expression in cancers can be found beyond leukaemias (Chapter 3 and Appendix 13). In literature, PADI4 has been studied in breast cancer[223, 227, 228], hepatocellular carcinoma[223], sarcoma[226, 229, 234], lung adenocarcinoma[229] and colon cancer[229] cell lines. Furthermore, PADI4 is detected in immunohistochemical stains of various primary cancer tissue samples[247, 248]. Together, this evidence suggests that cancers may aberrantly express *PADI4*. One explanation for this is its implicated role in p53 response downregulation. Indeed, depletion of PADI4 can push p53 activated cells to apoptosis[229, 230]. Alternatively, PADI4 may be a required protein for effective hypoxia-induced and HIF-dependent gene response. In primary breast cancers, PADI4 expression is associated with improved breast cancer vascularisation, whilst its knockdown in mouse models reduces xenograft progression[223]. Furthermore, PADI4 was implicated in facilitating mesenchymal-to-epithelial transition in colorectal cancer liver metastases. Cancer cells were found to produce PADI4 and release it via extracellular vesicles. Subsequently, citrullination of extracellular matrix proteins in the liver improved cancer cell transition and metastasis in mice[249]. Together, this evidence suggests PADI4 to have varied pro-oncogenic roles.

However, some literature suggests PADI4 to be a tumour suppressor instead. In PADI4^{KO} mice models, thymic cells were found to be more resistant to radiation-

induced apoptosis[234]. In line with this, the same group also showed that *PADI4* downregulation has a mild growth-promoting and anti-apoptotic effect in *in vitro* cancer cell line models[233]. This hypothesis is further supported by a proportion of cancer cell lines carrying inactivating *PADI4* mutations[234]. Hence, PADI4 function and significance in cancer remains unclear.

To summarise, *PADI4* encodes for a citrullinating enzyme with diverse functions in haematopoietic and extra-haematopoietic compartments. Its been implicated in cell transcriptional responses to stress, such as p53 and HIF signalling, as well as hormonal signalling. It was also investigated for its role in induction of pluripotency and haematopoietic stem and progenitor (HSPC) cell quiescence. Conversely, in a different context, it was shown to promote granulocytic cell differentiation and, in mature granulocytes, enable NETosis. Finally, PADI4 was investigated for its putative roles in cancer, where it was suggested to act as an oncogene and a tumour suppressor.

1.7 Thesis Aims

My *DPhil* project aimed to further investigate the discovered *PADI4* miHA response. Of all the 22 miHA responses, the *PADI4* miHA appeared to have the highest haematopoietic restriction across healthy tissues, suggesting that it may contribute to a GvL-specific effect. This was further suggested by the HLA class II restriction of the response.

Firstly, I aimed to discover the TCR(s) that mediate the *PADI4* miHA-specific response in patient OX866 and confirm that they are specific to the peptide encoded by the SNP variant genotype (recipient's) rather than the SNP reference genotype (donor's).

Using the antigen-specific TCR-transduced (PADI4 TCR) T cells, I wanted to establish if the *PADI4* miHA can be presented by leukaemia cell lines, and whether that induces T cell activation & killing. These and above aims are explored in Chapter 3.

Next, I wanted to establish if the PADI4 TCR T cells can become activated by primary leukaemia samples *in vitro*. In addition, given that HLA class II-restricted $CD4^+$ T cells were previously shown to interact with other immune cells to promote anti-cancer immunity (see above), I wanted to assess if monocyte-derived dendritic cells

(MDDCs) can also present the *PADI4* miHA and elicit PADI4 TCR T cell activation. These questions are explored in Chapter 4.

Finally, I aimed to answer if the *PADI4* miHA is leukaemia-specific by investigating if healthy haematopoietic cells can activate and induce a cytotoxic response in PADI4 TCR T cells (Chapter 5).

Chapter 2

Materials and Methods

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Details of all consumables, oligonucleotides, kits and plasmids mentioned in this chapter are listed in the Appendix.

2.1 Human Sample Collection and Processing

Patient AML samples were obtained from the HaemBio Biobank (research ethics committee approval 17/SC/0572) with informed consent. Healthy blood or bone marrow samples were obtained with informed consent either from the MARCH study (research ethics committee approval 17/YH/0382), NHS Blood and Transplant (under the non-clinical issue account T255) or healthy donors (Weatherall Institute of Molecular Medicine phlebotomy consent).

Samples were processed to isolate either: 1) the whole white blood cell (WBC) fraction using the ACK lysis buffer and according to the manufacturer recommendations or 2) peripheral blood mononuclear cells (PBMC) using a density separation method. For the latter, blood or bone marrow was diluted 1:1 with phosphate-buffered saline (PBS) and layered on top of Ficoll-Paque Plus reagent in a final ratio of 2(blood):1(Ficoll-Paque). The tube was centrifuged for 30 minutes at 400g with minimal acceleration and no brake. Buffy coat was collected, washed twice with PBS and either used immediately or cryopreserved for later use in the liquid nitrogen tanks using a freeze media of foetal bovine serum (FBS) with 10% DMSO.

To thaw, samples were immersed into a 37°C water bath until almost completely thawed and immediately diluted dropwise with FBS. After a wash using RPMI with 10% FBS, samples were counted either manually with trypan blue and a haemocytometer or using an automatic cell counter NucleoCounter NC-3000 (ChemoMetec).

2.2 Cell Line Culture

Cell line GDM-1 was obtained twice, first from the German Collection of Microorganisms and Cell Cultures (DSMZ) and then from the American Type Culture Collection (ATCC). HEK 293T and Jurkat TCR KO cell lines were gifted by Mai Vuong and Dr. Margarida Rei, respectively (University of Oxford). B-lymphoblastoid cell lines (BLCL) were obtained from Alastair Waugh (WIMM HIU).

All cell line stocks were tested for mycoplasma at the start of culture and then at least once more peri-cryopreservation to confirm maintenance of mycoplasma negative status. Tests were performed using either the MycoAlert luciferase-based assay or the MycoStrip isothermal PCR assay according to the manufacturer's recommendations. Any cell lines with positive mycoplasma status were discarded. Cell lines were cultured and preserved according to guidelines provided in the Appendix.

2.3 Lentiviral Transfer Plasmid Cloning and Gene Synthesis

Final pHRsin TCR constructs (used in this thesis) were produced using pMP71 γ-retrovirus constructs, which in turn were assembled using synthesised TCR fragment sequences.

Insert sequence ordering

V(D)J α and β variable sequences for TCR constructs were obtained from 10X single-cell RNA sequencing data (see the Appendix). Sequences were human codon optimised and ordered from GeneArt as separate fragments inserted into pMA-RQ (Ampicillin cassette) plasmids. In each case, variable fragments were flanked by sequences of pMP71 lentiviral transfer plasmid up to the restriction enzyme digestion sites.

PADI4 mRNA transcript with the wild-type single nucleotide polymorphism sequence was obtained from Ensembl (transcript ID ENST00000375448.4). Its DNA sequence was human codon optimised and ordered as a plasmid pUC19 PADI4-WT (Ampicillin cassette) from Gene Universal (see the Appendix).

pMP71 γ-retrovirus TCR transfer plasmid cloning

pMP71 retroviral transfer plasmid construction for each TCR was done by inserting one variable sequence at a time. Starting plasmid pMP71 CMV (gift from Hans Stauss, University College London) was digested with NotI-HF and SacII to excise the α variable fragment or with NcoI and PmlI to excise the β variable fragment (see the Appendix). A similar procedure was adopted for GeneArt variable fragment plasmids. Digestions were performed at 37°C for 90 minutes, followed by enzyme inactivation

at 60°C for 20 minutes. Subsequently, fragments and plasmid backbones were gel purified using QIAquick Gel Extraction kit.

T4 DNA ligase reactions were set up at 1:3 backbone-to-insert ratio using the NEBioCalculator and following the manufacturer's protocol. Reaction mixes were then transformed into NEB Stable *E. coli* according to the manufacturer's protocol and plated onto Luria Bertani plates with Ampicillin. After overnight 30°C incubation, colonies were picked and expanded overnight at 30°C in liquid Luria Bertani media with Ampicillin. Plasmids were miniprepped using the QIAprep Spin Miniprep kit.

α and β variable sequence inserts were verified by Sanger sequencing (WIMM Sequencing facility) with primers p3, p5, p20, p22 and p32. Sequence-confirmed plasmids were midiprepped using NucleoBond Xtra Midi EF kit and plasmid-carrying *E. coli* cryopreserved in Luria Bertani media with Ampicillin and 25% glycerol. Final plasmid maps can be found in the Appendix.

pHRsin lentivirus TCR transfer plasmid cloning

Starting plasmids pHRsin-SFFV-mCherry-TCRαConstant and pHRsin-SFFV-1G4 were a gift from Dr. Margarida Rei (University of Oxford). The former plasmid was linearised using BamHI-HF and NotI-HF, following the manufacturer's protocol, and gel purified as described above.

An overview of lentiviral plasmid construction is shown in Figure 2.1. The pHRsin-SFFV-1G4 plasmid was used to amplify and purify TCR β constant-P2A fragment with primers p34 and p35. α variable and β variable fragments for respective PADI4 TCR clones were amplified from pMP71 γ-retroviral plasmids using primers p36-p45. All amplification reactions were designed to introduce 20-33 nucleotide overhangs for seamless plasmid assembly. Amplification was done using Q5 polymerase, according to manufacturer's protocol, and reaction products were gel purified as described above.

The linearised plasmid backbone and the amplified TCR construct fragments were used to generate new pHRsin PADI4 TCR plasmids using HiFi assembly, according to manufacturer's protocol. *E. coli* were transformed, cultured and their plasmids purified as described above. Plasmids were first screened using junction PCRs with

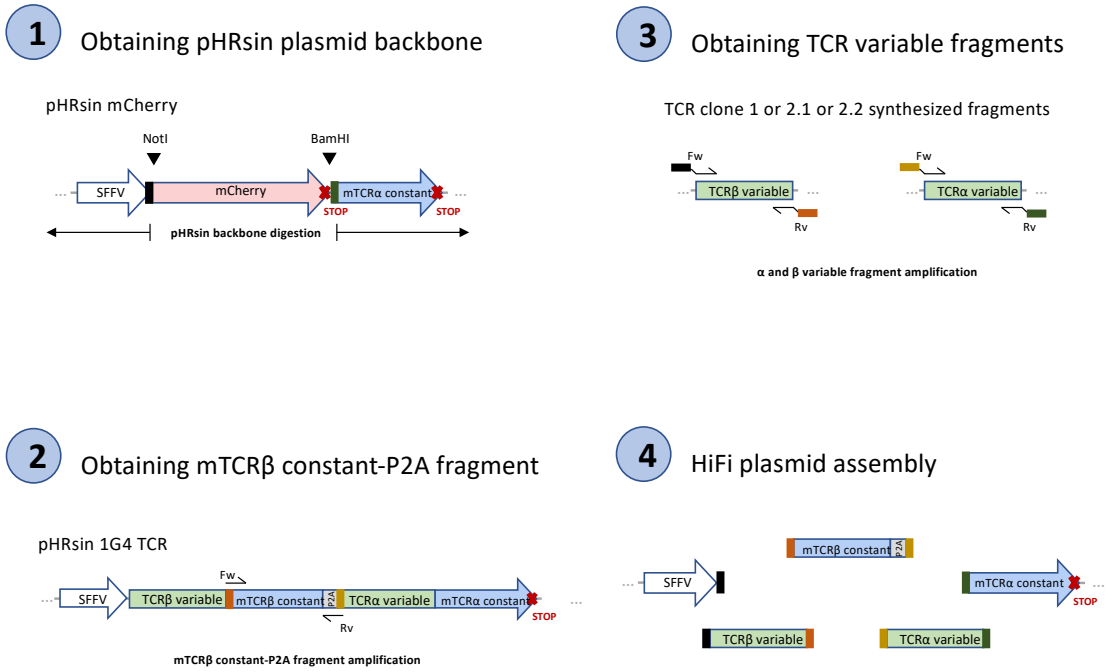

Figure 2.1: Overview of the TCR construct cloning for the pHRsin lentiviral transfer plasmid.

primers p54, p56 and p57. Final assemblies were confirmed with Sanger sequencing (WIMM Sequencing facility) of the insert using primers p56, p57, p59 and p61. Final plasmid maps can be found in the Appendix.

pL-PADI4-GFP lentiviral overexpression plasmid cloning

pUC19 PADI4-WT plasmid was first used to generate the pUC19 PADI4-Var plasmid with the site-directed mutagenesis kit and primers Q5SDM_6/7/2022_F and Q5SDM_6/7/2022_R, otherwise following the manufacturer's protocol. Successful mutagenesis was confirmed by Sanger sequencing (WIMM Sequencing facility) using primer p220. Starting plasmid pL-CRISPR.EFS.GFP (Addgene #57818) was digested with BamHI-HF and NheI-HF according to the manufacturer's protocol. Insert fragments PADI4 SNP-WT and PADI4 SNP-Variant were amplified from their respective pUC19 plasmids using overhang primers p224 and p225. Each insert and the plasmid backbone were gel purified and combined in a HiFi assembly reaction, following the manufacturer's protocol, to produce pL-PADI4-WT.EFS.GFP and pL-PADI4-Var.EFS.GFP plasmids. *E. coli* transformation, culture and plasmid purification were carried out as described

above. Insert identity was first checked with a restriction digest using BamHI-HF and NheI-HF. Later, plasmids were confirmed using Sanger sequencing (WIMM Sequencing facility) using primers p218-p221. Final plasmid maps can be found in the Appendix.

2.4 PADI4 SNP and HLA-DP Typing of Samples and Cell Lines

If required, gDNA was extracted using DNeasy Blood & Tissue Kit following the manufacturer's protocol.

PADI4 SNP (dbSNP ID rs1748020) genotyping was performed either by manually checking Sanger sequencing traces or by using a commercial SNP genotyping qPCR assay. For the former, gDNA was used to amplify the area of interest with primers PAD_geno_Fw and PAD_geno_Fw and Q5 PCR MasterMix, following manufacturer's recommendations. The amplified product was gel purified using the QIAquick Gel Extraction kit and sent for Sanger sequencing with primers PAD_geno_Fw and PAD_geno_Fw (WIMM Sequencing facility).

For SNP typing using qPCR, commercial PADI4 SNP genotyping assay was diluted to 20X using TE buffer and PCR reaction was assembled as follows: 1. 5µl of TaqMan Universal PCR Master Mix (2X); 2. 0.5µl of TaqMan genotyping assay mix (20X); 3. 2µl of sample gDNA (5-20ng in total); 4. 2.5µl of DNase-free water. MicroAmp™ Fast optical plates and MicroAmp™ optical adhesive film were used with QuantStudio 7 qPCR machine. It was programmed as follows: 1. Pre-run fluorescence capture at 60°C for 30 seconds; 2. Polymerase activation at 95°C for 10 minutes; 3. Denature at 95°C for 15 seconds; 4. Anneal & extend at 60°C for 60 seconds; (Steps 3 and 4 repeated for a total of 40 cycles) 5. Post-run fluorescence capture at 60°C for 30 seconds.

HLA-DP typing was performed by genotyping *HLA-DPB* gene only, due to strong genetic linkage with *HLA-DPA*. gDNA was sent to either WIMM Sequencing facility or NHS OUH Churchill Hospital Department of Clinical Haematology.

2.5 Primary T Cell Transduction

Overview of the method is set out in Figure 3.3b,c.

Lentiviral plasmid preparation and transfection

Plasmids required for lentivirus production, pHRsin (transfer), pQ8.91 (packaging) and pMD-G (VSV-G pseudotyping), were amplified from frozen glycerol stocks and purified as described above. For each preparation, either enzyme restriction or Sanger sequencing was performed to confirm plasmid/insert identity. For pHRsin, full insert sequencing was done, as described above. For pQ8.91 and pMD-G, enzyme restriction with BamHI and EcoRI was used according to New England Biolabs recommendations. Expected agarose gel bands post-restriction were 1.3kb and 11kb for pQ8.91 and 0.5kb, 0.8kb, 1.6kb, and 3kb for pMD-G. Subsequently, plasmids were aliquoted and frozen until later use.

For lentivirus production, HEK 293T cells were thawed and passaged at least once before transfection. When confluent, cells were detached by first carefully decanting the media, washing with PBS and then adding trypsin solution. After up to 5 minutes of incubation at 37°C, cells were resuspended by pipetting up and down with fresh media. Finally, cells were spun down and resuspended in fresh media at 0.5 million/ml concentration. 2 ml of cell suspension were aliquoted into each well of a 6-well plate and incubated overnight.

Next day, transfection solution was prepared, containing (per each well of a 6-well plate): 1. 1μg pHRsin; 2. 0.5μg pQ8.91; 3. 0.25μg pMD-G; 4. up to 100μl of OptiMEM media. After thorough mixing, 4.5μl of Turbofectin were added and the solution was mixed once again. After 30 minutes of room temperature (RT) incubation, 104.5μl of solution were added dropwise to each well and the plate was shuffled.

After exactly 2 days of incubation, supernatant from 6-well plates was collected and spun down to remove debris at 1500rpm for 5 minutes. The preparation was used immediately for T cell transduction.

T cell culture and transduction

PBMCs were isolated from healthy donor blood as described above. To activate T cells, PBMCs were placed in T cell media (RPMI, 5% h.i. Human AB serum, 1% Pen/Strep, 1% GlutaMAX, 1% sodium pyruvate, 1% non-essential amino acids, 1% HEPES, 0.1% β-mercaptoethanol, 1000 IU/mL IL-2 and 10ng/mL IL-15) and supplemented with

1:1 ratio of CD3/28 Dynabeads. After two days, cells were transduced (see below). After three further days, Dynabeads were removed. If CD4 and CD8 T cell separation was required, this was done on the same day, using MACS microbeads and following the manufacturer's protocol. For further culture expansion, cells were grown in plates (typically 24-well) and counted every 2-3 days. When needed, culture was supplemented with media to maintain 1.5-2 million/ml concentration.

Prior to the assays, transduced T cells were washed and rested for 24 hours in T cell media without cytokines. If CD4 and CD8 T cells were previously separated, they were combined immediately prior to the assay at 1:1 ratio.

For the transduction, non-tissue culture treated 24-well plates were coated overnight with 25 μg/ml Retronectin in PBS (300 μl/well). Next day, Retronectin was removed and 500 μl/well 2% BSA PBS was added for a 30-minute RT incubation. Next, 2% BSA PBS was removed and the wells were washed with PBS (600 μl/well). Immediately after PBS was removed, wells were filled to the top with viral supernatant ($\sim$3 ml; see previous section) and the plate was spun at 4°C 2000g for 90 minutes. Next, viral supernatant was discarded and 2 ml T cell culture at 0.5 million/ml were added. The plate was spun at 2000 rpm for 1 minute and returned to the incubator.

After three days (on the day of CD3/28 Dynabeads removal), T cell transduction level was measured. A sample of culture was taken and washed once with FACS buffer. Cells were resuspended in FACS TruStain FcX and incubated for 10 minutes at RT. CD3 PECy7, CD4 APC, CD8 FITC and mTCRβ BV711 antibodies were then added and the cells were incubated for 20 minutes in the fridge. Finally, cells were washed once and resuspended in FACS Hoechst for acquisition with BD Fortessa X-20. The level of TCR transduction was assessed by staining with the anti-mTCRβ antibody.

2.6 Cell Line Transduction for PADI4 Overexpression

Lentiviral plasmid preparation and transfection

pL-PADI4-WT-GFP, pL-PADI4-Var-GFP and pL-CRISPR-GFP transfer plasmid production and quality control is described above. Packaging plasmid psPAX2 (Addgene

#12260) and pseudotyping plasmid pMD2.G (Addgene #12259) stocks were received from Dr. Ryan Beveridge (WIMM Virus Screening Facility).

The wirus production method followed the same protocol as set out in the Primary T cell Transduction section, with the exception that transfection solution contained different plasmid ratios: 1µg pL-X-GFP, 0.75µg psPAX2 and 0.5µg pMD2.G.

To measure PADI4 overexpression rate and toxicity in HEK 293T, cells were trypsinised as described above and stained with propidium iodide and Hoechst 33342 for imaging and enumeration with NucleoCounter NC-3000 (ChemoMetec) (following ChemoMetec Application note No. 3023. Rev. 1.4).

Cell line transduction

Transduction was performed in 48-well tissue culture-treated plates. 1 ml reaction was assembled in each well with indicated viral supernatant dilution (see Figure 9) and with either 2 or 4 µg/ml polybrene. Each well contained 0.5 million cells. If condition required spinoculation, assembled plates were spun at 37°C 1000g for 90 minutes. For all conditions, plates were incubated for 72 hours before assessing transduction levels.

For this, cells were washed and resuspended in FACS Hoechst. Transduction efficiency was assessed by measuring the level of GFP positivity when compared to untransduced cell line controls. Transduction analysis was performed on BD Fortessa X-20. Cell sorting was done on Sony MA900 using a 100 µm sorting nozzle.

2.7 PADI4 SNP Knock-In with Cas9

3 µg of Cas9 nuclease and 1.6 µg of gRNA were combined together for ribonucleoprotein formation at 25°C for 10 minutes. If required, this was mixed with 100 pmol of ssODN. The final mixture was added to 0.2 million K562 cells in 20µl of SF nucleofector solution. For nucleofection efficiency control, 1 µg of plasmid pMaxGFP (Lonza) was added to cells instead. The reaction was transferred to a nucleofection strip and placed in the Lonza 4D Nucleofector System X-unit (FF120 programme). Afterwards, the cells were transferred to 1ml of pre-warmed RPMI 10% FBS.

Three days later, cells were washed twice with PBS and gDNA was extracted using the QuickExtract DNA Extraction Solution (following manufacturer's recommendations). 1 µl of the extract was used for a PCR with 12.5 µl of DreamTaq Green PCR MasterMix, 1µl each of primers pPAD1 and pPAD2 (10 µM stock concentration) and 9.5 µl of nuclease-free water. The following cycling programme was used: 1. Polymerase activation at 95°C for 3 minutes; 2. Denature at 95°C for 30 seconds; 3. Anneal at 63°C for 30 seconds; 4. Extend at 72°C for 60 seconds; (Steps 2-4 repeated for a total of 35 cycles) 5. Final extension at 72°C for 5 minutes. Final product was cleaned up using the MinElute PCR Purification kit (following manufacturer's recommendations) and sent for Sanger sequencing (WIMM Sequencing facility) using pPAD1 and pPAD2. Using the traces, KO and KI efficiency was estimated using Synthego ICE tool[250].

2.8 Western Blotting

Protein was extracted from a PBS-washed cell pellet using RIPA buffer supplemented with Halt protease inhibitor cocktail. Extracts were quantified using Pierce BCA assay. Immediately after, 1M DTT (1:10) and LDS sample buffer (1:4) were added and samples were denatured by incubating at 70°C for 10 minutes. If needed, samples were frozen for later use at this stage.

For gel electrophoresis, 15 µg per sample were loaded onto a NuPAGE 4-12% BisTris gel with PageRuler Plus Protein Ladder. Gel was run at 130-180V until leading dye reached the bottom and subsequently was transferred onto PVDF using semi-dry BioRad Power Pac system (60 minutes at 400mA). After a wash in TBST, PVDF membrane was blocked for 1 hour in TBST with 5% skimmed milk powder. Subsequently, the membrane was cut and the two halves were stained overnight at 4°C with either 1:1000 anti-PADI4 antibody or 1:5000 anti-GAPDH antibody in TBST with 5% skimmed milk powder.

Next day, the membrane was washed 5X with TBST and stained for 1 hour at RT with 1:10,000 secondary antibody in TBST with 5% skimmed milk powder. After five further washes with TBST, membrane was developed using the Amersham ECL detection kit (according to the manufacturer's protocol) and imaged using the BioRad ChemiDoc imaging system.

2.9 IFNγ ELISpot

Lymphocyte expansion culture

An overview of the expansion culture is shown in Figure 3.1a. Samples were thawed as described above with all washes performed using RAB-10 media (RPMI 10% h.i. human serum 1% Pen/Strep 1% sodium pyruvate 1% HEPES 1% NEAA 1% GlutaMAX 0.1% β-mercaptoethanol 25 U/ml Benzonase). Subsequently, cells were rested overnight in RAB-10.

Next day (day 0), cells were washed once and resuspended in fresh RAB-10 supplemented with 25 ng/ml IL-7 and 2 μg/ml of each peptide. Cells were cultured in 12-well plates and maintained at 1-2 million/ml density. On day 3, culture was supplemented with IL-2 at 1,800 U/ml final concentration. On day 7 and 10, additional RAB-10 media was added (typically 1 ml per each well) and IL-2 supplemented again at 1,800 U for each ml of culture. On day 12/13, the cells were washed 4X with PBS and rested for 24 hours in fresh RAB-10, before IFNγ ELISpot assay set-up.

IFNγ ELISpot

ELISpot plate was activated with 70% ethanol for 5 minutes, washed 3x PBS and coated with 10 μg/ml anti-human IFNγ antibody (clone 1-D1K) overnight at 4°C. Next day, plate was washed 3X with PBS and blocked for 3 hours with RAB-10. Rested cells were washed and 50,000 were added per well. For negative control, media was supplemented with 1% DMSO. For each peptide, 10 μM concentration was used. PADI4 variant condition contained two peptides (21 and 22), unless otherwise indicated (see the Appendix for all peptide sequences). After the set-up, the plate was incubated for 16-20 hours at 37°C 5% CO_2. Subsequently, wells were washed 6X with 0.05% Tween-20 PBS, coated with 1 μg/ml biotinylated anti-human IFNγ antibody (clone 7-B6-1) in 0.5% BSA PBS for 3 hours and washed 6X with 0.05% Tween-20 PBS. Then, the wells were coated with Vectastain avidin-peroxidase conjugate in 0.05% Tween-20 PBS for 1 hour. Finally, the plate was washed 3X with 0.05% Tween-20 PBS and

3X with PBS, followed by addition of 3-amino-9-ethylcarbazole (AEC) substrate (AEC tablet is dissolved in 2.5 ml of dimethylformamide, to which acetate buffer (43.7ml ddH_2O with 3.5ml 0.1M acetic acid and 280 µl 3M sodium acetate) is added, followed by 25 µl of 30% H_2O_2). 2-3 minutes later, the plate was washed with tap water, dried and imaged using the AID machine (Autoimmun Diagnostika).

2.10 Monocyte-Derived Dendritic Cell Production

Monocyte-derived dendritic cell (MDDC) generation protocol is summarised in Figure 4.10a. Freshly isolated healthy donor PBMCs (typically 50-75 million) with HLA-DPB*04:01 genotype (heterozygous or homozygous) were seeded in a T75 flask using complete media (RPMI 5% h.i. human serum 1% Pen/Strep 1% HEPES 1% NEAA). 2-3 hours later non-adherent cells were decanted and flask was carefully washed 3-5X with PBS. 15 ml of fresh media were added and supplemented with 300 U/ml IL-4 and 400 U/ml GM-CSF. At day 3, media was decanted and replaced with 10ml of fresh media with cytokines. The decanted media was spun down to retrieve any non-adherent cells. Supernatant was removed, cell pellet resuspended in 5 ml of fresh media with cytokines and this was added back to the flask. On day 6, media was decanted and 10ml of PBS were added. Cell scrapper was used to detach MDDC and PBS poured into the the tube with decanted media. This was repeated further 2-3 more times. Finally, the tube with all the cells was spun down and resuspended in T cell media (see above) for cytometry analysis and T cell assays.

For MDDC maturation, once the cells were resuspended in T cell media (±lysate), they were aliquoted 100,000 cells/well of a 96-well plate. Subsequently, indicated maturation cocktails (see Figure 4.13) were added and cells incubated for 24 hours before addition of T cells or cytometry analysis, unless otherwise indicated.

2.11 Normal Haematopoietic Cell Isolation

To isolate non-CD34 normal target cells, either cryopreserved CB mononuclear cells (CD34 cell depleted fraction) or healthy fresh blood donors were used, latter being

the only available source of granulocytes. For CD34 cells, either BM or CB samples were used, from MARCH study or NHSBT, respectively.

To fractionate fresh blood or BM, Ficoll density separation and mononuclear cell isolation was performed as described previously. For granulocyte isolation (referred to as 'CD15' in Chapter 5), red blood cell (bottom) layer from the Ficoll was isolated and lysed 3X with 10 ml of ACK buffer.

For fractionation into CD3, CD14, CD19 and CD34 cells, respective marker MACS bead separation was used, following manufacturer's protocol. CD34 cell separation was always performed first and $CD34^+$ as well as $CD34^-$ fractions were cryopreserved for later use. Using thawed $CD34^-$ cells or freshly isolated PBMC, CD14 cells were isolated first and the negative flow through was then used to isolate CD19. $CD14^-CD19^-$ flow through was finally used to isolate CD3 cells. Alternatively, $CD14^-CD19^-$ flow through was considered the 'untouched' CD3 fraction.

All isolated cell fractions were assessed for their purity with cytometry before any assays with T cells. Where possible, MACS bead selection marker was used to do so, however, for CD19 cells this was not successful (see Chapter 5) and instead secondary antibody was used to probe for CD19 MACS beads attached to B cell surface post-magnetic separation. To do so, samples of isolated cell fractions were taken and washed with MACS buffer (PBS 0.5% BSA 2mM EDTA). Then, cells were blocked with MACS TruStain FcX solution for 10 minutes at RT and one of the following antibodies were added: 1. CD3 PECy7; 2. Goat anti-mouse IgG AF488; 3. CD14 AF700; 4. CD15 PerCP; 5. CD34 APC. Samples were stained for 30 minutes in the fridge and subsequently washed and resuspended in FACS buffer with Hoechst for acquisition with BD Fortessa X-20.

2.12 T Cell Activation Assays

All target cells were washed and resuspended in T cell media prior to the T cell activation assay. Where indicated, T cell media also contained cytokines, ligands or cell lysate. Lysate was produced by first resuspending the cells at 2 million/ml concentration in T cell media and then placing the tube on dry ice until completely frozen. Then,

the tube was allowed to completely thaw at RT and the liquid was spun down to remove large debris (pellet). Viable target cells were resuspended in the supernatant (cell lysate), before plating with T cells.

Target cells were plated in 96-well plates and, if required, any 24-hour treatments were added for incubation. It is important to distinguish that all 24-hour target cell pre-treatments were not washed out from T cell media prior to T cell addition, with the exception of peptide pulses (where cells were washed 3X with PBS and resuspended in fresh T cell media prior to plating). All pre-treatments that lasted longer than 24 hours (*e.g.* IFNγ pre-treatment of cell lines) were washed out from media prior to target cell plating with T cells. Where applicable, the effect of a given treatment on the target cell surface HLA-DP expression was assessed prior to the T cell co-culture with flow cytometry (see below).

Peptide pulses for target cells were performed at 37°C either overnight or for 2 hours prior to the assay at the indicated molarity (for details, see each figure legend). In each case, cells were washed 2X prior to plating.

Once target cells were plated, T cells were added at 1:1 or 1:2 effector-to-target (E:T) ratio (indicated in each case). Incubations to allow T cell activation lasted 9, 24, 48 or 72 hours. For each case, CD107a and/or CD107b antibodies were added at 1:300 dilution in the last 9 hours of co-culture. In the last 8 hours, Monensin and Brefeldin A were added as well (1:1000 dilution for both).

Where T cells were peptide pulsed, peptide was added directly to the activation assay plate with T cells at the indicated molarity and at the start of the assay (hour 0 of co-cultures). PHA treatment of T cells was done similarly, at 10 μg/ml final concentration. At the end of co-culture, cells were processed for flow cytometry (see below).

2.13 T Cell Killing Assays

Imaging-based killing assays

Imaging-based killing assays were performed on cell lines only (Fig. 3.14, 11 and 3.19). After IFNγ pre-treatment (where indicated), cell lines were washed once and resuspended in T cell media for plating. Where the cell lines were pulsed with peptide, this was

done for 2 hours at 37°C and immediately prior to the assay. The excess peptide was then removed with 2X washes (for details see each figure legend). Using flat-bottom tissue culture-treated 96-well plates, 100,000 target cells were plated in each well for MONOMAC1, BOLETH and SUP-B15, and 50,000 cells for HEL. Subsequently, rested and washed T cells were plated in 1:1 E:T ratio.

For higher E:T ratios, 24-well tissue culture treated plates were used. 1:5 E:T ratio received 100,000 target cells and 1:10 E:T ratio received 50,000 target cells per well. Proportionately, the number of plated T cells was 500,000 for both conditions.

Once effector and target cells were plated, the wells were topped up with media and any remaining empty wells were filled with PBS, to mitigate drying over time. Plates were placed into the Incucyte Live-Cell Analysis system (Sartorius). It was set to capture imaging every 6 hours using 20X objective for phase and green fluorescence with the following parameters: 1. Maximum number of images per well (for 96-well plates, it is 9 images/well); 2. Non-adherent cell-by-cell imaging setting.

Cytometry-based killing assays

Cytometry-based killing assays were performed on normal haematopoietic cells only (Chapter 5). Isolated target cells (see above) were resuspended in T cell media and plated 50,000 in each well of a 96-well plate. Where target cells were pulsed with peptide, this was done in the 2 hours immediately prior to the assay, at 37°C (for details, see each figure legend). Subsequently, excess peptide was removed with 2X washes before plating.

Meanwhile, rested effector T cells were labelled with CellTrace Violet (staining dilution 1:2,500, otherwise following the manufacturer's protocol). This permitted to efficiently separate target and effector cells at the cytometry data analysis stage.

Labelled T cells were added into the wells with targets at indicated E:T ratios and the co-culture was incubated at 37°C for 6 or 24 hours before processing. Afterwards, CountBright counting beads were added, wells were washed once with FACS buffer and the cells were resuspended in FACS TruStain FcX solution for 10 minutes at RT. After, the HLA-DP PE antibody was added. If CD34 or 'untouched' CD3 target cells were investigated, CD34 APC or CD3 PECy7 antibodies were added as well,

respectively. Samples were stained for 20 minutes in the fridge and then washed once before resuspension in FACS 7-AAD for analysis on BD Fortessa X-20. To enumerate the total number of target cells in each well, the following formula was used:

$$\frac{(N_{\text{acquired target cells}} * N_{\text{added beads}})}{N_{\text{acquired beads}}} = N_{\text{total target cells}}$$

Then, the ratio of killing was calculated by first averaging the total number of target cells for technical replicates of all conditions with irrelevant TCR (1G4 10:1 pulsed, 1G4 10:1 and 1G4 1:1, calculated separately), referred to as $N_{\text{average targets in 1G4}}$. This was used to calculate the ratio of killing for each well in respective E:T ratio condition with PADI4 TCR T cells:

$$\frac{(N_{\text{total target cells}} * 100)}{(N_{\text{average targets in 1G4}} - 100)} = \text{Killing ratio (\%)}$$

Meanwhile, relative change of target cell HLA-DP MFI in PADI4 TCR conditions when compared to irrelevant TCR were calculated using a similar formula:

$$\frac{(X_{\text{target HLA-DP MFI}} * 1)}{(Y_{\text{average target HLA-DP MFI in 1G4}} - 1)} = \text{Relative change in MFI}$$

2.14 Flow Cytometry and Sorting

FACS buffer (IMDM h.i. 10% FBS 1%Pen/Strep) was used for sample washing, staining and acquisition, unless otherwise indicated. Cytometry data was analysed on FlowJo 10.8.1 (BD).

AML immunophenotyping

Once AML samples were thawed (see above), cells were washed and incubated with FACS TruStain FcX for 10 minutes at RT. Then the following antibodies were added: CD45 APC eFluor 780, CD34 AF700, CD117 BV650, CD3 PECy7, CD19 PerCP Cy5.5, CD14 FITC and HLA-DP PE. Samples were stained for 25 minutes in the fridge, washed and resuspended in FACS Hoechst for acquisition with Sony MA900.

IFNγ catch assay

An overview of this technique is shown in Figure 3.1. Overnight rested T cells from lymphocyte expansion culture (see Section 2.9) were stimulated with PADI4 variant peptides 21 and 22 for 6 hours at 10 μg/ml. Next, cells were washed with cold MACS buffer (PBS 0.5% BSA 2mM EDTA) (1-2 ml/million cells) and resuspended in cold RAB-10 (see Section 2.9) at 90 μl/million cells. 10 μl/million cells of IFNγ catch reagent were added and cells incubated on ice for 5 minutes. Afterwards, cells were diluted with warm RAB-10 to 1 million/ml concentration and the tube was incubated at 37°C for 45 minutes. The tube was turned every 5 minutes to prevent cell settlement.

Next, the tube was placed on ice and washed once with cold MACS buffer. All supernatant was removed and cells were resuspended in 90 μl/million cells of cold MACS TruStain FcX. Cells were incubated in the fridge for 10 minutes and 10 μl/million cells of IFNγ PE antibody were added (part of the Miltenyi IFNγ Catch kit). At this point, other antibodies for cell phenotyping were added as well: CD3 PECy7, CD4 APC and CD8 FITC. Cells were incubated in the fridge for 30 minutes and washed 3X with FACS buffer. Finally, the sample was suspended in FACS Hoechst and sorted using the BD Aria Fusion.

MDDC immunophenotyping

Harvested MDDCs (see Section 2.10) were washed once with FACS buffer and resuspended in staining mix, composed of the FACS buffer and the following reagents: L/D Aqua, HLA-DR BV605, CD80 BV650, HLA-DP BV711, CD86 AF488, CD40 PE, CD4 PETR, CD19 PE Cy5, CD3 PE Cy5 and CD83 APC. After a 20-minute incubation in the fridge, the cells were washed in PBS and resuspended in 2% PFA PBS. After a further 10 minute incubation in the fridge, the cells were washed once more and resuspended in PBS for acquisition with BD Fortessa X-50.

T cell activation

Where applicable, the effect of a given target cell pre-treatment prior to the co-culture with T cells was assessed separately, by measuring HLA-DP surface expression. Target

cells were washed once in FACS buffer and resuspended in staining buffer with HLA-DP PE. After the incubation for 30 minutes in the fridge, the cells were washed and resuspended in FACS buffer with Hoechst for acquisition with BD Fortessa X-20.

For assessment of T cell activation, T cell co-cultures received either CD107a BV650 antibody alone or CD107a BV421 and CD107b BV421 antibodies together at 9 hours prior to the end of the culturing period (see each experiment separately). One hour later, Brefeldin A and Monensin were added.

Eight hours later, cells were washed twice with PBS and resuspended in TruStain FcX PBS for 10 minutes at RT. Next, L/D Aqua was added and cells were incubated for 15 more minutes at RT. After one wash with PBS, cells were resuspended in surface staining mix (PBS): mTCRβ BV711, CD3 PE Cy7, CD4 PETR and CD8 APC Cy7. After 15 minutes at RT, cells were washed once with PBS and resuspended in BD Fix/Perm solution for 20-minute fixation in the fridge. Subsequently, cells were washed twice with BD Perm/Wash and resuspended in intracellular staining mix (BD Perm/Wash): CD3 PE Cy7, CD4 PETR, mTCRβ BV711, CD8 APC Cy7, TNFα FITC, CD137 PE, CD69 APC and CD134 BB700. After 30 minutes in the fridge, cells were washed twice with BD Perm/Wash and resuspended in PBS for acquisition with BD Fortessa X-50. During data analysis, activation markers were measured in the Singlet Live CD3$^+$ CD4$^+$/CD8$^+$ mTCRβ^+ gate.

2.15 Computational Analysis

Public dataset analysis

Gene expression data from cancer cell lines was retrieved from the Broad Institute Cancer Cell Line Encyclopedia. Downloaded data was already processed to display transcript per million (TPM) values. Cell lines were filtered to display only myeloid leukaemias (n=49) and several other cell types for comparison: HeLa (cervical cancer cell line), SUP-B15 and RS4;11 (B-cell aculte lymphoblastic leukaemia cell lines).

Gene expression data from primary cancers and acute myeloid leukaemias was derived from The Cancer Genome Atlas (TCGA) programme, BeatAML project[251] as well as

from an in-house AML cohort (data acquisition and analysis done by Connor Sweeney). Retrieved data was already analysed to display TPM values.

Gene expression data across healthy tissues was derived from the Human Protein Atlas Consensus Expression dataset[219, 220]. Retrieved data was already analysed to display TPM values. Gene expression across different haematopoietic cell types was derived from the Bloodspot database[252] as well as from Corces et al.[253] and Karamitros and Stoilova et al.[254] sequencing datasets. Data was already analysed to either display consensus $\log_2$expression values[252] or TPM.

10X single cell RNA sequencing with V(D)J enrichment

Single cell RNA sequencing data with V(D)J enrichment was collected and analysed by Connor Sweeney. Below provides a brief summary of data acquisition and analysis, adapted from Connor Sweeney's thesis.

Following INFγ catch assay (see section 2.14), up to 10,000 sorted CD4$^+$ and INFγ^+ or INFγ^- T lymphocytes were loaded into the 10X Chromium chip. Cells were processed using the Chromium Single Cell 5' Library and Gel Bead kit (10X Genomics) and following the provided protocol. After reverse transcription, cDNA was amplified for 14 cycles and resultant material split between V(D)J and transcriptome library preparation protocols. Former was done using the Human T cell Chromium Single Cell V(D)J Enrichment kit (10X Genomics). Library quality was assessed with the Bioanalyzer High Sensitivity DNA kit (Agilent Technologies), before being sent for sequencing with Illumina NovaSeq 6000 (Novogene, Cambridge). Sequencing aimed for 100,000 reads/cell for the transcriptome and 5,000 reads/cell for the V(D)J.

Received sequencing data was first mapped onto the human reference genome (GRCh38) and the unique transcripts counted using Cell Ranger. This both processed the transcriptome and the V(D)J data. For the latter, Cell Ranger V(D)J output provided TCR sequences of each cell and the frequency of each TCR. For the former, Cell Ranger transcriptome output was further analysed with RStudio. First, only cells with expression of 5,000-7,000 genes and lower that 10% abundance of mitochondrial genes were kept using Seurat. Next, expression data was normalised, scaled and transformed to perform

dimentionality reduction followed by UMAP (Uniform Manifold Approximation and Projection) visualisation and differential gene expression analysis using Seurat. Cell type identification on the UMAP was performed using SingleR and CIPR (Cluster Identity Predictor), using Monaco et al.[255, 256] and the Database of Immune Cell Expression (DICE)[257, 258] reference datasets, respectively.

Other

Diagrams shown in this thesis were generated with BioRender. Data cleanup and display was done with RStudio and GraphPad Prism. Plasmid cloning and display was done with SnapGene.

Chapter 3

PADI4 TCR Discovery, Target Cell Line Development and Testing

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3.1 Overview

After the initial discovery of the *PADI4* miHA response in patient OX866 (see Chapter 1), further study of this required to isolate the *PADI4* miHA-specific T cells and identify their TCRs. Thus, the first section of this chapter describes the capture of antigen-specific IFNγ^+ patient T cells, their transcriptome as well as V(D)J sequences. Importantly, the antigen-specific TCR was identified by cloning and transferring the sequenced TCRs to third-party primary T cells for activation assays using the PADI4 variant peptide. Meanwhile, a cell line model was required to test the anti-leukaemic efficacy of the transduced T cells *in vitro*. Thus, the second section of this chapter

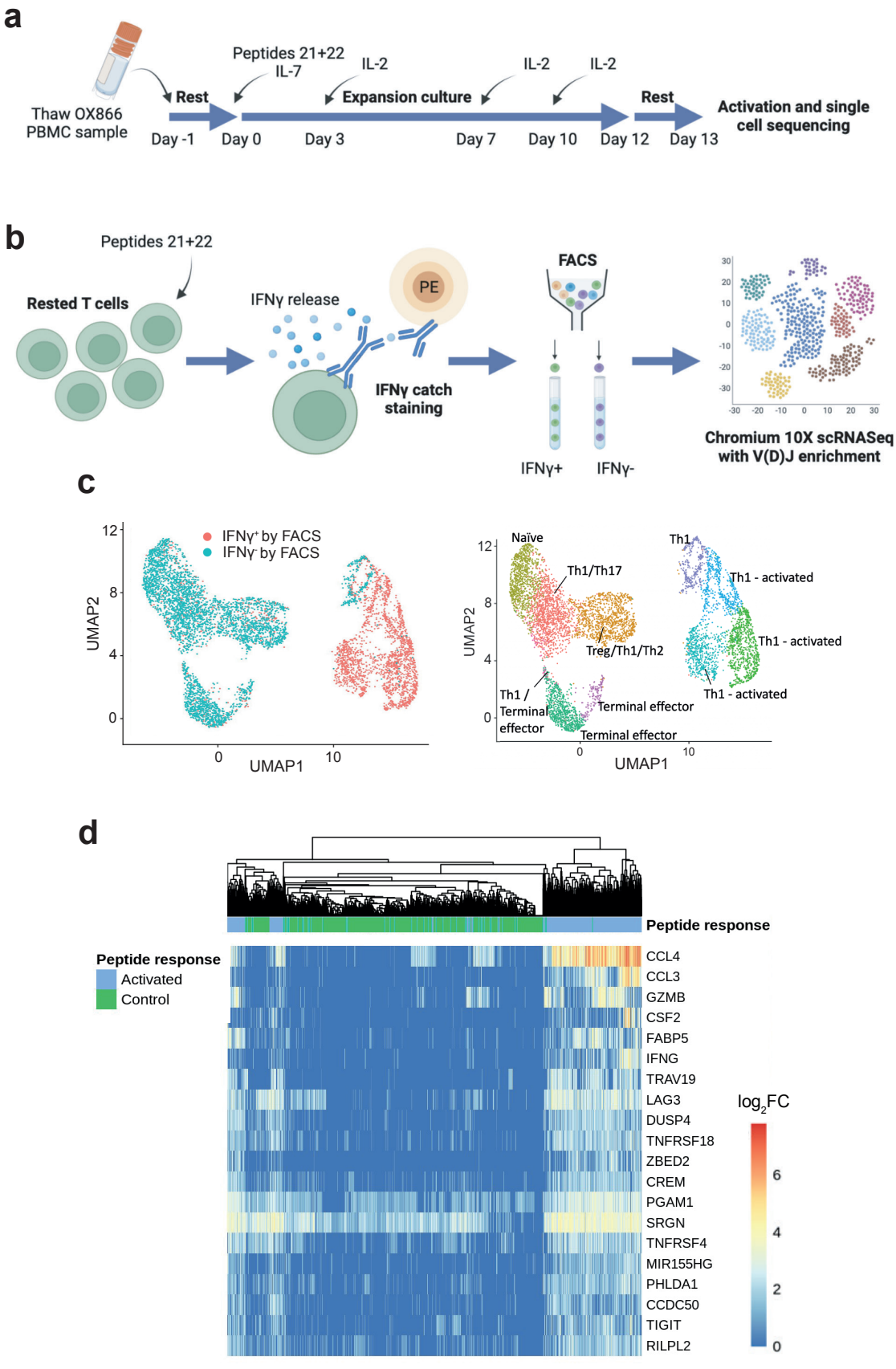
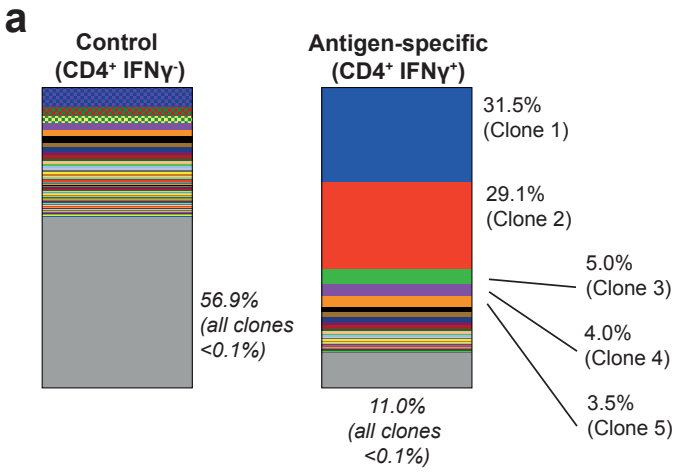

a
Thaw OX866 PBMC sample
Rest
Peptides 21+22
IL-7
IL-2
Expansion culture
IL-2
IL-2
Rest
Activation and single cell sequencing
Day -1
Day 0
Day 3
Day 7
Day 10
Day 12
Day 13
b
Peptides 21+22
Rested T cells
IFNγ release
PE
IFNγ catch staining
FACS
IFNγ+
IFNγ-
Chromium 10X scRNASeq with V(D)J enrichment
c
IFNγ+ by FACS
IFNγ- by FACS
UMAP2
UMAP1
Naïve
Th1/Th17
Treg/Th1/Th2
Th1 / Terminal effector
Terminal effector
Terminal effector
Th1
Th1 - activated
Th1 - activated
Th1 - activated
d
Peptide response
Activated
Control
CCL4
CCL3
GZMB
CSF2
FABP5
IFNG
TRAV19
LAG3
DUSP4
TNFRSF18
ZBED2
CREM
PGAM1
SRGN
TNFRSF4
MIR155HG
PHLDA1
CCDC50
TIGIT
RILPL2
log2FC

Figure 3.1: (Previous page) *PADI4* miHA responding T lymphocytes are successfully isolated from post-transplant PBMC sample. **a** Method of PBMC sample culture to enrich for peptide-specific T lymphocytes. **b** Method of peptide-specific T lymphocyte activation to capture IFNγ^+ cells. **c** UMAP dimensionality reduction of samples retrieved from the sorted IFNγ^+ and IFNγ^- cell populations, coloured by sample identity (left) and bioinformatically predicted cell phenotypes (right). **d** Hierarchically clustered gene expression heatmap showing top 20 differentially expressed genes (log2FC: log2 fold change) in IFNγ^+ (activated) and IFNγ^- (non-activated) cells. Columns represent individual cells, whilst rows represent genes. Data in this figure produced by Connor Sweeney.



b

Clone		Sequence			
		V	D	J	C
1	TRA	TRAV19		TRAJ32	TRAC
	TRB	TRBV2		TRBJ2-7	TRBC2
2	TRA (1)	TRAC12-2		TRAJ15	TRAC
	TRA (2)	TRAV19		TRAJ29	TRAC
	TRB	TRBV5-1	TRBD1	TRBJ1-2	TRBC1
3	TRA	TRAV8-1		TRAJ27	TRAC
	TRB	TRBV27	TRBD2	TRBJ2-2	TRBC2
4	TRA	same as clone 2 TRA (1)			
	TRB	same as clone 2 TRB			
5	TRA	TRAV23DV6		TRAJ45	TRAC
	TRB	TRBV18	TRBD1	TRBJ2-1	TRBC2

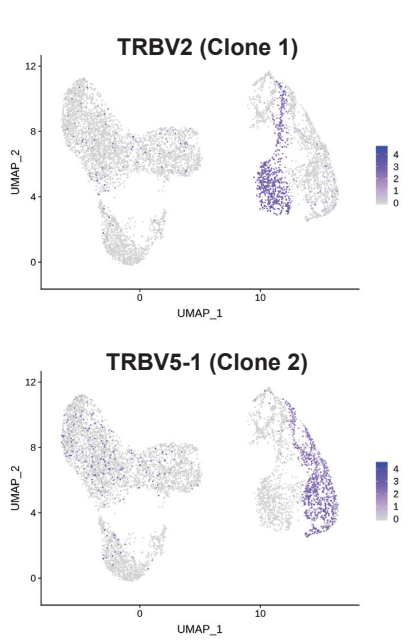


Figure 3.2: Sorted IFNγ^+ and IFNγ^- T lymphocyte fractions can be used to identify putative *PADI4* miHA specific TCR clones. **a** Column graph depicting the diversity of the TCR repertoire in both cell populations. Each colour represents a different TCR clone, arranged from most to least frequent (top to bottom). Frequency of a clone in the cell population is represented by the thickness of the coloured bar. Grey bars, where indicated, represent a sum of low frequency clones (<0.1%): 56.9% of the repertoire in IFNγ^- and 11.0% of the repertoire in IFNγ^+ cell populations. **b** Table showing V, D, J and C genes that comprise top 5 TCR α and β chains in the IFNγ^+ cell population. **c** Same UMAP dimensionality reduction plot as seen in Figure 3.1 coloured by expression of TRBV2 (top) and TRBV5-1 (bottom) genes that characterise IFNγ^+ clone 1 and IFNγ^+ clone 2, respectively. Data in this figure produced by Connor Sweeney.

discusses the genotype and gene expression requirements for a suitable target cell line and the steps taken to fulfull these criteria. Finally, the third section explores whether the developed cell lines can be targeted by the PADI4-specific TCR transduced (PADI4 TCR) T cells *in vitro*, using T cell activation in co-culture as well as target cell growth inhibition assays.

3.2 *PADI4* miHA Specific TCR Discovery

PADI4 miHA-activated patient T cells can be isolated and studied

To investigate the *PADI4* miHA response further, the same post-transplant patient sample originally used for the peptide screening pipeline (see Chapter 1) was thawed and cultured with the *PADI4* miHA peptides 21 & 22 to enrich for miHA-specific T cells in culture (Fig. 3.1a). After the expansion period and an overnight rest in cytokine and peptide-free media, lymphocytes were restimulated with peptide. The miHA-reactive population was isolated by capturing the released IFNγ on the activated cell surface and labelling it with a fluorescent antibody (IFNγ capture assay), followed by fluorescence-activated cell sorting (FACS) (Fig. 3.1b). Finally, both activated ($CD4^+$ $IFN\gamma^+$) and non-activated ($CD4^+$ $IFN\gamma^-$) sorted cell populations were subjected to RNA sequencing with V(D)J transcript analysis using the Chromium 10X single cell sequencing platform.

Single cell RNA transcriptome analysis of isolated cell populations showed that activated and non-activated lymphocytes cluster apart on the Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction plot, indicating that the two populations have distinct transcriptional profiles (Fig. 3.1b). Identified clusters could be further characterised using publicly available single cell RNA sequencing cluster cell identity annotation tools CIPR and SingleR[255–258]. This predicted $IFN\gamma^+$ population derived cell clusters to have activated type 1 T helper (Th1) cell phenotype, which describes $CD4^+$ T lymphocytes that are involved in cell-mediated immune responses and release IFNγ as well as IL-2. Meanwhile, $IFN\gamma^-$ population derived cell clusters had a mixture of predicted cell identities, including naïve, Treg, type 2 T helper (Th2), type 17 T helper (Th17) and terminal effector, but no activated phenotypes.

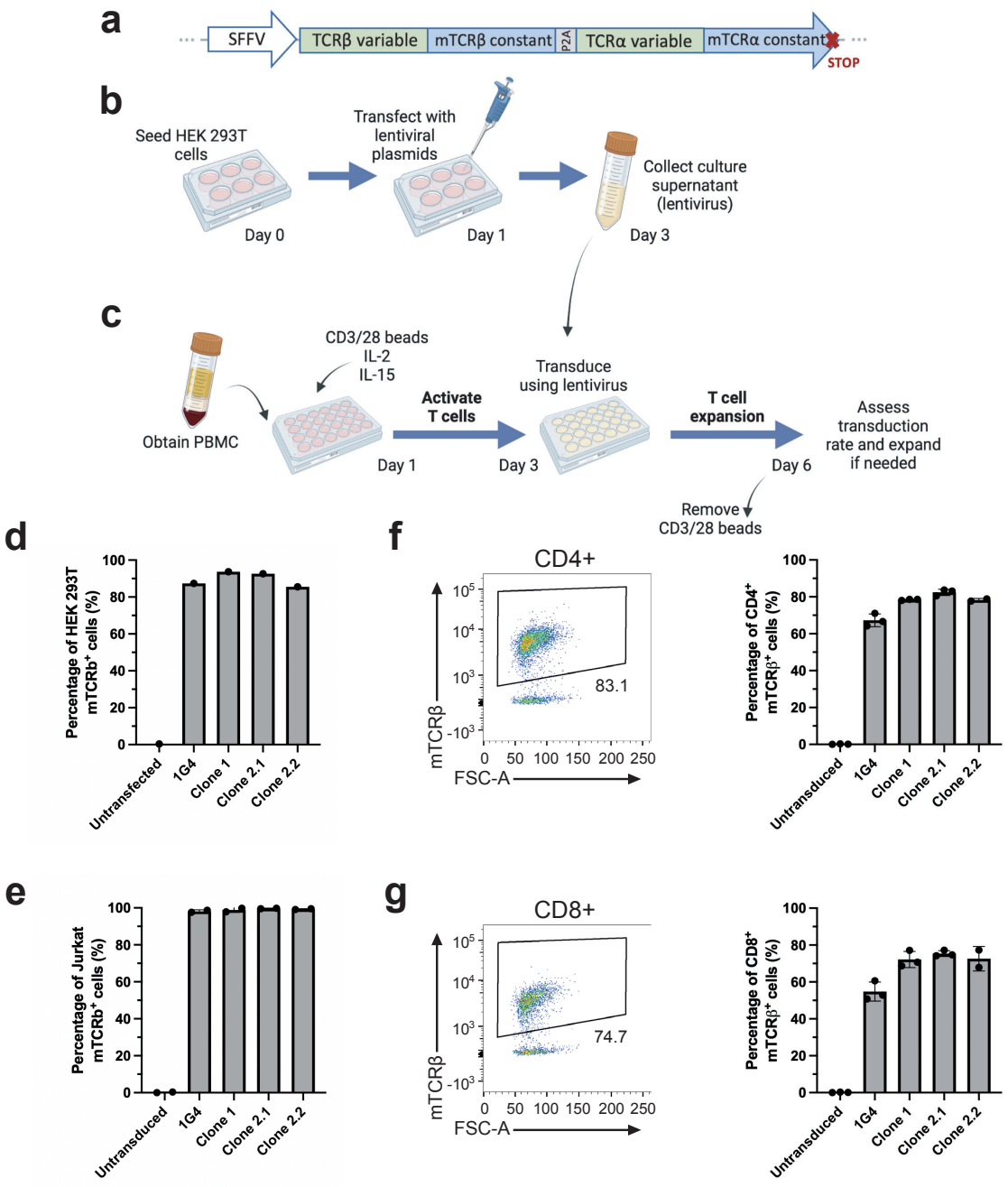


Figure 3.3: Primary human T lymphocytes can be genetically engineered using lentivirus to express a transgenic TCR of choice. **a** Lentiviral TCR insert map. **b** Method of lentivirus production. **c** Method of primary human T cell isolation, activation, and transduction. **d** Level of HEK 293T transfection as measured by surface and intracellular expression of the TCR construct at day 3 of the protocol. **e** Level of TCR transduction in Jurkat TCR KO at day 6 of the protocol. **f** and **g** Level of TCR transduction in $CD4^+$ and $CD8^+$ primary human T lymphocytes, respectively, at day 6 of the protocol. Number of independent repeats for each condition is indicated by dots in the column graphs (1-3). Error bars show standard deviation. In all depicted cases, transgenic TCR expression level is measured by fluorescent antibody staining for murine TRCβ constant (mTCRβ) region (a non-variable part of the construct seen in **a**.

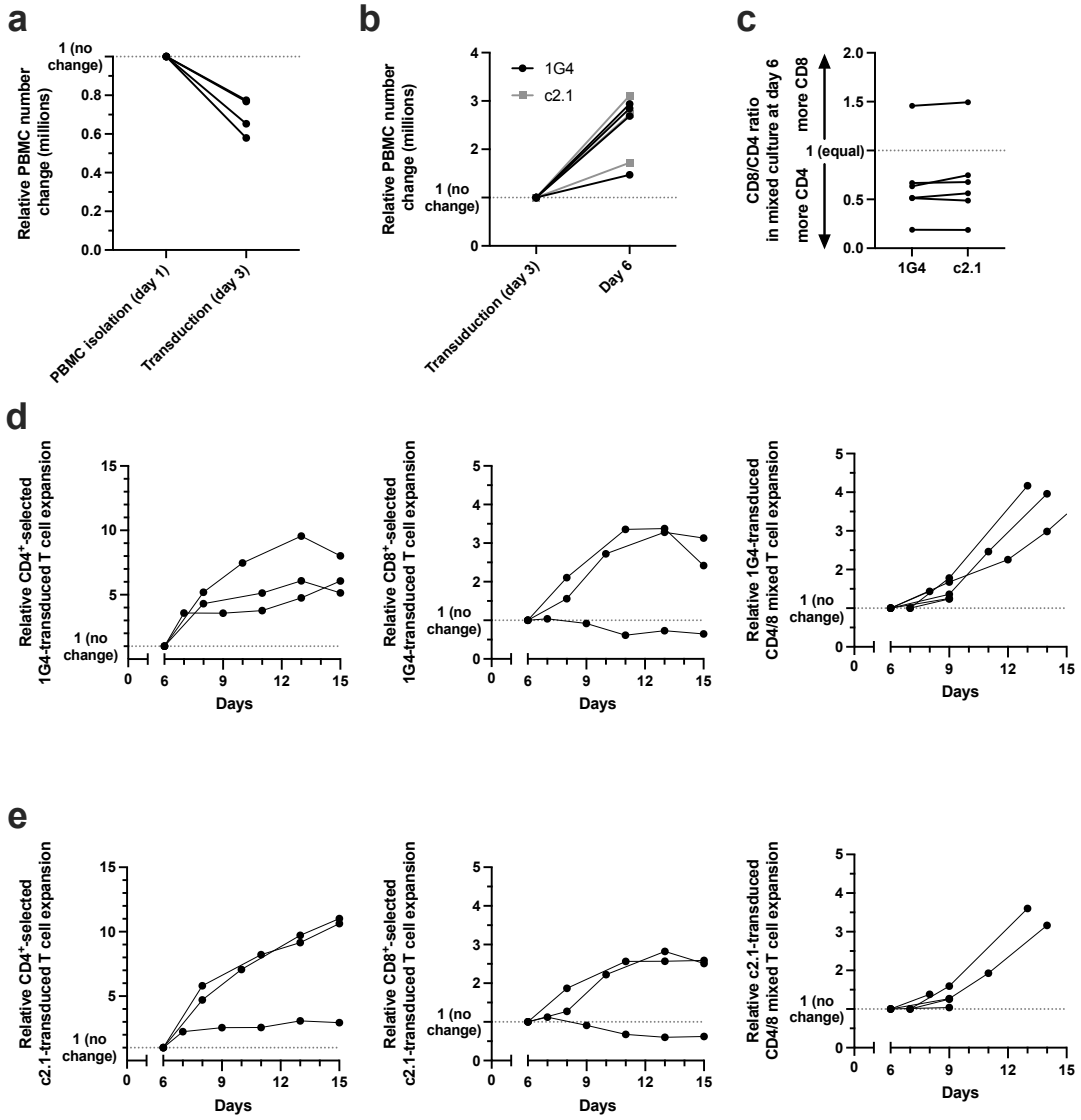


Figure 3.4: The dynamics of TCR-transferred primary human T cell lymphocyte expansion *in vitro*. **a** Graph showing relative cell number reduction in the two days of culture immediately post PBMC isolation from blood. **b** From day 3 (transduction day) to day 6 cell number rapidly increases. **c** At day 6, CD8 to CD4 ratio varies widely from donor to donor as measured by flow cytometry. **d** and **e** Show relative T cell expansion from day 6 onwards for cells transduced with 1G4 or c2.1 TCR constructs, respectively. Left and middle panels show how at day 6 magnetically separated $CD4^+$ or $CD8^+$ T lymphocytes expand in isolation, whilst right panel shows expansion of mixed $CD4^+$ and $CD8^+$ culture. Each line indicates an independent experiment.

PADI4 miHA-responsive lymphocytes could be further studied with differential gene expression analysis comparing activated and non-activated populations (Fig. 3.1d). Notable genes expressed in the responsive population include proinflammatory chemokines CCL3 (MIP-1α) and CCL4 (MIP-1β), cytokines IFNγ and CSF2 (GM-CSF), activation markers GZMB (granzyme B) and CD134 as well as exhaustion markers LAG3 and TIGIT. Interestingly, this transcriptional profile, combined with CD107a (degranulation marker) staining in cytometry-based peptide pulse experiments (Connor Sweeney's thesis), suggested that these cells may have cytotoxic properties. Indeed, CD4$^+$ cytotoxic cells have been previously described and studied in both cancers and infections[196, 197, 259, 260].

In terms of the TCR repertoire, the CD4$^+$ IFNγ^+ population shows strong enrichment of the select few TCR clones when compared to the CD4$^+$ IFNγ^- population (Fig. 3.2). Clones 1 and 2 comprise 31.5% and 29.1% of the whole CD4$^+$ IFNγ^+ population, respectively. Notably, clone 2 contains 1 TCRβ chain and 2 TCRα chains, a phenomenon termed as 'incomplete phenotypic allelic exclusion'[261]. This is partially explained by the mechanisms of α chain recombination during the thymic selection and may not necessarily mean that both TCR heterodimers are found on the surface of the T cells from which these sequences were derived, but a detailed discussion about this is beyond the scope of this work[261, 262]. Additionally, the much less frequent clone 4 (4.0%) carries a TCR identical to clone 2, however without the second TCRα chain (Fig. 3.2b and Appendix 1). It remains unknown, although unlikely, whether these two clones are truly distinct, and their existence could perhaps be explained by the technical dropout of the second TCRα chain sequence in clone 4. If so, combining the frequency of clone 2 and 4, leads to the identification of the most frequent TCR within the CD4$^+$ IFNγ^+ population (33.1%). Taken together, clone 1 and 2 TCRs are the most likely candidates to mediate the *PADI4* miHA specific response.

Candidate *PADI4* miHA-specific TCR can be cloned and transferred to third party T cells

Initially, γ-retrovirus pMP71 TCR transfer plasmids (Hans Stauss, London) were used to produce the constructs for the top 2 TCR clones found in the CD4$^+$ IFNγ^+ population

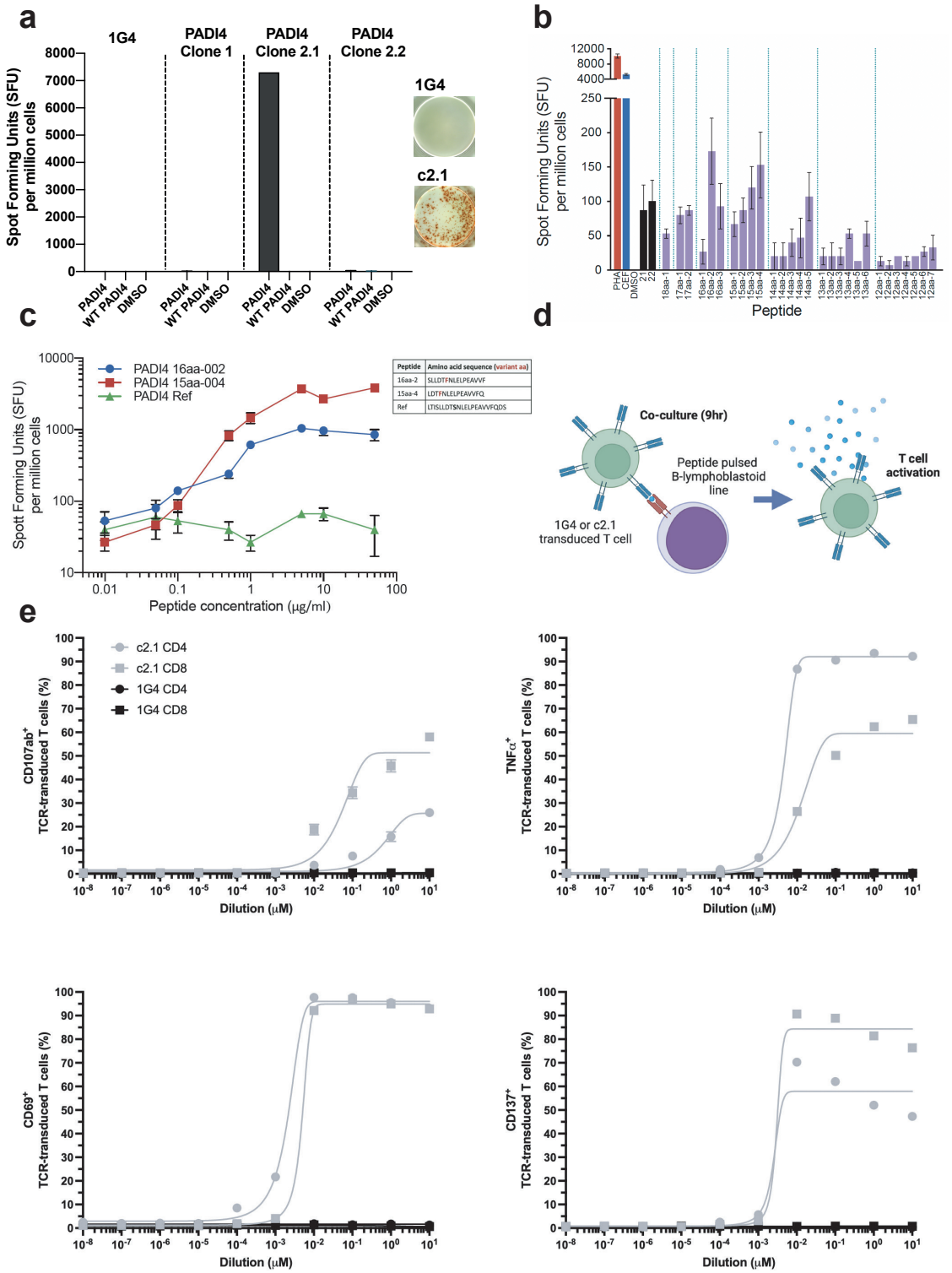

a
1G4
PADI4 Clone 1
PADI4 Clone 2.1
PADI4 Clone 2.2
Spot Forming Units (SFU) per million cells
PADI4
WT PADI4
DMSO
1G4
c2.1
b
Spot Forming Units (SFU) per million cells
Peptide
c
PADI4 16aa-002
PADI4 15aa-004
PADI4 Ref
Peptide
Amino acid sequence (variant aa)
16aa-2
SLLDTFNLELPEAVVF
15aa-4
LDTFNLELPEAVVFQ
Ref
LTISLLDTSNLELPEAVVFQDS
Spott Forming Units (SFU) per million cells
Peptide concentration (µg/ml)
d
Co-culture (9hr)
Peptide pulsed B-lymphoblastoid line
1G4 or c2.1 transduced T cell
T cell activation
e
c2.1 CD4
c2.1 CD8
1G4 CD4
1G4 CD8
CD107ab+ TCR-transduced T cells (%)
TNFα+ TCR-transduced T cells (%)
CD69+ TCR-transduced T cells (%)
CD137+ TCR-transduced T cells (%)
Dilution (µM)

Figure 3.5: (Previous page) Clone 2.1 TCR mediates *PADI4* miHA specific response in TCR-transferred primary human T lymphocytes. a IFNγ ELISpot on TCR-transferred T lymphocytes show Clone 2.1 reactivity and specificity to PADI4 variant peptides 21 & 22 added at final 2 μg/ml concentration. Images on the right show representative well views from the ELISpot assay. **b** and **c** Show IFNγ ELISpot on post-transplant patient PBMC cultured using the method depicted in Figure 3.5. Different length peptides covering the same PADI4 peptide variant were synthesised to map the most optimal peptide sequence for triggering antigen-specific T cell activation (see peptide sequences in the Appendix). **d** Schematic showing the set-up for a peptide titration experiment, using professional antigen presenting B-lymphoblastoid cell line BOLETH (homozygous for required HLA-DPB1*04:01 allele). **e** Results of the peptide titration experiment using four markers to read out T cell activation: CD107a and CD107b co-stain, TNFα, CD69 and CD137. Optimal epitope peptide PADI4 15aa-004 is used in this experiment. Non-linear regression is fit to approximate a dose-response curve. All results in this figure show one independent experiment. Error bars show standard deviation between technical replicates. Data shown in **b** and **c** are produced by Connor Sweeney.

(Chapter 2 and the Appendix). For Clone 2, where two TCRα chains were found, different TCR constructs were made for each TCRα chain (denoted as clone 2.1 and 2.2 TCRs). In addition, the following modifications were introduced to the TCR chains to aid effective gene transfer: 1) TCR variable regions were human codon optimised; 2) human TCR constant regions were swapped for the murine ones. The latter modification is intended to aid effective transgenic TCR chain pairing by reducing interactions with endogenous TCR in the primary T cells. Unfortunately, primary T cell transduction rates using the γ-retrovirus vector were variable as well as low (data not shown).

Next, the TCR constructs were cloned into the lentiviral pHRsin transfer plasmid (Margarida Rei, Oxford; Fig. 3.3a, see also Chapter 2 and the Appendix). Following the lentivirus production protocol (Fig. 3.3b and Chapter 2), virus producing cell line HEK 293T can be efficiently transfected (Fig. 3.3d) and the resultant viral supernatant can easily transduce Jurkat TCR KO cell line (Fig. 3.3e). Importantly, when using the protocol for primary T cell transduction (Fig. 3.3b), both human $CD4^+$ and $CD8^+$ T cells can be reproducibly transduced at high levels (Fig. 3.3f,g).

To effectively use TCR-transferred human primary T cells in further assays, an efficient post-transduction T cell expansion protocol was required. In the first few days after isolation from peripheral healthy blood, the cell number in culture contracted but then rapidly expanded owing to T cell proliferation induced by CD3/28 beads, IL-2

and IL-15 (Fig 3.4a,b). At day 6 of culture, T cells had highly varied $CD4^+$ to $CD8^+$ ratios across different healthy donors. More often $CD4^+$ T cells dominated the culture (Fig. 3.4c). To address this, $CD4^+$ and $CD8^+$ T cell magnetic separation at day 6 was used to test if separated cells can efficiently expand in isolation (Fig. 3.4d,e). This appeared to be donor dependent, although generally $CD4^+$ T cells expanded better than $CD8^+$. Moreover, the magnetic separation process itself was associated with decreased cell viability and significant cell loss (data not shown). Taken together, the magnetic separation approach did not prove useful to boost the $CD8^+$ T cell expansion and so T cell culture was mostly carried out with mixed, unperturbed $CD4^+$ and $CD8^+$ populations. Due to steady expansion and minimised cell loss, these mixed cultures rarely reached their expansion plateau within the time kept in culture (Fig. 3.4d,e).

Clone 2.1 TCR mediates *PADI4* miHA specific response

Once the method of TCR-transfer in human primary T cells was established, an IFNγ ELISpot was used to show that the TCR clone 2.1 (c2.1) can specifically recognise the variant PADI4 peptide and not its reference (also referred to as 'wild-type') version, therefore inducing T cell activation, measured by IFNγ release (Fig. 3.5a).

Previously, cultured T lymphocytes from the patient OX866 had been used to map the optimal PADI4 variant peptide length and sequence for T cell activation (Fig. 3.5b,c). Based on this work, the epitope mapped PADI4 15aa-004 peptide was used in a peptide titration experiment where TCR-transferred T cells were incubated with a professional antigen presenting cell line in order to approximate the dose-response curve for c2.1 TCR (Fig. 3.5d,e). Despite the HLA-DP restriction of the response, both $CD4^+$ and $CD8^+$ T cells responded to the presenting cell line. For most activation markers used, half maximal effective concentration ($_{EC}50$) was reached at 10^{-3} to 10^{-2} μmolar concentrations after 9 hours of co-culture.

3.3 Target Cell Line Model Development

After the successful discovery of the PADI4 variant peptide specific TCR (PADI4 TCR) and the establishment of a reproducible TCR-transfer protocol, the first steps in

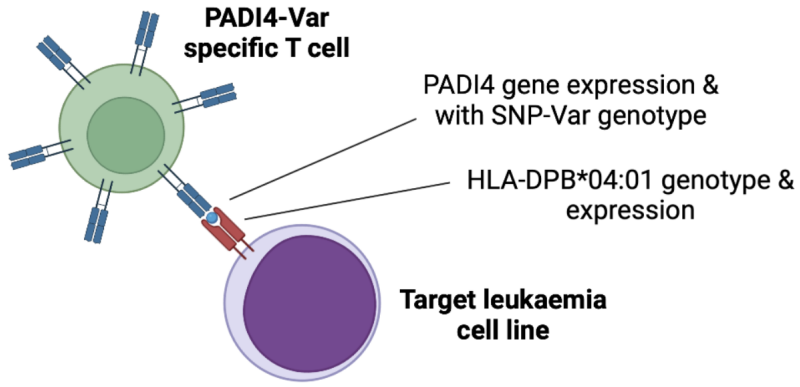


Figure 3.6: Conditions for an appropriate leukaemia cell line model to test the efficacy of PADI4 TCR. To be recognised by the PADI4 variant peptide specific TCR (PADI4 TCR), an appropriate target cell should fulfil the following non-exhaustive, but essential criteria: 1) PADI4 SNP variant (SNP-Var) genotype; 2) Restricting HLA-DPB1*04:01 genotype (heterozygous or homozygous); 3) Expression of the *PADI4* gene; 4) Expression of *HLA-DPA1*, *HLA-DPB1* and the associated MHC class II machinery.

the study of PADI4 TCR antileukaemic efficacy were *in vitro* assays using suitable leukaemic cell lines.

For this, an ideal target cell should fulfill the following non-exhaustive criteria, owing to the nature of TCR-mediated antigen recognition: 1) PADI4 variant SNP genotype (SNP-Var); 2) HLA-DPB1*04:01 restricting allele genotype (heterozygous or homozygous); 3) *PADI4* expression; 4) *HLA-DPA1*, *HLA-DPB1* and related MHC class II machinery expression (Fig. 3.6).

Of all available leukaemic/haematopoietic origin cell lines tested (n=21), only one cell line was found to have the PADI4 SNP variant heterozygous genotype. However, this cell line (RS4;11) is of lymphoid lineage, whereas *PADI4* expression is known to have myeloid restriction (Chapter 5). Therefore, the next step was to explore genetic engineering strategies to introduce the PADI4 SNP-Var genotype into a cell line of choice.

PADI4 SNP variant knock-in approach

The first strategy explored was to introduce the PADI4 SNP-Var genotype in a cell line that already expresses *PADI4* and has both the correct HLA-DP allele and expresses MHC class II.

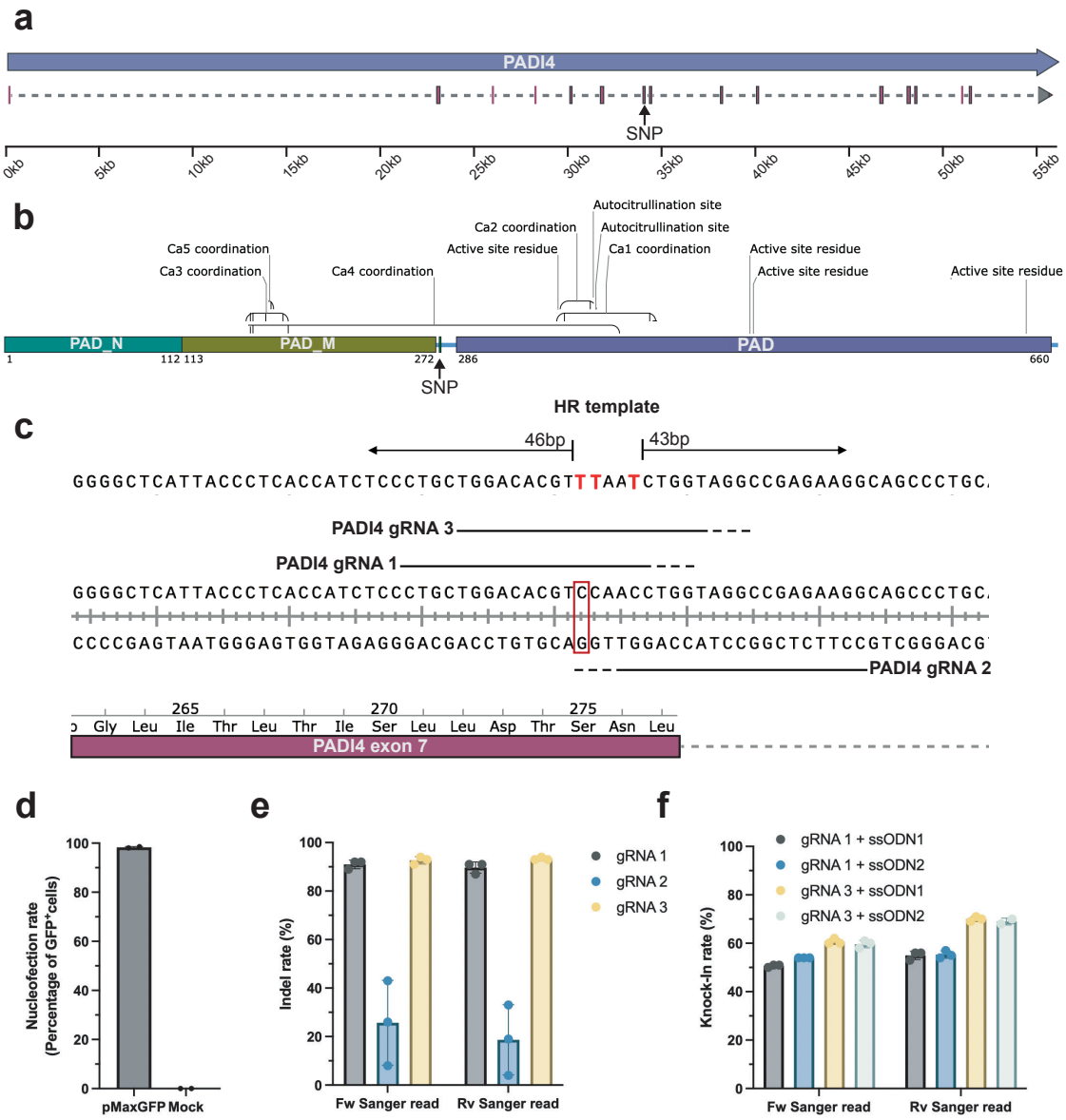


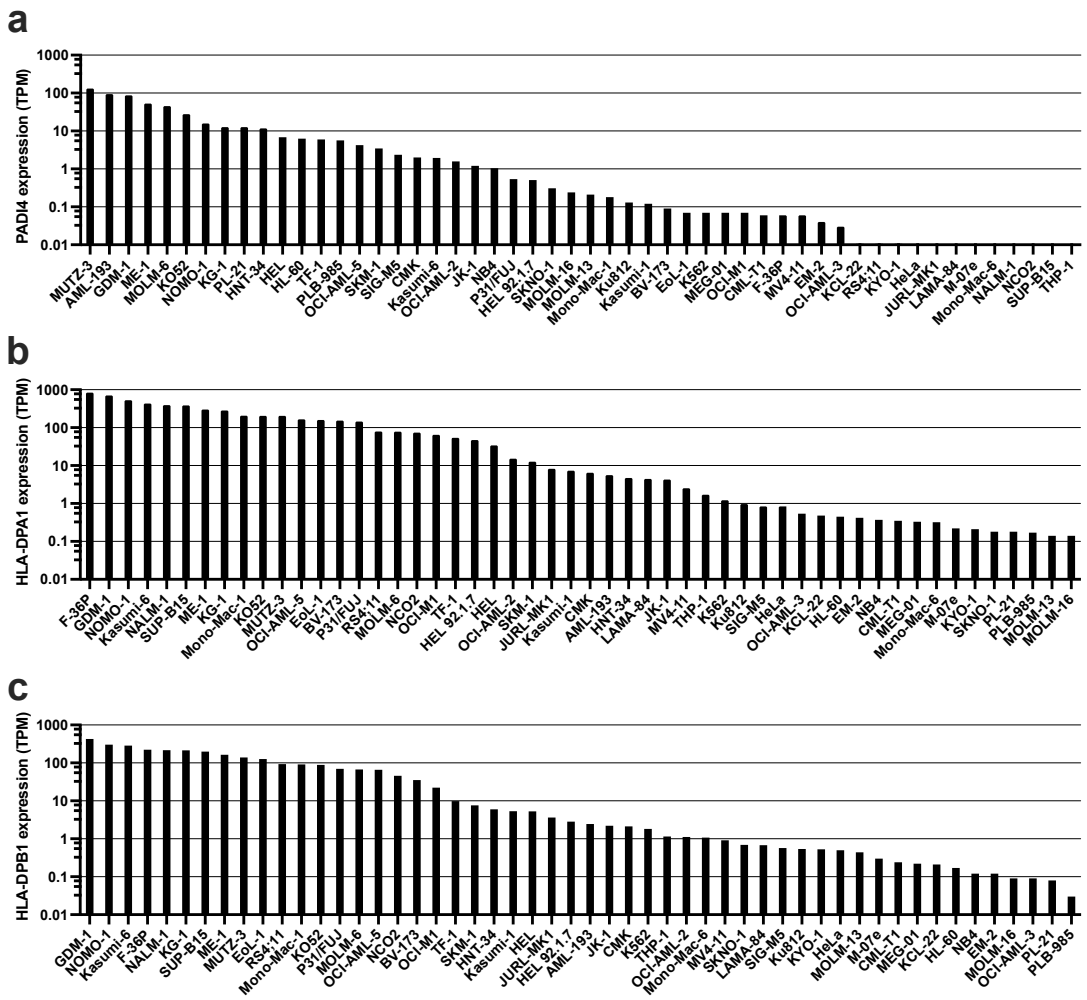
Figure 3.7: A strategy to knock-in the variant SNP genotype into the *PADI4* gene using Cas9 gene editing. **a** Overall structure of the *PADI4* gene, depicting the distribution of introns (dashed grey line) and exons (solid purple bars). The SNP of interest, indicated by an arrow, is located at the end of exon 7. **b** Protein translation of the *PADI4* gene with protein domains and key amino acid residues annotated. SNP of interest lies at the region in between two protein domains, indicated by an arrow. **c** Nucleotide-level view of the *PADI4* exon 7. Red box marks the SNP in its reference sequence. Above and below are three bioinformatically designed guide RNA (gRNA), where dashed line indicates the protospacer adjacent motif (PAM) sequence. On top, the designed single stranded oligodeoxynucleotide (ssODN) sequence (homology donor) is shown, with knock-in sequence highlighted in red. **d** Nucleofection efficiency with a GFP-carrying plasmid in K562 cell line measured after 24 hours of incubation. **e** Predicted gRNA cutting efficiency in K562 cell line 72 hours post-nucleofection with ribonucleoprotein (RNP). **f** Predicted knock-in efficiency in K562 cell line 72 hours post-nucleofection with RNP and ssODN. Predictions of knock-out and knock-in efficiencies were carried out by analysis of forward and reverse Sanger sequencing traces from extracted bulk gDNA.

The SNP of interest lies at the end of *PADI4* exon 7 (Fig. 3.7a-c). It is not known to have any clinical significance on the dbSNP database (NIH NCBI) as well as does not lie in any known protein domains or in/around known important structural/functional amino acids. Thus, it is not expected that introduction of the variant SNP genotype should perturb gene expression or resultant protein function in any way.

To introduce the desired amino acid sequence change, several guide RNA (gRNA) were designed as close as possible to the SNP site, in order to increase editing efficiency[263] (Fig. 3.7c). Because required genetic edits are minor, homologous directed repair (HDR) approach with single stranded oligonucleotide (ssODN) was chosen, which has been previously described to work optimally when donor length is around 90-100bp[263–265]. Previous publications have also described optimal ssODN donor strandedness as well as homology arm symmetry[263–265], however with conflicting results regarding donor strandedness effect on knock-in (KI) efficiency. Thus, HDR template was designed in two versions, different only in their strandedness. Finally, KI design was intended to not only introduce the desired change in amino acid sequence but to also: 1) introduce blocking mutations that could prevent edited sequence re-cutting[263]; 2) allow fast and easy edited cell screening with restriction enzyme MseI, which would specifically cut successfully edited DNA sequence 'TTAA'.

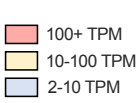
Designed KI strategy was tested in K562 cell line using ribonucleoprotein (RNP) delivery with chemically modified ssODN and gRNA to enhance their stability[266, 267]. Nucleofection method was shown to be highly efficient using a GFP-expressing plasmid and optimal guides were determined by predicted knock-out (KO) efficiency (Fig. 3.7d,e). Finally, most optimal gRNA were co-delivered with different ssODN designs to show high predicted KI efficiency in all tested conditions (Fig. 3.7f).

With the editing strategy established, a suitable cell line had to be selected . Bioinformatic analysis of the publicly available RNA sequencing data on myeloid leukaemia cell lines (Cancer Cell Line Encyclopaedia) showed which candidates have highest expression of *HLA-DPA1*, *HLA-DPB1* as well as *PADI4* (Fig. 3.8a-c). Next, overlap analysis of highest expressing cell lines in all three genes showed which candidates are most suitable to test in-house (Fig. 3.8d,e).



d

	PADI4	HLA-DPA1	HLA-DPB1
1	MUTZ-3	F-36P	GDM-1
2	AML-193	GDM-1	NOMO -1
3	GDM-1	NOMO-1	Kasumi-6
4	ME-1	Kasumi-6	F-36P
5	MOLM -6	NALM-1	NALM-1
6	KO52	*SUP-B15*	KG-1
7	NOMO-1	ME-1	*SUP-B15*
8	KG-1	KG-1	ME-1
9	PL-21	Mono-Mac-1	MUTZ-3
10	HNT-34	KO52	EoL-1
11	HEL	MUTZ-3	*RS4;11*
12	HL-60	OCI-AML-5	Mono-Mac-1
13	TF-1	EoL-1	KO52
14	PLB-985	BV-173	P31/FUJ
15	OCI-AML-5	P31/FUJ	MOLM-6
16	SKM-1	*RS4;11*	OCI-AML-5
17	SIG-M5	MOLM-6	NCO2
18	CMK	NCO2	BV-173
19		OCI-M1	OCI-M1
20		TF-1	TF-1
21		HEL 92.1.7	SKM-1
22		HEL	HNT-34
23		OCI-AML-2	Kasumi-1
24		SKM-1	HEL
25		JURL-MK1	JURL-MK1



e

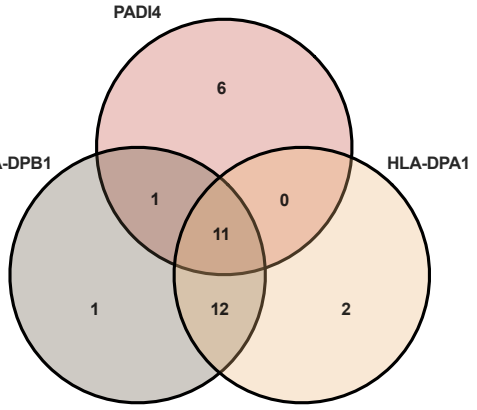


Figure 3.8: (Previous page) Bioinformatic analysis of publicly available cell line RNA sequencing data reveals most suitable myeloid leukaemia cell lines for the knock-in of the variant SNP genotype in *PADI4*. **a**, **b** and **c** Show expression of *PADI4*, *HLA-DPA1* and *HLA-DPB1* genes, respectively. 49 myeloid leukaemia cell lines, 2 B-cell acute lymphoblastic leukaemia (B-ALL) cell lines (SUP-B15 and RS4;11) and 1 cervical cancer cell line (HeLa) are shown and ranked in expression from highest to lowest (left to right). **d** Table of top 25 myeloid leukaemia cell lines expressing *PADI4*, *HLA-DPA1* and *HLA-DPB1* genes. Minimum expression of 2 TPM was used as a cut-off. Colours indicate gene expression level. Cell lines underlined and in bold are B-ALL. **e** Venn diagram showing numbers of cell lines that are top 25 expressors for the indicated genes.

Of the identified most suitable cell lines, only few were available in-house and even fewer carry the correct HLA-DP restricting allele (Fig. 3.9a). The remaining cell lines as well as all other in-house available myeloid cell lines were assessed for HLA-DP and PADI4 expression at protein level (Fig. 3.9b-d). This identified only one available cell line that fulfilled all the criteria to use in the KI approach - GDM1. Unfortunately, this cell line has proven difficult to expand in culture. After extensive effort and attempted culture from three separate sources, alternative strategies for the development of a target cell line model were explored.

PADI4 overexpression approach

An alternate strategy for target cell line development is to lentivirally-transfer a full length *PADI4* gene transcript with the desired SNP genotype and an overexpression promoter (Fig. 3.10a). Hence, this approach only requires a cell line that has the restricting HLA-DP allele and expresses MHC class II.

Since *PADI4* gene encodes for a functional arginine deiminase enzyme, its over-expression could result in toxicity. Nevertheless, transient transfection of HEK 293T cells with the lentiviral transfer plasmid did not result in increased cell death, despite successful transcript expression and PADI4 production (Fig. 3.10b,c).

Next, all available leukaemic/haematopoietic cell lines (that had not been previously screened) were assessed for their HLA-DP expression and allele (Fig. 3.11). Since the overexpression approach is not restricted to the myeloid lineage candidates (as *PADI4* expression is typically myeloid-restricted), far more HLA-DP expressing cell lines were available to choose from. In the end, four cell lines were selected to take forward, based

a

	Cell Lines	Cell Line Availability	HLA-DPB1* 04:01
1	MUTZ-3	No	
2	GDM-1	Yes	+/-
3	ME-1	No	
4	MOLM-6	No	
5	KO52	No	
6	NOMO-1	Yes	-/-
7	KG-1	Yes	-/-
8	HNT-34	No	
9	HEL	Yes	+/-
10	TF-1	No	
11	OCI-AML-5	No	
12	SKM-1	No	

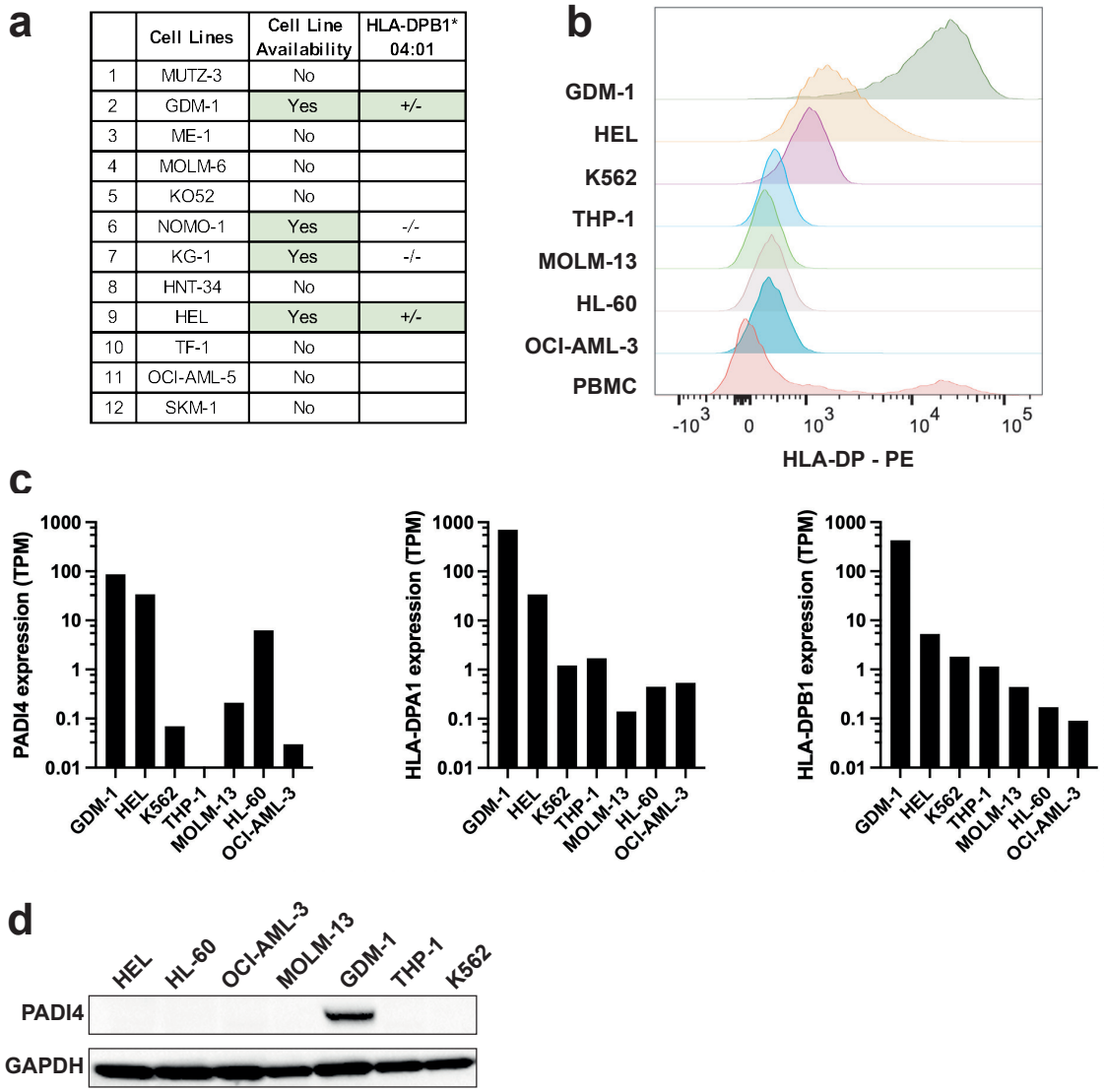


Figure 3.9: GDM-1 is the only available and suitable cell line for the *PADI4* variant SNP genotype knock-in strategy. a Table showing 12 myeloid leukaemia cell lines with highest expression of *PADI4* and *HLA-DPB1* and/or *HLA-DPA1* (as shown in 3.8e). It indicates which lines are available in-house and if so, whether they carry the required HLA-DPB1*04:01 allele. **b** All myeloid leukaemia cell lines available in-house were stained for HLA-DP expression by cytometry. PBMCs were used as a reference, as it contains T cells (mostly HLA-DP negative), B cells (mostly HLA-DP positive). **c** Shows Cancer Cell Line Encyclopaedia (CCLE) RNA expression data for *PADI4*, *HLA-DPA1* and *HLA-DPB1* in myeloid leukaemia cell lines available in-house. **d** Western blot for PADI4 on myeloid leukaemia cell lines available in-house.

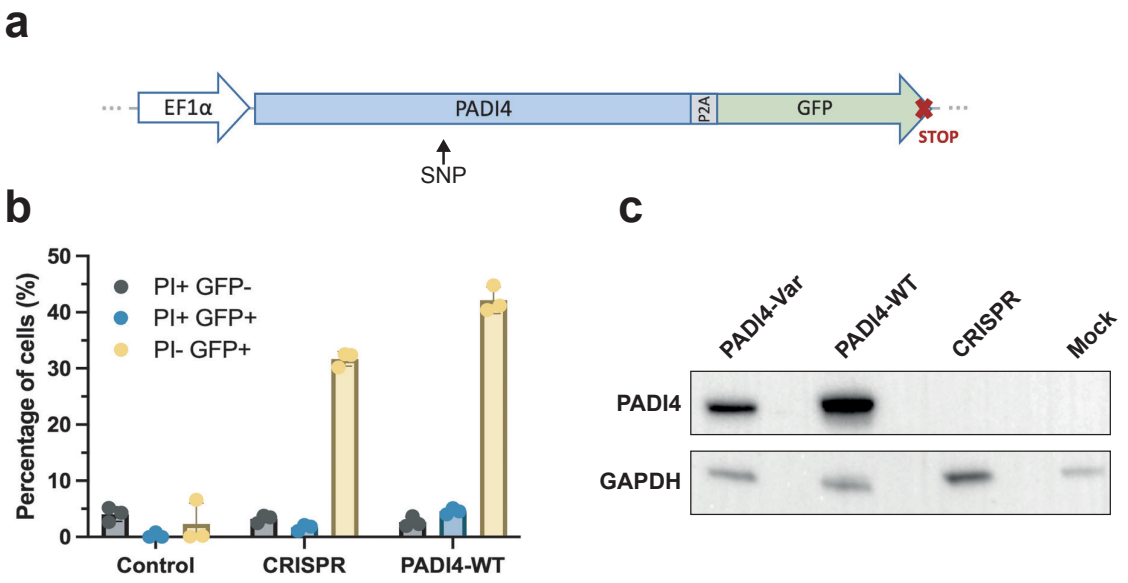


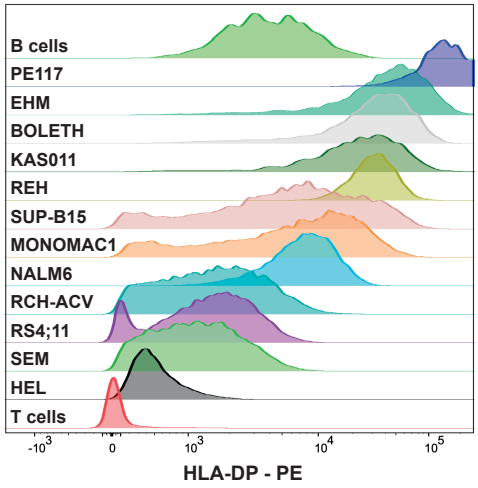
Figure 3.10: PADI4 overexpression provides an alternative to knock-in strategy. a Lentiviral PADI4 overexpression vector insert map. SNP location is indicated with an arrow. **b** Graph showing HEK 293T transfection efficiency (GFP^+) and staining with propidium iodide (PI) 48 hours after adding the lentiviral transfer plasmid. **c** PADI4 western blot on HEK 293T cells 48 hours post-transfection with the indicated lentiviral transfer plasmids.

on their lineage, HLA-DP allele and expression properties: 1) HEL, a myeloid leukaemia cell line with low expression of the correct restricting HLA-DPB1 allele; 2) MONOMAC1, a myeloid leukaemia cell line with high expression of the correct restricting HLA-DPB1 allele; 3) BOLETH, a professional antigen presenting cell (APC) line with high expression of the correct restricting HLA-DPB1 allele ; 4) SUP-B15, a lymphoid leukaemia cell line with high expression of the incorrect HLA-DPB1 allele.

To begin with, the lentiviral transduction protocol was optimised for all four cell lines separately (Appendix Fig. 9). Each had a different capacity for transduction, with BOLETH by far being the most difficult to transduce (Appendix Fig. 9a-d). Furthermore, post-transduction cell viability was different for each cell line, with SUP-B15 being the most sensitive to increasing viral supernatant doses (Appendix Fig. 9e-h). In the end, the most efficient as well as tolerable transduction conditions were chosen for each cell line separately.

Subsequently, cell lines were successfully transduced with vectors carrying *PADI4* either with the wild-type (PADI4-WT) or the variant SNP (PADI4-Var). Transduced populations (GFP^+) were purified by sorting. Successful delivery and function of the

a



b

	Cell Lines	Cell Line Identity	Cell Line Availability	HLA-DPB1* 04:01
1	PE117	BLCL	Non viable	+/+
2	EHM	BLCL	Mycoplasma positive	+/-
3	BOLETH	BLCL	Yes	+/+
4	KAS011	BLCL	Non viable	+/-
5	REH	B-ALL	Yes	+/-
6	SUP-B15	B-ALL	Yes	-/-
7	MONOMAC1	AML	Yes	+/-
8	NALM6	B-ALL	Yes	+/+
9	RCH-ACV	B-ALL	Yes	+/+
10	RS4;11	B-ALL	Yes	+/-
11	SEM	B-ALL	Yes	-/-
12	HEL	AML	Yes	+/-

c

	Cell Line Identity	HLA-DP allele	HLA-DP expression
HEL	AML	Correct	Low
MONOMAC1	AML	Correct	High
BOLETH	BLCL	Correct	Very high
SUP-B15	B-ALL	Incorrect	High

Figure 3.11: Cell line screening for the HLA-DP genotype and expression identifies several candidates for PADI4 overexpression. **a** Cell lines available in-house were stained for HLA-DP expression by cytometry. **b** Table showing cell lines available in-house, their identity and HLA-DPB1 allele. **c** Summary table of the cell lines selected for *PADI4* overexpression strategy.

construct was confirmed with PADI4 Western blot (Fig. 3.12a). Expectedly, different cell line capacities for transduction were correlated with different PADI4 protein band intensities post-transduction. Furthermore, as the overexpression construct linked PADI4 and GFP expression with a self-cleaving P2A peptide, PADI4 expression could be enumerated by comparing relative GFP MFI for each cell line (Fig. 3.12b). This showed that the HEL cell line by far had the highest level of overexpression compared to the other three cell lines studied.

Of note, PADI4-WT transduction at GFP and PADI4 protein level appeared higher than PADI4-Var (Fig. 3.12a,b). This may indicate that the SNP-Var genotype affects either the gene expression or the protein half-life. However, the multiplicity of infection (MOI) at the time of transduction was not measured. Therefore, it is also possible that this difference is due to unequal viral preparation qualities. To investigate this, further analysis of viral integration sites would be required.

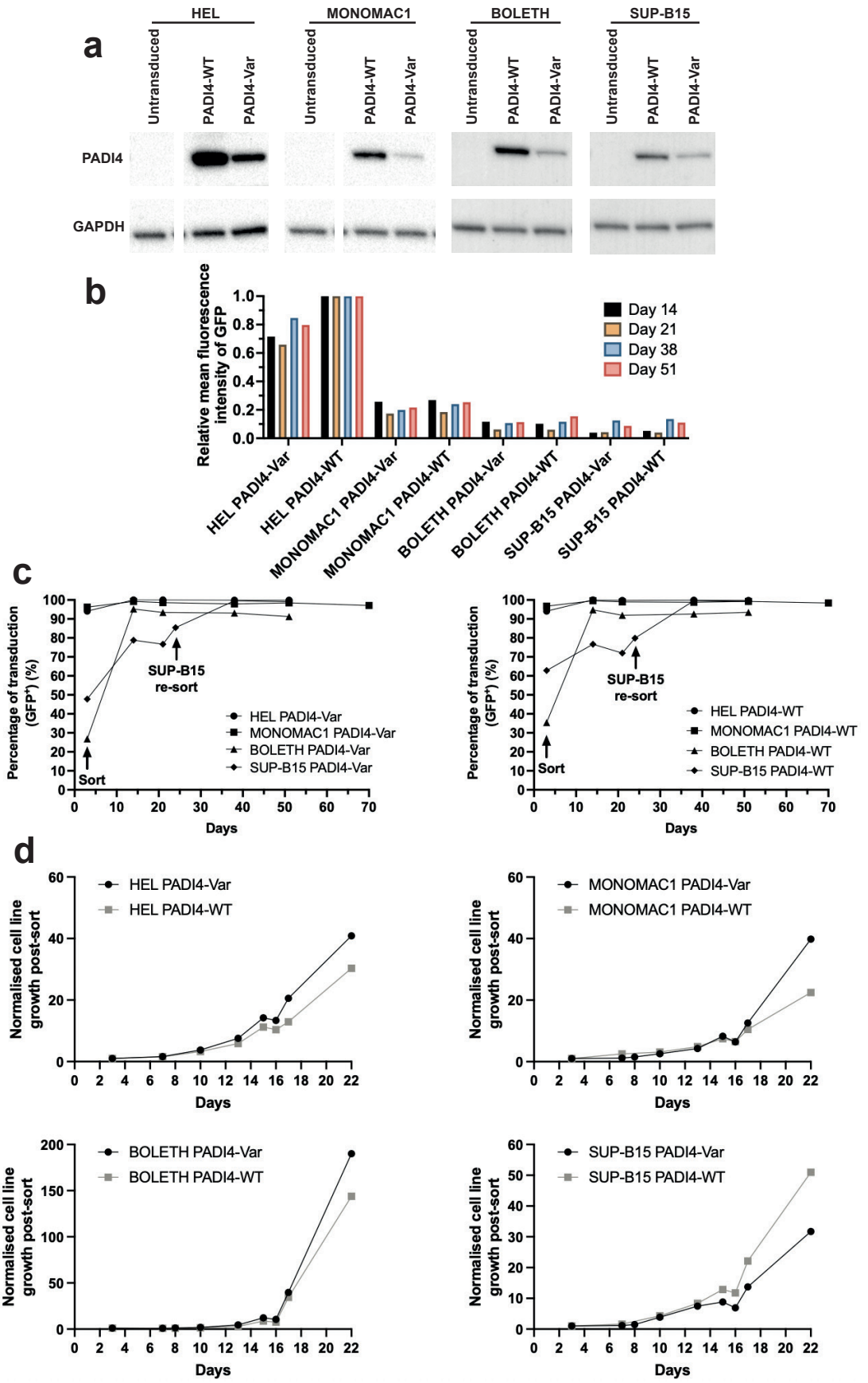

a
HEL
MONOMAC1
BOLETH
SUP-B15
Untransduced
PADI4-WT
PADI4-Var
PADI4
GAPDH
b
Relative mean fluorescence intensity of GFP
Day 14
Day 21
Day 38
Day 51
HEL PADI4-Var
HEL PADI4-WT
MONOMAC1 PADI4-Var
MONOMAC1 PADI4-WT
BOLETH PADI4-Var
BOLETH PADI4-WT
SUP-B15 PADI4-Var
SUP-B15 PADI4-WT
c
Percentage of transduction (GFP+) (%)
SUP-B15 re-sort
Sort
Days
d
Normalised cell line growth post-sort
Days

Figure 3.12: (Previous page) Cell line transduction with PADI4-WT-GFP and PADI4-Var-GFP lentiviral constructs. a PADI4 western blot on transduced cell lines at day 16 post-transduction. **b** Relative mean fluorescence intensity (MFI) of GFP in transduced cell lines, normalised for each timepoint to the highest MFI value across cell lines. **c** Percentage of GFP^+ cells in culture over time in transduced cell lines, plotted separately by lentiviral construct. **d** Growth of transduced cell lines over time, normalised to starting cell number for each condition and plotted separately by cell line.

Finally, PADI4 overexpression was stable over time and did not abolish either cell line's ability to expand (Fig. 3.12b-d). With the exception of SUP-B15, one purification sort post-transduction was sufficient to maintain high levels of transduction over time. For SUP-B15, second purification sort was required to achieve the same.

3.4 PADI4 TCR Testing in Overexpression Cell Line Models

PADI4 overexpression in a professional APC is sufficient for PADI4 TCR T cell recognition

Once PADI4 overexpression cell lines were produced, primary T cells were transduced with the PADI4 TCR and expanded. After an overnight rest in cytokine-free media, T cells were placed in co-culture with overexpression cell lines to investigate T cell activation. To ensure any activation is antigen and TCR specific, two different controls were used: 1) T cell co-culture with PADI4 SNP-WT overexpressing cell lines; 2) Target cell line co-culture with irrelevant TCR (1G4) transferred T cells (Fig. 3.13). BOLETH, an Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell line (BLCL), activated PADI4 TCR T cells when transduced with the PADI4 SNP-Var overexpression construct. The PADI4-WT overexpression and irrelevant TCR controls indicated that PADI4 TCR response is indeed antigen as well as TCR specific. This result showed, for the first time, that the PADI4 variant peptide can be produced from a full length PADI4 protein and successfully presented on the HLA-DP for TCR-mediated T cell recognition.

Expectedly, neither HEL nor SUP-B15 cell line were able to activate PADI4 TCR T cells, owing to low HLA-DP expression levels and incorrect restricting HLA-DP allele, respectively.

However, MONOMAC1, a myeloid leukaemia cell line, did not elicit PADI4 TCR T cell activation, despite its favourable HLA-DP expression and genotype characteristics.

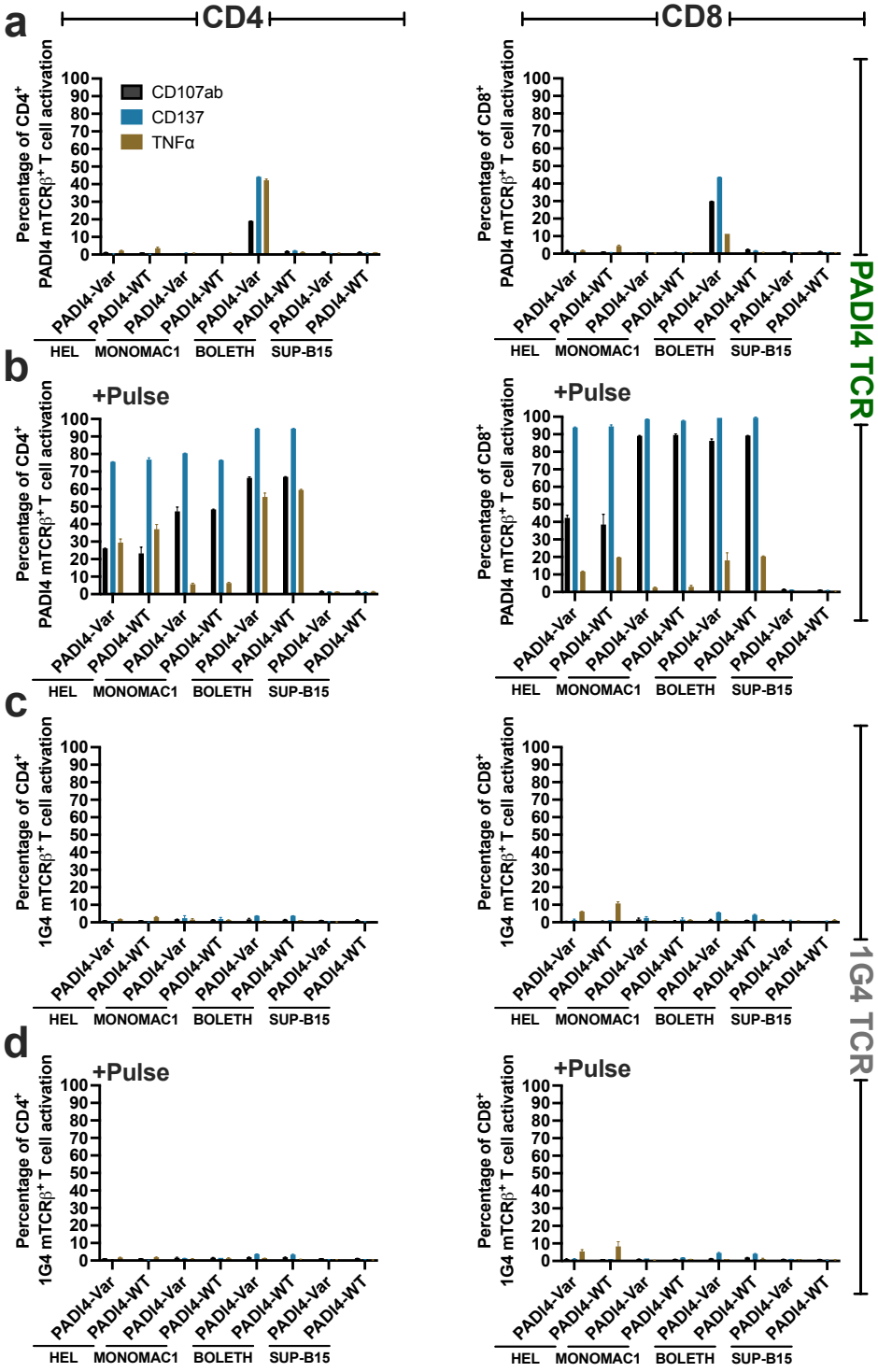


Figure 3.13: PADI4 TCR T cells are activated by PADI4-transduced BOLETH in an antigen-specific manner after 24 hours of co-culture. Panels **a** and **b** show T cells transferred with PADI4 TCR, while panels **c** and **d** show T cells transferred with irrelevant 1G4 TCR. Columns show $CD4^+$ (left) and $CD8^+$ (right) T cells. In panels **c** and **d**, target cell lines were pulsed with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates. Similar activation with BOLETH cell line has been observed in 2 additional independent experiments (data not shown).

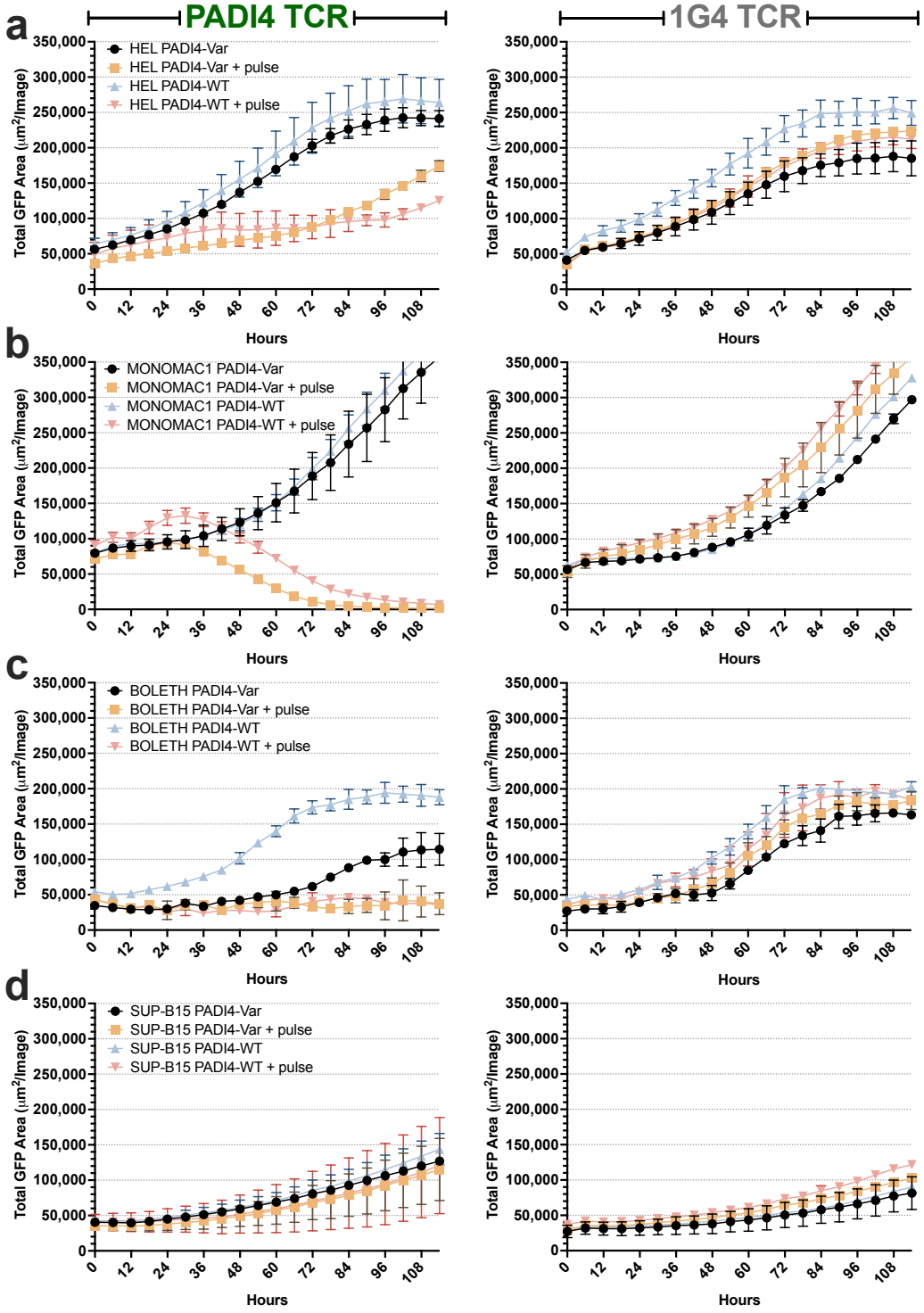


Figure 3.14: PADI4 TCR T cells can inhibit PADI4-transduced cell line growth in an antigen-specific manner when cells are mixed in 1:1 ratio. Panels **a**, **b**, **c** and **d** show HEL, MONOMAC1, BOLETH, and SUP-B15, respectively. Growth over time is measured by calculating the total GFP^+ area at the bottom of a cell culture dish using a live cell imaging system. Columns distinguish between target cell line co-culture with T cells carrying different constructs: PADI4 TCR (left) and irrelevant 1G4 TCR (right). 'Pulse' indicates that target cells were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Graphs show one experiment and error bars represent standard deviation between technical replicates.

This finding suggests an unknown biological difference between MONOMAC1 and a professional APC BOLETH. For one, it may be that the antigen processing machinery in MONOMAC1 is different. Alternatively, the cell line could have different surface receptor/ligand constitution, which ultimately prevents PADI4 TCR T cell activation.

Antigen-specific T cell activation to BOLETH PADI4-Var was further characterised using a co-culture time course(Appendix Fig. 10). It measured transduced T cell expression of CD107a & CD107b (co-stain), CD137 and TNFα after 9, 24 or 48 hours of co-culture. This suggested that the most optimal T cell activation was at 24 hours of co-culture, where both $CD4^+$ and $CD8^+$ TCR-transduced T cells were $>$10% positive for most of the assayed markers. At 9 hours, activation is low ($<$10%), while at 48 hours, the peak of the marker expression had passed. In addition, the background positivity for CD137 was increased. These activation dynamics were in line with CD107a, CD137 and TNFα being previously described as early activation markers[268–270].

Observed PADI4 TCR T cell activation closely correlated with overexpression cell line growth inhibition when in co-culture with antigen-specific T cells (Fig. 3.14). Where target cell lines with the restricting HLA-DP allele were pulsed with the variant PADI4 peptide, co-culture with PADI4 TCR T cells resulted in marked growth inhibition. Only HEL, a cell line with low HLA-DP expression, showed partial recovery from this during the period of the assay. Importantly, even at 1:1 effector-to-target ratio, mild growth inhibition of BOLETH PADI4-Var cell line was seen when compared to BOLETH PADI4-WT or non-specific TCR conditions. This was also observed at higher effector-to-target ratios (Appendix Fig. 11), however, in these assays, the dynamic range was decreased due to the lower starting concentrations of target cells (see reduced y-axis scale).

PADI4 TCR T cell recognition of myeloid leukaemia additionally requires target cell IFNγ pre-treatment

Next, I further investigated the conditions that could facilitate PADI4 TCR T cell activation to MONOMAC1 PADI4-Var cell line. As mentioned previously, a possible explanation for the initial negative result could be inadequate antigen processing machinery activity, leading to insufficient or completely absent variant peptide presentation on HLA-DP. Indeed, multiple types of cancers have been described to modulate MHC class II

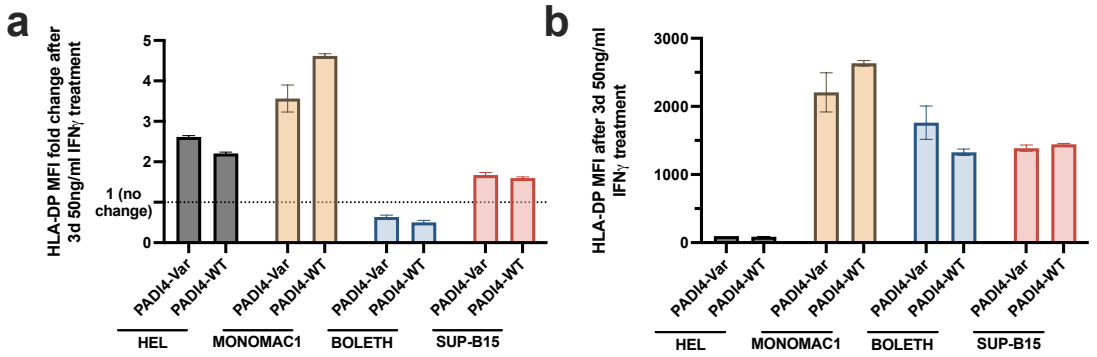


Figure 3.15: PADI4-transduced target cell lines change their HLA-DP expression upon 3 days of 50ng/ml IFNγ treatment. Cell lines were treated with IFNγ following the Christopher et al.[125] protocol. **a** Relative change in HLA-DP MFI at the end of IFNγ treatment for each cell line. **b** Absolute HLA-DP MFI at the end of IFNγ treatment for each cell line. Graphs show one experiment and error bars represent standard deviation between technical replicates.

antigen processing machinery[271], including acute myeloid leukaemia[272] (see Chapter 1). Meanwhile, it is known that IFNγ can act to upregulate MHC class II machinery, both in healthy cells[273] as well as cancers[271]. More specifically, this has also been shown in primary acute myeloid leukaemias[125, 274] and myeloid leukaemia cell lines [274].

Thus, MONOMAC1 cell line was IFNγ treated following a previously published protocol (3 day incubation at 50ng/ml) [125] to assess if this can increase MHC class II expression and, in turn, activate PADI4 TCR T cells. Indeed, MONOMAC1 was highly responsive to this treatment, showing $\sim$3.5-4.5 fold increase in HLA-DP expression at 72 hours (Fig. 3.15). However, PADI4 TCR T cell activation was still very low, if not completely absent (Fig. 3.16).

This activation assay result, combined with the high MONOMAC1 sensitivity to IFNγ treatment, prompted to further investigate if the IFNγ treatment protocol could be optimised for higher PADI4 TCR T cell activation. Therefore, the next experiment investigated HLA-DP expression dynamics in IFNγ treated cell lines over time (Fig. 3.17a,b). Indeed, in all cell lines except BOLETH, the highest HLA-DP expression was observed with maximal length of IFNγ treatment (6 days). In this experiment, HEL, MONOMAC1, BOLETH and SUB-B15 HLA-DP expression saw $\sim$3-4, $\sim$10-11, $\sim$0.6 and $\sim$3 fold change at 6 days, respectively. Arguably, HLA-DP expression in response to IFNγ

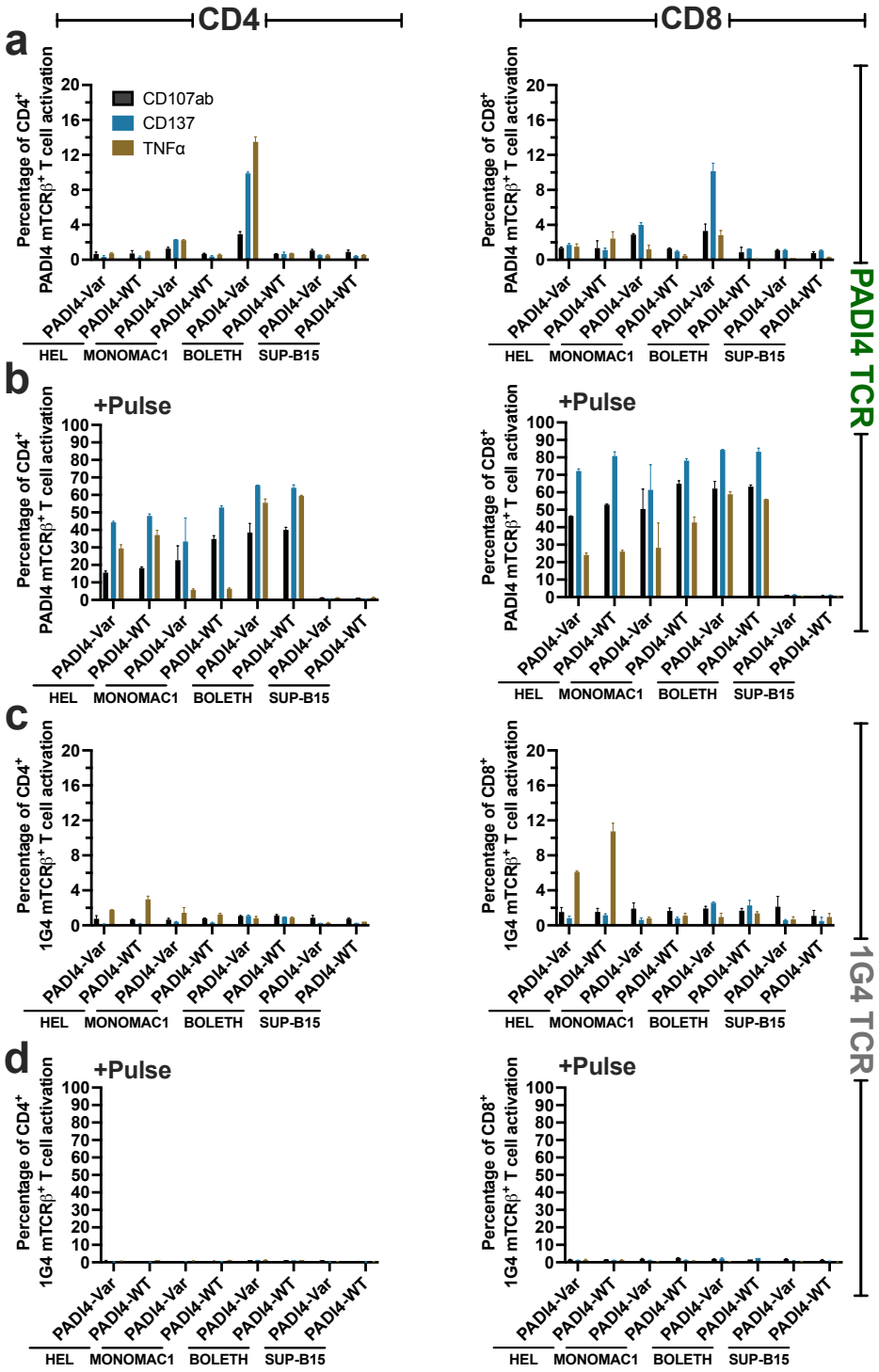


Figure 3.16: PADI4 TCR T cell response to IFNγ pre-treated PADI4-transduced cell lines after 9 hours of co-culture. All cell lines were pre-treated with 50ng/ml IFNγ for 3 days prior to the co-culture with T cells. Panels **a** and **b** show T cells transferred with PADI4 TCR, while panels **c** and **d** show T cells transferred with irrelevant 1G4 TCR. Columns show $CD4^+$ (left) and $CD8^+$ (right) T cells. In panels **b** and **d**, target cell lines were pulsed with PADI4 variant peptides 21 and 22 (1μM) prior to the co-culture with T cells. Cells are mixed 1:1 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

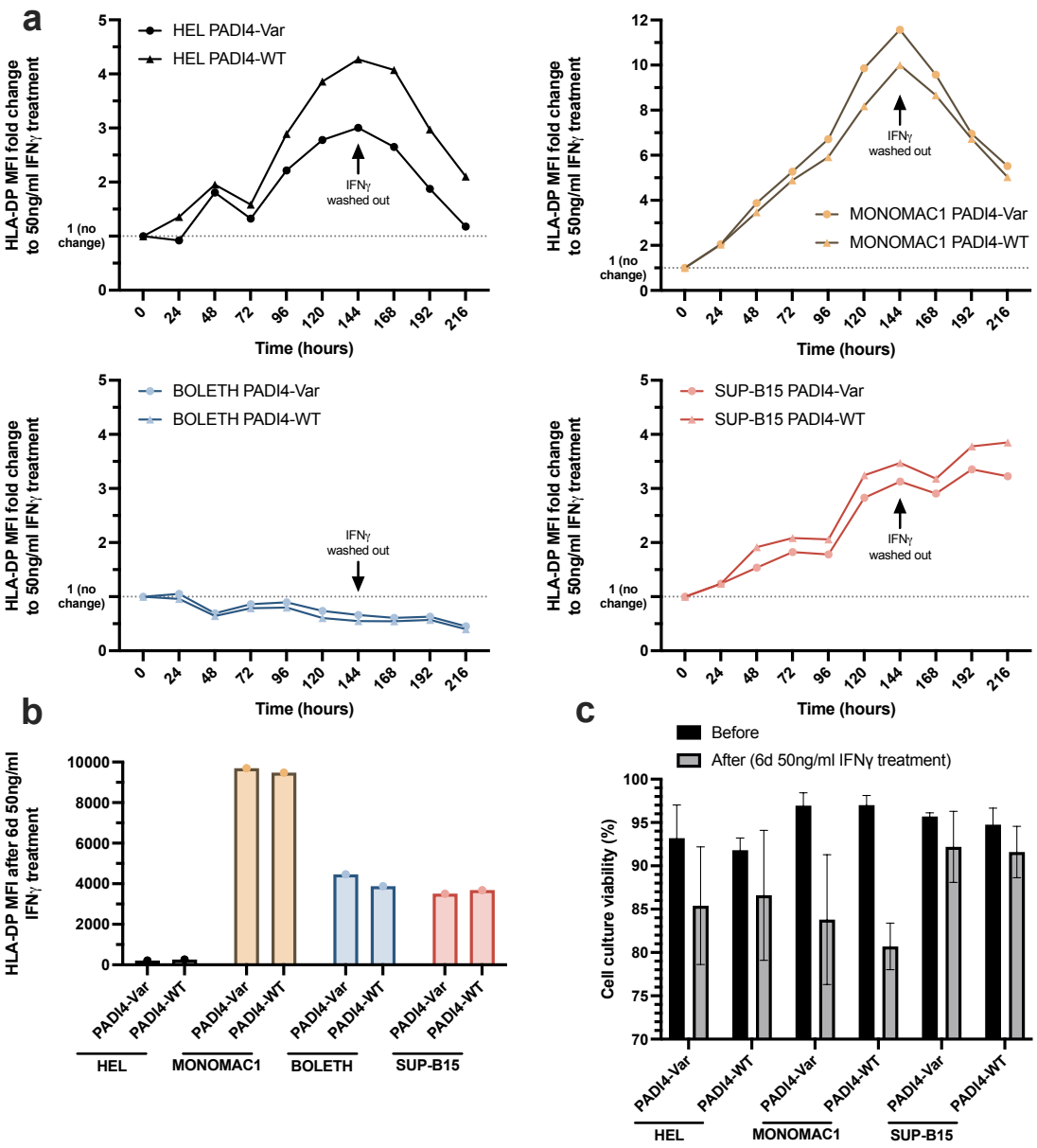


Figure 3.17: Different PADI4-transduced cell lines have different response dynamics to IFNγ treatment. All cell lines were treated with 50ng/ml IFNγ for 6 days (144 hours) and then the cytokine was washed out from culture. **a** Relative change in HLA-DP MFI throughout the IFNγ treatment for each cell line. **b** Absolute HLA-DP MFI at the end of the IFNγ treatment for each cell line. **c** Cell viability at the start and end of the IFNγ treatment for each cell line. Graphs show one experiment and error bars represent standard deviation between technical replicates.

did not reach a plateau for MONOMAC1, suggesting that incubation may be improved even further. However, even with the 6-day treatment, notable cell viability decrease was observed (Fig. 3.17c), limiting the usefulness of further incubation with the cytokine.

Notably, for BOLETH, the treatment did not upregulate HLA-DP expression at any point, perhaps owing to the unique biology of an EBV-transformed BLCL line. Because of this finding as well as prevously seen convincing BOLETH PADI4-Var PADI4 TCR T cell activation without any pre-treatment, BOLETH was excluded from further IFNγ treatment experiments.

Using the improved 6-day IFNγ pre-treatment protocol, convincing PADI4 TCR T cell activation to MONOMAC1 PADI4-Var was finally seen (Fig. 3.18). Similarly to BOLETH, the response was both antigen and TCR specific. Moreover, a time course for T cell activation also showed a peak at 24 hours co-culture (Appendix Fig. 12).

However, when growth inhibition experiment was attempted under these conditions, MONOMAC1 showed complete growth non-recovery (Fig. 3.19). This indicated that the previously seen loss in cell culture viability (Fig. 3.17c), perhaps combined with the non-specific toxicity of the *in vitro* cultured T lymphocyte incubation, lead to a level of toxicity that prevented the observation of any potential growth inhibition effect specifically from the PADI4 TCR.

To summarise, MONOMAC1 can activate PADI4 TCR T cells in an antigen-specific way. This finding showed, for the first time, that the PADI4 variant peptide antigen can be presented by myeloid leukaemia cells themselves and lead to direct antigen-specific T cell activation. However, in order to achieve that, the target cell line requires a long, high-dose IFNγ pre-treatment that disturbs cell viability and growth capacity. Indeed, other studies, where IFNγ was used to upregulate MHC Class II, often opted for shorter and lower concentration IFNγ treatments[275–278]. More so, in other cases, similar or even lower IFNγ doses to the one used here were employed to induce apoptosis in cancer cell lines[279–282]. This raises a question: how does the IFNγ pre-treatment facilitate activation of PADI4 TCR T cells against MONOMAC1 PADI4-Var?

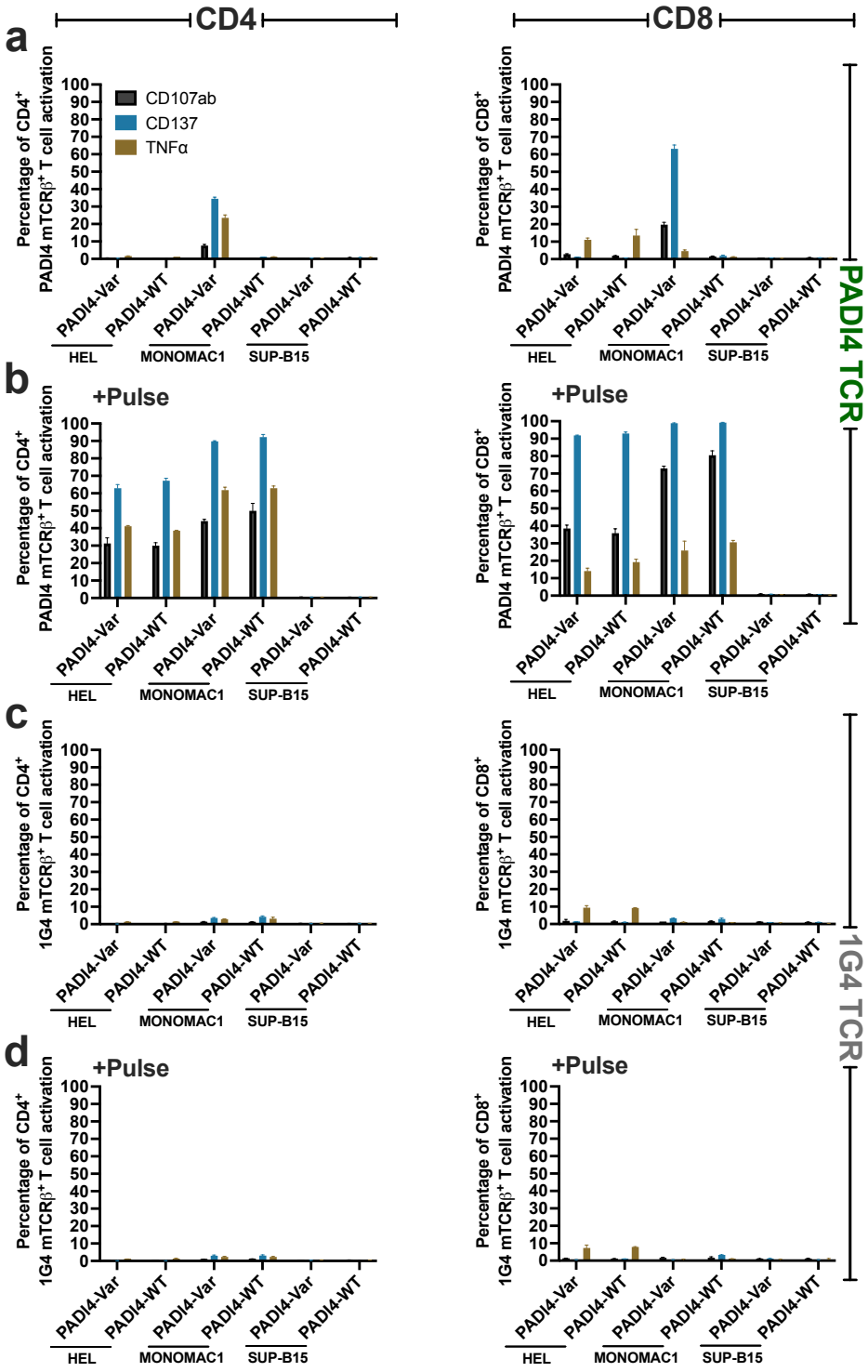


Figure 3.18: PADI4 TCR T cells are activated by IFNγ-treated PADI4-transduced MONOMAC1 cell line in an antigen-specific manner after 24 hours of co-culture. Cell lines were treated with 50ng/ml IFNγ for 6 days prior to T cell co-culture. Panels **a** and **b** show T cells transferred with PADI4 TCR, while panels **c** and **d** show T cells transferred with irrelevant 1G4 TCR. Columns show $CD4^+$ (left) and $CD8^+$ (right) T cells. In panels **b** and **d**, target cell lines were pulsed with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

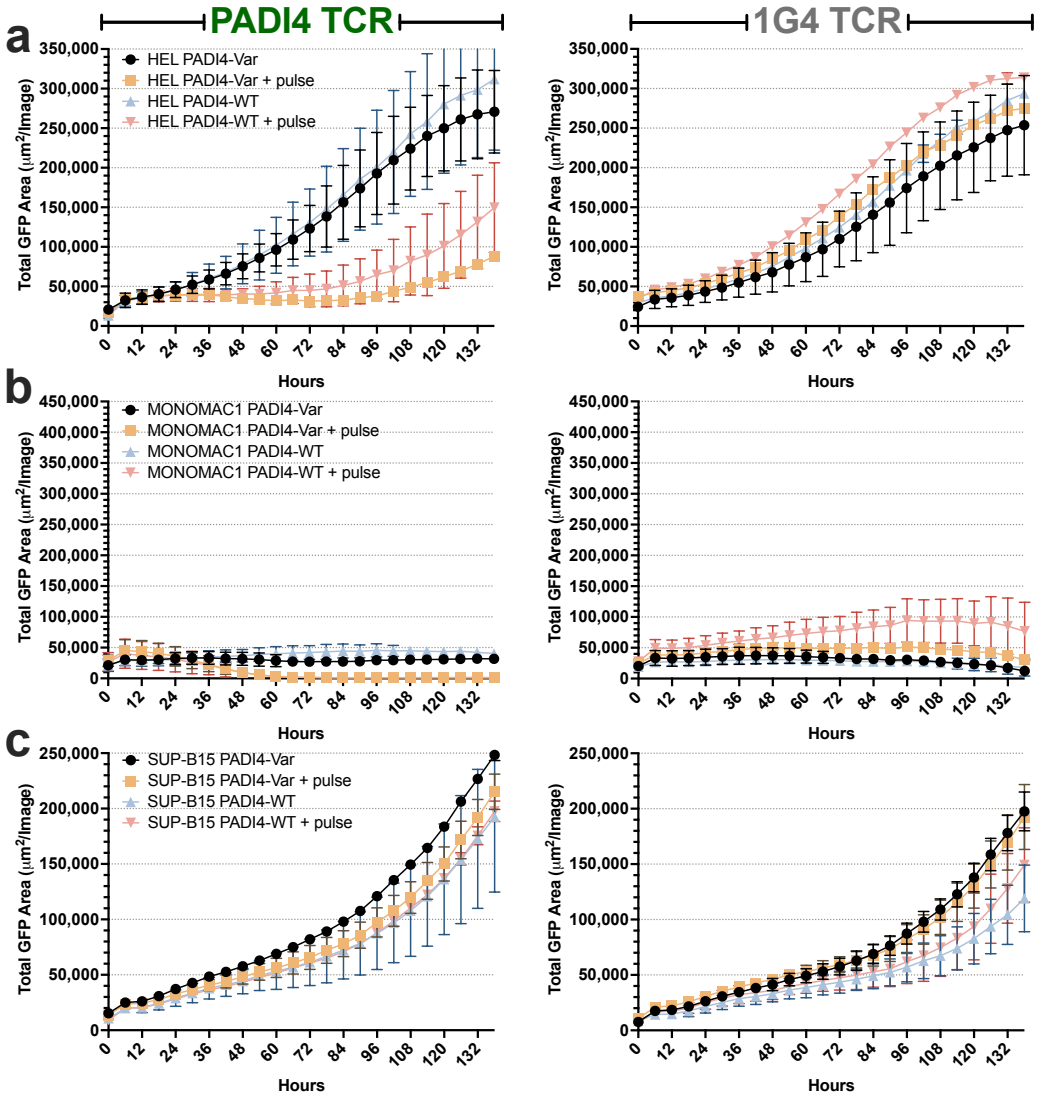


Figure 3.19: IFNγ-treated PADI4-transduced MONOMAC1 does not grow in co-culture with TCR-transduced T cells mixed in 1:1 ratio. Cell lines were treated with 50ng/ml IFNγ for 6 days prior to T cell co-culture. Panels **a**, **b**, and **c** show HEL, MONOMAC1 and SUP-B15, respectively. Growth over time is measured by calculating the total GFP$^+$ area at the bottom of a cell culture dish using a live cell imaging system. Columns distinguish between target cell line co-culture with T cells carrying different constructs: PADI4 TCR (left) and irrelevant 1G4 TCR (right). 'Pulse' indicates that the target cells were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Graphs show one experiment and error bars represent standard deviation between technical replicates.

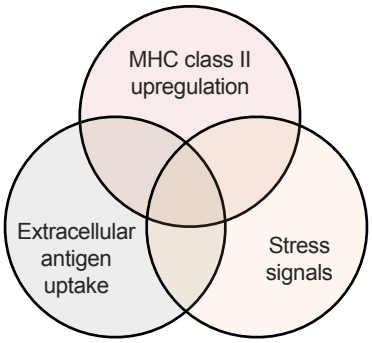


Figure 3.20: IFNγ pre-treatment may facilitate PADI4 TCR T cell recognition of MONOMAC1 PADI4-Var through several different mechanisms or their combinations.

IFNγ pre-treatment enables PADI4 TCR T cell recognition through an unknown mechanism

Although IFNγ pre-treatment successfully enabled MONOMAC1 PADI4-Var to activate PADI4 TCR T cells, its required length and dosage significantly affected target cell viability. Hence, even though IFNγ treatment was initially intended to improve MHC class II expression, two alternative hypotheses emerged that could explain why IFNγ treatment actually worked.

Firstly, knowing that the canonical MHC class II antigen presentation pathway presents extracellularly-derived antigen, the IFNγ treatment of MONOMAC1 may have worked because it induced cell death. Indeed, cell debris could have provided sufficient extracellular antigen for the surviving cells to take it up and subsequently present it on HLA-DP (see Chapter 1). To support this, MONOMAC1 cell line has been previously described to possess phagocytic activity[283]. If that is the case, MONOMAC1 cell line induced PADI4 TCR T cell activation could be dependent on extracellular antigen availability.

Secondly, IFNγ-induced cell stress could have allowed antigen-specific T cell activation via some other, stress-dependent mechanism. For example, IFNγ is known to activate autophagy during infection[284, 285], a process that permits intracellular protein trafficking to the MHC class II antigen presentation pathway[286–288]. If this mechanism is insufficiently active at baseline and is then increased upon high-dose IFNγ treatment, it could explain why MONOMAC1 PADI4-Var induced activation is only seen when the cells are sufficiently 'stressed'. Another example of a stress-dependent response

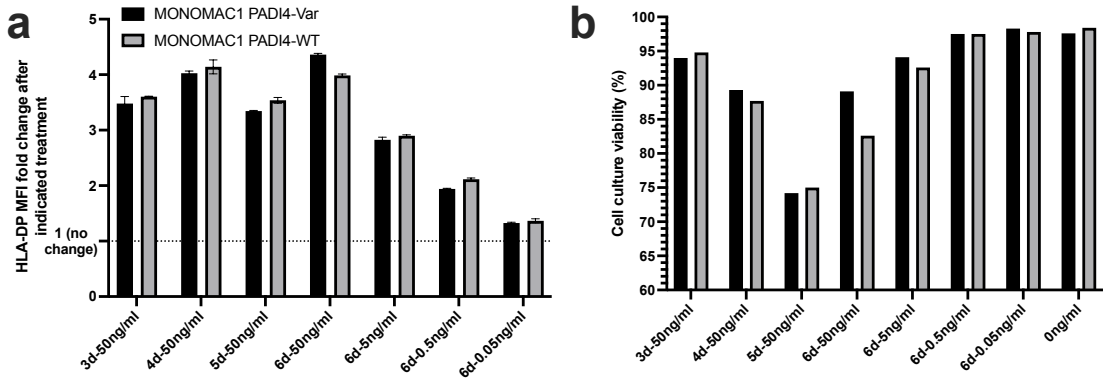


Figure 3.21: PADI4-transduced MONOMAC1 HLA-DP expression and viability is dependent on length and concentration of IFNγ treatment. a Relative change in HLA-DP MFI, and **b** cell viability of MONOMAC1 with different IFNγ treatment regimens. Graphs show one experiment and error bars represent standard deviation between technical replicates.

could be an IFNγ-dependent change in presented immune receptors/ligands[289, 290] or paracrine signals[291]. These changes could ultimately satisfy 'signal 2 and/or 3' for successful T cell activation in presence of the required peptide-MHC (pMHC) complex ('signal 1') (see Chapter 1).

To summarise, IFNγ could allow MONOMAC1 PADI4-Var-induced T cell activation via any of the three described processes or indeed their combination (Fig. 3.20). Hence, to begin with, it was first important to determine whether it is possible to observe antigen-specific T cell activation in absence of IFNγ pre-treatment induced loss of MONOMAC1 cell viability.

To address this, an experiment was designed to titrate different doses and lengths of IFNγ pre-treatment by reading out target cell line viability, HLA-DP expression and antigen-specific T cell activation to MONOMAC1 PADI4-Var. In this experiment, MONOMAC1 HLA-DP expression increased proportionally with the IFNγ concentration to about $\sim$3-4 fold increase at the maximum dose of 50 ng/ml. However, the length of the IFNγ pre-treatment did not correlate with the increased HLA-DP expression, with both 3-day and 6-day treatments showing similar $\sim$3-4 fold increase in the surface HLA-DP expression (Fig. 3.21). This result contradicts what was seen previously (Fig. 3.17a). Meanwhile, cell viability was only affected in highest doses of 5 ng/ml and especially 50 ng/ml. In addition, viability correlated with the length of the IFNγ

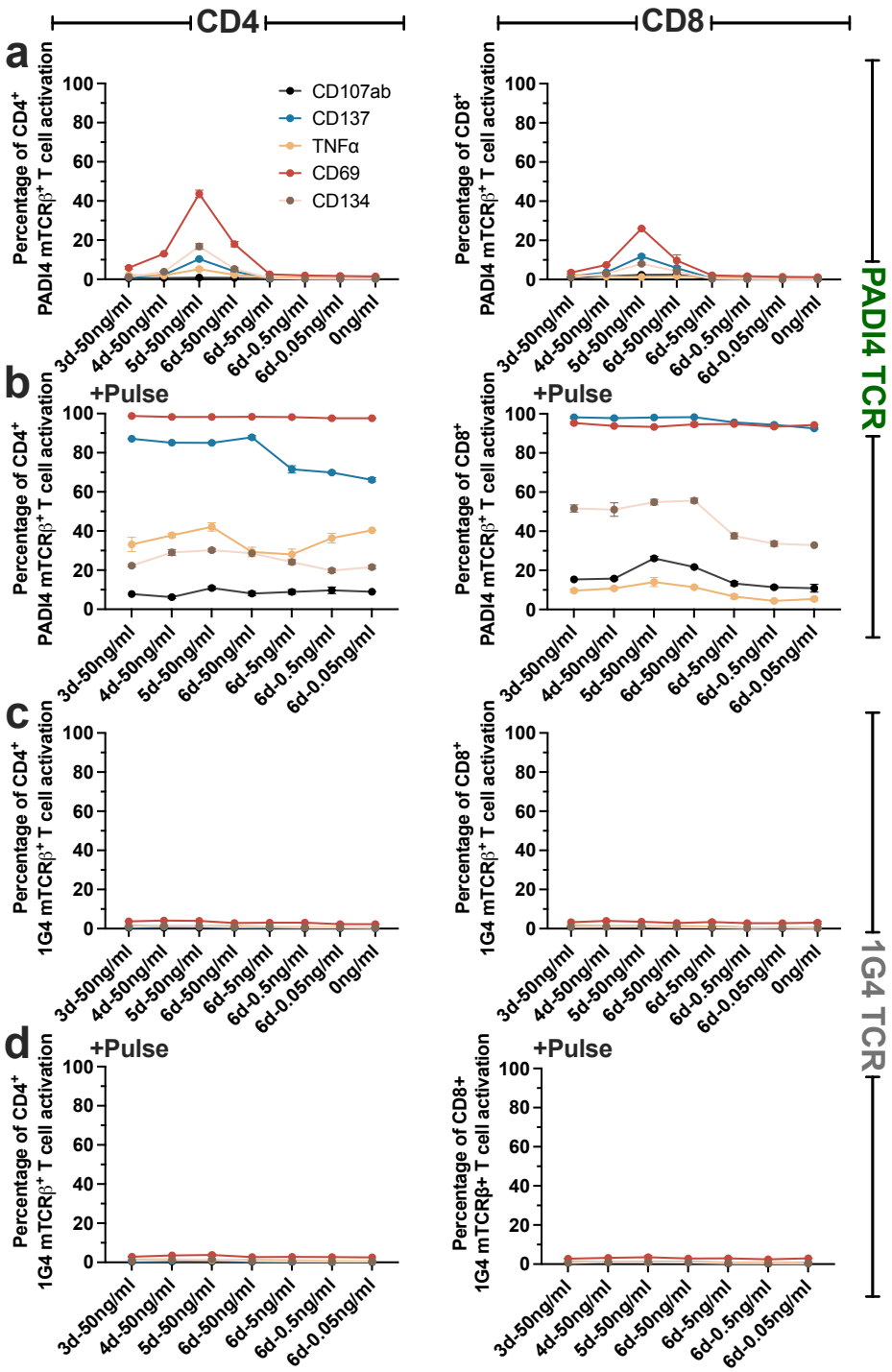


Figure 3.22: PADI4 TCR T cell activation to IFNγ-treated PADI4-transduced MONOMAC1 closely correlates with MONOMAC1 viability as well as HLA-DP expression (PADI4-Var data). MONOMAC PADI4-Var cell line was placed in co-culture with TCR-transduced T cells for 24 hours. Labels on X-axis indicate cell line treatments with IFNγ prior to the co-culture. Panels **a** and **b** show T cells transferred with PADI4 TCR, while panels **c** and **d** show T cells transferred with irrelevant 1G4 TCR. Columns show CD4$^+$ (left) and CD8$^+$ (right) T cells. In panels **b** and **d**, target cell line was pulsed with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

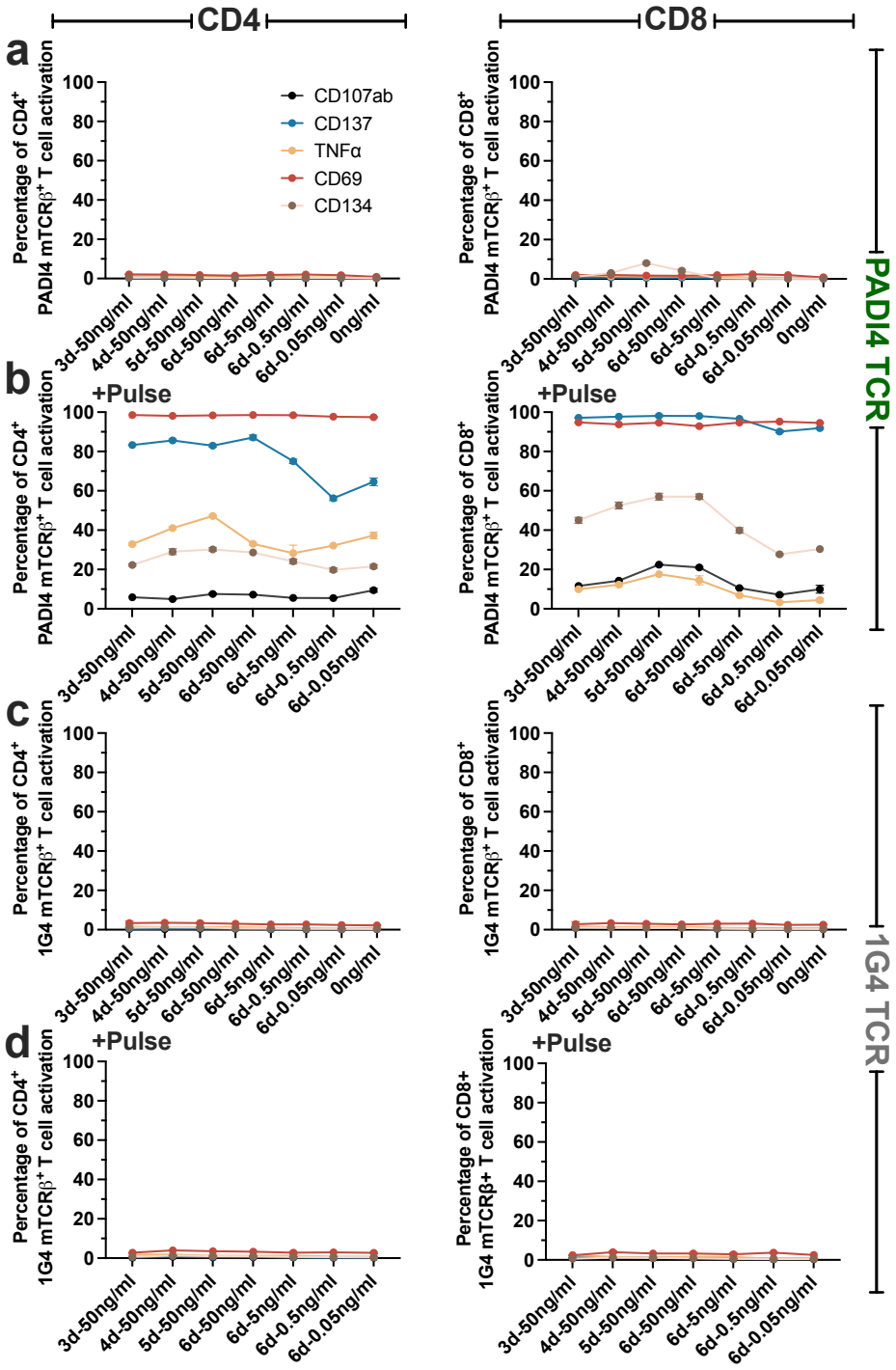


Figure 3.23: PADI4 TCR T cell activation to IFNγ-treated PADI4-transduced MONOMAC1 closely correlates with MONOMAC1 viability as well as HLA-DP expression (PADI4-WT data). MONOMAC PADI4-WT cell line was placed in co-culture with TCR-transduced T cells for 24 hours. See further explanation in Figure 3.22

treatment only somewhat, with lowest seen for 5-day treatment (∼74%). But, for the 6-day treatment, cell viability was slightly higher, at ∼82-89% (Fig. 3.17b). Interestingly, T cell activation results showed a tight correlation with the decreased target cell line viability (Fig. 3.22 and 3.23), rather than highest levels of surface HLA-DP.

This result further suggested that target cell death and/or stress plays an important role in mediating antigen-specific T cell activation. If so, other methods of inducing these conditions should allow PADI4 TCR recognition as well. Cell death (and presence of extracellular antigen) can be mimicked by producing a freeze-thaw lysate of a PADI4 overexpressing cell line and incubating other viable target cells in it. Meanwhile, cell stress can encompass many different physiological responses and signalling pathways. Without *ad hoc* knowledge of which specific stress-related pathway IFNγ activates in order to facilitate T cell activation, it is difficult to successfully mimic it with other treatments. That said, a proinflammatory signal cocktail (named "DC6", see Figure 4.13) may be able to recapitulate this condition as it provides a variety of Toll-like receptor (TLR) ligands and cytokines. It was previously used to induce dendritic cell maturation, a crucial step for successful T cell activation (discussed in Chapter 4).

Thus, an experiment was set up to test whether treatment with lower dose IFNγ (MHC class II upregulation), cell lysate (extracellular antigen presence) or proinflammatory signal and TLR ligand cocktail DC6 (stress signals) can recapitulate T cell activation seen with long and high dose IFNγ pre-treatment. To account for a possibility that successful T cell activation may require several factors to come together (summarised in Fig. 3.20), different treatments were done in different combinations as well as in isolation. Importantly, to test the effects of MHC class II upregulation without incurring considerable cell death, this experiment used a lower IFNγ concentration of 5 ng/ml.

Firstly, any condition receiving the 5 ng/ml dose of IFNγ had ∼3 times higher surface HLA-DP expression (Fig. 3.24a). Also, the highest toxicity to the target cells was seen in the conditions with all three treatments at the same time (*i.e.* lysate with 5 ng/ml IFNγ and DC6), showing ∼70-75% cell viability (Fig. 3.24b). Surprisingly, the T cell activation assay showed a single condition in which antigen-specific T cell response

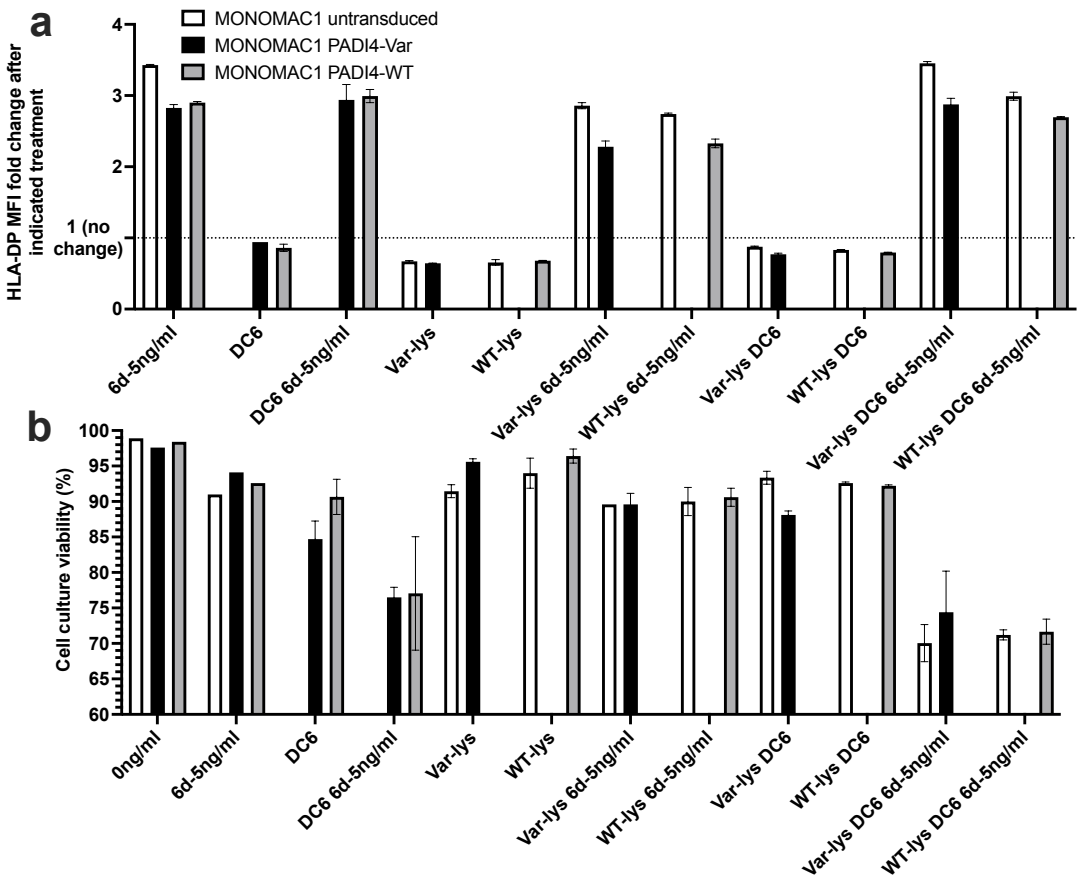


Figure 3.24: Combinatorial effects of IFNγ treatment, stress signals and extracellular antigen availability on PADI4-transduced MONOMAC1 HLA-DP expression and viability. MONOMAC1 was subjected to three types of treatment or their combination: 1) '6d 5ng/ml' – 6 day 5ng/ml IFNγ treatment; 2) 'DC6' – incubation for 24 hours with dendritic cell maturation cocktail 6, see Figure 4.13; 3) 'Var-lys' or 'WT-lys' – incubation for 24 hours with freeze-thaw cell lysate of MONOMAC1 cells transduced with PADI4-Var or PADI4-WT lentivirus, respectively. **a** Relative change in HLA-DP MFI, and **b** cell viability of MONOMAC1 with different treatment regimens. Graphs show one experiment and error bars represent standard deviation between technical replicates.

was seen - in co-culture with MONOMAC PADI4-Var pre-treated with 5 ng/ml IFNγ and its own lysate (Fig. 3.25 and 3.26). In this condition, $CD4^+$ and $CD8^+$ T cells expressed CD69 marker the highest, at $\sim$25% and $\sim$15%, respectively. This would suggest a synergistic effect between extracellular antigen presence and IFNγ-induced MHC class II upregulation. However, a crucial control - untransduced MONOMAC1 treated with the MONOMAC1 PADI4-Var lysate and 5 ng/ml IFNγ, did not induce T cell activation. This contradicts the hypothesis of extracellularly-derived PADI4 antigen presentation on target cells and suggests that intracellular presence of PADI4-Var is necessary.

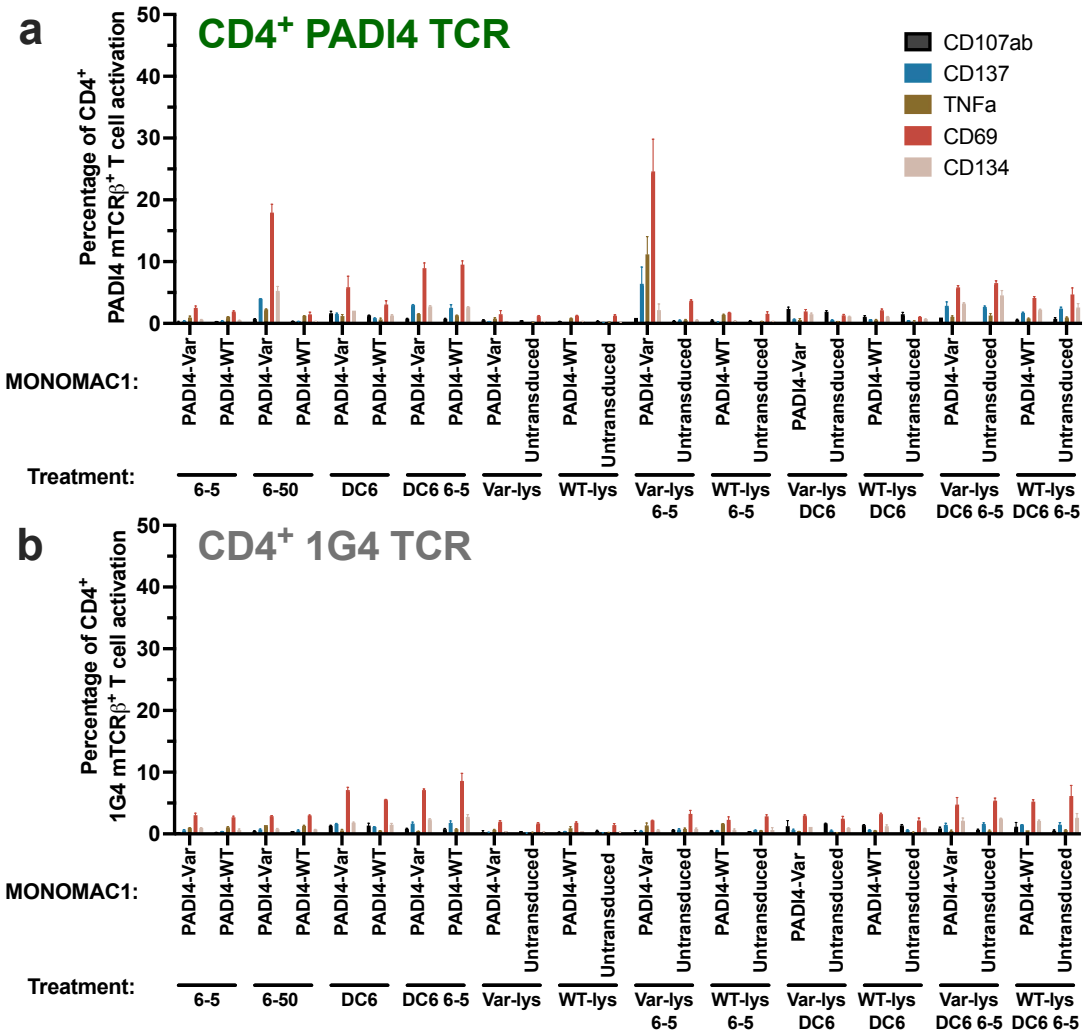


Figure 3.25: PADI4 TCR T cell activation to PADI4-transduced MONOMAC1 may depend on both HLA-DP-upregulating IFNγ as well as other non-specific stress signals (CD4$^+$ T cell activation data). Experiment shows pre-treated MONOMAC1 co-culture with TCR-transduced T cells for 24 hours. For MONOMAC1 treatment explanation, see figure 3.23 legend. **a** and **b** show PADI4 TCR and 1G4 TCR transduced CD4$^+$ T cells, respectively. Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

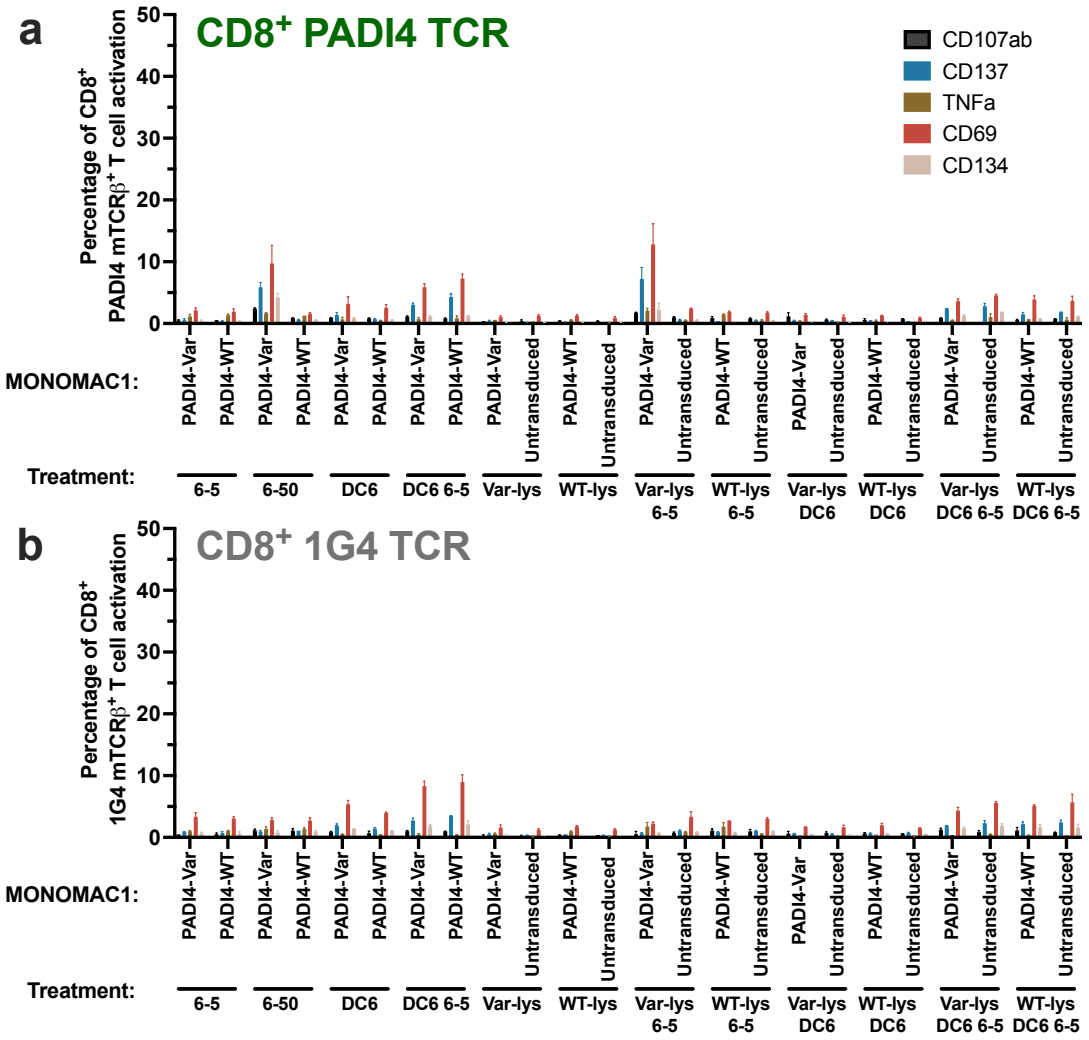


Figure 3.26: PADI4 TCR T cell activation to PADI4-transduced MONOMAC1 may depend on both HLA-DP-upregulating IFNγ as well as other non-specific stress signals (CD8$^+$ T cell activation data). Same as Figure 3.25, but graphs show CD8$^+$ T cell data.

3.5 Closing Remarks

In this chapter, several experimental aims were addressed. Firstly, the *PADI4* miHA specific TCR was identified by isolating the IFNγ^+ T cells from a post-transplant patient OX866 cultured lymphocyte sample. The isolated cells were studied at the single cell level, to highlight their distinct, Th1-like, transcriptome and strong enrichment for select few TCRs as compared with the non-activated IFNγ^- T cell population. Subsequent TCR cloning into a lentiviral transfer vector as well as successful primary T cell transduction and expansion allowed to confirm that the clone denoted as c2.1 is *PADI4* miHA specific.

Secondly, suitable cell line models were investigated in order to test the anti-leukaemic efficacy of the PADI4 TCR. For this purpose, several criteria were proposed to guide cancer cell line screening: 1) *PADI4* SNP-Var genotype; 2) restricting HLA-DPB1*04:01 allele; 3) expression of *PADI4*; 4) expression of the MHC class II machinery. Unfortunately, none of the available leukaemia cell lines fulfilled this. To address the requirement for a PADI4 SNP-Var genotype, a Cas9-guided knock-in strategy was designed. However, only one available cell line fulfilled the remaining three criteria required for this approach and the cell line failed to expand culture. Thus, the main bottleneck for a suitable cell line model was identified as both the *PADI4* SNP-Var genotype as well as *PADI4* expression. To overcome this, a lentiviral PADI4 overexpression vector was designed and several cell lines with varying HLA-DP expression as well as different HLA-DPB1 alleles were stably transduced.

Finally, it remained to assess if the developed cancer cell line models can be targeted with primary PADI4 TCR T cells. Indeed, in co-culture experiments, PADI4-transduced BOLETH cell line induced antigen-specific T cell activation and target cell growth inhibition. Moreover, the diversity of available PADI4 overexpression cell lines allowed to explore some of the conditions that determine successful PADI4 TCR T cell targeting. For one, PADI4 TCR could only recognise PADI4 antigen with the variant SNP genotype (SNP-Var). Secondly, this required the correct restricting HLA-DPB1*04:01 allele and its expression on the target cell. Finally, for one cell line, MONOMAC1, PADI4 TCR

recognition was dependent on the target cell line pre-treatment with IFNγ. It remains unknown what changes this treatment incurs to permit antigen-specific T cell activation. However, preliminary experiments shown here suggested that non-specific stress signals as well as surface MHC class II upregulation may contribute to this.

Chapter 4

PADI4 TCR Testing with Primary Acute Myeloid Leukaemia Samples

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4.1 Overview

This chapter attempts to further describe PADI4 TCR ability to control AML through use of primary patient leukaemia samples *in vitro*. Firstly, suitable target cells were found by systematically screening the biobank of available cryopreserved patient samples. Similarly to cell lines, genotype and expression criteria were applied to *PADI4* and HLA-DP. In addition, samples were investigated for their predicted blast purity and overall quality, to determine which are most suitable for *in vitro* T cell co-culture assays.

Next, *PADI4* miHA presentation by primary leukaemia cells was investigated using a series of activation assays with the PADI4 TCR T cells. Similarly to investigation in leukaemia cell lines, several different conditions were assessed to investigate the effect of extracellular PADI4 antigen, proinflammatory, MHC class II upregulating or other signals on the ability of target cells to activate antigen-specific T cells.

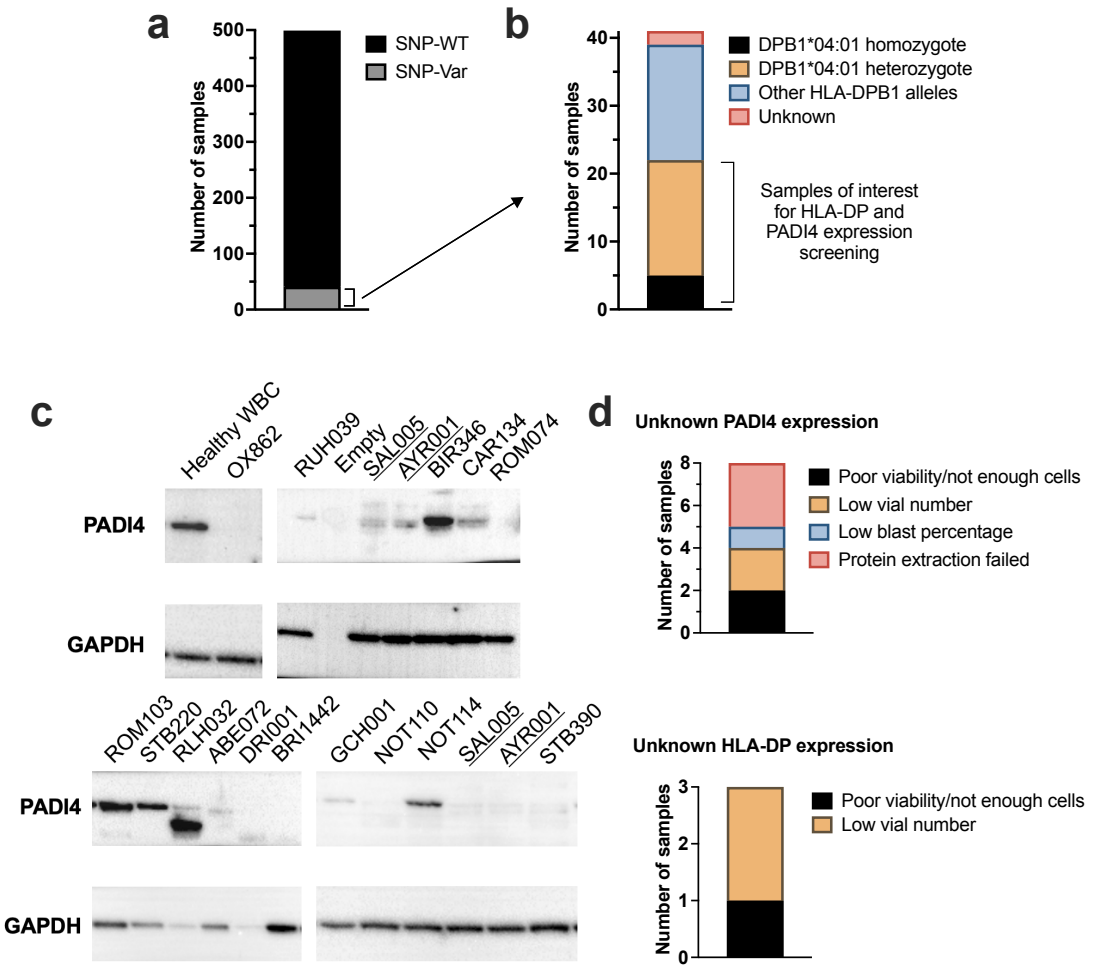

Figure 4.1: Primary AML sample screening identifies several candidates for use in PADI4 TCR T cell experiments. a Column graph showing all samples screened by their *PADI4* SNP genotype. **b** HLA-DP genotype of samples with *PADI4* SNP-Var. **c** Western blot for PADI4 on samples with *PADI4* SNP-Var and restricting HLA-DP allele. Underlined samples have *PADI4* SNP-WT genotype. **d** Reasons for unknown PADI4 expression by western blot (top) or unknown HLA-DP expression by cytometry (bottom) in samples with *PADI4* SNP-Var and restricting HLA-DP allele.

Finally, primary leukaemia cell derived PADI4 antigen presentation by professional antigen presenting cells was investigated. Healthy donor monocytes were used to produce monocyte-derived dendritic cells (MDDCs), which were subsequently treated with primary leukaemia cell lysate. Next, MDDC co-culture assays with PADI4 TCR T cells were performed with or without proinflammatory MDDC maturation signals, to determine optimal MDDC pre-treatment conditions for eliciting antigen-specific T cell activation.

4.2 AML Sample Screening and Initial Testing with PADI4 TCR

Minority of primary AML samples are suitable for PADI4 TCR testing

I aimed to test the ability of PADI4 TCR T cells to recognize primary AML samples. To start with, suitable samples were needed with the following criteria: 1) *PADI4* SNP-Var genotype; 2) *PADI4* expression; 3) restricting HLA-DPB1*04:01 allele; 4) expression of MHC class II machinery (summarised in Fig. 3.6). Luckily, both MHC Class II[125, 126] and PADI4 (Appendix Fig. 13) are commonly expressed in AML.

As the *PADI4* SNP-Var genotype is the most restrictive requirement due to the low variant genotype frequency in the general population ($\sim$5%)[292], all available primary AML samples were first genotyped for it (Fig. 4.1a). This identified 41 SNP-Var samples out of a total of 499 (8.2%). These were further genotyped for HLA-DPB1 and those with the required restricting allele were finally investigated for PADI4 expression by western blot (Fig. 4.1b,c).

Next, flow cytometry was used to further investigate samples with *PADI4* SNP-Var genotype, PADI4 expression and HLA-DPB1*04:01 allele in order to assess: 1) predicted blast purity; 2) HLA-DP surface expression (Fig. 4.2, 4.3). Unlike cell lines, primary samples have highly variable purity of cancer cells. Sample purification is challenging as well, due to the overall fragility of primary leukaemia cells and the relatively high cell number requirement for future activation assays. Thus, blast purity prediction is especially important in assessing sample quality.

In addition, cytometry showed the heterogeneity of HLA-DP expression both between different samples and between different predicted blast sub-populations within a given

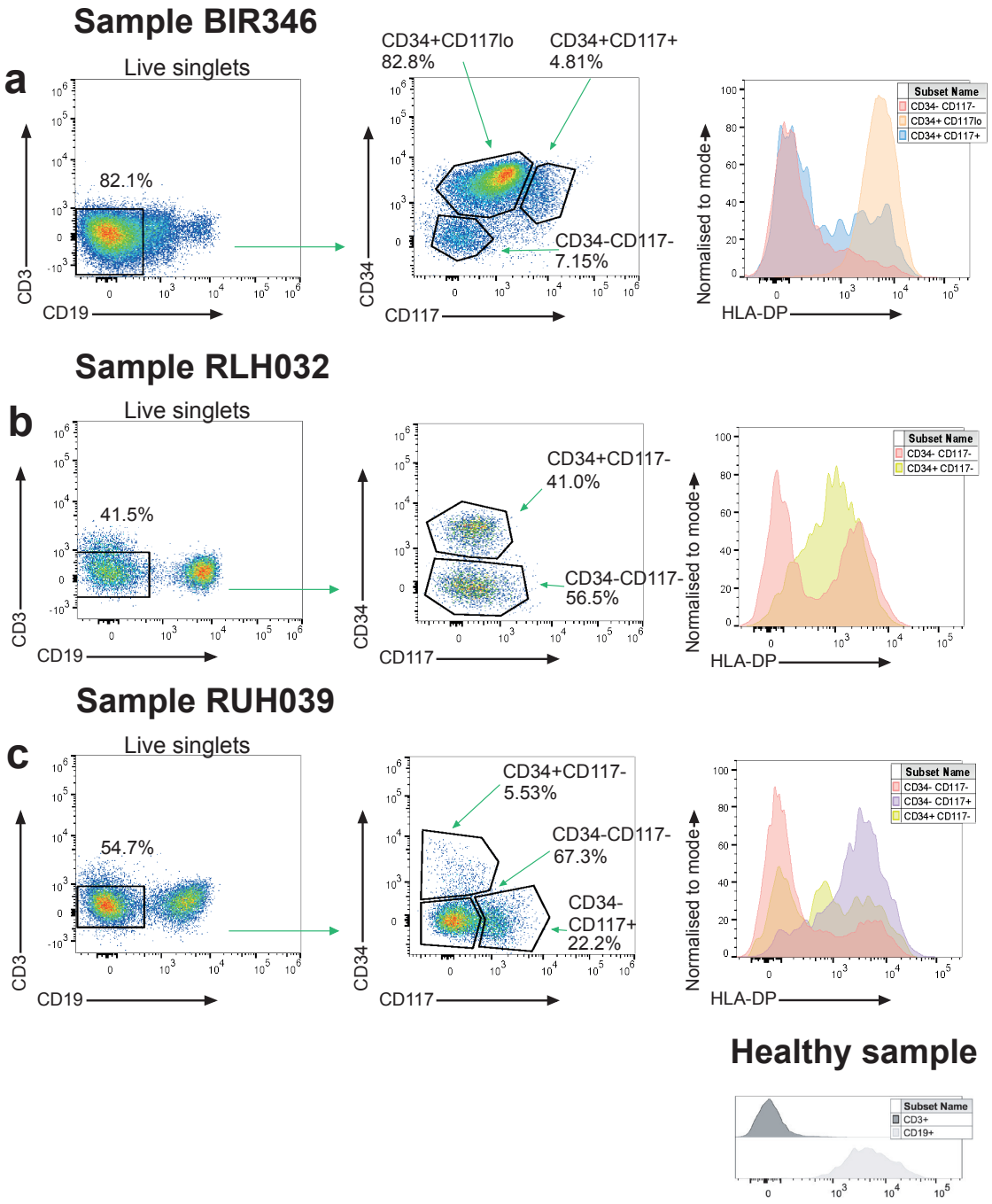

Figure 4.2: Patient AML samples have heterogenous HLA-DP expression. **a**, **b** and **c** Show samples BIR346, RLH032 and RUH039, respectively. $CD3^{-}CD19^{-}$ gates (left) show predicted AML blast purities, whilst CD34 and CD117 gates (middle) allow to stratify AML and show heterogeneity in different sub-population HLA-DP expression (right). Bottom right shows HLA-DP expression of $CD3^{+}$ and $CD19^{+}$ cells from healthy PB.

HLA-DP and PADI4 expression	Sample	Viability at thaw (%)	Viability at cytometer (%)	PADI4 WB	$CD3^-$ ($CD19^-$)	$CD3^-$ ($CD19^-$) $HLA\text{-}DP^+$ (%)	$CD3^-$ ($CD19^-$) HLA-DP MFI
Both	BIR346	96.3	98.6	Band	82.1	87.3	5704
	CAR134	93	98.3	Band	90.2	18.6	438
	RUH039	62.3	92.7	Faint band	54.7	44.7	2526
	ABE072	26.4	32.5	Faint band	64.5	18	881
	RLH032	57	95	Band	41.5	57.5	1597
	STB220	66.8	97.7	Band	90.8	8.6	441
	NOT114	83	-	Band	3.5	-	-
	GCH001	73	-	Faint band	41	10.2	-
PADI4 only	ROM103	96	98.6	Band	87.7	1.86	184
	STB390	83.9	97.4	Faint band	73.4	4.55	331
HLA-DP only	STB319	60.9	90.7	-	9.06	63.1	3856
	CUH018	91.5	96.4	-	98.9	19.9	544
	STB270	29.4	76.8	-	90.9	44.8	1059
	ROM074	85.9	93.1	No band	83.7	60.6	4943
	BRI1442	88.2	98.3	No band	83.7	77.9	2346
	AYL065	60.3	89.2	-	45.3	27.7	742
	NOT110	66	89.3	No band	53.2	70.3	6110
	OX862	-	97.9	No band	85	95.9	-
Neither	DRI001	41.3	1.19	-	Low viability at thaw		
	BRI2122	38.7	33.3	-	37.7	2.31	170
	BRI2348	16 million cells or fewer available					
	GEO006						

HLA-DP Controls	$CD3^+$ HLA-DP MFI	$CD19^+$ HLA-DP MFI
Healthy PBMC	67.5	8060

Figure 4.3: Summary of all available patient AML samples with the restricting *PADI4* SNP-Var and HLA-DPB1*04:01 genotypes.

sample (Figs. 4.2, 4.3 and Appendix Fig. 14). Finally, not all candidate samples were fully characterised, either due to low predicted blast purity, overall viability or cell number (Fig. 4.1d).

Taking all these factors into consideration, only 8 candidate samples emerged for their favourable characteristics out of total 41 with the PADI4-Var genotype (Fig. 4.3). Out of those, 3 had higher HLA-DP expression (BIR346, RLH032 and RUH039) and thus were most suitable for the experiments.

AML samples are not able to activate PADI4 TCR directly

Next, it was tested whether PADI4 TCR T cells can become activated by primary AML samples alone. In order to do so, thawed primary samples were co-cultured with overnight rested TCR-transferred T cells for 9 hours in the presence of cytokine release-blocking Brefeldin and Monensin A. To test whether any activation seen is antigen-specific, co-cultures were performed with both PADI4 TCR and irrelevant 1G4 TCR transduced T cells. At the end, T cells were analysed using flow cytometry to assess staining for CD107a & CD107b (co-stain), CD137, TNFα, IFNγ and IL-2 (Fig. 4.4 and 4.5). Out of all samples tested (n=4), none showed an ability to activate PADI4 TCR T cells directly. This result may suggest that the target cells do not efficiently present intracellularly derived PADI4 antigen on their own HLA-DP. To investigate if antigen-specific activation can be seen in the presence of extracellular PADI4 antigen, primary AML samples were incubated in their own freeze-thaw lysate for 24 hours prior to T cell co-culture. Similarly, no activation was seen in conditions with lysate pre-treatment either ((Fig. 4.4a and 4.5a).

Absence of any activation in experimental conditions could indicate a technical fault, *e.g.* PADI4 TCR T cells are non-functional. However, PADI4 TCR T cells could efficiently activate themselves in monoculture when presented with the PADI4 variant peptide, indicating that the they are indeed functional (Fig. 4.4a and 4.5a).

Peptide response in T cell monoculture is elicited when T cells present the antigen to each other. This relies on presence of restricting HLA-DP allele and its expression. To fulfill these criteria, donors with the restricting HLA-DP*04:01 allele were used for T cell

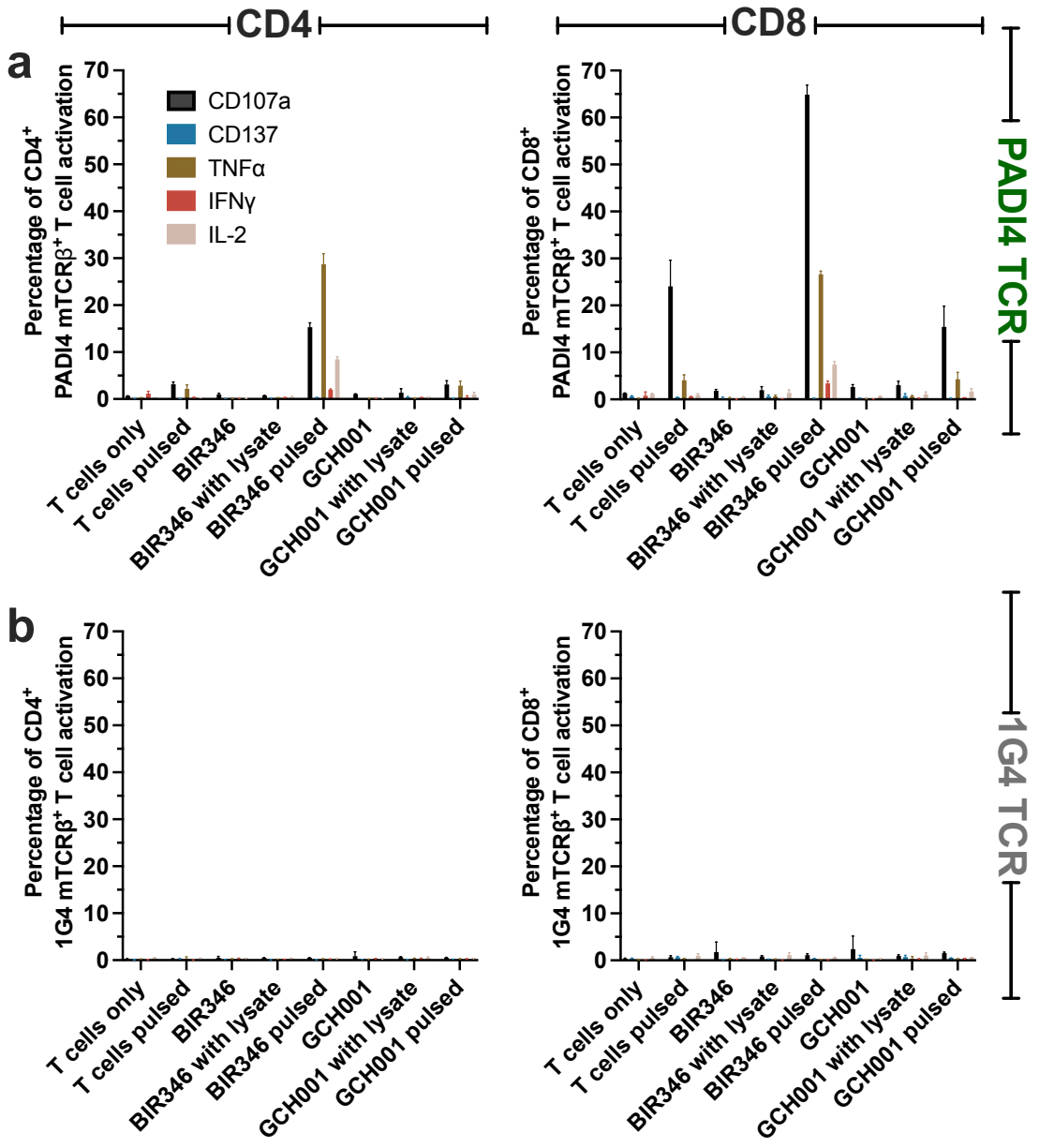

Figure 4.4: PADI4 TCR T cells activate when co-cultured with PADI4 variant peptide pulsed patient AML samples BIR346 and GCH001. a Shows T cells transferred with PADI4 TCR, whilst **b** shows T cells transferred with 1G4 (irrelevant) TCR after 9 hours of co-culture with target cells. $CD4^+$ (left) and $CD8^+$ (right) T cells are plotted separately. Pulsed condition indicates target cells or T cells themselves were incubated with PADI4 variant peptides 21 and 22 (10μM). In conditions with lysate, target cells were incubated with their own freeze-thaw lysate for 24 hours prior to T cell co-culture. Error bars indicate standard deviation between technical replicates.

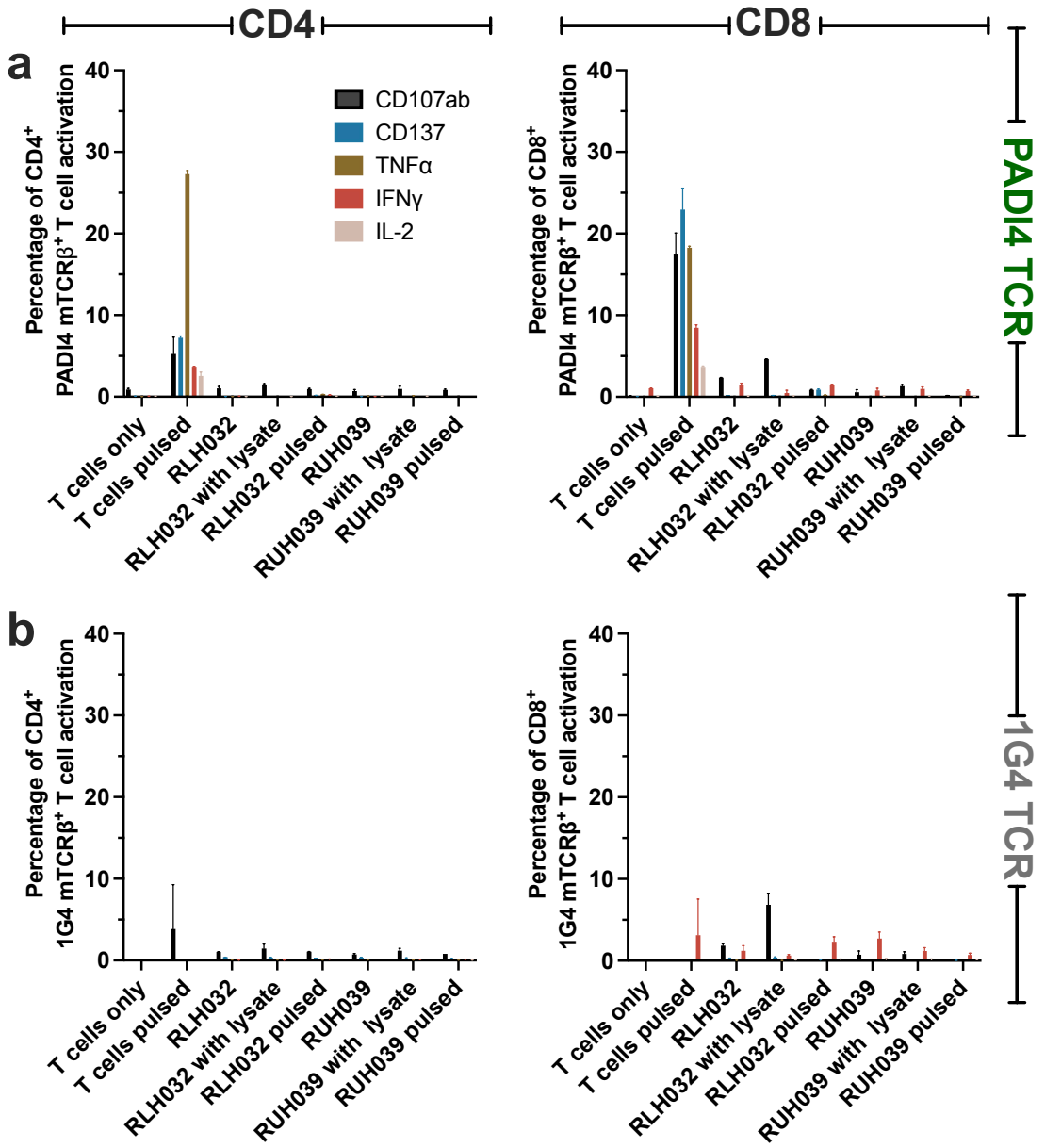

Figure 4.5: PADI4 TCR T cells do not activate when co-cultured with PADI4 variant peptide pulsed patient AML samples RLH032 and RUH039. For figure explanation, see figure 4.4 legend. Pulsed condition indicates target cells or T cells themselves were incubated with PADI4 variant peptides 21 and 22 (1μM).

production and although T cells normally do not express MHC class II, it is upregulated in response to expansive T cell culture during the transduction protocol (data not shown).

Similarly to T cell monoculture, primary AML samples should also evoke T cell activation when pulsed with the PADI4 variant peptide immediately prior to the co-culture. Indeed, such is seen with pulsed samples BIR346 and GCH001 (Fig. 4.4a). However, some samples (RLH032 and RUH039) failed to do this (Fig. 4.5a). This could indicate that these samples are overall more fragile and thus may not tolerate co-culture with TCR-transduced T cells. It should be noted that where peptide pulse response to AML was seen for BIR346 and GCH001 (Fig. 4.4a), its magnitude closely correlated with overall HLA-DP expression level of the sample (Fig. 4.3).

Because for some PADI4 variant peptide pulsed AML samples antigen-specific T cell response in co-culture was absent, T cell activation results seen when these samples were unpulsed (*i.e.* the condition of direct AML activation of PADI4 TCR T cells) are invalid. Further, any other conditions where AML is in any way manipulated (*e.g.* lysate pre-treatment) are also invalid. Thus, further investigation of any conditions that could permit *in vitro* PADI4 TCR T cell activation to AML directly (*i.e.* where cancer cell serves as the APC) are limited to samples that can efficiently evoke the positive control response using the variant peptide - BIR346 and GCH001.

4.3 Effects of Different AML Sample Pre-Treatments on PADI4 TCR Activation

Although PADI4 TCR T cells did not show evidence of activation when co-cultured with AML samples, there are several questions to explore based on this result:

1. Has the activation (co-culture) period been sufficient? After all, in cell line experiments (see Chapter 3), optimal activation period was 24 hours, while this work so far had only used 9 hours.

2. Could PADI4 antigen be presented only when it is extracellularly-derived? Have the conditions used to test this so far been optimal (Fig. 4.4 and 4.5)? MHC class II machinery typically presents extracellular antigens. However, previous findings in cell lines have suggested that intracellular antigen may be sufficient (see Chapter 3).

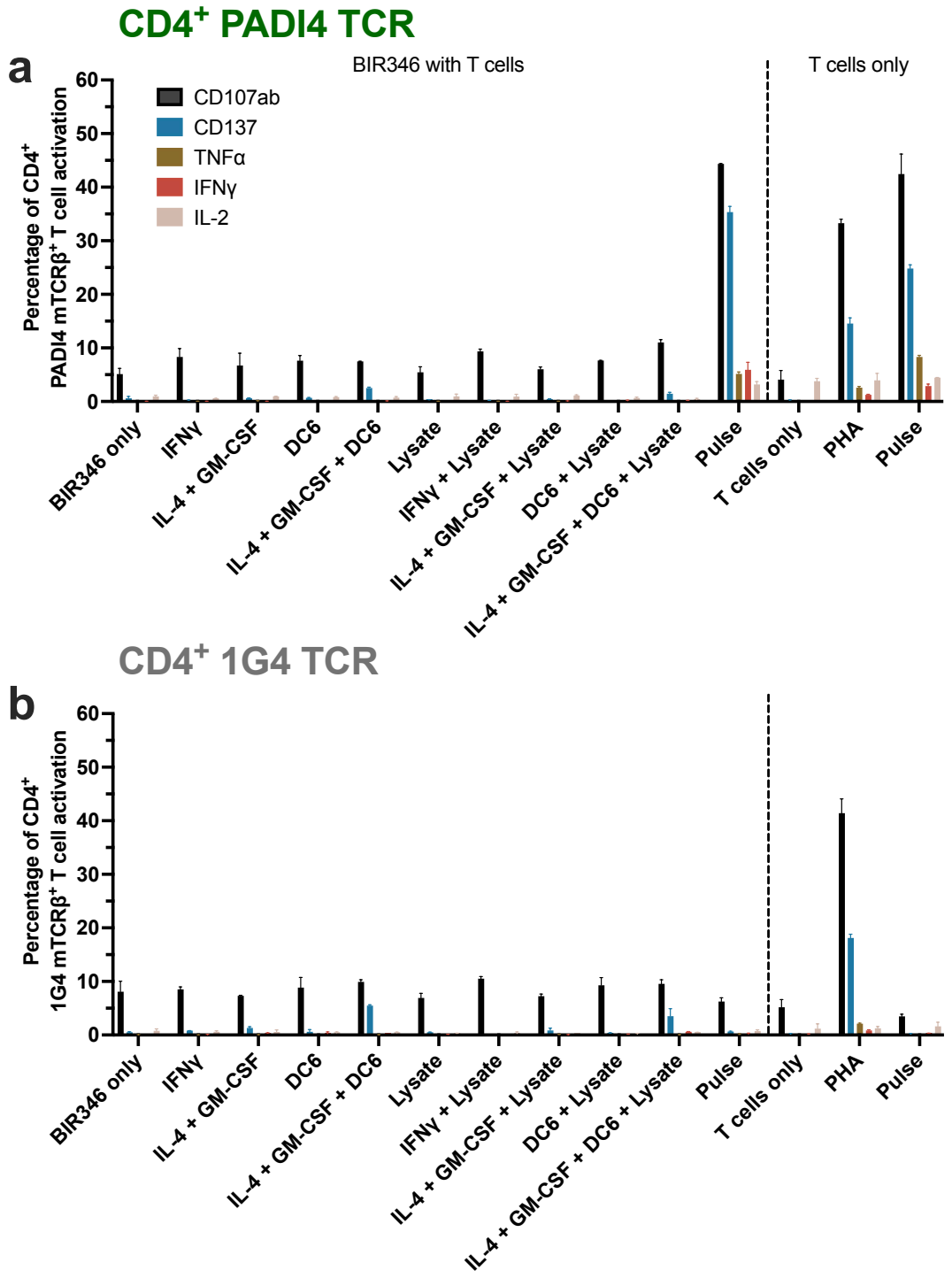

Figure 4.6: Neither stress, maturation, or MHC-II upregulation signals nor extracellular antigen availability prompt BIR346 to activate PADI4 TCR T cells (24-hour co-culture, CD4$^+$ T cell data). a and **b** Show T cells transferred with PADI4 TCR and 1G4 (irrelevant) TCR, respectively. BIR346 was treated with indicated conditions for 24 hours before co-culture with T cells. IFNγ treatment concentration is 0.25ng/ml. DC6, dendritic cell maturation cocktail 6, see Figure 4.13. Lysate condition indicates BIR346 treated with its own freeze-thaw lysate. Pulse indicates cells were incubated with PADI4 variant peptides 21 and 22 (1μM). PHA, phytohaemagglutinin. Error bars indicate standard deviation between technical replicates.

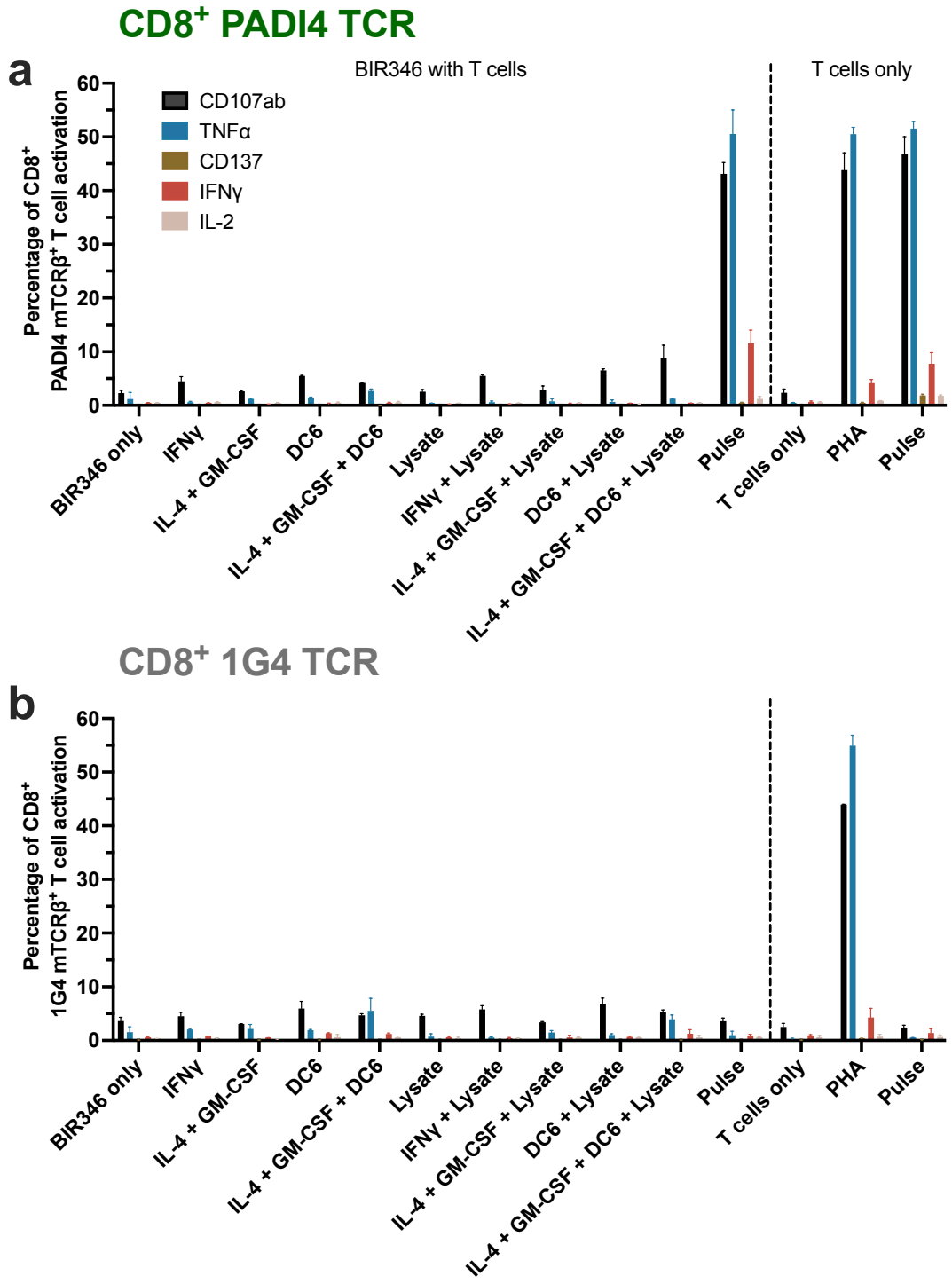

Figure 4.7: Neither stress, maturation, or MHC-II upregulation signals nor extracellular antigen availability prompt BIR346 to activate PADI4 TCR T cells (24-hour co-culture, CD8$^+$ T cell data). For figure explanation, see figure 4.6 legend.

3. Does primary AML require IFNγ pre-treatment? This was found to be neccessary for T cell activation to myeloid leukaemia cell line MONOMAC1 PADI4-Var (see Chapter 3).

4. Could stress in target cells prompt T cell activation? Stress signals are known to upregulate autophagy and change presentation of immune receptors/ligands[284–291](see Chapter 3).

5. Could leukaemia cells require dendritic cell lineage differentiation? Primary AML cells have been previously matured into dendritic-like cells, which show a more favourable immune receptor profile[293].

Any of these factors or indeed their combination could permit PADI4 TCR T cell activation. In fact, this framework is very similar to that proposed with the cell line MONOMAC1 (see Chapter 3 and Fig. 3.20), where pre-treatment with IFNγ was found to be a necessary albeit likely insufficient factor in permitting antigen-specific T cell activation.

To explore these ideas in a primary AML, BIR346 sample was chosen due to a more favourable HLA-DP expression profile as well as higher purity and viability *in vitro* (see previous section and Fig. 4.3). The sample was pre-treated with indicated regimens for 24 hours until addition of T cells into culture (Fig. 4.6 to 4.9). As the treatments were not removed from media, they continued to act on cells in culture for the whole activation period of 24 (Fig. 4.6 and 4.7) or 72 hours (Fig. 4.8 and 4.9). The exception for this are the PADI4 variant peptide pulses of BIR346 - to ensure that the peptide is presented by the target cells rather than the effector T cells themselves, the peptide was washed out of the media prior to the co-culture with T cells.

To summarise, neither 24 nor 72 hour activation assays showed any evidence that AML sample could activate PADI4 TCR T cells in any of the conditions tested. Positive controls using phytohaemagglutinin (PHA) or PADI4 variant peptide pulse, either in T cell monoculture or in co-culture with target cells, were positive, indicating that the T cells are functional and the AML sample is able to present the peptide. Expectedly, both PHA and peptide pulse induced T cell activation decreased over time, with 72-hour time point showing generally lower activation (Fig. 4.8 and 4.9).

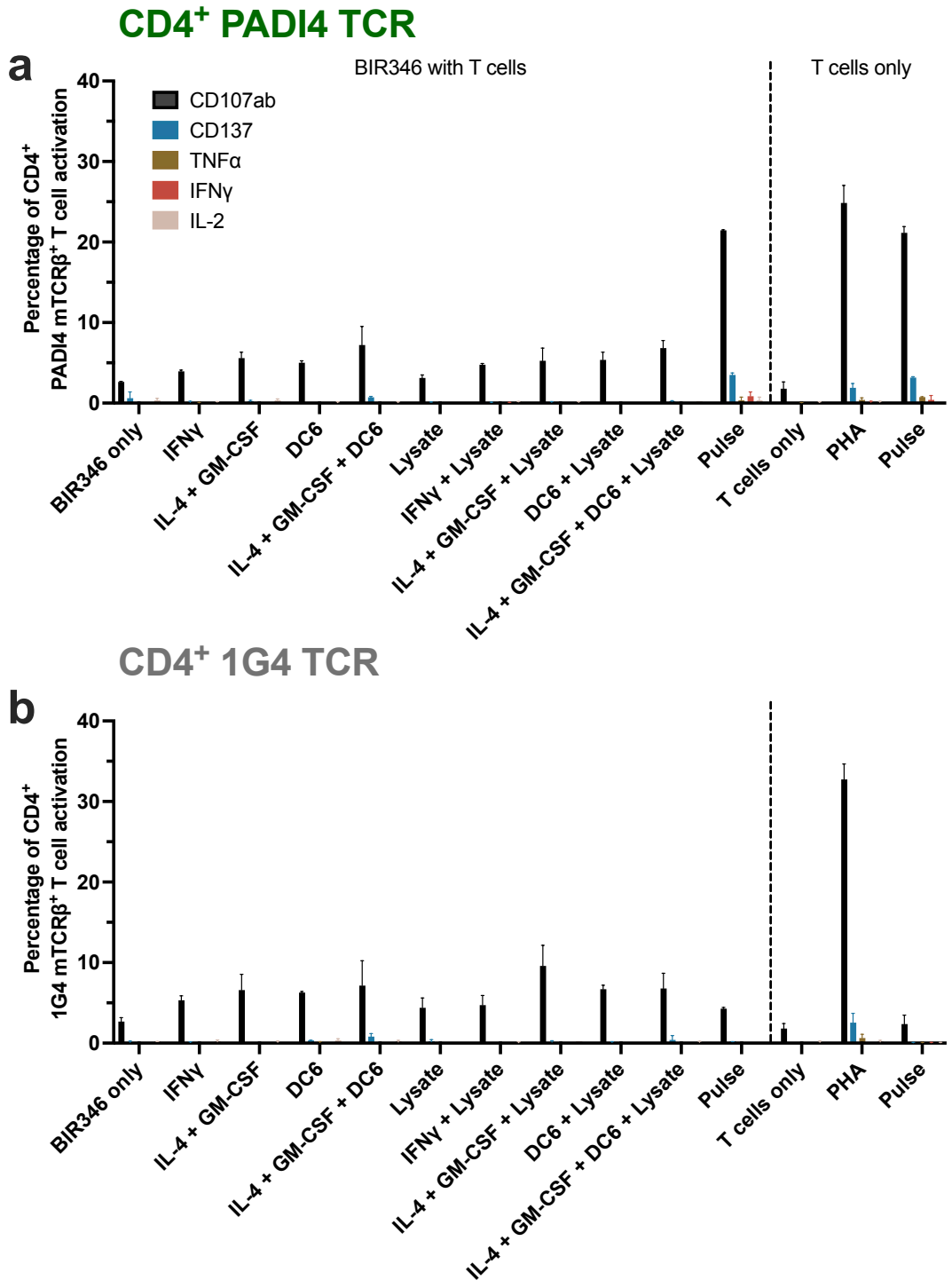

Figure 4.8: Neither stress, maturation, or MHC-II upregulation signals nor extracellular antigen availability prompt BIR346 to activate PADI4 TCR T cells (72-hour co-culture, CD4$^+$ T cell data). For figure explanation, see figure 4.6 legend.

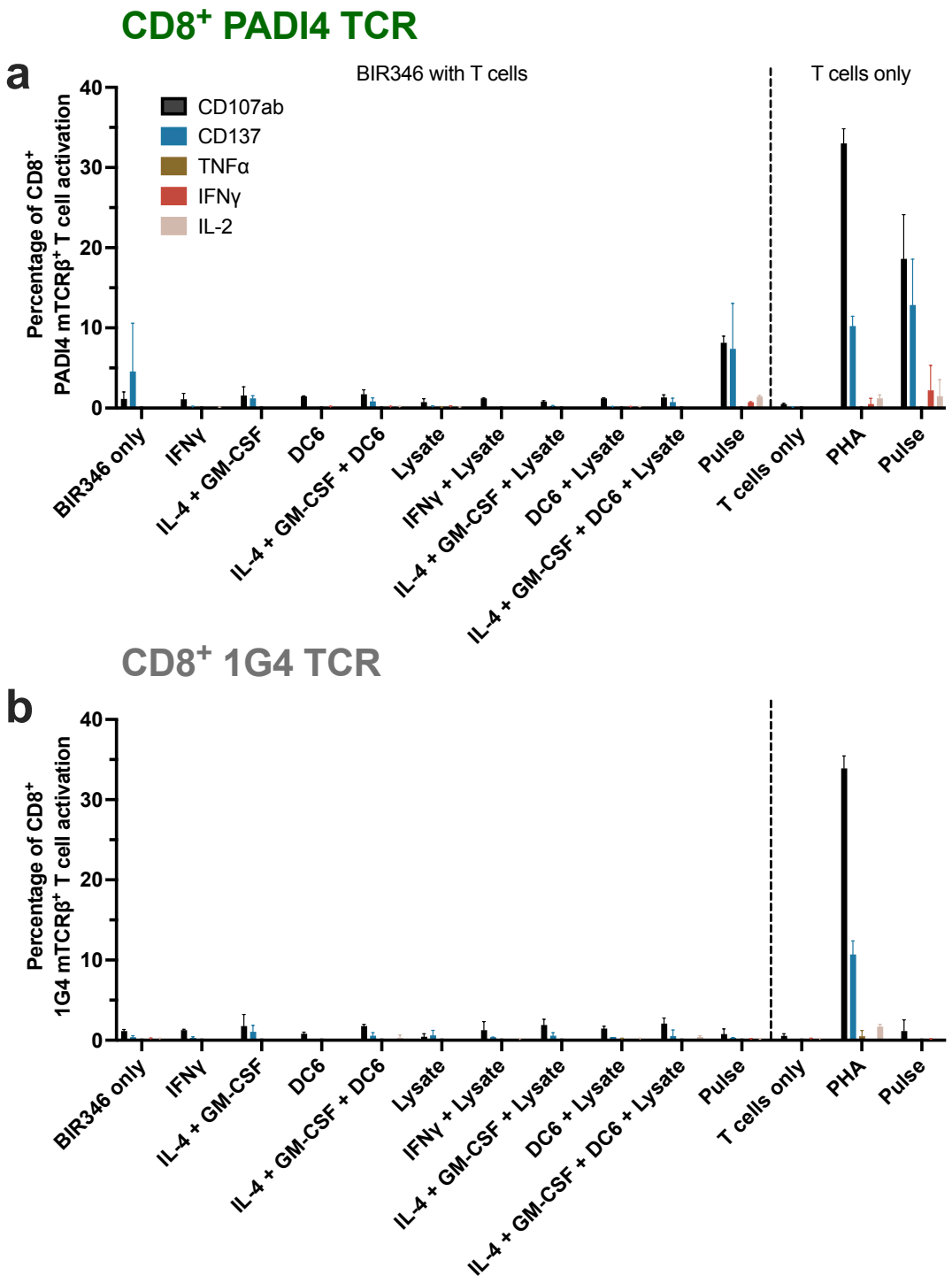

Figure 4.9: Neither stress, maturation, or MHC-II upregulation signals nor extracellular antigen availability prompt BIR346 to activate PADI4 TCR T cells (72-hour co-culture, CD8$^+$ T cell data). For figure explanation, see figure 4.6 legend.

Presence of lysate in co-culture had no effect, either alone or in combination with the IFNγ treatment. This is in contrast to MONOMAC1 PADI4-Var cell line, where the combination treatment was able to induce antigen-specific T cell activation. However, due to heavily limited ability to culture primary sample *in vitro*, IFNγ treatment was significantly shorter (up to 4 days in the 72 hour condition) and lower concentration (0.25 ng/ml) when compared to the MONOMAC1 cell line treatment that previously yielded PADI4 TCR T cell activation (6 days at 5 ng/ml, see Chapter 3).

The condition with GM-CSF and IL-4 treatment intended to assess the effects of providing dendritic cell (DC) differentiation cytokines on the ability of primary leukaemia cells to present antigen. Later in this chapter, the same cytokines are successfully used to differentiate primary monocytes into DC (Fig. 4.10). However, in the primary AML samples, addition of the cytokines showed no effect on T cell activation. Unlike the healthy monocyte differentiation protocol of 6 days, the cytokines were acting on primary leukaemia for up to 4 days in the 72 hour activation assay. Although the shorter time period may have been sufficient to upregulate certain DC-related markers, it is impossible to know this without a cytometry-based assessment of the leukaemia sample[293].

Previous leukaemia cell DC-like differentiation regimens have also used additional proinflammatory and/or 'danger' signals, such as TNFα, IL-1β or different TLR agonists. These are intended to further push leukaemia cells towards a more mature DC phenotype, which, similarly to actual DCs, may be critical for efficient antigen presentation to T cells[293, 294]. Furthermore, even in this work, maturation of MDDCs has been crucial to achieve reproducible and high antigen-specific T cell activation (see later in this chapter). That is why, in a separate condition, IL-4 and GM-CSF treatment was combined with the DC6 maturation cocktail (Fig. 4.6 to 4.9). Alas, this has proven unsuccessful as well.

4.4 PADI4 TCR Engagement via a Professional Antigen Presenting Cell

Several considerations prompted me to explore the use of professional APCs in PADI4 TCR T cell activation assays. Firstly, it is a suitable practical approach to determine whether leukaemia-derived PADI4 antigen can be produced and presented

to antigen-specific T cells in a primary cell setting. Importantly, antigen presentation by APCs does not require leukaemia sample to remain viable and functional *in vitro*, which has been the major hurdle to the assays described before. Secondly, use of professional APCs eliminates the variability in HLA-DP expression and MHC class II functionality in primary AML samples. *In vitro* derived APCs would be sourced from healthy donor cells and are expected to have fully functional antigen-presentation machinery as well as high HLA-DP expression.

Crucially, use of professional APC to present a leukaemia-derived antigen has biological rationale. PADI4 TCR is MHC class II restricted. As discussed in Chapter 1, MHC class II restricted T cell therapies could have anti-cancer functions beyond direct cancer cell cytotoxicity. This is based on numerous studies describing wide-ranging functions $CD4^+$ T cells can have in anti-cancer and anti-infection immune responses (see Chapter 1). Therefore, this section explores the ability of primary professional APC to present leukaemia-derived PADI4 antigen.

MDDC can present AML sample derived PADI4 antigen

Dendritic cells are considered a crucial link between innate and adaptive immunity. Although originating from bone marrow, they migrate to both lymphoid and non-lymphoid tissues where they have the capacity to sample the antigenic environment as well as respond to inflammatory cues. Upon inflammation and/or 'danger' signal induced maturation, DC functionality and phenotype changes, allowing not only CCR7-dependent migration to lymphoid tissues but also optimal T cell activation. This is achieved through improved antigen processing and presentation on MHC, increased costimulatory molecule expression and paracrine proinflammatory signalling. Together, these changes enable naïve T cell activation in lymphoid tissue by providing the required signals[294, 295].

As such, DCs are an ideal APC to study antigen-specific T cell activation. However, their frequency in healthy adult blood is very low, being just around 1% of the total PBMC fraction. To overcome this scarcity, various different DC production strategies have been developed. For one, different precursor cells, such as cord blood or adult $CD34^+$[296–299] or adult PB circulating $CD14^+$ (monocytic) cells[300], can

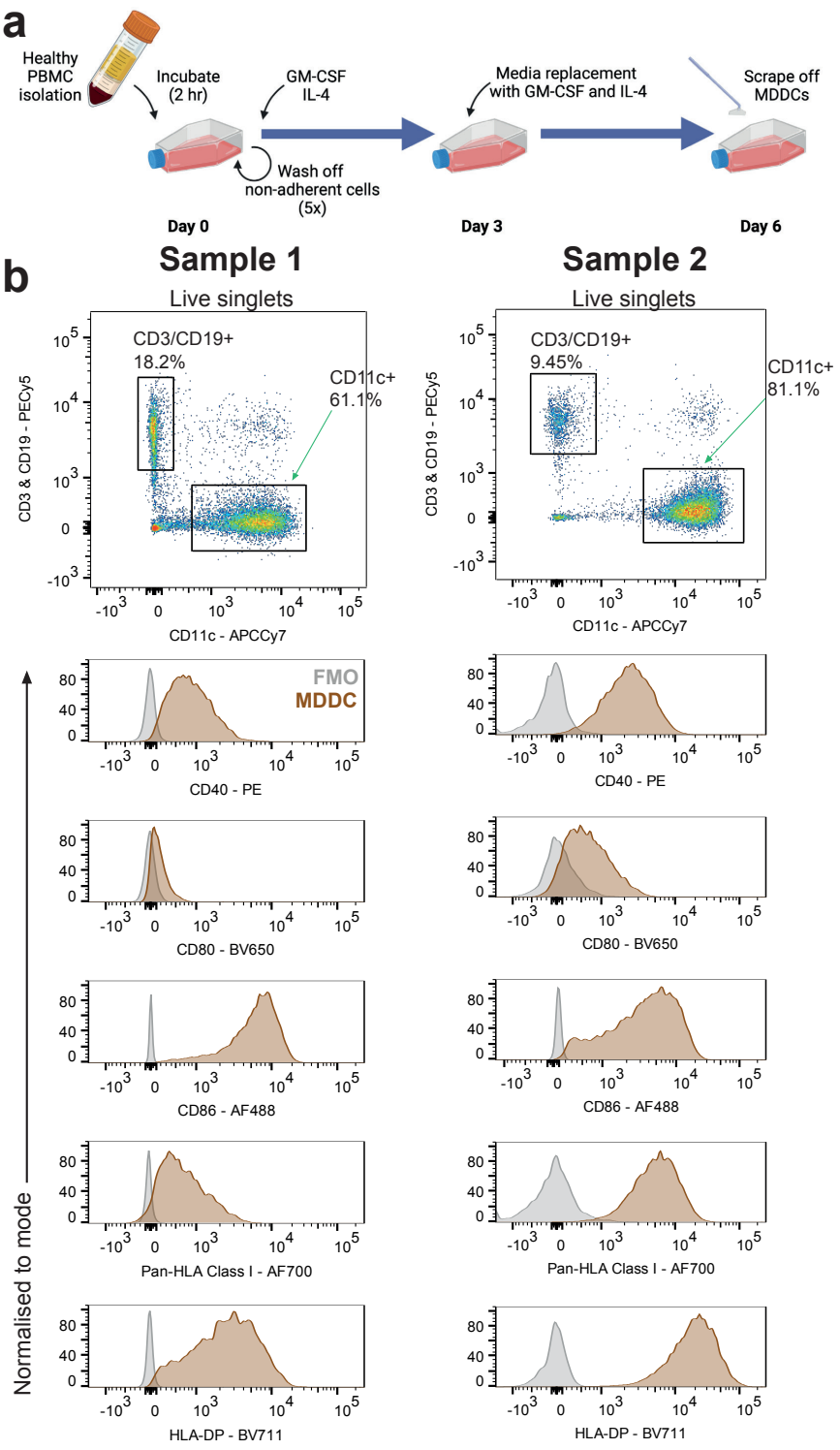

Figure 4.10: Healthy peripheral blood monocytes can be used to produce monocyte-derived dendritic cells (MDDC). a Overview of MDDC production protocol. **b** Two representative examples of MDDC product at day 6 of protocol. Purity is indicated by $CD11c^{+}$ fraction.

be directly differentiated into DCs. In addition, fibroblasts can be either directly transdifferentiated[301] or initially dedifferentiated into inducible pluripotent stem cells (iPSC) followed by differentiation into DCs[302, 303].

Of these methods, use of monocytes for DC production is the most practical, due to easy access (fresh or cryopreserved PB), relatively high monocyte frequency in collected samples (∼10% in PBMC fraction) and a fast differentiation protocol. *In vitro* monocyte differentiation into DCs was first described nearly 30 years ago[304–307] and has been widely used ever since[295, 300]. In addition, this method mimics a process that can occur *in vivo* - since monocytes are known to differentiate into DCs under inflammatory conditions[308–310]. Thus, for further experiments, I opted to develop and use monocyte-derived dendritic cells (MDDCs).

To begin with, healthy donors with the restricting HLA-DPB1*04:01 genotype were chosen for MDDC production. Fresh blood was used to isolate PBMCs and monocytes were further enriched by allowing their attachment to the tissue culture flask (Fig. 4.10a). This was followed by a 6-day dendritic cell differentiation culture using IL-4 and GM-CSF. At the end, MDDC were scrapped off the tissue culture flask and their purity as well as DC-related marker profile were assessed using flow cytometry (Fig. 4.10b).

In order to assess PADI4 TCR T cell activation through professional APCs, MDDC product was incubated with freeze-thaw lysates of primary AML samples for 24 hours before activation assay co-culture with PADI4 TCR T cells (Fig. 4.11). This, for the first time, showed evidence that antigen-specific T cells can recognise leukaemia-derived PADI4 antigen. However, the activation level with the lysate treatment was low, despite a high positive control (PADI4 variant peptide pulse) response. In fact, the example shown in Fig. 4.11 is the highest antigen-specific T cell activation that was observed with the use of lysate treated MDDCs (see further examples in Fig. 4.14, 4.15 and 4.17). I hypothesised that the low and difficult to reproduce T cell activation may be due to an insufficient MDDC maturation.

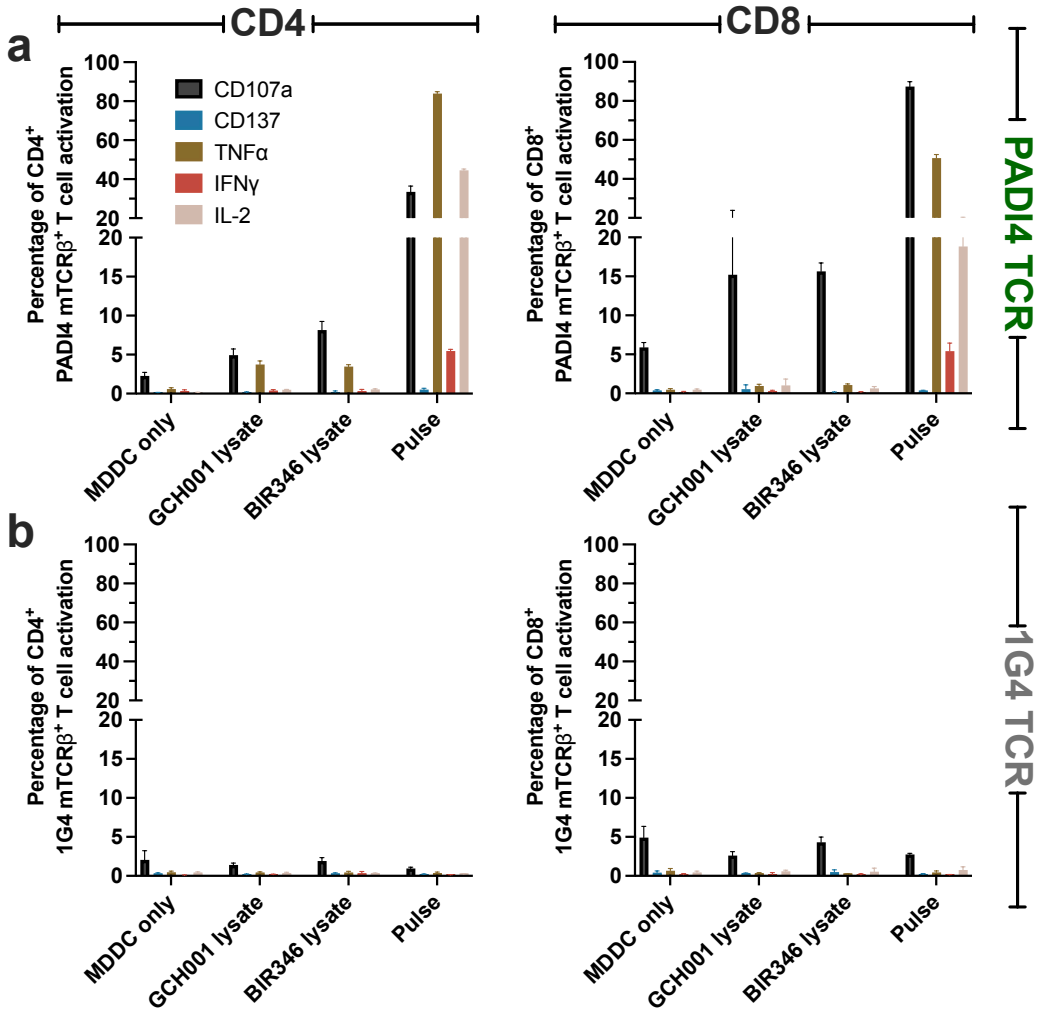

Figure 4.11: Immature MDDC can induce low PADI4 TCR T cell activation when pre-treated with primary AML sample lysate. a and **b** Show PADI4 TCR and 1G4 (irrelevant) TCR transferred T cells, respectively, after 9 hours of co-culture with target cells. $CD4^+$ (left) and $CD8^+$ (right) T cells are plotted separately. Lysate condition indicates MDDC were treated with AML freeze-thaw lysate for 24 hours prior to T cell co-culture. Pulsed condition indicates MDDC were incubated with PADI4 variant peptides 21 and 22 (10μM) prior to T cell co-culture. Error bars indicate standard deviation between technical replicates.

Efficient PADI4 TCR engagement by MDDC requires dendritic maturation

Given the importance of proinflammatory and 'danger' signals in DC phenotype and function[294, 295], I hypothesised that the current MDDC production protocol does not provide the correct signals for mature DC phenotype induction required for optimal antigen specific T cell activation (Fig. 4.12a). Indeed, MDDC production literature describes a wide variety of different maturation protocols that follow GM-CSF & IL-4 differentiation steps.

For example, initial reports describing *in vitro* MDDC production already attempted to introduce an additional step in the protocol to allow DC maturation[304–307]. This showed that simply supplementing culture with the proinflammatory cytokine TNFα or soluble CD40 ligand improves DC phenotype and/or their capacity to activate T cells. Soon after, a publication by Jonuleit et al.[311] has proposed a more advanced maturation cocktail (often referred to as the 'gold standard') that includes IL-1β, IL-6, TNFα and Prostaglandin E2 (PGE_2). This combination of cytokines was shown to be superior in activating T cells and polarizing $CD4^+$ cells towards a Th1 phenotype. Subsequently, a variety of further studies have compared different cytokine cocktails and proposed different solutions for optimal DC maturation, cytokine release profile and subsequent T cell activation[312–317]. In these studies, the maturation step typically lasts 24 or 48 hours after GM-CSF & IL-4 differentiation, and there is some evidence suggesting neither incubation period is inferior[318].

DC maturation state is often measured by change in costimulatory molecule expression by flow cytometry. Molecules such as CD80, CD83, CD86, CD40, MHC Class I and II are expected to increase upon maturation[294]. CD4, conversely, is expected to decrease[319]. Studies also complement this with measurements of 'Signal 3' cytokine release, especially IL-12p70 (Th1 polarising) cytokine[312–314, 316, 317]. Importantly, some studies directly assess T cell activation capacity of matured DC[311–317], which does not always correlate with costimulatory marker profile[312–314, 316]. This indicates that although cytometry-measured costimulatory profile is useful in assessing whether maturation has occurred, it may not accurately predict the ability of mature DCs to activate T cells.

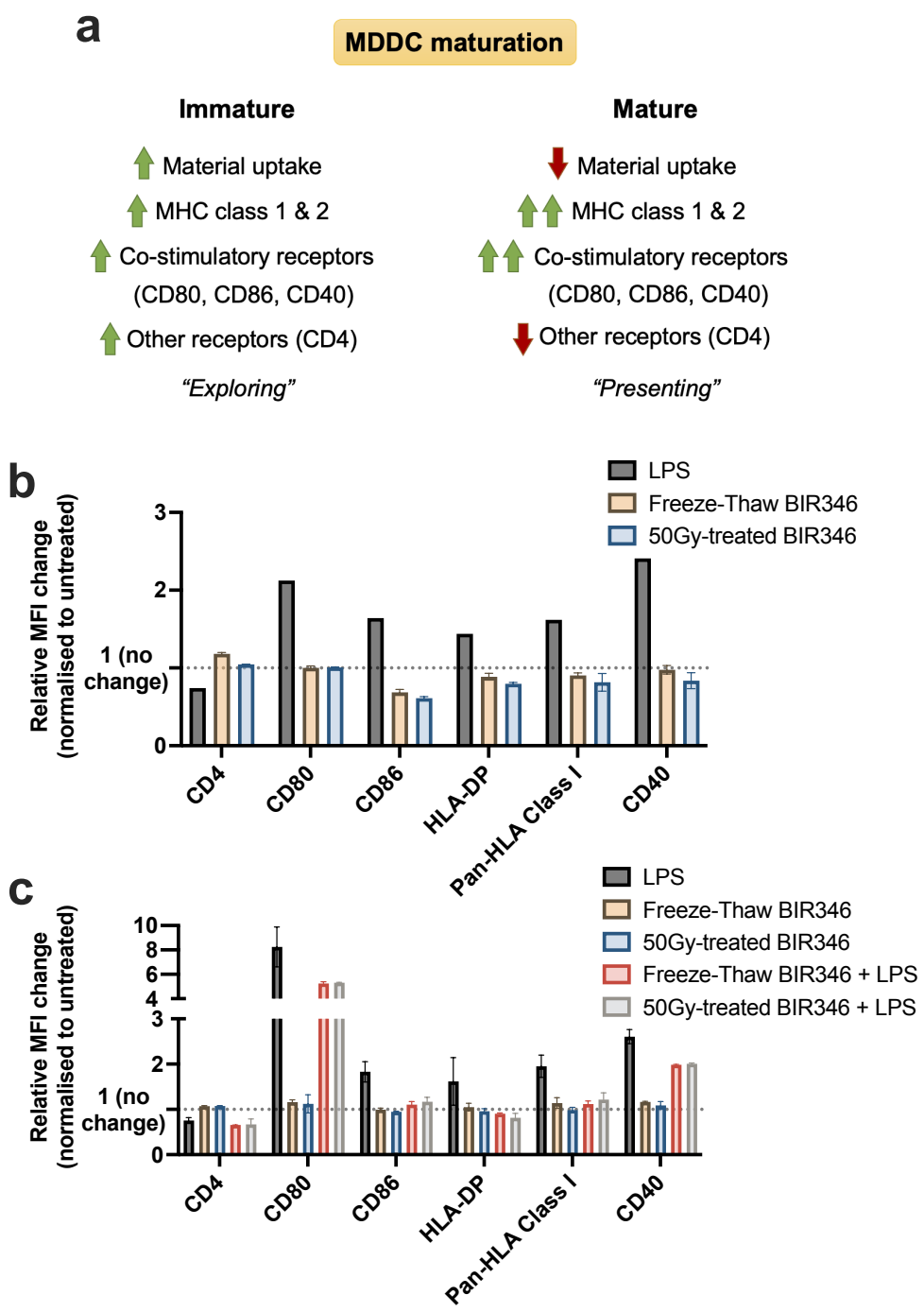

Figure 4.12: MDDC maturation does not occur when cells are treated with AML lysate only. **a** Overview of distinctions between mature and immature MDDC. **b** and **c** Show relative change in indicated cell surface markers upon 24-hour MDDC treatment with different sources of extracellular antigen or combination of maturation agent (LPS) with extracellular antigen. LPS, lipopolysaccharide used at 100ng/ml. Gy (Gray), unit of ionizing radiation. Each plot shows an independent experiment. Error bars indicate standard deviation between technical replicates.

Cocktail	Cytokines	TLR ligands	Other Additives
DC1		100 ng/ml LPS	
DC2	0.05 ng/ml IFNγ	100 ng/ml LPS	
DC3	0.05 ng/ml IFNγ	100 ng/ml LPS + 20 µg/ml pI:C + 2.5 µg/ml R848	
DC4		2.5 µg/ml R848 + 20 µg/ml pI:C	1 µg/ml PGE_2
DC5	10 ng/ml TNFα + 10 ng/ml IL-1β + 0.25 ng/ml IFNγ	100 ng/ml LPS + 20 ng/ml pI:C + 1 µg/ml R848	
DC6	10 ng/ml TNFα + 10 ng/ml IL-1β + 0.25 ng/ml IFNγ	100 ng/ml LPS + 20 ng/ml pI:C + 1 µg/ml R848	250 ng/ml PGE_2

Figure 4.13: Overview of different MDDC maturation cocktails used in this work.

Thus, to start with, I wanted to assess if AML debris treated MDDCs are mature when compared to positive control MDDCs treated with LPS (Fig. 4.12b,c). Two types of antigen source were tested - freeze-thawed and high-dose irradiated cancer cells, to assess if either is sufficient in providing the necessary maturation signals. However, neither version of AML debris treated MDDCs were mature according to the expression of cell surface proteins. Moreover, costimulatory receptor expression did not fully improve when AML debris treatment was combined with LPS itself, suggesting that the AML debris may inhibit DC maturation (Fig. 4.12c). Interestingly, a similar inhibitory effect on DC maturation has been reported before[320].

Importantly, although the costimulatory marker profile was affected by the AML debris treatment even in presence of LPS, it is unclear if that would still prevent antigen-specific T cell activation. Thus, further experiments were designed to read out the effects of MDDC maturation on PADI4 TCR T cell activation directly.

First, a wider range of different maturation cocktails was designed based on previous literature[312, 315–317]. Since the cocktails typically have a combination of proinflammatory cytokines, TLR ligands and/or possibly other additives (such as PGE_2), different cocktails in this work were designed to represent the variety of different ingredient class combinations and complexities (Fig. 4.13).

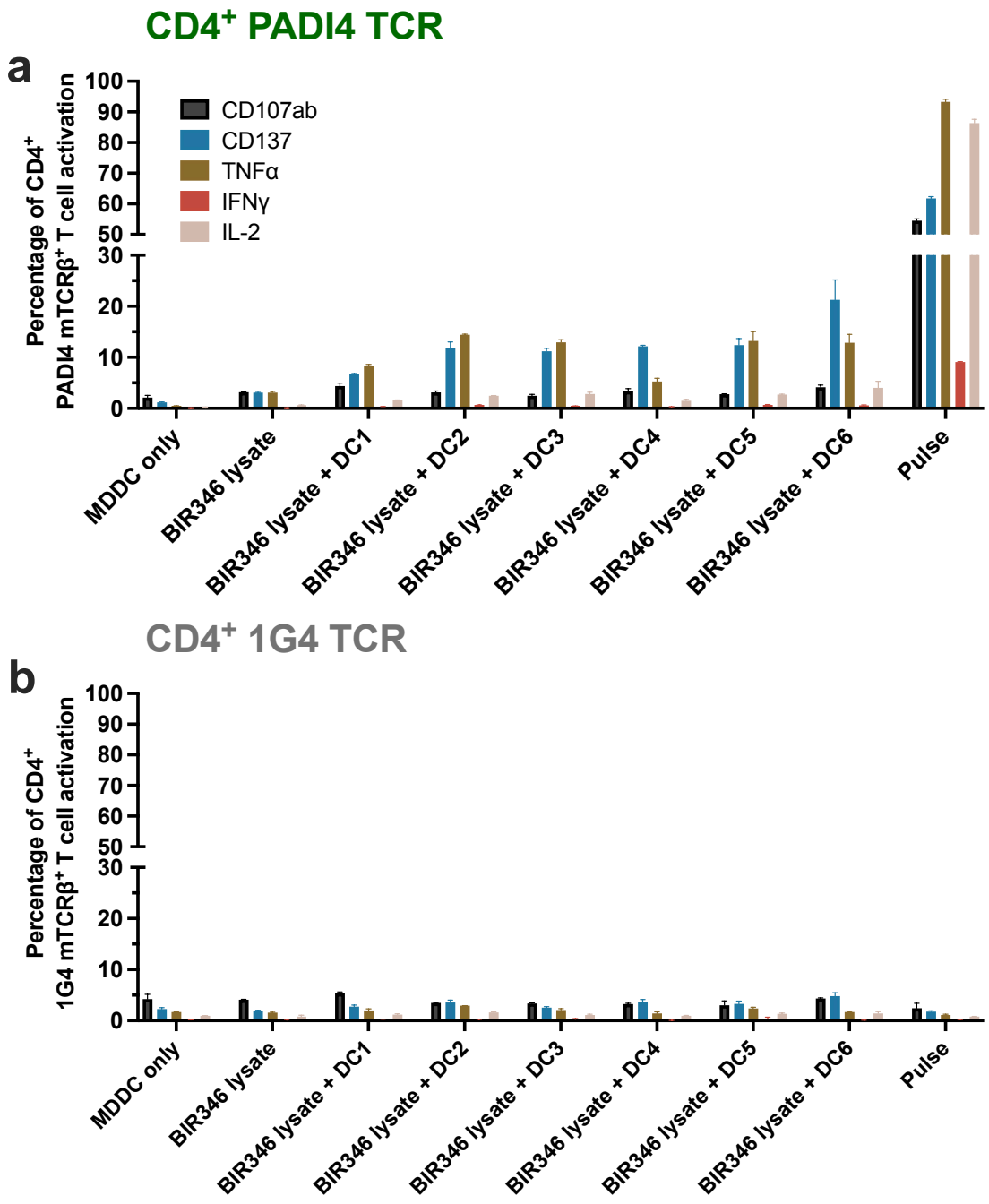

Figure 4.14: MDDC maturation improves PADI4 TCR T cell activation to AML-derived PADI4 antigen ($CD4^+$ T cell data). **a** and **b** show PADI4 TCR and 1G4 (irrelevant) TCR transferred T cells, respectively, after 9 hours of co-culture with target cells. MDDC were pre-treated with lysate and maturation cocktails 24 hours prior to T cell co-culture. Pulsed condition indicates MDDC were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to T cell co-culture. For maturation cocktail explanation see Figure 4.13. Error bars indicate standard deviation between technical replicates.

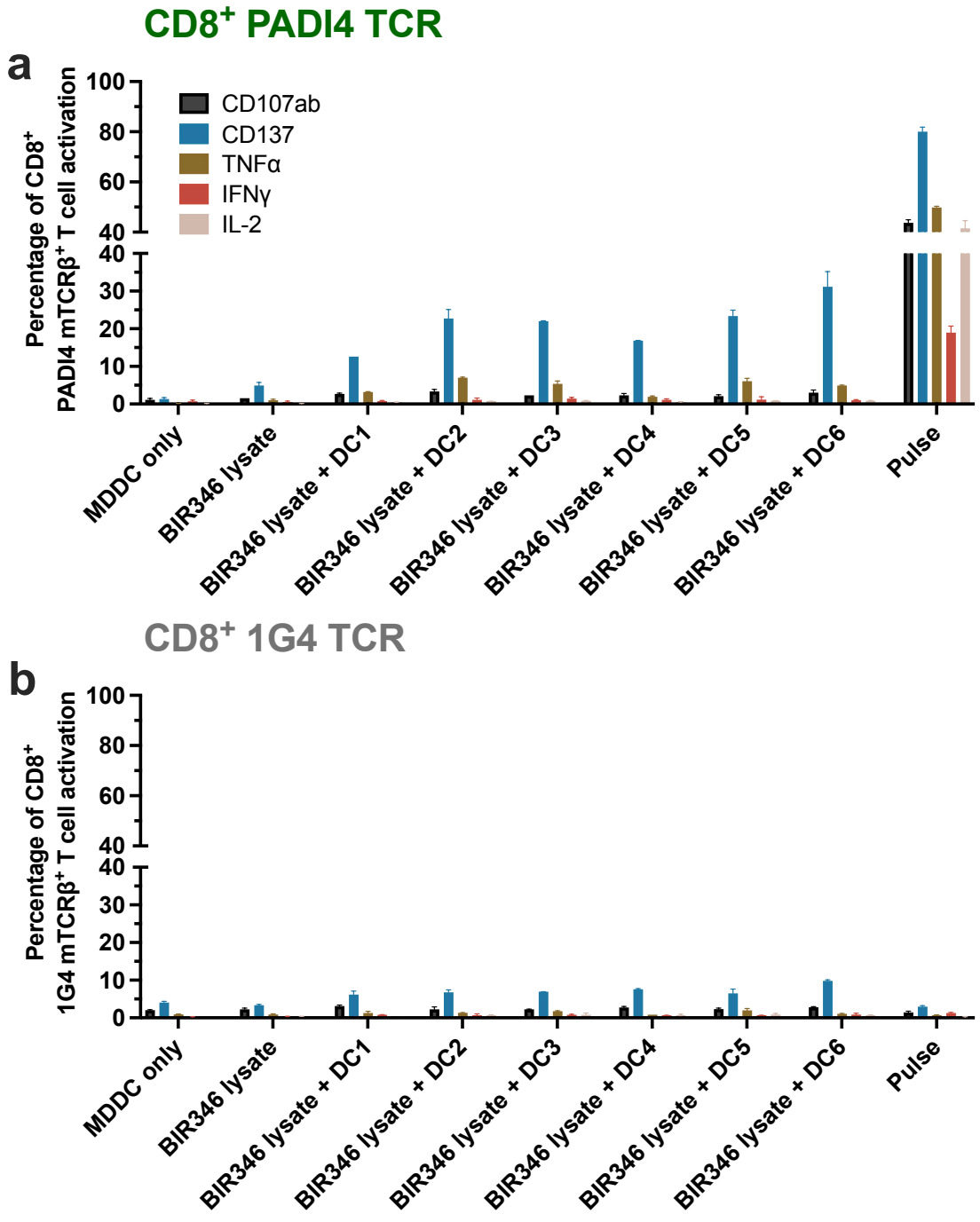

Figure 4.15: MDDC maturation improves PADI4 TCR T cell activation to AML-derived PADI4 antigen ($CD8^+$ T cell data). For figure explanation, see figure 4.14 legend.

Next, MDDCs were treated with either AML lysate only or in combination with different maturation cocktails for 24 hours. Then, TCR-transferred T cells were added and activation was read out by flow cytometry (Fig. 4.14 and 4.15). All maturation cocktails potentiated T cell activation, supporting the hypothesis that effective PADI4 antigen presentation by MDDC requires a maturation step. Arguably, DC6 cocktail produced highest T cell activation and so further experiments were done with this maturation cocktail.

Two crucial aspects remained unclear from these experiments. For one, is the maturation cocktail-dependent T cell activation due to the maturation effect on MDDC or rather a direct effect on T cells themselves? Upon MDDC exposure to DC cocktails and lysate, neither were removed from culture before T cells were added. Given that the cocktails contain proinflammatory signals, one could argue this may affect T cell functionality directly. Although irrelevant TCR transduced T cell controls provide reasonable evidence that such is not the case (Fig. 4.14b and 4.15b), PADI4 TCR T cell incubation with MDDC and DC cocktail (in absence of AML lysate) would provide the most relevant control to rule this out.

Secondly, the data shown here does not fully confirm that the PADI4 TCR T cell activation is leukaemia-derived PADI4 antigen dependent. Although the sample used for AML lysate has been investigated for PADI4 genotype and expression (Fig. 4.3), it is still unconfirmed that PADI4 TCR recognises the PADI4 variant peptide presented on MDDC. A suitable control to test this would be to use an AML sample with the *PADI4* SNP-WT genotype as a lysate for MDDCs.

Thus, the next experiment introduced these controls (Fig. 4.16) and confirmed that PADI4 TCR T cell activation is antigen and MDDC maturation-dependent. Furthermore, antigen specificity was also shown previously in cancer cell lines overexpressing PADI4 with either SNP-Var or SNP-WT genotype (see Chapter 3).

Finally, to show that this is applicable beyond the one *PADI4* SNP-Var$^+$ sample used so far (BIR346), lysates of samples RLH032 and RUH039 were also used in the same T cell activation experiment set-up (Fig. 4.17). Although for CD4$^+$ T cells it showed similar antigen-specific activation results as seen with the sample BIR346, CD8$^+$

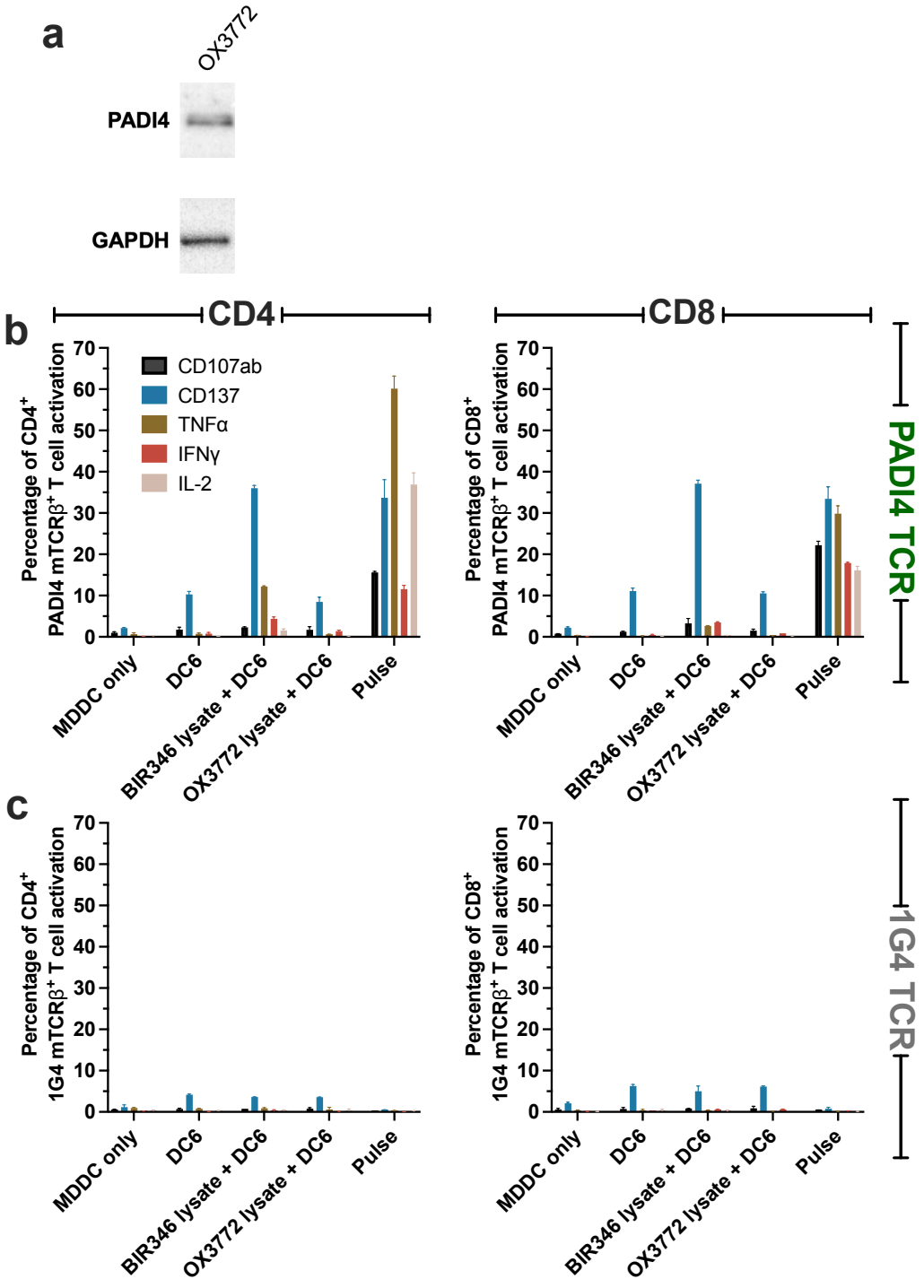

Figure 4.16: MDDC-mediated PADI4 TCR T cell activation is AML-derived PADI4 antigen dependent. **a** Western blot of OX3772, an AML sample with the *PADI4* SNP-WT genotype. **b** and **c** show PADI4 TCR and 1G4 (irrelevant) TCR transferred T cells, respectively, co-cultured with MDDC for 9 hours. $CD4^+$ (left) and $CD8^+$ (right) T cells are plotted separately. MDDC were treated with indicated AML freeze-thaw lysate and/or DC6 for 24 hours prior to T cell co-culture. Pulsed condition indicates MDDC were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to T cell co-culture. Error bars indicate standard deviation between technical replicates.

T cell compartment had a lot of background activation in all conditions treated with DC6 cocktail (Fig. 4.17b). Since this was never seen before (see Fig. 4.16 and 4.15), the result is likely an outlier. Unfortunately, due to time constraints and limited sample availability, this experiment could not be repeated.

4.5 Closing Remarks

To summarise, this chapter aimed to assess PADI4 TCR anti-leukaemic efficacy in primary AML samples *in vitro*. To start with, suitable target samples were identified by not only screening for the *PADI4* and *HLA-DPB1* restricting genotypes and expression but also sample viability, predicted blast purity and available biobanked cell number. Out of 499 screened samples, 22 fulfilled the *PADI4* and *HLA-DPB1* restricting genotype criteria, and, of those, three had markedly higher expression of both PADI4 and HLA-DP at protein level (BIR346, RLH032 and RUH039). Finally, only one sample out of the three (BIR346), had high cell viability at thaw and persistence in cell culture, as measured by the ability to activate PADI4 TCR T cells when pulsed (Fig. 4.4).

To begin with, AML sample co-cultures with TCR-transduced T cells aimed to assess if antigen-specific activation can be achieved by direct recognition of the target. When this has proven unsuccessful, several AML sample treatment regimens, as well as a prolonged co-culture period, were tested to see if direct PADI4 TCR T cell activation can be seen then. The treatment regimens explored whether MHC class II upregulation (IFNγ), proinflammatory signals (DC6 cocktail), extracellular PADI4 protein (target cell lysate), dendritic cell differentiation cytokines (GM-CSF & IL-4) or different treatment combinations can facilitate efficient PADI4 antigen presentation in target cells. Alas, no condition showed evidence of antigen-specific T cell activation.

This suggested that the PADI4 antigen may be more efficiently presented to antigen-specific T cells by a professional APC. Monocyte derived dendritic cells (MDDC) were produced from healthy peripheral blood and subsequently treated with AML sample lysate to reveal that indeed antigen-specific T cell activation can be achieved via an APC. Furthermore, higher and more reproducible activation of PADI4 TCR T cells was seen when the MDDCs were matured. These findings finally revealed that the

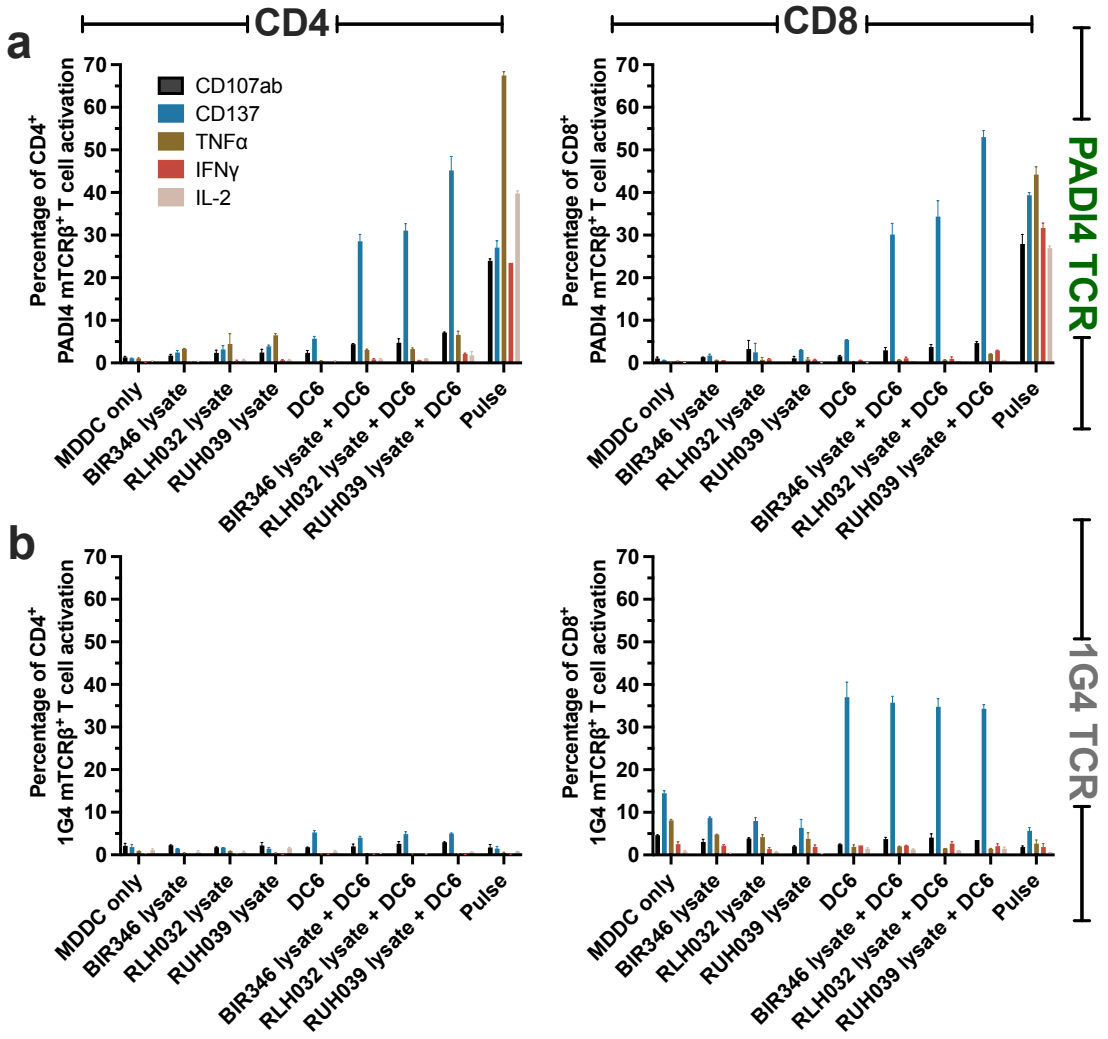

Figure 4.17: Matured MDDC can present PADI4 antigen to PADI4 TCR T cells from different primary AML samples with PADI4-Var genotype and expression. a and **b** show PADI4 TCR and 1G4 (irrelevant) TCR transferred T cells, respectively, co-cultured with MDDC for 9 hours. $CD4^+$ (left) and $CD8^+$ (right) T cells are plotted separately. MDDC were treated with indicated AML freeze-thaw lysate and/or DC6 for 24 hours prior to T cell co-culture. Pulsed condition indicates MDDC were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to T cell co-culture. Error bars indicate standard deviation between technical replicates.

PADI4 antigen can be produced and presented from primary AML at sufficient levels to elicit PADI4 TCR T cell recognition.

Chapter 5

Investigation of PADI4 TCR Toxicity to Normal Tissues

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5.1 Overview

So far PADI4 TCR has been investigated for its efficacy to recognise leukaemia-derived antigen and exert an anti-leukaemic effect. However, it remains unclear what, if any, toxicity this TCR could have to healthy tissues. Indeed, the original study to identify putative GvL-specific T cells from post-allo-HSCT patients did not restrict the pool of screened allogeneic peptides based on their specificity to leukaemia (see Chapter 1). Thus, it remains crucial to identify if the PADI4 TCR is capable of recognising healthy tissues, therefore mediating GvHD as well. In addition, evaluation of the TCR toxicity to healthy tissues would inform if PADI4 antigen is a good candidate for targeted immunotherapy.

To begin with, available public RNA expression datasets from healthy tissues were

investigated. Similarly to the identification of suitable leukaemia samples or cell lines, it was assumed that the most likely target cells are those with the highest expression of both the *PADI4* and the *HLA-DPA1 & HLA-DPB1* genes.

These criteria have identified healthy haematopoietic cells as the most likely targets, and thus further investigation was limited to this compartment. To obtain suitable samples, several sources of both cryopreserved and fresh healthy PB or BM samples were screened for restricting *PADI4* and HLA-DPB1 genotypes. In addition, optimal target cell purification protocols were established.

Finally, PADI4 TCR-transduced T cells were placed in co-culture with different purified healthy haematopoietic cells. The ability of target cells to activate PADI4 TCR T cells in an antigen-specific manner, with or without exogenous PADI4 variant peptide pulse, was assessed. Furthermore, antigen-specific T cell cytotoxicity to the targets was measured using cytometry-based *in vitro* killing assays.

5.2 PADI4 Expression Across Healthy Tissues and Cell Types

To start with, publicly available consensus RNA expression data across healthy tissue types was downloaded from The Protein Atlas database. The data was plotted to show *PADI4* expression almost exclusively in the bone marrow and spleen, suggesting haematopoietic restriction (Fig. 5.1a)[219]. Further haematopoietic cell type *PADI4* expression pattern was investigated by using public single cell or FACS sorted bulk RNA sequencing datasets (Fig. 5.2)[252–254]. This showed that within the haematopoietic compartment, *PADI4* expression is highest in mature neutrophils, immediate neutrophil precursors (promyelocytes, myelocytes) and monocytes. Meanwhile, expression in haematopoietic stem and progenitor cell compartments (abbreviated as HSPC and is composed of HSC, MPP, CMP, GMP, MEP, LMPP, CLP) is generally lower, whilst more mature lymphoid and erythroid compartments express *PADI4* the lowest. Taken together, this suggests myeloid lineage bias for the expression of this gene.

Besides *PADI4* expression in the bone marrow and spleen, some expression was also seen in the lungs (Fig. 5.1a). Upon careful investigation of different tissue RNA expression datasets on The Human Protein Atlas[219, 220], virtually every tissue

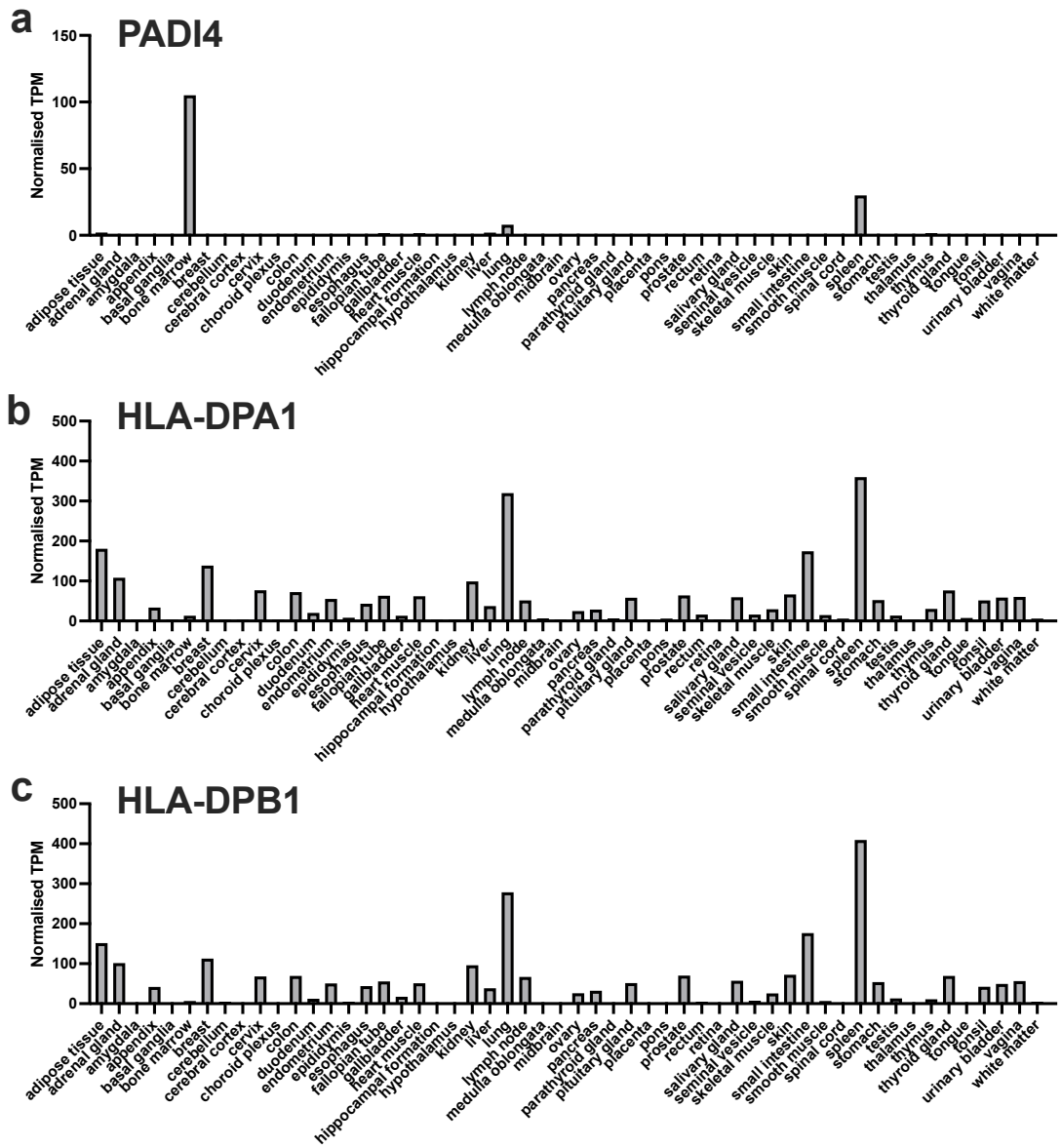

Figure 5.1: The *PADI4* gene is expressed exclusively in the haematopoietic compartment. a, **b** and **c** show *PADI4*, *HLA-DPA1* and *HLA-DPB1* consensus RNA expression across healthy tissues, respectively. The data is derived from The Human Protein Atlas Consensus Tissue Expression dataset[219, 220] (see Chapter 2). TPM, transcripts per million.

had some expression of *PADI4* because of the tissue-resident haematopoietic cells - typically macrophages, other histiocytes (such as Kupffer and Hofbauer cells) as well as granulocytes (including basophils and eosinophils).

However, PADI4 TCR would only recognise antigen presented on the HLA-DP heterodimer. MHC class II is typically expressed in professional APCs, which are of haematopoietic origin (dendritic cells, macrophages, B cells). Indeed, spleen was the biggest source of expression across different tissues (Fig. 5.1b,c). Expectedly, within the haematopoietic compartment, expression of both *HLA-DPA1* and *HLA-DPB1* genes was highest in professional APCs. However, most other haematopoietic cell types also expressed some *HLA-DPA1* and *HLA-DPB1*, with expression being lowest in T cells, neutrophils and erythroblasts (immediate erythrocyte precursors) (Fig. 5.3). Indeed, this pattern of expression is conserved at the protein level as well (Fig. 5.4).

HLA-DP gene expression appeared to be less restricted to the haematopoietic tissue sites (bone marrow and spleen) than *PADI4* (Fig. 5.1). However, closer inspection of the RNA expression datasets on The Human Protein Atlas showed that most of the *HLA-DPA1* and *HLA-DPB1* gene expression across different tissues and cell types was derived from tissue resident macrophages, B cells, dendritic cells (*e.g.* Langerhans cells). However, there were examples of non-haematopoietic sources of expression as well, such as endothelial cells and enterocytes[219, 220]. Thus, the *HLA-DPA1* and *HLA-DPB1* gene expression pattern is generally more diffuse than the *PADI4*, but mostly restricted to the cells of haematopoietic origin.

Nevertheless, it is important to consider this data in the context of existing literature on *PADI4* gene expression and function. It is known that RNA expression can have a variable correlation with protein levels[321]. Thus, any functional or protein level data on PADI4 may more accurately indicate which cells could be the primary targets of PADI4 TCR on-target off-tumour toxicity.

As discussed in Chapter 1, PADI4 has diverse roles within the haematopoietic compartment. It is particularly important in granulocytes, where it is essential for NETosis[240], but has also been implicated in the regulation of granulocyte differentiation[236]. This is in line with the *PADI4* expression being the highest in neutrophils and their immediate

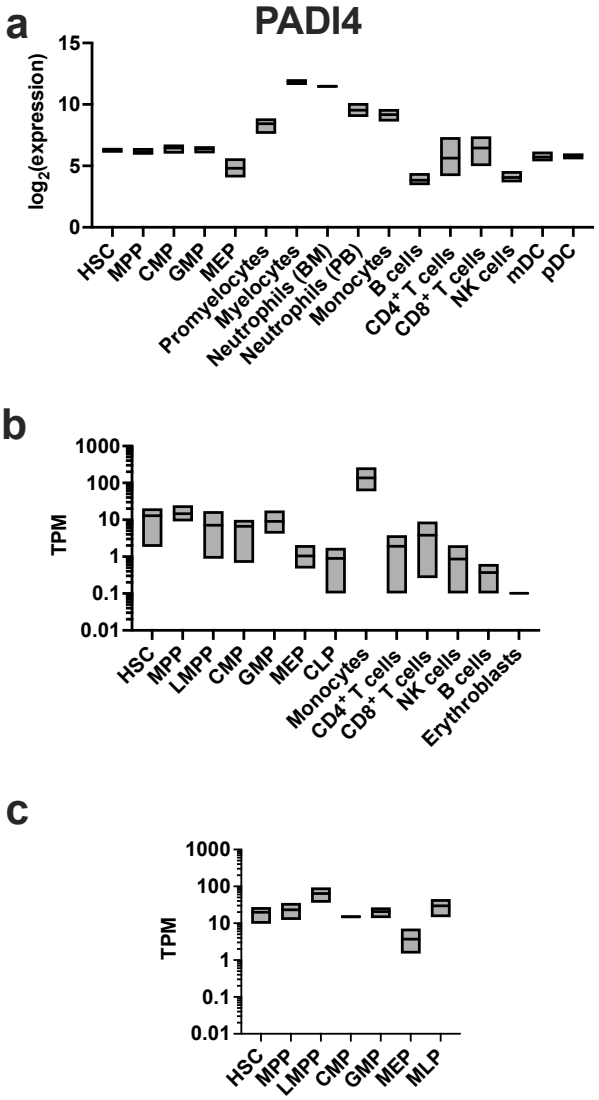

Figure 5.2: The *PADI4* gene is mostly expressed in the granulocytic and monocytic lineages. **a**, **b** and **c** show RNA expression of *PADI4* across haematopoietic cell types from the Bloodspot[252], Corces et al.[253] and Karamitros, Stoilova et al.[254] datasets, respectively. TPM, transcripts per million. Bars show mean with minimum and maximum values. n= 3-14 samples per condition.

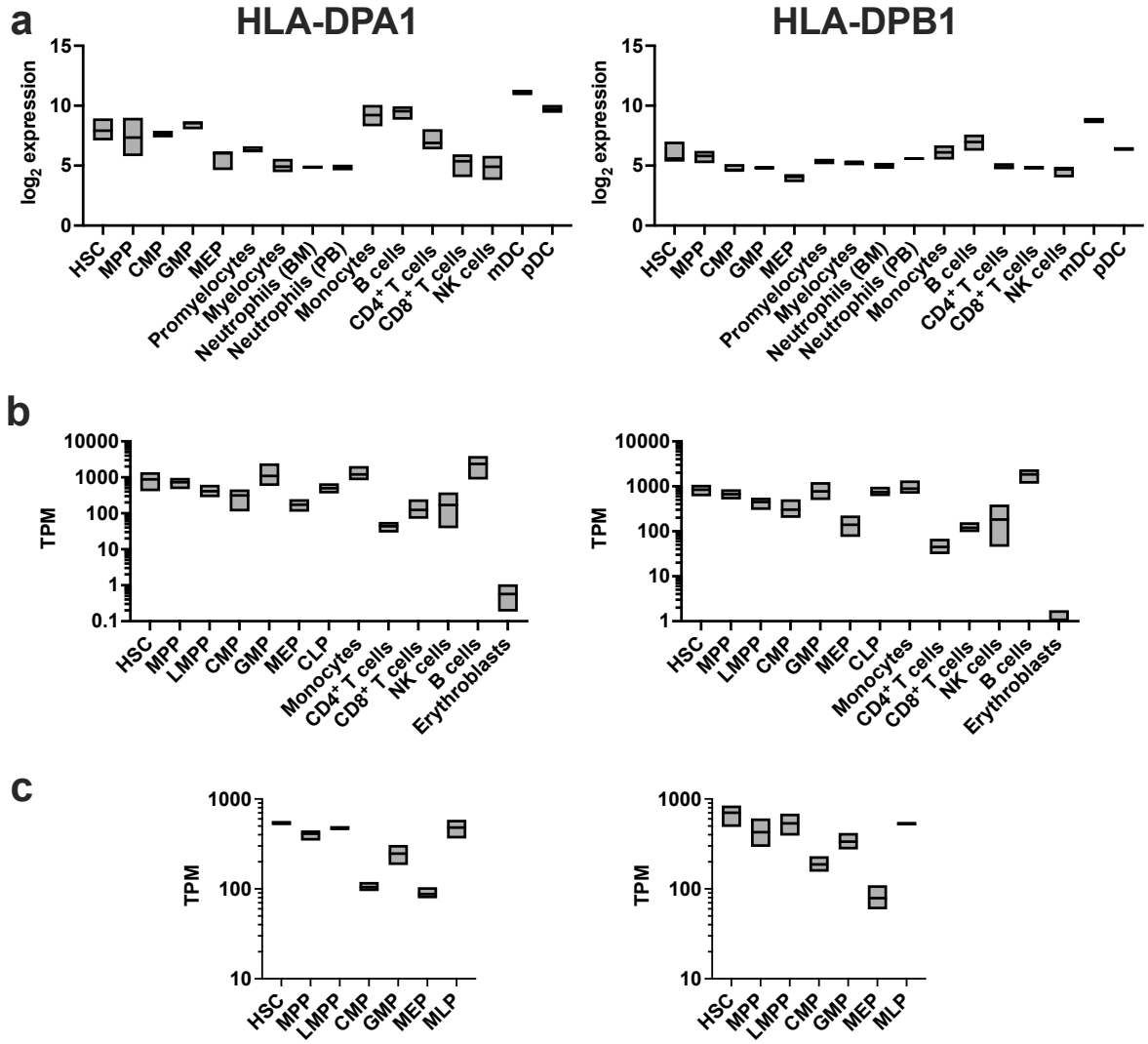

Figure 5.3: *HLA-DPA1* and *HLA-DPB1* expression is highest in professional APCs. a, **b** and **c** show RNA expression of *HLA-DPA1* (left) and *HLA-DPB1* (right) across haematopoietic cell types from the Bloodspot[252], Corces et al.[253] and Karamitros, Stoilova et al.[254] datasets, respectively. TPM, transcripts per million. Bars show mean with minimum and maximum values. n= 3-14 samples per condition.

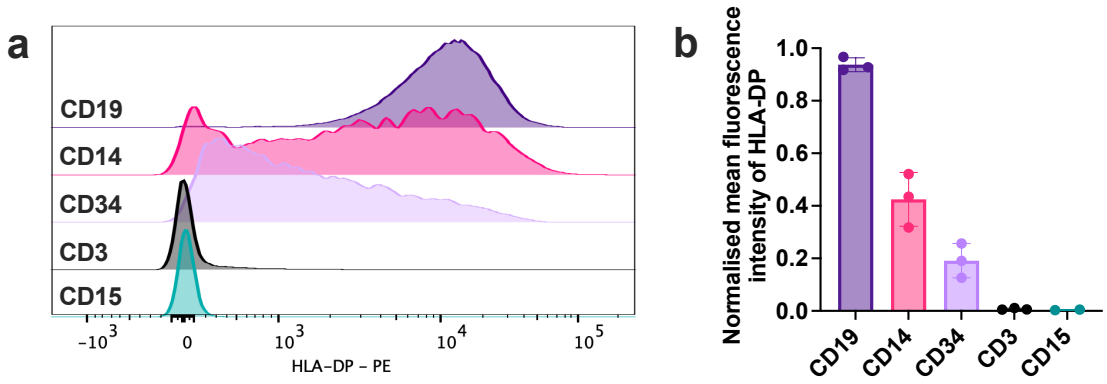

Figure 5.4: HLA-DP surface expression by cytometry confirms public RNA sequencing data. **a**, Representative HLA-DP surface expression profiles of indicated healthy haematopoietic populations *ex vivo*. **b**, Relative surface HLA-DP mean fluorescence intensity (MFI). CD19: B cells, CD14: monocytes, CD34: haematopoietic stem and progenitor cells (HSPCs), CD3: T cells, CD15: neutrophils. Numbers are normalised to the highest value across technical replicates within an experiment. Each dot represents an independent experiment. Error bars show standard deviation.

precursors (Fig. 5.2). *PADI4* has also been proposed to regulate stemness and quiescence of haematopoietic and other stem cells[235, 237] and indeed some *PADI4* expression is seen in the HSPC subsets as well (Fig. 5.2). Moreover, monocytes are amongst the cells with highest *PADI4* expression (Fig. 5.2). Similarly to granulocytes, its expression has been described as increasing with differentiation[222]. Furthermore, western blot confirmed PADI4 protein presence in purified monocytes from healthy donor PB[243]. Although, to my knowledge, the significance of PADI4 in monocytes is unclear, there is some evidence to suggest that macrophages can increase histone H3 citrullination upon stimulation with LPS. In turn, PADI4 inhibition with Cl-amidine reduced proinflammatory cytokine secretion from LPS-activated macrophages[322]. Thus, it may be that PADI4 has a role in macrophage activation. Finally, although lymphocytes are amongst the lowest *PADI4* expressing haematopoietic cell types (Fig. 5.2), there is evidence that both B and T cells have PADI4 protein[323]. Its role in these cell types is poorly characterised, apart from a few reports suggesting PADI4 promotes T cell function in autoimmune diseases. However, it is unclear if this effect is cell autonomous[324, 325].

Therefore, both public RNA expression datasets and previously published literature on *PADI4* suggest haematopoietic restriction of the PADI4 antigen. Next, I decided

to test the potential PADI4 TCR toxicity to the haematopoietic compartment. As all major blood and bone marrow populations (HSPCs, T cells, B cells, monocytes and neutrophils) have been shown to both express *PADI4* as well as contain its protein product, it is important to assess any potential toxicity to all of them. Of course, HLA-DP expression in these cell types is highly divergent at steady state (Fig. 5.4), which may be the determining factor for direct PADI4 TCR recognition. Alas, it is unknown what minimum level of MHC Class II expression would be required for antigen-specific T cell activation. Hence, this was not used as a criterion to exclude putative target cell populations from *in vitro* testing.

5.3 Healthy Primary Cell Sourcing and Processing

Multiple sources of healthy haematopoietic cells are available

Similarly to the PADI4 TCR experiments in leukaemia cell lines and primary AML samples, healthy haematopoietic cells require to fulfill *PADI4* SNP-Var and HLA-DPB1*04:01 genotype criteria to be considered potential targets (Fig. 3.6). Unlike in leukaemias, where both HLA-DP and PADI4 protein levels are considered variable (*cf.* Fig. 3.8, 3.9, 4.1, 4.3 and Appendix Fig. 13), healthy *ex vivo* haematopoietic cells are assumed to have relatively invariable expression levels (see previous section). Thus, in this chapter, none of the samples or cell subsets were screened and selected for minimum protein-level expression of PADI4 or HLA-DP before use in experiments.

Multiple sources were available to obtain purified fractions of HSPCs ($CD34^+$), B cells ($CD19^+$), monocytes ($CD14^+$) and T cells ($CD3^+$) because these cell types can be readily cryopreserved for later use. For neutrophils ($CD15^+$), only fresh PB samples could be used as these cells have a short half-life[326] and poorly tolerate cryopreservation. In total, three different sources were screened to identify potential samples: 1) MARCH study collection of cryopreserved PB and BM samples from volunteers that underwent a hip replacement; 2) National Health Service Blood and Transplant (NHSBT) collection of cryopreserved CB; 3) Healthy fresh PB donors at the Weatherall Institute of Molecular Medicine (WIMM).

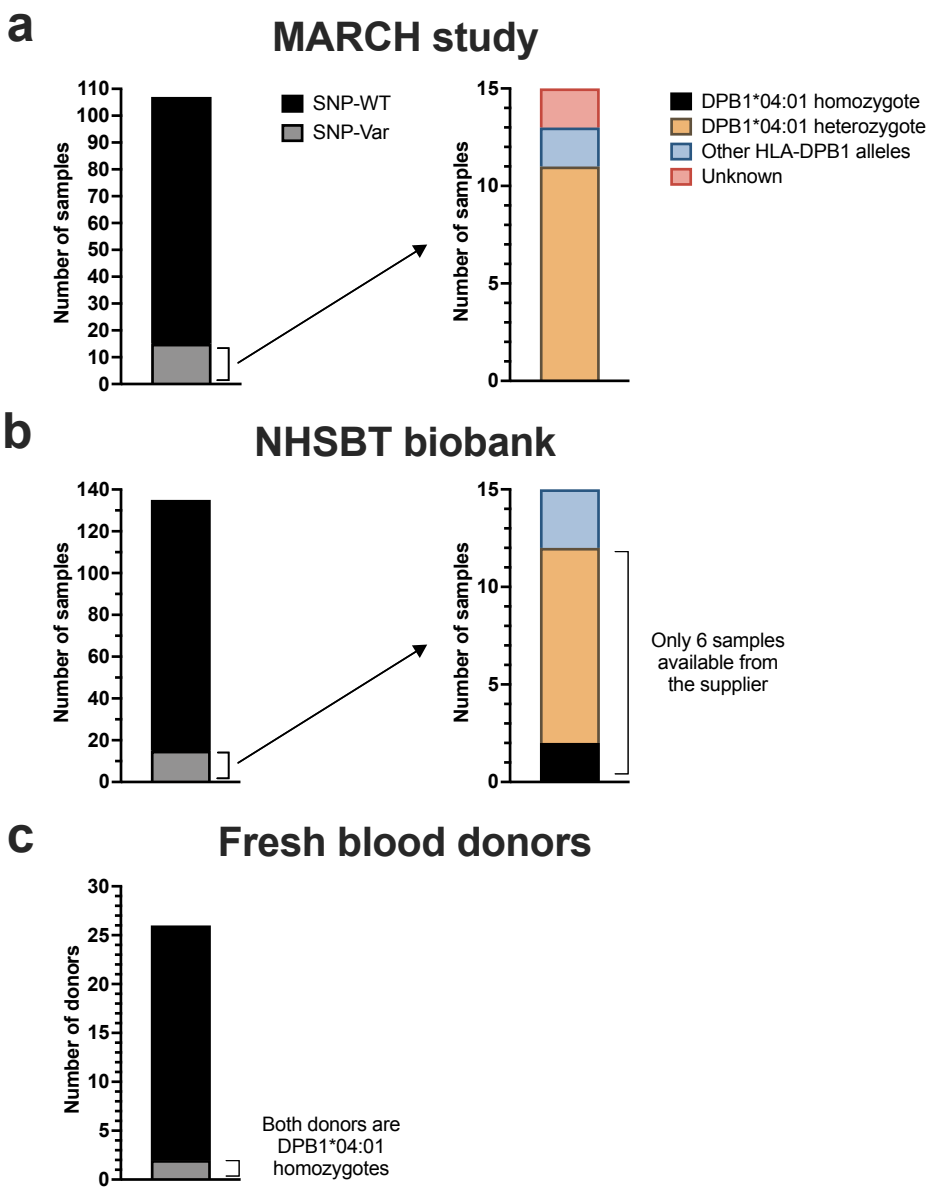

Figure 5.5: Different healthy haematopoietic sample sources are available for PADI4-TCR T cell toxicity investigation. **a**, **b** and **c** show MARCH study BM samples, NHSBT CB samples and available PB donors, respectively. All available samples/donors were first genotyped for *PADI4* SNP (left). Subsequently, all *PADI4* SNP-Var samples/donors were genotyped for the restricting HLA-DPB1*04:01 allele (right).

In all cohorts, multiple PADI4 SNP-Var samples/donors with the HLA-DPB1*04:01 (heterozygous/homozygous) allele were identified (Fig. 5.5). For MARCH collection, out of 107 samples screened, 15 were identified as *PADI4* SNP-Var heterozygotes and, out of those, 11 as HLA-DPB1*04:01 heterozygotes. For NHSBT collection, out of 135 samples screened, 15 were identified as *PADI4* SNP-Var heterozygotes and, out of those, 10 as HLA-DPB1*04:01 heterozygotes and two as HLA-DPB1*04:01 homozygotes. While all identified MARCH study samples were available in-house, only 6/12 NHSBT samples were available for purchase from the supplier. Finally, due to the limited number of available fresh PB donors, only two were identified to carry the *PADI4* SNP-Var genotype (heterozygous). Fortunately, both samples were also HLA-DPB1*04:01 homozygotes (Fig. 5.5c). As healthy fresh PB donors are the only source for neutrophils, this limited all *PADI4* SNP-Var experiments with neutrophils to two samples (independent repeats) in total.

Target cell populations require purification from primary samples

Concomitantly with suitable sample identification, methods for the purification of target cell populations were developed. For HSPC, magnetic enrichment using $CD34^+$ selection beads was done prior to the cryopreservation of mononuclear cell samples from CB and BM. For all other mononuclear cell populations, enrichment with respective magnetic beads was done either using cryopreserved and CD34-depleted mononuclear fraction or density separated fresh PBMC. For neutrophil enrichment, fresh blood samples were density separated and the entire bottom (erythrocyte) layer was incubated with the red cell lysis solution to produce a white pellet.

For the assessment of population purities post-enrichment, samples were stained with antibodies and acquired at the cytometer. As the purified populations would subsequently be co-cultured with effector T cells for up to 24 hours, it is also important to assess if the target cells can be efficiently captured and enumerated at 24 hours in culture. This unveiled a technical difficulty in the CD3-enriched cell population (Fig. 5.6). It appeared that although magnetic purification was efficient, the $CD3^+$ cell purity could only be captured immediately after enrichment (Fig. 5.6b) but not 24 hours after

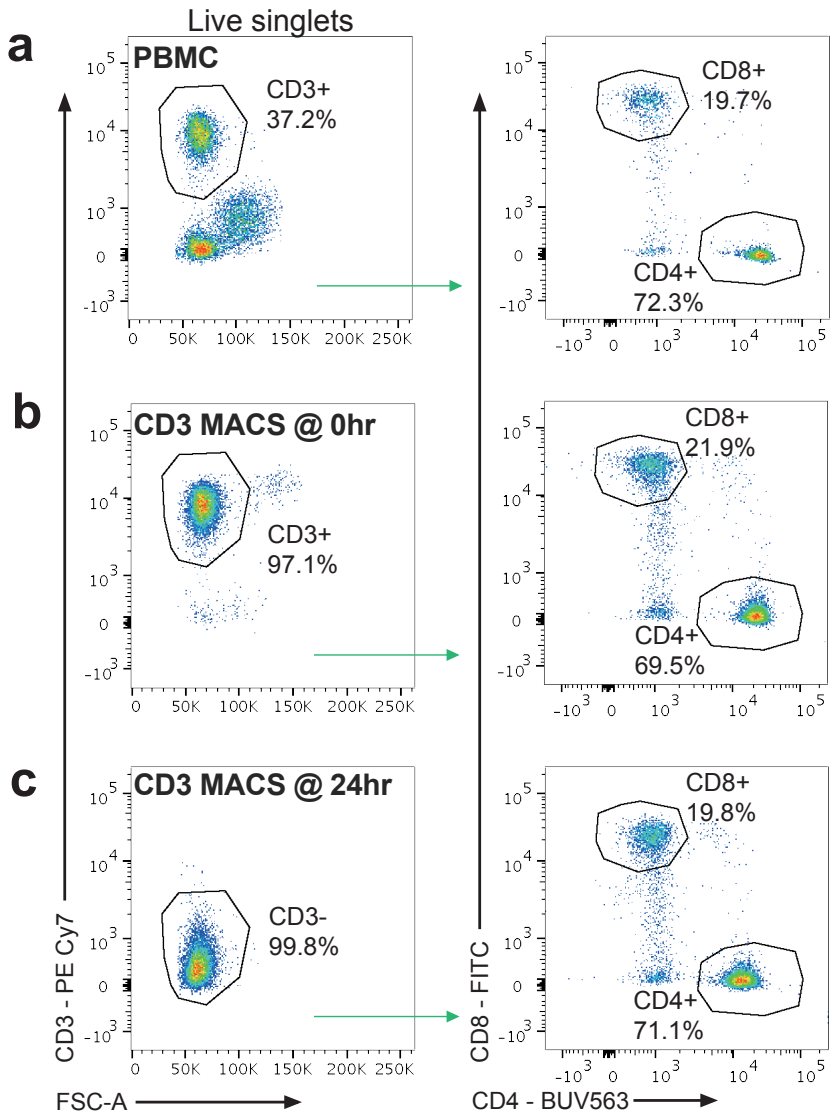

Figure 5.6: Magnetic CD3$^+$ cell separation interferes with cytometric CD3$^+$ purity assessment. Healthy PBMC were used for magnetic CD3$^+$ cell separation. **a**, Starting CD3$^+$ cell purity in the PBMC sample. **b**, CD3$^+$ purity immediately after magnetic separation. **c**, CD3 staining is largely negative at 24 hours post-magnetic separation.

(Fig. 5.6c). Although the reasons for this are unclear, it could be caused by the CD3 selection beads themselves. During the purification protocol, they are not removed from the final purified cell fraction. It could be that the beads act as a stimulation signal in T cells, eventually leading to increased intracellular TCR-CD3 complex retention[327]. In turn, this could explain markedly lower CD3 signal seen at 24 hours in culture (Fig. 5.6c).

Another difficulty emerged with the purity measurement of CD19-enriched cell population. Staining with CD19 antibody did not work even immediately after purification. This was only partially improved when antibody was added in at the same time as magnetic beads (Fig. 5.7a). Based on this, I hypothesised that the magnetic separation beads and the anti-CD19 antibody compete for the same epitope.

Next, I tested several alternatives for B cell purity measurement. Anti-IgM staining, which labels antigen naïve B cells and a minority of antigen stimulated cells[328], could only capture a proportion of B cells when compared to CD19 staining in the pre-purification PBMC sample. Unsurprisingly, post-purification, IgM marker did not perform well either, with only around half of the cells staining positive (Fig. 5.7b). Anti-CD20 staining, which also labels antigen naïve B cells[329], performed better. For one, similar proportion of pre-purification PBMC were labelled with CD20 antibody as with the CD19. Reassuringly, post-purification, around 90% of cells were $CD20^+$ (Fig. 5.7c). Finally, I tried using secondary anti-mouse IgG staining. Since CD19 magnetic beads are not removed from the purified cell fraction, I hypothesised that the beads themselves could be labelled immediately post-purification whilst still on the surface of the purified cells. Indeed, this staining proved most efficient at enumerating cell purity, with around 94% of cells labelled as positive (Fig. 5.7d).

Thus, two changes in the target cell purification protocol were implemented. Firstly, cells were assessed for purity immediately post-purification in order to avoid any loss in the marker of interest. Reassuringly, in all target cell populations except HSPC the resultant purity was typically 90% or above. For HSPC, $CD34^+$ purity was was highly sample dependent as well as typically lower, varying between 50% and 90%. Secondly, B cell population purity after magnetic enrichment was assessed using the secondary anti-mouse IgG antibody stain. Together, these changes allowed to reproducibly enrich

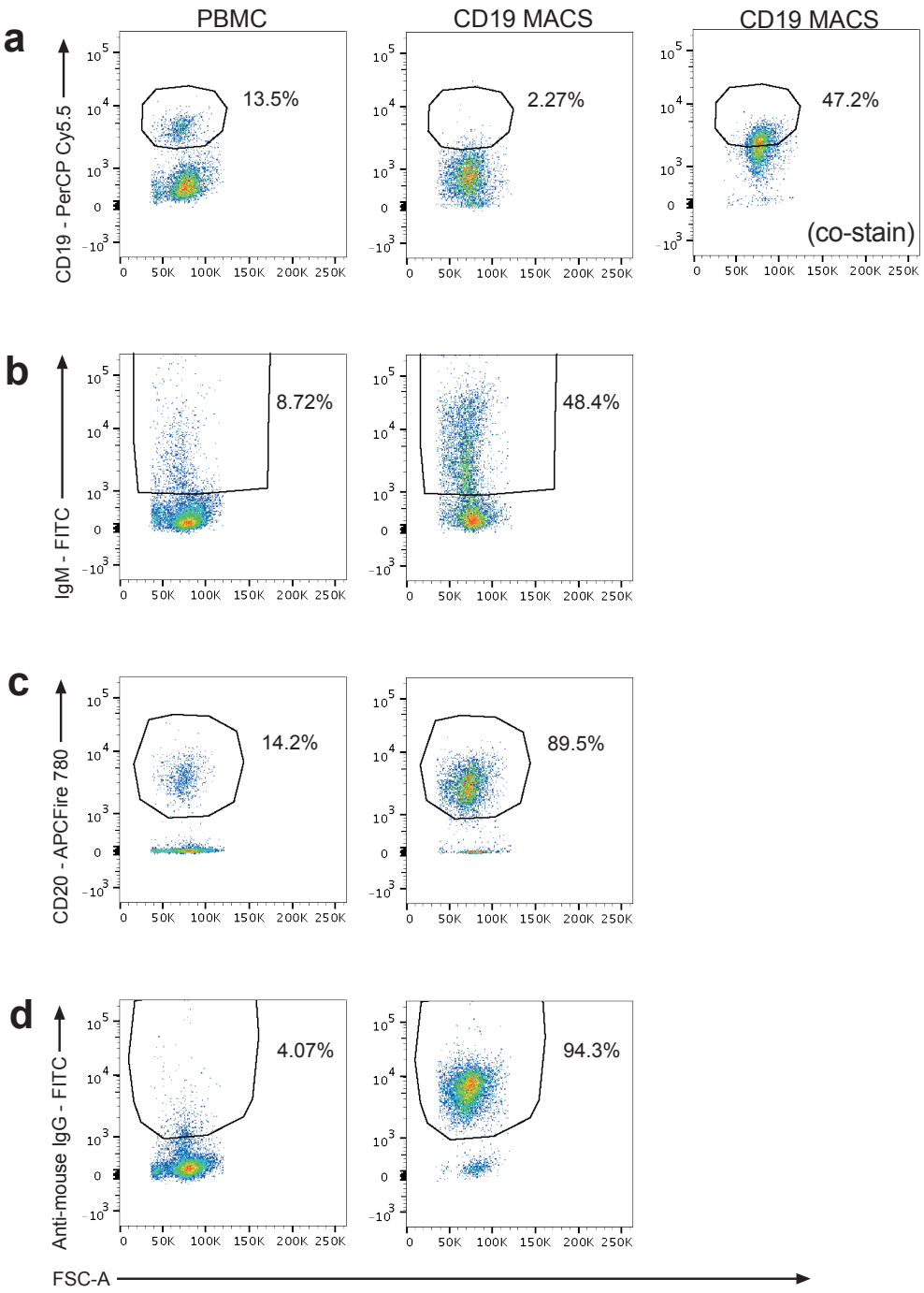

Figure 5.7: Magnetic CD19$^+$ cell separation interferes with cytometric CD19$^+$ purity assessment. Healthy PBMC were used for magnetic CD19$^+$ cell separation. **a**-**c** show sample stains with different antibodies targeting common B cell markers: CD19, IgM and CD20, respectively. **d** shows sample staining with secondary anti-mouse IgG antibody, which targets CD19 magnetic beads attached to the purified B cell surface. Left column shows starting marker prevalence in the PBMC sample. Centre column shows marker prevalence immediately post-magnetic separation. Right column (in **a** only) shows CD19 marker positivity when CD19 PerCP Cy5.5 antibody is added to the cells at the same time as CD19 magnetic beads (co-stain), rather than after (the middle column).

for and measure the purity of all target populations going into co-culture assays with PADI4 TCR T cells (Appendix Figure 15).

Purified cell populations differ in their *in vitro* persistence

Finally, persistence of purified target cell populations in short-term *in vitro* cultures was assessed. Given that activation to target cells in previous chapters 3 & 4 was typically seen at 9-24 hours in co-culture, normal haematopoietic target cells must remain viable in culture for at least this period. For neutrophils this is especially relevant due to their short life span - it is estimated that the circulating PB neutrophil half-life is less than 1 day[326]. Furthermore, it is important to consider monocyte adherence to the tissue culture flask, which may affect overall target cell recovery post-culture at the cytometer. This would be especially relevant for PADI4 TCR T cell killing assays, where the remaining target cells must be carefully enumerated.

Thus, once the target cell populations were isolated and assessed for their purity (Appendix Figure 15), 50,000 cells were plated in each well of a tissue culture plate for either a 6 or a 24-hour culture (Fig. 5.8). After, absolute cell counts were calculated using cytometry counting beads. Considering that CD3 marker decreases in culture (see previous section) and all purified cell populations except HSPC are typically at least 90% pure, target cells were gated as Singlet/Live. However, for lower purity HSPC samples, CD34 marker was used to enumerate the cells more precisely.

The total number of recovered cells was highly dependent on the sample/individual experiment (Fig. 5.8a). Reassuringly, both neutrophils and monocytes were recovered after culture, although the absolute number of cells at 6 hours decreased to around half at 24 hours. The numbers of available HSPC and B cells were always limiting, thus the data on their persistence is mostly available for the 24-hour culture only. Nevertheless, both B cells and HSPC were efficiently recovered too, although on average the recovered HSPC number was lower, at around 30,000. This is mostly attributed to the impurity of CD34-enriched target cell samples (Appendix Figure 15). Overall, all the purified target cell populations were efficiently recovered at both timepoints. Thus, this experimental setup permits measurement of T cell-related toxicity.

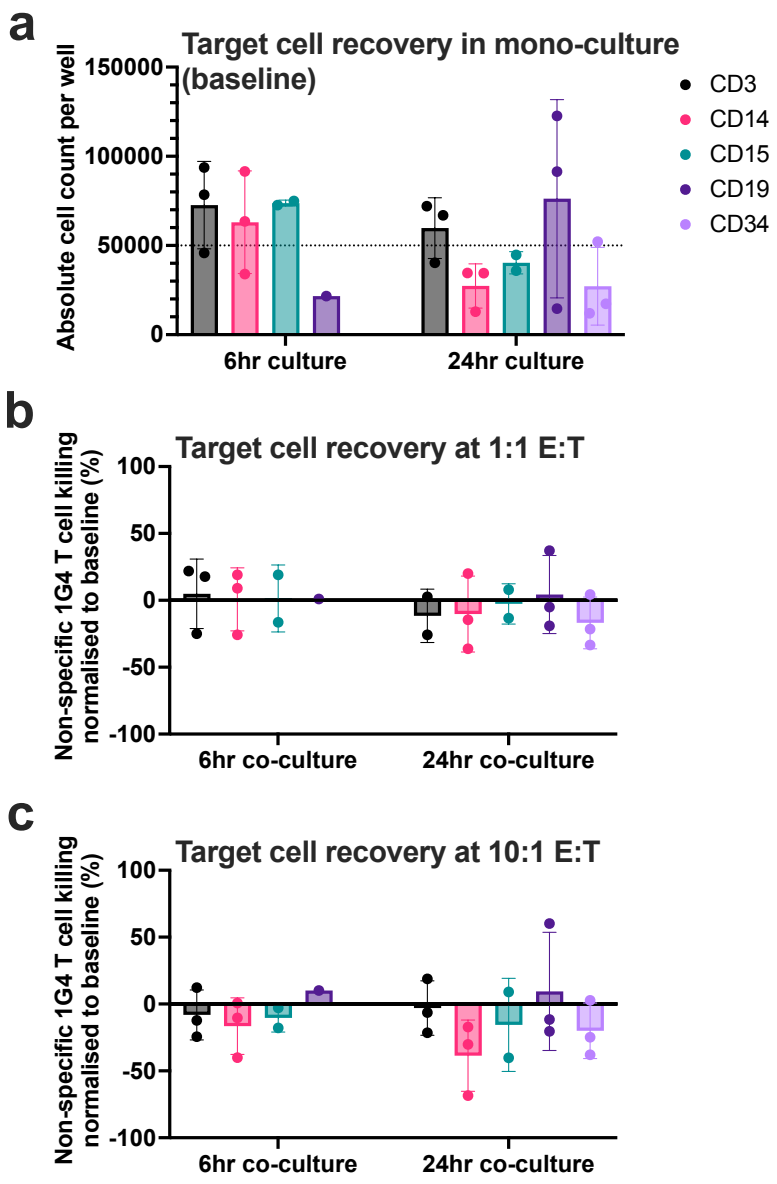

Figure 5.8: Healthy haematopoietic target cells have different persistence *in vitro*. Purified healthy target cells were cultured for 6 or 24 hours. After, their absolute cell numbers were enumerated using cytometry counting beads. **a**, Absolute cell numbers. Dashed line indicates added cell number into each well at timepoint 0 hours. **b** and **c** show the change in absolute target cell number when they are co-cultured with non-specific TCR-transduced T cells (1G4). **b** shows 1:1 and **c** shows 10:1 E:T ratio. Each dot represents an independent experiment. Error bars show the standard deviation.

Next, I wanted to assess how target cell recovery may be affected by the co-culture with irrelevant TCR-transduced T cells. The killing of targets in this condition could occur as the TCR-transduced T cells are non-specifically activated during the TCR transduction protocol. To assess this, purified target cell populations were plated alone or in co-culture with 1G4 TCR T cells at either 1:1 or 10:1 effector-to-target ratio (Fig. 5.8b,c). Reassuringly, no toxicity was seen in any of the target populations at 1:1 ratio. At 10:1 ratio, some toxicity was seen in the 24-hour co-culture condition with monocytes, where recovery of the target cells was around 40% lower than in the condition where monocytes were cultured alone. Overall, this experiment showed that the target cells can be co-cultured with TCR-transduced T cells for activation and killing assays with the PADI4 TCR.

5.4 *In Vitro* PADI4 TCR T cell Assays with Healthy Haematopoietic Target Cells

Activation assays show antigen-specific T cell activation to monocytes

To begin with, isolated *PADI4* SNP-Var haematopoietic target cells with the HLA-DPB1*04:01 allele were co-cultured with TCR-transduced T cells for 24 hours to read out any PADI4 antigen-specific activation using T cell activation markers CD107a & CD107b (co-stain), CD137, TNFα, CD134 and CD69 (Fig. 5.9). Activation in PADI4 TCR T cells was seen to both target T cells ($CD3^+$) and monocytes ($CD14^+$). For the former, the highest marker was CD69 in $CD4^+$ T cells (8.4%) and CD137 in $CD8^+$ T cells (18.5%). For the latter, the highest marker was CD69 in $CD4^+$ T cells (41.0%) and CD134 in $CD8^+$ T cells (21.0%). However, the irrelevant 1G4 TCR condition showed similar levels of activation in response to target T cells, suggesting effector T cell activation for that condition is not antigen-specific (Fig. 5.9a,c). Activation to monocytes, however, had a low background in the irrelevant 1G4 TCR condition, suggesting this response was mediated by the presence of the PADI4 antigen.

Non-antigen specific T cell activation to $CD3^+$ targets was reproducible (n=2). This prompted to investigate the reason for this effect. I hypothesised that this may be a consequence of the positive magnetic bead separation of $CD3^+$ cells prior to

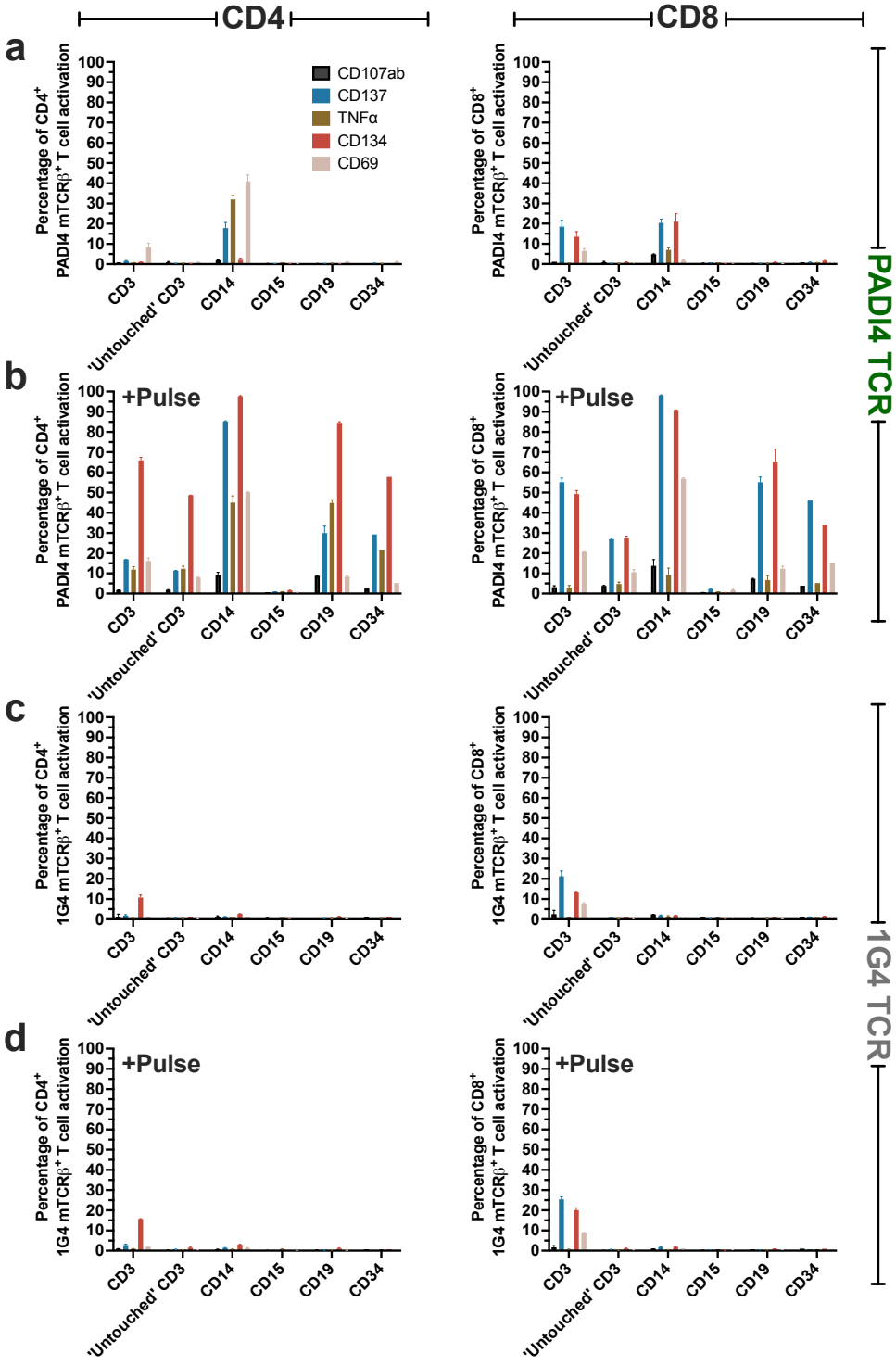

Figure 5.9: *PADI4* SNP-Var monocytes activate PADI4-TCR T cells. Panels **a** and **b** show T cells transferred with PADI4 TCR, while panels **c** and **d** show T cells transferred with 1G4 TCR. All targets have PADI4-Var and HLA-DPB1*04:01 genotypes. Columns show $CD4^+$ (left) and $CD8^+$ (right) T cells. In panels **b** and **d**, target cells were pulsed with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Cells are mixed at 1:2 E:T ratio and co-cultured for 24 hours. Graphs show one experiment and error bars represent standard deviation between technical replicates. This assay was independently repeated once more (data not shown).

the co-culture with the effector T cells. As mentioned previously, the purification process did not remove magnetic beads from the isolated population. Hence, the remaining anti-CD3 beads may activate not only the target cells but also the effector cells. To test this, I enriched for $CD3^+$ target cells by sequentially depleting $CD19^+$ and $CD14^+$ cells from PBMC (*i.e.* negative selection). This yielded a lower purity $CD3^+$ population of 71.4%, named 'untouched' CD3 (*cf.* 99.2% purity in the positively selected population; Appendix Figure 15). As expected, 'untouched' CD3 targets no longer activated TCR-transferred T cells (Fig. 5.9), confirming that previously seen activation in $CD3^+$ targets is not antigen-specific. Instead, this result suggested it was an artifact of the target cell purification protocol.

Target cell pulsing with the PADI4 variant peptide showed the different propensities target cells have for activating PADI4 TCR T cells in conditions of surplus antigen (Fig. 5.9b,d). PADI4 TCR T cell activation in pulsed targets was highest in monocytes (97.8% $CD134^+$ in $CD4^+$ and 90.9% $CD134^+$ in $CD8^+$). It was somewhat lower in B cells (84.5% $CD134^+$ in $CD4^+$ and 65.3% $CD134^+$ in $CD8^+$) and lower still in HSPC (57.8% $CD134^+$ in $CD4^+$ and 34.0% $CD134^+$ in $CD8^+$). It was comparably low in $CD3^+$ (66.0% $CD134^+$ in $CD4^+$ and 49.3% $CD134^+$ in $CD8^+$), although 'untouched' $CD3^+$ was lower still (48.7% $CD134^+$ in $CD4^+$ and 27.3% $CD134^+$ in $CD8^+$), in line with lower non-specific activation background (Fig. 5.9d). Finally, pulsed neutrophils were completely unable to activate T cells.

Interestingly, different target cell propensities to activate PADI4 TCR T cells when peptide pulsed correlated with the corresponding target cell surface HLA-DP expression only partially (Fig. 5.4). Despite pulsed monocytes being the best at activating T cells, they did not express the highest level of HLA-DP. In addition, both T cells and neutrophils showed very similar surface expression of HLA-DP, but only T cells were able to activate PADI4 TCR T cells when pulsed with the peptide. These discrepancies could indicate differences in target cells beyond HLA-DP, such as presence of adequate costimulatory signals on the cell surface. However, it must be noted that HLA-DP expression comparison in these targets does not account for differences in cell size,

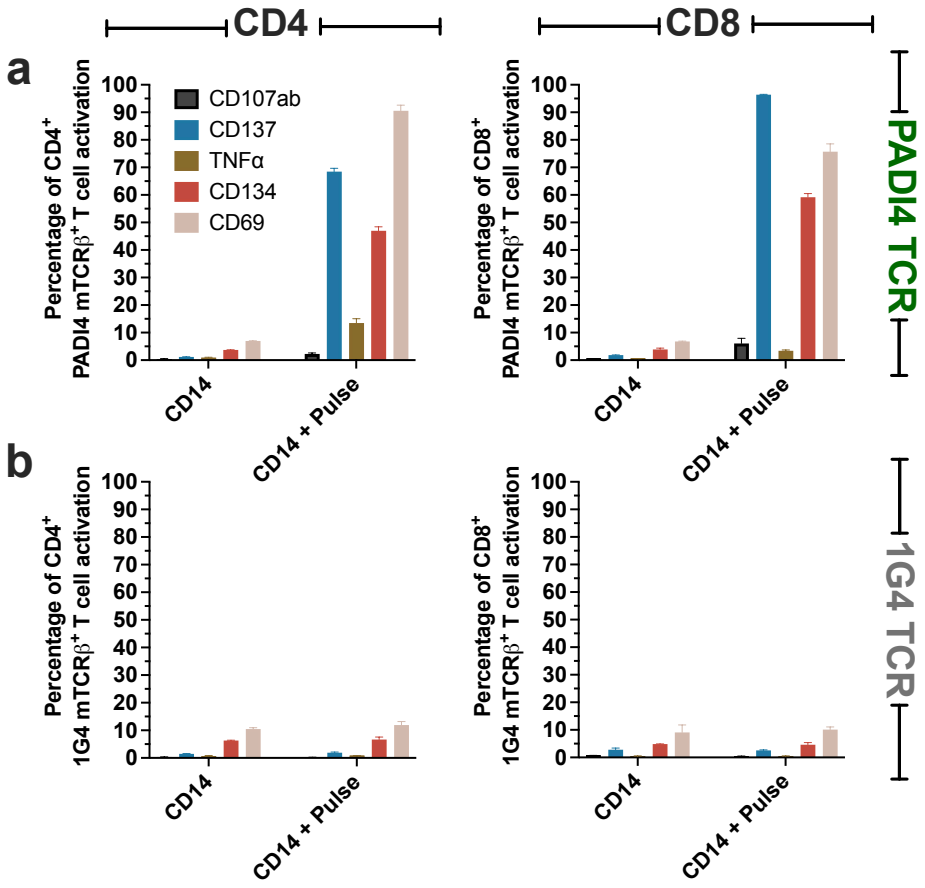

Figure 5.10: *PADI4* SNP-WT monocytes do not activate PADI4-TCR T cells. Panels **a** and **b** show T cells transferred with PADI4 TCR or 1G4 TCR, respectively. Columns show $CD4^+$ (left) and $CD8^+$ (right) T cells. 'Pulse' indicates that the target cells were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Cells are mixed at 1:2 E:T ratio and co-cultured for 24 hours. Graphs show one experiment and error bars represent standard deviation between technical replicates. This assay was independently repeated once more (data not shown).

e.g. neutrophils are much larger than T cells, despite these two cell types staining similarly for HLA-DP surface expression.

Lastly, in order to assess if the PADI4 TCR T cell activation to monocytes is PADI4 SNP-Var specific, I set up an experiment using *PADI4* SNP-WT monocytes with the restricting HLA-DPB1*04:01 allele. If the hypothesis is true, the PADI4 TCR T cells would no longer activate. Indeed, no response was seen to the *PADI4* SNP-WT monocytes in either the PADI4 TCR or the irrelevant 1G4 TCR conditions (Fig. 5.10). Importantly, when pulsed with the PADI4 variant peptide, these monocytes were still capable of inducing strong PADI4 TCR T cell activation. This result further

confirmed that the PADI4 TCR T cells can specifically recognise endogenous PADI4 antigen presented on healthy monocytes.

Killing assays suggest no antigen-specific toxicity without a peptide pulse

Although PADI4 TCR T cell activation assays have shown that the PADI4 antigen is produced and presented by healthy monocytes with the restricting genotype, this may not necessarily result in direct cytotoxicity and target cell death. To test this, 50,000 purified target cells were incubated for either 6 or 24 hours with the TCR-transferred T cells. At the end of culture, remaining target cells were quantified in the Singlet/Live or Singlet/Live/$CD34^+$ (HSPCs only) gate and total cell counts were calculated using absolute counting beads. Next, percentage killing by PADI4 TCR T cells was calculated for each condition by normalising the absolute cell number to the equivalent condition with the irrelevant 1G4 TCR T cells. In this way, data was normalised between independent experiments, removing some variability in cell handling, sample quality, target cell persistence and purity. Furthermore, normalisation to the equivalent irrelevant 1G4 TCR condition accounted for any non-specific T cell toxicity (see previous section).

The *in vitro* killing data showed no evidence that any non-pulsed target cell populations were killed in an antigen-specific manner (Fig. 5.11). Neither 1:1 nor 10:1 effector-to-target ratio was effective for either of the different purified haematopoietic target cells. However, it is important to note the high variability of data, as reflected by the large standard deviations. This may be a result of error in cell counting and pipetting of both cells and absolute counting beads. Reassuringly, where target cell killing was seen in the PADI4 variant peptide pulse conditions, the data variability was low and the killing effect was reproducible across independent experimental repeats. This confirmed that the assay has the required sensitivity to capture antigen-specific target cell killing.

Interestingly, although target T cells and monocytes activated PADI4 TCR T cells when pulsed (Fig. 5.9), they were not killed. In fact, only B cells and HSPCs were killed with the PADI4 variant peptide pulse (Fig. 5.11). Based on this, target cell killing did not correlate with the level of PADI4 TCR T cell activation. Indeed, pulsed

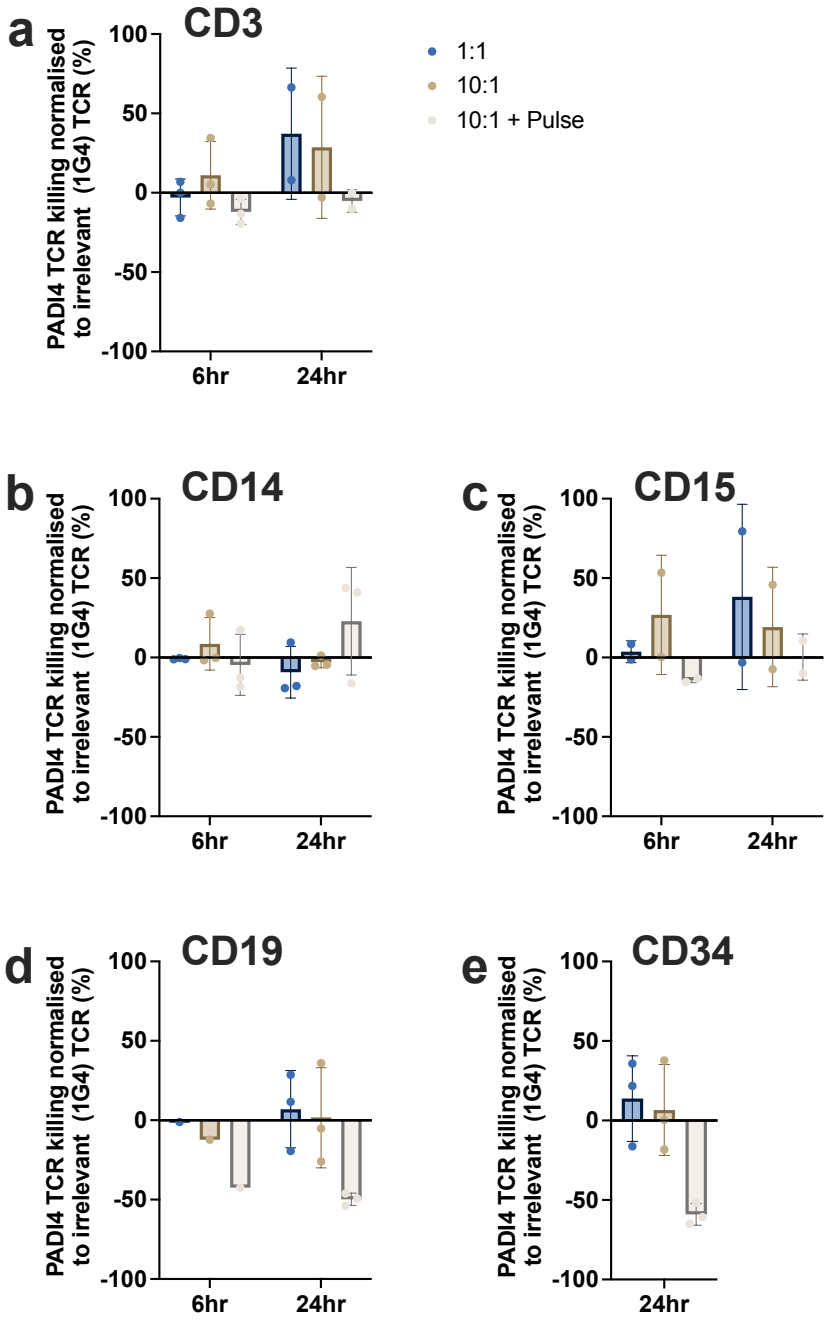

Figure 5.11: No *in vitro* killing is observed in normal *PADI4* SNP-Var haematopoietic targets. a-e show $CD3^+$, $CD14^+$, $CD15^+$, $CD19^+$ and $CD34^+$ purified target cells with restricting HLA-DPB1*04:01 allele, respectively. In each case, plots show the change in absolute target cell number after indicated period of co-culture with the PADI4 TCR T cells. The change in target cell number is normalised to control conditions where targets were co-cultured with irrelevant TCR-transduced T cells (1G4). Different bar colours indicate different E:T ratios. 'Pulse' indicates that the target cells were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Each dot represents an independent experiment and the error bars indicate standard deviation.

monocytes could activate PADI4 TCR T cells the most out of all five target cell populations studied (see previous section).

PADI4 TCR recognition elicits variable changes in target cell surface HLA-DP

To investigate the results of the *in vitro* killing experiment further, I decided to assess the target cell HLA-DP surface expression after co-culture with the TCR-transferred T cells. Although it is known what levels of HLA-DP each target cell population has at the baseline (Fig. 5.4), it is unclear how these change upon culture with effector T cells. Importantly, it may be that this changes upon antigen-specific effector T cell recognition rather than non-specific co-culture with T cells. In turn, this could further inform how different target cells respond to co-culture with the PADI4 TCR T cells.

To achieve this, mean fluorescence intensity (MFI) of HLA-DP was measured in remaining live (and $CD34^{+}$, if in HSPCs) target cells after 6 or 24-hour co-culture with effector T cells. To visualise any changes related only to the antigen-specific PADI4 TCR T cell recognition, MFI of target cells in co-culture with the PADI4 TCR T cells was normalised to the equivalent condition with irrelevant 1G4 TCR T cells. Similarly to the *in vitro* killing experiment results discussed above, no change in HLA-DP expression was seen in any experimental conditions without the PADI4 variant peptide pulse (Fig. 5.12). This includes unpulsed monocytes (Fig. 5.12b), which were shown to elicit PADI4 TCR T cell activation previously (Fig. 5.9), but were not killed by the antigen-specific T cells (Fig. 5.11).

However, some target cell populations, when pulsed with the PADI4 variant peptide, appeared to change their HLA-DP MFI in response to the PADI4 TCR T cells. Pulsed B cells and HSPCs increased their HLA-DP MFI after 24 hours of co-culture with the antigen-specific PADI4 TCR T cells. In contrast, pulsed monocytes and T cells showed somewhat lower HLA-DP MFI in the same condition. This dichotomy in the regulation of surface HLA-DP correlates with the *in vitro* killing assay results, where neither pulsed monocytes nor T cells were killed, but pulsed B cells and HSPCs were. Overall, this suggests that different target cells respond differently to antigen-specific recognition by TCR-transferred T cells. Indeed, different regulation of HLA-DP surface levels in these

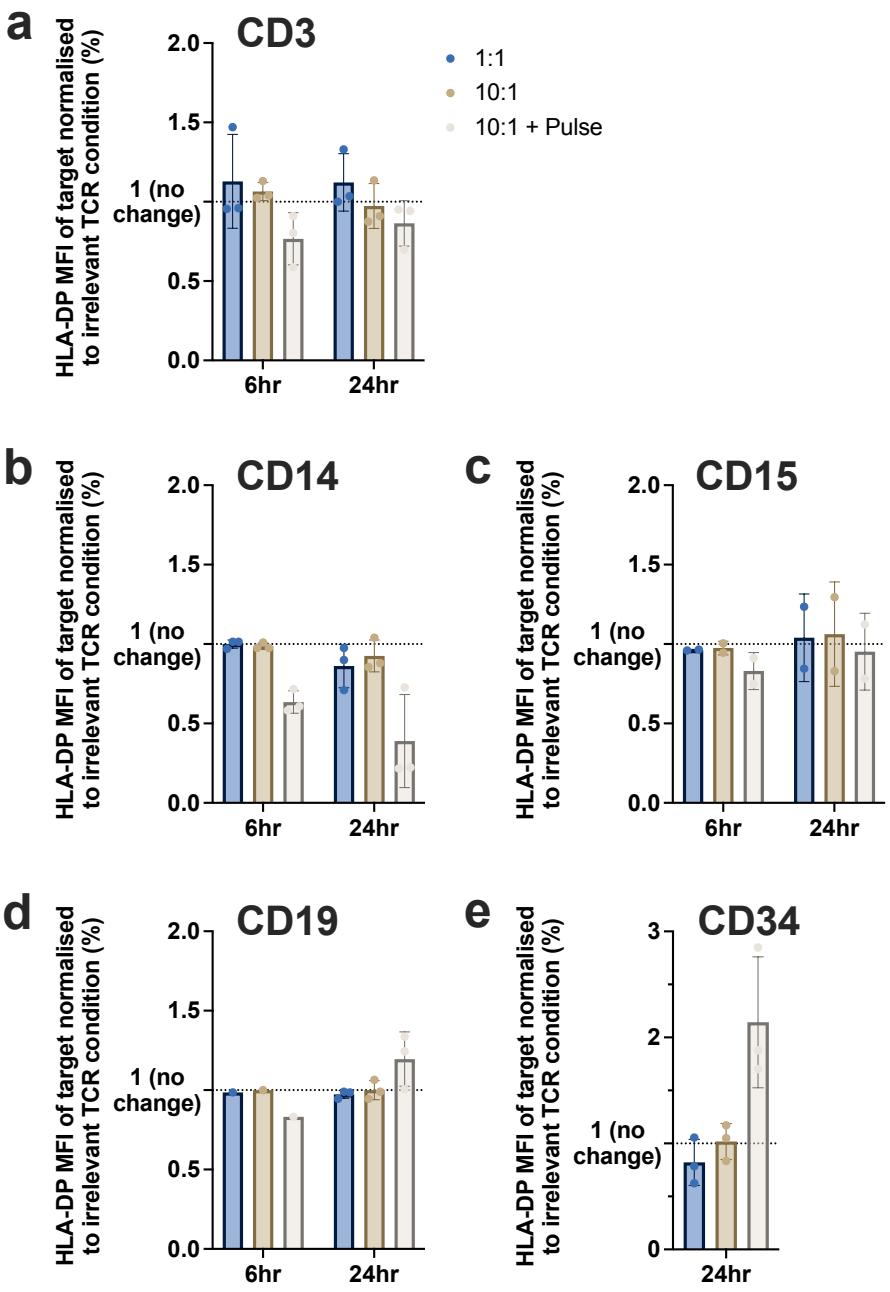

Figure 5.12: HLA-DP surface levels change in response to antigen-specific PADI4 TCR T cell recognition. a-e show $CD3^+$, $CD14^+$, $CD15^+$, $CD19^+$ and $CD34^+$ purified target cells with restricting HLA-DPB1*04:01 genotype, respectively. In each case, plots show the change in HLA-DP mean fluorescence intensity (MFI) after indicated period of co-culture with PADI4 TCR T cells. The change in MFI is normalised to control conditions where targets were co-cultured with irrelevant TCR-transduced T cells (1G4). See Figure 5.11 for further details.

experiments may allude to the mechanism which determines whether a given target cell population is killed after a pulse with the PADI4 variant peptide. However, it must be noted that these results are obtained from a small number of independent experiments and any further analysis would require more experimental repeats.

5.5 Closing Remarks

This chapter aimed to investigate putative healthy tissue toxicity of the PADI4 TCR. To start with, publicly available RNA expression data was analysed to identify healthy cell types with expression of both *PADI4* as well as *HLA-DPA1* & *HLA-DPB1*. Reassuringly, highest expressing cell types were largely restricted to the haematopoietic lineage. Furthermore, *PADI4* expression was mostly seen along the myeloid differentiation trajectory and was highest in neutrophils and monocytes. This evidence suggested that the most likely healthy tissue targets for the PADI4 TCR are haematopoietic, especially myeloid, cells.

This information supports the idea that the PADI4 TCR was mediating a GvL effect in the patient OX866, where it was originally identified. It also suggests that the transduced PADI4 TCR T cell activity should be restricted to the native haematopoietic compartment in any potential leukaemia patients who would receive it. This means that in a *PADI4* SNP mismatched allo-HSCT, where the recipient is SNP-Var and the donor is SNP-WT, the toxicity of the PADI4 TCR T cells to the healthy tissues would likely be limited, as the donor haematopoiesis is established post-transplant. However, if the PADI4 TCR T cells were used in patients without allo-HSCT or in those where both the recipient and the donor are *PADI4* SNP-Var, it would be required to establish that it does not target normal haematopoiesis at all.

Hence, this chapter further investigated if the PADI4 TCR toxicity is seen *in vitro* to healthy haematopoietic cells with the restricting genotype. Firstly, suitable primary samples were identified. Unlike in leukaemia cell lines and primary AML samples, it was assumed that the protein levels of PADI4 and HLA-DP are relatively invariant across steady state healthy cells. Thus, the samples were screened for the *PADI4* SNP-Var and the restricting HLA-DPB1*04:01 genotypes only.

Major BM and PB haematopoietic cell populations were selected for experimental investigation - T cells, B cells, monocytes, neutrophils and haematopoietic stem and progenitor cells. Upon identification of suitable samples, target cell populations were purified using magnetic beads and their purities measured using optimised cytometry methods. Importantly, short-term cultures showed that all target cell populations were able to persist for at least 24 hours, a requirement for the activation and killing assays with the PADI4 TCR.

Finally, the activation assays showed antigen-specific PADI4 TCR T cell recognition of monocytes. No other target cell was recognised without the PADI4 variant peptide pulse. Interestingly, subsequent killing assays showed no evidence of *in vitro* monocyte or any other tested target cell killing. Furthermore, only HSPCs and B cells were killed with the PADI4 variant peptide pulse, suggesting that antigen-specific T cell killing of healthy haematopoietic cells requires other factors beyond the baseline presence of the required pMHC. To investigate this further, analysis of the killing experiment results suggested that different target cells respond to the PADI4 TCR recognition differently in terms of the changes to their surface HLA-DP expression.

Chapter 6

Discussion

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6.1 Summary

To summarise, this work investigated a novel HLA-DP-restricted allogeneic T cell response to a peptide antigen produced by a non-synonymous SNP in the *PADI4* gene. To start with, the antigen-specific TCR was identified by single cell RNA sequencing of peptide pulsed patient-derived T cells. Subsequently, the TCR was cloned into a lentiviral vector for transfer to third party primary T cells. This allowed to investigate transduced T cell capacity to recognise the antigen using leukaemia cell line models, primary leukaemia samples, primary professional APCs loaded with leukaemia-derived antigen and healthy haematopoietic cells. This showed that the antigen peptide can be produced and presented on the HLA-DP complex by professional APCs and, in turn, this resulted in PADI4 TCR T cell activation and cytotoxic function. However, direct T cell activation to myeloid leukaemia cell lines and primary samples proved nuanced. Moreover, PADI4 TCR T cells were activated in response to healthy primary monocytes, suggesting putative anti-haematopoietic toxicity, however, this did not translate into any measurable *in vitro* killing. Overall, this work supports the idea that the isolated T cell response could have a specific GvL effect with minimal reactivity

against healthy tissues. In this chapter, I aim to further discuss these findings and suggest potential future research avenues.

6.2 PADI4 TCR Discovery

PADI4 TCR was successfully isolated from patient OX866 PBMC at 6 years post-transplant. Although the patient was diagnosed with intermediate risk acute myeloid leukaemia, was refractory to first line chemotherapy, received reduced intensity conditioning and subsequently went into relapse post-transplant, the disease eventually went into long-term remission after successful DLI. Up to the time of writing this, the disease has not come back (>7 years) and patient never experienced GvHD. Thus, this patient offered a rare chance to study successful and specific GvL.

The isolated response was to a myeloid lineage restricted antigen (see Chapter 5) presented on MHC class II, which further suggested its putative GvL specificity. Single-cell RNA sequencing of antigen-specific T cells even showed a cytotoxic phenotype, suggesting the TCR may enable T cells to recognise pMHC directly on cancer and enact target cell killing themselves.

However, only one TCR and one antigen (*PADI4* miHA) were discovered in patient OX866. Although this may be a result of technical limitations in the screen, from an immunological perspective that may not be unexpected. Previous studies on viral infections have indicated that out of numerous possible antigens, T cell responses usually develop to a few select antigens across different individuals, a phenomenon called immunodominance[330, 331]. Further, only few or even a single antigen-specific TCR may be found, especially in the context of chronic infection[332]. Immunodominance can also be affected by the starting naive TCR repertoire diversity[333–335]. This factor may play an especially important role in the GvL, where the TCR diversity is low in the first few years after transplantation[336, 337]. Limited evidence also suggests that some known anti-leukaemic T cell responses may be immunodominant in post-transplant leukaemia patients[163, 175]. Lastly, the GvL specific effect may even be distinguished from the GvL effect with the GvHD by the lower diversity and frequency of alloreactive antigen-specific responses post-transplant[172] (see Chapter 1).

6.3 PADI4 TCR Testing with Cell Lines

Cell line model development was limited by the available resources. Firstly, there were no cell lines that would satisfy all the required criteria of *PADI4* SNP-Var genotype, restricting HLA-DPB1*04:01 allele and the expression of both (Fig. 3.6). Next, most conservative, strategy was to specifically knock-in the *PADI4* SNP-Var genotype into a cell line otherwise satisfying all other criteria. However, this did not succeed as no viable *PADI4*-expressing myeloid leukaemia cell lines were available. Fortunately, the development of overexpression cell lines was successful, and a few different leukaemia cell lines were used for this approach.

Perhaps the biggest bottleneck for cell line models was PADI4 protein expression. Cell line characterisation in laboratory was limited to Western blot, which is a qualitative measure. In the future, development of intracellular flow cytometry for PADI4 would greatly benefit further work. For one, it could allow to precisely measure how overexpression cell lines compare to patient leukaemia samples. Also, it could help to assess how variable PADI4 protein levels are across different myeloid leukaemia cell lines and patient samples, and how that correlates with PADI4 TCR activation.

Finally, T cell activation experiments with the PADI4 overexpression cell lines showed that the PADI4 antigen can be produced by the MHC class II processing machinery, presented on the restricting HLA-DP allele and can successfully engage antigen-specific T cells. For BOLETH cell line, the data shows that in the presence of antigen, PADI4 TCR can induce T cell activation as well as inhibition of the target cell growth *in vitro*. However, the conditions for T cell activation by the myeloid leukaemia cell line MONOMAC1 were indeterminate.

At baseline, no activation to MONOMAC1 PADI4-Var was seen. However, after long, high-dose IFNγ treatment (6 days 50 ng/ml), MONOMAC1 PADI4-Var was able to activate antigen-specific T cells. Several hypotheses were proposed to explain what IFNγ may do to target cells in order to facilitate PADI4 TCR T cell activation. For one, it may upregulate MHC class II expression in target cells, which in turn allows for efficient

PADI4 antigen presentation. Secondly, as the high-dose IFNγ elicits MONOMAC1 cell death, it may result in accumulation of extracellular PADI4 protein from dead cells, which then enters the endosomal-lysosomal antigen-processing compartment in the remaining alive cells, where the MHC Class II complex loading occurs. Lastly, IFNγ may act to promote cellular stress signalling pathways, which could change the target cell immunogenicity by altered surface immune receptor/ligand constitution. To deconvolute this, MONOMAC1 PADI4-Var was exposed to different treatments, showing only one condition in which PADI4 TCR T cell activation was seen - MONOMAC1 PADI4-Var pre-treated with lower dose (5 ng/ml) IFNγ and its own lysate. Meanwhile, 5 ng/ml IFNγ by itself was shown to be insufficient in isolation, and the treatment of a control cell line MONOMAC1 PADI4-WT with the lysate from MONOMAC1 PADI4-Var and lower dose IFNγ did not elicit T cell activation either. This would suggest that the combination of cell lysate and IFNγ pre-treatment acts on MONOMAC1 PADI4-Var to permit intracellularly derived PADI4-Var antigen presentation.

If that is the case, why was the lysate treatment of MONOMAC1 PADI4-Var a necessary factor for T cell activation? One could argue that the lysate, produced by rapid freeze-thaw of PADI4-Var overexpressing MONOMAC1 cells, also contained numerous other molecules that could affect target cell ability to activate T cells, *e.g.* damage associated molecular patterns (DAMPs).

Indeed, immunogenic cancer death (ICD) has been long thought to promote immune system's response through release of DAMPs[338, 339]. Conventionally, presence of DAMPs is thought to promote dendritic cell maturation, which improves their antigen presentation and skews them towards a proinflammatory response[338]. However, in truth, ICD may promote tumour control by any of the following three ways[340]: 1) improved immune cell function and their skewing towards a proinflammatory phenotype; 2) improved immunogenicity of cancer cells themselves; 3) improved antigen presentation by cancer cells. Neither of these factors are mutually exclusive and may even be required in combination. In line with this, this experimental result indicates that any of the three proposed effects from ICD are possible, including the proinflammatory skewing

of the PADI4 TCR T cells themselves, as neither the cell lysate nor the IFNγ were washed off before T cells were added to the culture.

Having discussed the potential effect of ICD and DAMPs in mediating PADI4 TCR T cell ability to recognise MONOMAC1 PADI4-Var, one could ask why the proinflammatory cocktail DC6 did not induce the same effect. After all, PAMP and DAMP induced signalling pathways overlap considerably and can even share the same surface receptors[341]. For one, the cocktail may simply not provide the right signals due to its relatively limited composition. On the other hand, reduced target cell viability (Fig. 3.24b) as well as higher T cell activation background (Fig. 3.25 and 3.26) in all DC6 conditions suggests significant cell death. Simply, the cocktail may have been too toxic for the target cells.

Having discussed that, if true, ICD may offer another level of allogeneic T cell specificity to cancer. Since bone marrow transplantation occurs after a conditioning regimen that reduces the bulk of the cancer using chemotherapeutic agents, it is not unreasonable to suggest that chemotherapy-induced proinflammatory environment contributes to successful GvL[340]. This may also have been the mechanism that facilitated establishment of a long-persisting anti-PADI4 T cell response in patient OX866. Thus, further investigation of suggested conditions that may augment T cell recognition of leukaemia would be merited. However, it is important to note that only one myeloid leukaemia cell line with the required genotype and expression criteria was tested in this work (MONOMAC1). Thus, it is unclear to what extent the observed features are shared with other cell lines and primary leukaemias.

6.4 PADI4 TCR Testing with Primary AML Samples

To investigate the ability of primary leukaemia cells to activate PADI4 TCR, similar target cell screening criteria were applied as in the previously described cell line screening (summarised in Fig. 3.6). However, the imposed PADI4 and HLA-DP expression criteria were rather arbitrary. It is unknown what levels of expression for either of those genes are required to elicit PADI4 TCR T cell activation. In addition, PADI4 WB screening in primary samples is affected by sample purity, as the protein extraction

was performed on all cells in the sample. Thus, it is unknown to which extent PADI4 protein comes from healthy contaminating cells rather than leukaemia. Reassuringly, the largest source of PADI4 protein, granulocytes (see Chapter 5), were eliminated by density separation before AML samples were investigated.

The ideal sample to use in this work would have been the original patient OX866 leukaemia - the same patient from which the original PADI4-specific T cell was isolated 6 years post-transplant (Fig. 3.1 and 3.2). This would have answered whether the PADI4 TCR T cells, and thus the original donor-derived alloreactive T cells, could have used their TCR to exert direct cytotoxicity on cancer. Furthermore, this leukaemia could have been studied for its PADI4 and HLA-DP expression to better understand how these can correlate with T cell response. Unfortunately, there are no samples of this leukaemia available.

Nevertheless, PADI4 TCR T cell activation assays were carried out using other suitable primary AML samples. Although direct antigen-specific T cell activation was not seen, it was possible to show that in the presence of supra-physiological PADI4 variant peptide, leukaemia cells could induce T cell activation. To better understand why direct activation was not seen, several different pre-treatments of target AML cells were explored: 1) IFNγ treatment; 2) AML sample lysate treatment; 3) Proinflammatory cocktail treatment (DC6); 4) Dendritic-like maturation treatment (GM-CSF & IL-4). By using these, I hoped to deconvolute if MHC class II upregulation, presence of extracellular PADI4 protein or different proinflammatory/maturation signals are required for antigen presentation and T cell activation. Moreover, the treatments were added together in different combinations, to assess if antigen-specific T cell activation requires more than one. Nevertheless, neither combination showed any evidence of PADI4 TCR T cell activation.

However, one could argue that the effect of these treatments to the leukaemia sample is unknown. *E.g.* has the treatment with IFNγ successfully upregulated MHC class II machinery in BIR346? As shown in Chapter 3, different cancer cell lines have different sensitivities to IFNγ when HLA-DP is measured. A similar effect has been shown in primary samples as well and was attributed to varied immune evasion mechanisms[271].

Moreover, it is unknown to what extent BIR346 MHC class II machinery is functional or what other immune evasion mechanisms it may have (see Chapter 1). Thus, to truly assess the effect of IFNγ on primary leukaemia antigen presentation via MHC class II, a careful titration of different doses and lengths of the treatment would be required. It is likely that similar would be true for all other treatment types as well.

The absence of PADI4-specific, direct T cell activation to leukaemia cells could be either a feature of cancer immune evasion or normal biology. *I.e.* PADI4 antigen may not be efficiently presented on the cell surface of a leukaemia either because of its immune evasion mechanisms or because the intracellular PADI4 antigen does not undergo efficient trafficking into the MHC Class II pathway in general. In the latter case, we would see normal cells with the required genotypes and expression of PADI4 and HLA-DP to also struggle with PADI4 antigen presentation, but we know that healthy monocytes with these characteristics activate PADI4 TCR T cells (see Chapter 5). Another clue supporting this has been the direct PADI4 TCR T cell activation to the BLCL cell line BOLETH. Given that it is an immortalised professional APC, rather than an *in vivo* developed cancer that was later immortalised *in vitro*, this result suggests that the unperturbed antigen presentation machinery does not struggle with intracellular PADI4 antigen presentation on HLA-DP. Therefore, the absence of PADI4 TCR T cell response to primary AML cells points towards immune evasion mechanisms.

The most important limitation of these findings is that they are based on a single AML sample. Thus, further investigation of direct PADI4 antigen presentation by different AML samples is needed. This would require more samples not only with the suitable genotype and expression characteristics, but also with good viability, purity and cell number. One way to overcome some of these limitations is by engrafting the favoured samples into immunodeficient mice, which allows to amplify the cells *in vivo* (termed as 'primagraft'). Once a cohort of suitable samples is available, direct presentation to T cells can be investigated to gauge the requirements for T cell activation, any necessary pre-treatments to cancer cells and required PADI4 and HLA-DP expression levels in leukaemia. All of these observations would be valuable to eventually prospectively predict which leukaemias could be efficiently targeted with the PADI4 TCR.

6.5 Presentation of Primary AML-Derived Antigen by MDDCs

The only condition that allowed reproducible antigen-specific T cell activation in the context of primary AML samples was with AML lysate-treated MDDCs. Moreover, the maturation of MDDCs was crucial for this response. *In vivo*, DC maturation allows for optimal priming of naïve T cells in lymphoid tissues. However, T cells used in these experiments were not naïve. In fact, not only do they overexpress PADI4 TCR but are also highly activated prior to functional assays to allow efficient TCR transduction. Hence, this raises a question for why the activation of PADI4 TCR T cells was highly MDDC maturation-dependent.

This may be due to a number of changes, or even their combinations, such as higher levels of endocytic processing in mature MDDC and/or higher presentation of costimulatory molecules. However, the knowledge of the exact mechanisms that lead to the T cell activation could be highly valuable to understand and predict reasons for why in some cases T cell activation does not occur, *e.g.* when leukaemia cell acts as the APC. This could also be investigated by comparing the ability of different professional APCs to present antigen, such as B cells, macrophages and DC. The benefit of this approach is that it may also inform what other functions PADI4 TCR T cells could readily perform beyond direct cytotoxicity.

With better knowledge of the conditions that determine effective PADI4 TCR T cell activation, the potential application of the PADI4 TCR and the way it could exert anti-cancer immune effects would become more clear. *E.g.* if the T cell cannot recognise antigen on the leukaemia cell surface directly, what other immune cells are needed for the anti-cancer effect to occur?

6.6 PADI4 TCR Toxicity to Normal Tissues

Chapter 5 aimed to evaluate the potential of the PADI4 TCR to mediate a reaction against healthy tissues rather than cancer. This question is particularly relevant due to

the allogeneic context from which this TCR was originally discovered - the post-allo-HSCT cultured lymphocyte sample. Furthermore, the PADI4 antigen is produced by a germline SNP rather than a cancer specific mutation. Therefore, all nucleated cell types have the required genotype to potentially produce the required pMHC complex.

Thus, the investigation of healthy tissue toxicity began with the analysis of publicly available RNA expression data to establish which healthy cell types are the most likely PADI4 TCR targets, based on *PADI4*, *HLA-DPA1* and *HLA-DPB1* expression. HLA-DP genes, like other MHC class II complexes, were broadly haematopoietically restricted. Furthermore, *PADI4* expression appeared to be exclusively haematopoietic, with highest levels in neutrophilic and monocytic lineages.

Knowledge of any toxicity to healthy haematopoietic tissues is useful to guide potential further applications of the PADI4 TCR. If the T cell response is seen to the healthy haematopoietic cells, along with the leukaemia, this TCR may only be useful in the peri-transplant context. In such case, the donor bone marrow would require a PADI4 SNP-WT genotype, but the recipient would be PADI4 SNP-Var. Hence, the toxicity to the healthy cells should be self-limiting as the donor haematopoiesis reconstitutes within the bone marrow. However, if the PADI4 TCR were to be used outside of the PADI4 SNP mismatched allo-HSCT context, it should have limited to no toxicity to any healthy haematopoietic cells at all.

Therefore, the chosen populations for *in vitro* experimentation were most frequent PB and BM populations (neutrophils, monocytes, B cells and T cells), as well as HSPCs, due to their critical role in haematopoiesis. Out of all tested populations, only monocytes were able to activate the PADI4 TCR T cells in an antigen-specific manner. This is not unexpected, as the RNA expression data suggested monocytes to be the only tested cell type that has similarly high expression levels of both *PADI4* as well as MHC class II. However, when the abundance of the PADI4 antigen was made equal across different target cell types by using a peptide pulse, HLA-DP surface expression was clearly not the sole factor determining a given target cell's ability to activate PADI4 TCR T cells. Indeed, although monocytes did not appear to have the highest surface levels of HLA-DP (it was B cells instead), they were the most capable of activating T cells when pulsed.

The reason for this is unknown, but one hypothesis could be that this is determined by the different costimulatory and coinhibitory receptor profiles on the target cells.

Interestingly, subsequent *in vitro* killing assays did not show any toxicity to the target cell populations investigated, even the monocytes. It is unknown how monocytes are evading death, despite their ability to activate T cells. This effect is even more clear when the monocytes are pulsed with the peptide - even in supra-physiological abundance of the antigen, monocytes remain intact, while other target cell types (HSPCs, B cells) are killed. Ultimately, the reason for this is unknown, but several hypotheses could be raised. For one, the nature of T cell interaction with monocytes may prevent killing mechanisms to occur in the first place. It may be that monocytes have inappropriate costimulatory or coinhibitory receptors to mediate successful killing, such as expression of PD-L1. Ultimately, this may mean that TCR signal strength is not sufficient to elicit an effective cytotoxic T cell response.

Alternatively, monocytes may have the ability to evade T cell killing. For example, they may have a resistance to Fas (death receptor) signalling. Indeed, it has been previously documented that monocytes express apoptosis suppressing protein Bcl-x[342], which has also been shown to counteract Fas signalling[343]. However, some research suggests that *ex vivo* monocytes are nevertheless sensitive to Fas[344]. Instead, it could be that the proinflammatory cytokines released by the antigen-specific T cells can act on the monocytes and reduce their sensitivity to Fas signalling. In support of this, experiments presented in this chapter do show that PADI4 TCR T cells release TNFα upon antigen recognition (Fig. 5.9). In fact, it is known that proinflammatory cues, such as TNFα, can activate monocytes and thus reduce their sensitivity to pro-apoptotic signals[344–346]. In addition, T cell membrane-bound proteins can have similar functions - CD40 stimulation in monocytes by the T cell bound CD40L was previously shown to have anti-apoptotic effects[347]. Nevertheless, it remains unclear how monocytes avoid death by the PADI4 TCR T cells and further careful experimentation would be required to suggest mechanisms that determine this.

Finally, results of the killing experiment also highlighted the dichotomy in surface HLA-DP regulation between cells that were killed with the PADI4 variant peptide

pulse and those that were not. After co-culture with the PADI4 TCR T cells, surface HLA-DP expression increased in pulsed B cells and HSPCs, but decreased in pulsed monocytes and T cells. Former target cells were killed, whilst latter were not. It's unclear what significance this finding has or why, in this case, different target cells changed their surface HLA-DP expression differently upon antigen-specific PADI4 TCR T cell recognition. This may simply highlight that different targets activated effector T cells differently, eliciting varied functional responses. *E.g.* IFNγ released from the activated T cells is known to upregulate MHC class II machinery in target cells. This may have been the reason pulsed B cells and HSPCs increased their surface HLA-DP expression after co-culture with antigen-specific T cells. However, IFNγ release in these experiments was not measured.

6.7 Further Investigations on Healthy Tissue Toxicity

Although the experiments performed to date provide insight into the potential off-tumour toxicity for the PADI4 TCR, several key questions remain unanswered and beyond the scope of this work. To further investigate the risks of on-target off-tumour toxicity, additional *in vitro* experiments may be considered. For one, target cell choice in this work was influenced by what cell populations are prevalent as well as easily accessible for experimentation. However, many lower frequency cell types had measurable expression of both *PADI4* and *HLA-DP* genes, such as dendritic cells, NK cells, and various subsets of HSPCs. Indeed, although toxicity to the HSPCs was measured in this work, it is important to acknowledge the high heterogeneity of this population. Not only do different subsets of HSPCs have different differentiation potentials, but they also express different levels of *PADI4* and *HLA-DP*, sometimes with up to a $\log_{10}$ difference in between them (Fig. 5.2 and 5.3). HSPC fraction ($CD34^+$) from the bone marrow is expected to be mostly comprised of CMPs ($\sim$35%), MEPs ($\sim$15%) and GMPs ($\sim$18%), with the rest being lower frequency populations such as HSC and LMPP[254]. Given the low target cell numbers used in the killing experiments so far, as well as the sparsity of these different HSPC sub-populations, it is not possible to effectively assess PADI4 TCR toxicity at this resolution. Thus, alternate approaches

for investigating this would be required. For example, HSPC *in vitro* killing assays could be coupled with colony-forming unit (CFU) assays to assess any loss in lower frequency progenitors or their differentiation capacity. *In vivo* human HSPC engraftment in immunodeficient mice could offer an alternate approach, although haematopoiesis reconstitution in mice is heavily lymphoid-biased[348]

However, further experiments like this need to be balanced against the potential clinical implications a specific target cell toxicity may have. Of course, toxicity to the HSPC compartment could have serious complications due to the important role these cells have in sustaining or establishing haematopoiesis. Another possible concern, given the *PADI4* gene expression pattern, is neutropenia due to potential toxicity to neutrophils or their precursors. Experiments with mature neutrophils from healthy donors did not suggest any toxicity. This result was not unexpected, because neutrophils have very little, if any, HLA-DP (Fig. 5.3 and 5.4). Yet, potential toxicity to myelocytes or promyelocytes was not investigated. These committed neutrophil precursors express *PADI4* as well as some *HLA-DP*, especially in earlier, promyelocyte stage. Thus, it is unclear whether PADI4 TCR would target these cells instead. Assessment of this would require prospective isolation of these cell types from the bone marrow, where their average frequency is around 7-8% for both[349]. Alternatively, $CD34^+$ cell differentiation cultures may be used to produce neutrophils and their precursors *in vitro*[350].

In addition to investigating PADI4 TCR T cell toxicity to healthy cells at steady state, changes during non-specific inflammation and/or immune activation should be considered. Although it is unclear if PADI4 protein levels change under different conditions, it is known that neutrophils release PADI4 into the extracellular space during NETosis (see Chapter 1). Thus, sites of inflammation may have extracellular PADI4 protein, such as seen in rheumatoid arthritis[243, 244]. Unlike in previous Chapters 3 and 4, normal tissue toxicity investigation in Chapter 5 did not assess the ability of professional APC to present healthy cell derived PADI4 antigen to the PADI4 TCR T cells; through previous experiments using primary AML samples, it has already been established that MDDCs possess such ability *in vitro*. However, it must be noted that the possibility of toxicity to healthy tissues through 'indirect' antigen presentation

(where extracellular antigen is taken up by the APC) is not excluded. Importantly, and as shown by experiments with the primary AML samples in Chapter 4, the impact of this process to the *in vivo* PADI4 TCR toxicity will also depend on the inflammatory cues that the APCs receive. This may be particularly relevant in the context of the tissue-damaging chemotherapy used in AML patients.

Moreover, it is known that under conditions of immune activation many different cell types can upregulate MHC class II expression through IFNγ signalling. Interestingly, even short-lived neutrophils can present MHC class II restricted antigen to T cells and activate them both *in vitro* and *ex vivo*[351]. Thus, it is important to consider which cells may become potential targets due to context-dependent MHC class II upregulation. This may even be T cells themselves. During the TCR transduction protocol, T cells upregulate surface HLA-DP due to the activation signals in tissue culture media (IL-2, IL-15, CD3 and CD28 stimulation). Furthermore, it is not uncommon for some studies to measure T cell activation using surface HLA-DR staining. Therefore, PADI4 TCR T cells themselves could recognise each other, provided there is *PADI4* expression and its genotype is SNP-Var. If true, T cell fratricide may pose a serious limitation in gene-transferred T cell production and expansion. For the aforementioned reasons, further experimentation should assess if the PADI4 TCR T cells can target healthy cells, or even themselves, under inflammatory conditions.

To summarise, several further experimental avenues may be pursued to better understand potential PADI4 TCR toxicity to healthy tissues. Suggested here is closer investigation of functionally relevant, but lower frequency haematopoietic populations as well as haematopoietic cell populations under inflammatory conditions. Nevertheless, the suggested work is not comprehensive, *e.g.* any potential off-target off-tumour toxicity would remain unknown. Ultimately, most reliable toxicity and tolerability information would come from first-in-human clinical trials. But, for the purposes of establishing whether this TCR contributed to the GvL versus GvHD response in the original patient from which it was isolated, evidence presented in Chapter 5 supports the hypothesis that PADI4 TCR recognises haematopoietically-restricted, if not leukaemia-associated, antigen.

6.8 Augmenting The GvL Effect

This work considers the first out of the 22 newly discovered miHA responses in AML patients successfully treated with allo-HSCT (Chapter 1). The initial screen aimed to learn more about which long-term persisting alloreactive T cell responses correlate with the clinical disease remission and limited GvHD. Surprisingly, most of the discovered responses were $CD4^+$-mediated and MHC class II restricted, and the *PADI4* miHA response is one of them.

Although just one response was investigated in this work, these results raise important considerations for broader research on the GvL effect. It remains unclear how important the MHC class II restricted antigens are for a successful GvL effect, but this research suggests that their persistence correlates with long-term remission. Moreover, by investigating the *PADI4* miHA response, I was able to show that an antigen-specific TCR isolated from this context can recognise leukaemia-derived antigen.

Given the large phenotypic diversity of the $CD4^+$ T cell compartment, it is not only important to investigate the cancer-specific TCRs that they possess, but also the phenotype that they have *in vivo*. Investigation of the latter within this body of work is challenging - the long-term persisting responses are low frequency and require expansive *in vitro* culture before isolation of antigen-specific cells. Based on the phenotype (transcriptional and flow cytometry-based) seen at that point, the original *PADI4* miHA response was hypothesised to be $CD4^+$ cytotoxic. However, it remains important to assess what phenotypes were seen in the other 21 newly discovered responses, and ideally, what phenotypes the antigen-specific cells have directly *ex vivo*. This might provide important clues on what roles allogeneic anti-leukemic $CD4^+$ T cells carry out in the context of a successful GvL effect. Furthermore, any coupling of this data to functional read outs, such as killing assays and cytokine release assays, in presence of antigen, would be highly valuable.

Knowledge of this could help to direct our search of an effective GvL-specific T cell therapy - *e.g.* while this work primarily focused on TCR-transduced T cell direct

cytotoxicity, perhaps assays to measure the capacity for myeloid cell recruitment and activation might be more or equally appropriate. My own results described in Chapter 4 allude to this - while direct activation from leukaemia samples was not achieved, a professional APC could reproducibly activate T cells by leukaemia-derived antigen presentation. Hence, could the PADI4 TCR T cells recruit and enable the innate myeloid compartment to kill leukaemia? This could present an important killing mechanism, independent of cancer's own MHC class II expression[211] (see Chapter 1).

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.1 Supplemental Materials and Methods

List of consumables

Reagent or Resource	Final concentration or required dilution	Source	Catalog number
Antibodies			
Goat anti-mouse IgG [Poly4053]	1:100	Biolegend	405307
Mouse anti-human CD3 PE Cy5 [HIT3a]	1:100	Biolegend	300310
Mouse anti-human CD3 PE Cy7 [HIT3A]	1:50 or 1:100	Biolegend	300310
Mouse anti-human CD3 BV711 [UCHT1]	1:100	Biolegend	300463
Mouse anti-human CD4 APC [OKT4]	1:200	Biolegend	317415
Mouse anti-human CD4 APC Cy7 [OKT4]	1:50	Biolegend	317417
Mouse anti-human CD4 PE [OKT4]	1:400	Biolegend	317416
Mouse anti-human CD4 PETR [SK3]	1:50 or 1:100	Biolegend	344640
Mouse anti-human CD8 FITC [SK1]	1:100	Biolegend	344704
Mouse anti-human CD8 AF700 [RPA-T8]	1:200	BD	561026
Mouse anti-human CD8 APC Cy7 [SK1]	1:50 or 1:100	BD	557834
Hamster anti-mouse TCRb APC [H57-597]	1:80	BD	561080
Hamster anti-mouse TCRb BV711 [H57-597]	1:80	BD	563135
Mouse anti-human CD117 BV650 [104D2]	1:40	BD	563859
Mouse anti-human CD117 AF700 [104D2]	1:40	Biolegend	313245
Mouse anti-human CD14 AF700 [63D3]	1:40	Biolegend	367114
Mouse anti-human CD14 FITC [63D3]	1:33	Biolegend	367115
Mouse anti-human CD15 PerCP [W6D6]	1:50	Biolegend	323018
Mouse anti-human CD19 PerCP Cy5.5 [HIB19]	1:160	Biolegend	302229
Mouse anti-human CD19 PE Cy5 [HIB19]	1:100	Biolegend	302210
Mouse anti-human HLA-DP BV711 [B7/21]	1:200	BD	750870
Mouse anti-human HLA-DP PE [B7/21]	1:40	BD	566825
Mouse anti-human HLA-DR BV605 [G46-6]	1:200	BD	562845
Mouse anti-human CD34 APC [581]	1:50	Biolegend	343510
Mouse anti-human CD34 AF700 [581]	1:40	Biolegend	343525
Mouse anti-human CD40 PE [5C3]	1:50	Biolegend	334308
Mouse anti-human CD45 FITC [HI30]	1:40	ThermoFisher	11-0459-41
Mouse anti-human CD45 APC eFluor780 [HI30]	1:40	ThermoFisher	47-0459-42
Mouse anti-human CD69 APC [FN50]	1:200	Biolegend	310910
Mouse anti-human CD80 BV650 [2D10]	1:25	Biolegend	305227
Mouse anti-human CD83 APC [HB15e]	1:50	Biolegend	305312
Mouse anti-human CD86 AF488 [IT2.2]	1:200	Biolegend	305414
Mouse anti-human CD107a BV650 [H4A3]	1:90	Biolegend	328637
Mouse anti-human CD107a BV421 [H4A3]	1:300	Biolegend	328626
Mouse anti-human CD107b BV421 [H4B4]	1:300	Biolegend	354308
Mouse anti-human CD134 BB700 [ACT35]	1:50	BD	746071
Mouse anti-human CD137 PE [4B4-1]	1:40	Biolegend	309803
Mouse anti-human IFNγ PE Cy7 [B27]	1:50	BD	560924
Mouse anti-human TNFa FITC [MAb11]	1:500	BD	562082
Rat anti-human IL-2 PerCP eFluor710 [MQ1-17H12]	1:50	Thermofisher	46-7029-42
Rat anti-mouse Ter119 PB [TER119]	1:200	Biolegend	116232
Mouse anti-human CD33 BV605 [P67.6]	1:50	Biolegend	366612
Rat anti-mouse CD45 APC Fire750 [30-F11]	1:100	Biolegend	103154
Mouse anti-human IFNγ antibody [1-D1K] (ELISpot)	1:10000	Mabtech	3420-3-250
Mouse anti-human biotinylated IFNγ [7-B6-1] (ELISpot)	1:1000	Mabtech	3420-6-250
Mouse anti-human PADI4 (Western blot)	1:1000	Abcam	ab128086
mouse anti-human GAPDH (Western blot)	1:5000	Santa Cruz Biotech	sc-47724
Anti-mouse HRP-conjugated (Western blot)	1:10000	Affinity Biosciences	#s0002

Reagent or Resource	Final concentration or required dilution	Source	Catalog number
Chemicals, peptides and cell culture			
RPMI	N/A	ThermoFisher	21875-034
MycoAlert	N/A	Lonza	LT07-118
MycoStrip	N/A	InvivoGen	rep-mys-10
DMEM	N/A	ThermoFisher	11960-044
α-MEM	N/A	ThermoFisher	22571-020
IMDM (no phenol red)	N/A	ThermoFisher	21056-023
IMDM (with phenol red)	N/A	ThermoFisher	12440-053
GlutaMAX	1:100	ThermoFisher	35050061
Sodium pyruvate	1:100	Sigma-Aldrich	S8636-100ML
Non-essential amino acids	1:100	ThermoFisher	11140-035
β-mercaptoethanol	1:1000	ThermoFisher	31350-010
DMSO	N/A	Sigma-Aldrich	D2650-100ML
TruStain FcX block	1:100	Biolegend	422301
Ficoll-Paque PLUS	N/A	Cytiva	17144002
fetal bovine serum (FBS)	N/A	Sigma-Aldrich	F7524
Nucleobond Xtra Midi EF	N/A	Macherey-Nagel	740420.10
Human AB serum	N/A	Sigma-Aldrich	H4522
IL-2 (Proleukin)	1000U/ml	Pharmacy, OUH NHS or Peprotech	N/A or 200-02
IL-4	300U/ml	Miltenyi Biotec	130-093-919
IL-15	10ng/ml	Peprotech	200-15
GM-CSF	100U/ml	Miltenyi Biotec	130-093-865
CD3 MACS microbeads	N/A	Miltenyi Biotec	130-050-101
CD4 MACS microbeads	N/A	Miltenyi Biotec	130-045-101
CD8 MACS microbeads	N/A	Miltenyi Biotec	130-045-201
CD14 MACS microbeads	N/A	Miltenyi Biotec	130-050-201
CD19 MACS microbeads	N/A	Miltenyi Biotec	130-050-301
CD34 MACS microbeads	N/A	Miltenyi Biotec	130-046-702
CD3/28 Dynabeads	1:1 bead-to-cell	ThermoFisher	11131D
Retronectin	25µg/mL	Takara Bio	T100B
Turbofectin	N/A	Origene	TF81001
RIPA buffer	N/A	ThermoFisher	89900
Halt protease inhibitor cocktail	1:100	ThermoFisher	87786
NuPAGE 4x LDS sample buffer	1:4	ThermoFisher	NP0008
NuPAGE 4-12% BisTris gel	N/A	ThermoFisher	NP0321BOX
PageRuler Plus Protein Ladder 10 to 250 kDa	N/A	ThermoFisher	26619
Immobilon -P PVDF membrane	N/A	Sigma-Aldrich	IPVH00010
CellTrace Violet	1:2500	ThermoFisher	C34557
L/D near IR	1:1000	ThermoFisher	L34975
L/D Aqua	1:1000 for T cells 1:500 for MDDC	ThermoFisher	L34957
Brefeldin A (GolgiPlug)	10µg/ml	BD	555029
Monensin (GolgiStop)	2µM	BD	554724
IL-7	25ng/ml	Peprotech	200-07
Benzonase	1:1000	Merck	70746-10KUN
3-Amino-9-ethylcarbazole (AEC) tablets	N/A	Sigma-Aldrich	A6926-50TAB
Hoechst 33258	1:10000	ThermoFisher	H3569
Countbright Counting Beads	N/A	Thermofisher	C36950
7-AAD	1:100	BD	559925
Ampicillin	100 µg/mL	Merck	A9518-5G
TE buffer	N/A	Thermofisher	12090015
Trypsin-EDTA	N/A	Thermofisher	25200056
Opti-MEM	N/A	Thermofisher	31985062
HEPES	1:100	Sigma-Aldrich	H0887-100ML
Pen/Strep (Penicillin and Streptomycin)	1:100	Thermofisher	15140-122
BSA 7.5% in DPBS	N/A	Sigma-Aldrich	A8412-100ML
Polybrene	See protocol	Merck	TR-1003-G
LCK lysing buffer	N/A	ThermoFisher	A1049201

Reagent or Resource	Final concentration or required dilution	Source	Catalog number
Commercial kits, assays and enzymes			
HiFi Assembly Master Mix	N/A	New England Biolabs	E2621S
MS columns	N/A	Miltenyi Biotec	130-042-201
ELISpot 96-well plates	N/A	Merck	MSIPS4510
Vectastain Elite ABC-HRP kit	N/A	Vector laboratories	PK-6100
TaqMan *PADI4* SNP genotyping assay ID C 7541150 10	1:40	ThermoFisher	4351379
TaqMan Universal PCR Master Mix	N/A	ThermoFisher	4304437
SF cell line nucleofector X Kit S	N/A	Lonza	V4XC-2032
Cas9 nuclease	See protocol	IDT	1081058
Pierce BCA protein assay	N/A	ThermoFisher	23225
Amersham ECL detection reagent	N/A	Merck	GERPN2232
BD Fix/Perm and BD Perm/Wash	N/A	BD	554714
NotI-HF	See protocol	New England Biolabs	R3189S
SacII	See protocol	New England Biolabs	R0157S
NcoI	See protocol	New England Biolabs	R0193S
PmlI	See protocol	New England Biolabs	R0532S
QIAquick Gel Extraction kit	N/A	Qiagen	28704
T4 DNA ligase	See protocol	New England Biolabs	M0202S
QIAprep Spin Miniprep kit	N/A	Qiagen	27104
Q5 polymerase	See protocol	New England Biolabs	M0491S
Q5 MasterMix	1:2	New England Biolabs	M0492S
TaqMan Universal PCR Master Mix	1:2	Thermofisher	4304437
DreamTaq Green Matermix	1:2	Thermofisher	K1081
QuickExtract DNA Extraction Solution	N/A	Biosearch technologies	N/A
MinElute PCR Purification kit	N/A	Qiagen	28004
Stable Competent *E.coli*	N/A	New England Biolabs	C3040H
IFNγ catch kit	N/A	Miltenyi Biotec	130-054-202
Dneasy Blood & Tissue Kit	N/A	Qiagen	69504
Plasmids			
pHRsin 1G4 TCR	N/A	Margarida Rei (Oxford)	N/A
pHRsin mCherry		Margarida Rei (Oxford)	
pHRsin PADI4 clone 1 TCR		This project	
pHRsin PADI4 clone 2.1 TCR		This project	
pHRsin PADI4 clone 2.2 TCR		This project	
pMP71 CMV TCR		Hans Stauss (London)	
pMP71 PADI4 clone 1 TCR		This project	
pMP71 PADI4 clone 2.1 TCR		This project	
pMP71 PADI4 clone 2.2 TCR		This project	
pQ8.91		Margarida Rei (Oxford)	
pMD.G		Margarida Rei (Oxford)	
psPAX2		Ryan Beveridge (Oxford)	
pMD2.G		Ryan Beveridge (Oxford)	
pUC19 PADI4-WT		This project	
pUC19 PADI4-Var		This project	
pL-CRISPR-GFP		Philip Hublitz (Oxford)	
pL-PADI4-WT-GFP		This project	
pL-PADI4-Var-GFP		This project	
Miscellaneous			
MicroAmp™ Optical Adhesive Film	N/A	ThermoFisher	4311971
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	N/A	ThermoFisher	4346906

List of oligonucleotides

Oligonucleotide names	Sequence (5' to 3')
Primers	
p3	CTCAGGTTCTGGAAGTTCAGG
p5	CACTTGTCCTCTTCGCTCAG
p20	CGTTCTGTGTTGTCTCTGTC
p22	GGCTTCAACCTGCTGATGAC
p32	GGGTACGTGGCTTTGTTG
p34	GAAGATCTACGTAACGTGACACCAC
p35	CGGGGTTTTCCTCCACATCT
p36	CTCCGACAGACTGAGTCGCCCGGGGCCGCCACCATGGATACCTGGCTCGTG
p37	GTGGTGTCACGTTACGTAGATCTTCTGTCACGGTCAGTCTTGTGC
p38	AGATGTGGAGGAAAACCCCGGCCCTATGCTTACAGCTTCTCTGCTGAG
p39	CGGCGGGCTCGGGGTTCTGGATATCAGGTTGAACAGCCAGCAGT
p40	CTCCGACAGACTGAGTCGCCCGGGGCCGCCACCATGGGCAGCAGACTGC
p41	GTGGTGTCACGTTACGTAGATCTTCGACCACTGTCAGTCTGGTG
p42	AGATGTGGAGGAAAACCCCGGCCCTATGAAGTCTCTGAGAGTGCTGC
p43	CGGCGGGCTCGGGGTTCTGGATATCGCTGGACACGCTCAGTGTT
p44	AGATGTGGAGGAAAACCCCGGCCCTATGCTTACAGCTTCTCTGCTGAG
p45	GGCGGGCTCGGGGTTCTGGATATCGGCGATCACAGACAGTCTGG
p54	CAGTCCTCCGACAGACTGAGT
p56	GTTCTTTCCAGATCACGTGGAGC
p57	GGCACAGAGTACTATCCTGAGATCG
p59	AAGCTTGCATGCCTGCAG
p61	GTACACGACATCACTTTCCCAG
p218	CTTGCCGGTTCTTGTGATATCG
p219	GGTGATCAGGCCAGGGAAATC
p220	GAATCCAGTCCGCTGATGC
p220	GAATCCAGTCCGCTGATGC
p221	CGATGCATCTCTCCACGAAGG
p224	GCCGCCAGAACACAGGTGTCGTGACGCGGGATCCGCCACCATGGCCCAGGGCAC
p225	GGCTGAAGTTGGTGGCGCCGCTGCCGCTAGCAGGCACCATGTTCCACCAC
PAD_geno_Fw	CTCCAAGTGCAGCGTAGTCT
PAD_geno_Rv	TGGAACACCACAGCCTCG
pPAD1	GCTGCAAGGGGTTCTCTGAT
pPAD2	ACGTAGCCTCAGCTGCATC
Q5SDM_6/7/2022_F	CTGGACACGTTCAACCTGGAG
Q5SDM_6/7/2022_R	CAGGGAGATGGTGAGGGT
gRNA	
PADI4 gRNA A1	CCTGCTGGACACGTCCAACC
PADI4 gRNA A2	GCCTTCTCGGCCTACCAGGT
PAdi4 gRNA A3	CTGGACACGTCCAACCTGGT
ssODN	
PADI4 ssODN1	GACTTCCCGGGGCTCATTACCCTCACCATCTCCCTGCTGGACACGTTTAATCTGGTAGG CCGAGAAGGCAGCCCTGCATCGGGGGCCTGGGCTT
PADI4 ssODN2	AAGCCCAGGCCCCCGATGCAGGGCTGCCTTCTCGGCCTACCAGATTAAACGTGTCCAG CAGGGAGATGGTGAGGGTAATGAGCCCCGGGAAGTC

Cell line culture conditions

Cell line	Base Media	Additives	Culture density (*10^6/ml)	Freeze media
BOLETH	RPMI	20% FBS	0.5-1	FBS 10% DMSO
EHM	RPMI	20% FBS	0.5-1	-
GDM-1	RPMI	10-20% FBS	0.5-1	-
HEK 293T	DMEM	10% FBS 1X GlutaMAX 1X Sodium pyruvate	2 million seeded to T75 flask	FBS 10% DMSO
HEL	RPMI	10% FBS	0.5-1	FBS 10% DMSO
HL-60	RPMI	10% FBS	0.5-1	FBS 10% DMSO
Jurkat TCR KO	RPMI	10% FBS	0.5-1	FBS 10% DMSO
K562	RPMI	10% FBS	0.5-1	FBS 10% DMSO
KAS011	RPMI	20% FBS	0.5-1	-
MOLM-13	RPMI	10% FBS	1-2	FBS 10% DMSO
MONOMAC1	RPMI	10% FBS 1X Non-essential amino acids 1X Sodium pyruvate	0.3-1	FBS 10% DMSO
NALM6	RPMI	10% FBS	1-2	FBS 10% DMSO
OCI-AML-3	α-MEM	20% FBS	0.5-2	FBS 10% DMSO
PE117	RPMI	20% FBS	0.5-1	-
Pheonix Ampho	IMDM	10% FBS 1X GlutaMAX	2 million seeded to T75 flask	FBS 10% DMSO
RCH-ACV	RPMI	10% FBS	0.5-2	FBS 10% DMSO
REH	RPMI	20% FBS	2-5	FBS 10% DMSO
RS4;11	α-MEM	20% FBS	0.5-1	FBS 10% DMSO
SEM	IMDM	10% FBS	0.5-2	FBS 10% DMSO
SUP-B15	IMDM	20% FBS 1X β-mercaptoethanol	1-2	FBS 10% DMSO
TF1	RPMI	20% FBS 5ng/ml GM-CSF	0.5-1	FBS 10% DMSO
THP-1	RPMI	10% FBS	0.5-1	FBS 10% DMSO

Peptide sequences

Peptide name	Peptide sequence
PADI4 variant peptide 21	LTISLLDTFNLELPEAVVFQ
PADI4 variant peptide 22	ISLLDTFNLELPEAVVFQDS
PADI4 wild-type peptide	LTISLLDTSNLELPEAVVFQDS
PADI4 18aa-001-var	ISLLDTFNLELPEAVVFQ
PADI4 17aa-001-var	ISLLDTFNLELPEAVVF
PADI4 17aa-002-var	SLLDTFNLELPEAVVFQ
PADI4 16aa-001-var	ISLLDTFNLELPEAVV
PADI4 16aa-002-var	SLLDTFNLELPEAVVF
PADI4 16aa-003-var	LLDTFNLELPEAVVFQ
PADI4 15aa-001-var	ISLLDTFNLELPEAV
PADI4 15aa-002-var	SLLDTFNLELPEAVV
PADI4 15aa-003-var	LLDTFNLELPEAVVF
PADI4 15aa-004-var	LDTFNLELPEAVVFQ
PADI4 14aa-001-var	ISLLDTFNLELPEA
PADI4 14aa-002-var	SLLDTFNLELPEAV
PADI4 14aa-003-var	LLDTFNLELPEAVV
PADI4 14aa-004-var	LDTFNLELPEAVVF
PADI4 14aa-005-var	DTFNLELPEAVVFQ
PADI4 13aa-001-var	ISLLDTFNLELPE
PADI4 13aa-002-var	SLLDTFNLELPEA
PADI4 13aa-003-var	LLDTFNLELPEAV
PADI4 13aa-004-var	LDTFNLELPEAVV
PADI4 13aa-005-var	DTFNLELPEAVVF
PADI4 13aa-006-var	TFNLELPEAVVFQ
PADI4 12aa-001-var	ISLLDTFNLELP
PADI4 12aa-002-var	SLLDTFNLELPE
PADI4 12aa-003-var	LLDTFNLELPEA
PADI4 12aa-004-var	LDTFNLELPEAV
PADI4 12aa-005-var	DTFNLELPEAVV
PADI4 12aa-006-var	TFNLELPEAVVF
PADI4 12aa-007-var	FNLELPEAVVFQ

Lentiviral TCR constructs

Patient T cell sequencing-derived TCR sequences

Following are single cell RNA sequencing-assembled TCR sequences of α and β TCR mRNA transcripts.

Asterisk (*) denotes boundaries between V,D and J (see Figure 3.2b). Vertical bar (|) indicates boundaries for 5' or 3' UTR.

PADI4 Clone 1 TCRα

CAGAAGCCTCACACAGCCCAGTAACTTTGCTAGTACCTCTTGAGTGCAAGG
TGGAGAATTAAGATCTGGATTTGAGACGGAGCACGGAACATTTCACTCAGGGGA
AGAGCTATGAAC|ATGCTGACTGCCAGCCTGTTGAGGGCAGTCATAGCCTCCAT
CTGTGTTGTATCCAGCATGGCTCAGAAGGTAACTCAAGCGCAGACTGAAATTTC
TGTGGTGGAGAAGGAGGATGTGACCTTGGACTGTGTGTATGAAACCCGTGATA
CTACTTATTACTTATTCTGGTACAAGCAACCACCAAGTGGAGAATTGGTTTTC
CTTATTCGTCGGAACTCTTTTGATGAGCAAAATGAAATAAGTGGTCGGTATTC
TTGGAACTTCCAGAAATCCACCAGTTCCTTCAACTTCACCATCACAGCCTCACA
AGTCGTGGACTCAGCAGTATACTTCTGTGCTCTGAGTGAACGGC*CGTATGGTG
GTGCTACAAACAAGCTCATCTTTGGAACTGGCACTCTGCTTGCTGTCCAGCCAA
*ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGT
GACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAA
AGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCT
ATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCA
TGTGCAAACGCCTTCA... Consensus ends before completion of α constant region ...
ACGCCTTCA...ACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGAA
AGTTCCTGTGATGTCAAGCTGGTCGAGAAAAGCTTTGAAACAGATACGAACCTA
AACTTTCAAAACCTGTCAGTGATTGGGTTCCGAATCCTCCTCCTGAAAGTGGCC
GGGTTTAATCTGCTCATGACGCTGCGGCTGTGGTCCAGCTGA|GATCTGCAAGA
TTGTAAGACAGCCTGTGCTCCCTCGCTCCTTCCTCTGCATTGCCCCTCTTCTCC
CTCTCCAAACAGAGGGAACTCTCCTACCCCCAAGGAGGTGAAAGCTGCTACCAC
CTCTGTGCCCCCCCGGCAATGCCACCAACTGGATCCTACCCGAATTTATGATTA

AGATTGCTGAAGAGCTGCCAAACACTGCTGCCACCCCCTCTGTTCCCTTATTGC
TGCTTGTCACTGCCTGACATTCACGGCAGAGGCAAGGCTGCTGCAGCCTCCCCT
GGCTGTGCACATTCCCTCCTGCTCCCCAGAGACTGCCTCCGCCATCCCACAGAT
GATGGATCTTCAGTGGGTTCTCTTGGGCTCTAGGTCCTGCAGAATGTTGTGAG
GGGTTTATTTTTTTTTAATAGTGTTCATAAAGAAATACATAGTATTCTTCTTC
TCAAGACGTGGGGGGAAATTATCTCATTATCGAGGCCCTGCTATGCTGTGTATC
TGGGCGTGTTGTATGTCCTGCTGCCGATGCCTTCATTAAAATGATTTGGAAGA
GCAGA

PADI4 Clone 1 TCRβ

AAAATGCCCCTCCTTTCCTCCACAGGACCAGATGCCTGAGCTAGGAAAGGCC
TCATTCCTGCTGTGATCCTGCC|ATGGATACCTGGCTCGTATGCTGGGCAATTT
TTAGTCTCTTGAAAGCAGGACTCACAGAACCTGAAGTCACCCAGACTCCCAGCC
ATCAGGTCACACAGATGGGACAGGAAGTGATCTTGCGCTGTGTCCCCATCTCTA
ATCACTTATACTTCTATTGGTACAGACAAATCTTGGGGCAGAAAGTCGAGTTT
CTGGTTTCCTTTTATAATAATGAAATCTCAGAGAAGTCTGAAATATTCGATGA
TCAATTCTCAGTTGAAAGGCCTGATGGATCAAATTTCACTCTGAAGATCCGGTC
CACAAAGCTGGAGGACTCAGCCATGTACTTCTGTGCCAGCAGTGAACGCAGGAC
GCAAC*CTGCCTACGAGCAGTACTTCGGGCCGGGCACCAGGCTCACGGTCACAG*
AGGACCTGAAAAACGTGTTCCCACCCGAGGTCGCTGTGTTTGAGCCATCAGAAG
CAGAGATCTCCCACACCCAAAAGGCCACACTGGTATGCCTGGCCACAGGCTTCT
ACCCCGACCACGTGGAGCTGAGCTGGTGGGTGAATGGGAAGGAGGTGCACAGTG
GGGTCAGCACAGACCCGCAGCCCCTCAAGGAGCAGCCCGCCCTCAATGACTCCA
GATACTGCCTGAGCAGCCGCCTGAGGGTCTCGGCCACCTTCTGGCAGAACCCCC
GCAACCACTTCCGCTGTCAAGTCCAGTTCTACGGGCTCTCGGAGAATGACGAGT
GG... Consensus ends before completion of β constant region ...GAGTGG...ACCCAGG
ATAGGGCCAAACCTGTCACCCAGATCGTCAGCGCCGAGGCCTGGGGTAGAGCAG
ACTGTGGCTTCACCTCCGAGTCTTACCAGCAAGGGGTCCTGTCTGCCACCATCC
TCTATGAGATCTTGCTAGGGAAGGCCACCTTGTATGCCGTGCTGGTCAGTGCCC
TCGTGCTGATGGCCATGGTCAAGAGAAAGGATTCCAGAGGCTAG|CTCCAAAAC

CATCCCAGGTCATTCTTCATCCTCACCCAGGATTCTCCTGTACCTGCTCCCAAT
CTGTGTTCCTAAAAGTGATTCTCACTCTGCTTCTCATCTCCTACTTACATGAAT
ACTTCTCTCTTTTTTCTGTTTCCCTGAAGATTGAGCTCCCAACCCCCAAGTACG
AAATAGGCTAAACCAATAAAAAATTGTGTGTTGGGCCTGGTTGCATTT

PADI4 Clone 2 TCRα (1)

AAAGCAGATTCTTTTTATGATTTTTAAAGTAGAAATATCCATTCTAGGTGC
ATTTTTTAAGGGTTTAAAATTTGAATCCTCAGTGAACCAGGGCAGAGAAGAAT
G|ATGAAATCCTTGAGAGTTTTACTAGTGATCCTGTGGCTTCAGTTGAGCTGGG
TTTGGAGCCAACAGAAGGAGGTGGAGCAGAATTCTGGACCCCTCAGTGTTCCAG
AGGGAGCCATTGCCTCTCTCAACTGCACTTACAGTGACCGAGGTTCCCAGTCCT
TCTTCTGGTACAGACAATATTCTGGGAAAAGCCCTGAGTTGATAATGTCCATAT
ACTCCAATGGTGACAAAGAAGATGGAAGGTTTACAGCACAGCTCAATAAAGCCA
GCCAGTATGTTTCTCTGCTCATCAGAGACTCCCAGCCCAGTGATTCAGCCACCT
ACCTCTGTGCCGT*CCGGGGGCAGGCAGGAACTGCTCTGATCTTTGGGAAGGGA
ACCACCTTATCAGTGAGTTCCA*ATATCCAGAACCCTGACCCTGCCGTGTACCA
GCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGA
TTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAA
AACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTG
GAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCA... Consensus ends before completion of α constant region

PADI4 Clone 2 TCRα (2)

CAGAAGCCTCACACAGCCCAGTAACTTTGCTAGTACCTCTTGAGTGCAAGG
TGGAGAATTAAGATCTGGATTTGAGACGGAGCACGGAACATTTCACTCAGGGGA
AGAGCTATGAAC|ATGCTGACTGCCAGCCTGTTGAGGGCAGTCATAGCCTCCAT
CTGTGTTGTATCCAGCATGGCTCAGAAGGTAACTCAAGCGCAGACTGAAATTTC
TGTGGTGGAGAAGGAGGATGTGACCTTGGACTGTGTGTATGAAACCCGTGATA
CTACTTATTACTTATTCTGGTACAAGCAACCACCAAGTGGAGAATTGGTTTTC
CTTATTCGTCGGAACTCTTTTGATGAGCAAAATGAAATAAGTGGTCGGTATTC

TTGGAACTTCCAGAAATCCACCAGTTCCTTCAACTTCACCATCACAGCCTCACA
AGTCGTGGACTCAGCAGTATACTTCTGTGCTCTCCC*GGCAGGAAACACACCTC
TTGTCTTTGGAAAGGGCACAAGACTTTCTGTGATTGCAA*ATATCCAGAACCCT
GACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGC
CTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGA
TGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAG
CAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTT
CA... Consensus ends before completion of α constant region

PADI4 Clone 2 TCRβ

CTCAGAGGACCAGTATCCCTCACAGGGTGACACCTGACCAGCTCTGTCCCAC
CTGGCCATGGGCTCCAGGTACCTCTGATGGGAAGACCTTTGTCTCTTGGGAACA
AGTGAATCCTTGGCACAGGCCCAGTGGATTCTGCTGTGCAGAACAGAGAGCAGT
GGACCTCAGGAGGCCTGCAAGGGGAGGACATAGGACAGTGACATCACAGTATGC
CCCTCCCACCAGGAAAAGCAAGGCTGAGAATTTAGCTCTTTCCCAGGAGGACCA
AGCCCTGAGCACAGACACAGTGCTGCCTGCCCCTTTGTGCC|ATGGGCTCCAGGC
TGCTCTGTTGGGTGCTGCTTTGTCTCCTGGGAGCAGGCCCAGTAAAGGCTGGAG
TCACTCAAACTCCAAGATATCTGATCAAAACGAGAGGACAGCAAGTGACACTGA
GCTGCTCCCCTATCTCTGGGCATAGGAGTGTATCCTGGTACCAACAGACCCCAG
GACAGGGCCTTCAGTTCCTCTTTGAATACTTCAGTGAGACACAGAGAAACAAAG
GAAACTTCCCTGGTCGATTCTCAGGGCGCCAGTTCTCTAACTCTCGCTCTGAG
ATGAATGTGAGCACCTTGGAGCTGGGGGACTCGGCCCTTTATCTTTGCGCCAG
CAGCTT*GACTGGGGG*AAACTATGGCTACACCTTCGGTTCGGGGACCAGGTTA
ACCGTTGTAG*AGGACCTGAACAAGGTGTTCCCACCCGAGGTCGCTGTGTTTGA
GCCATCAGAAGCAGAGATCTCCCACACCCAAAAGGCCACACTGGTGTGCCTGGC
CACAGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGTGGGTGAATGGGAAGG
AGGTGCACAGTGGGGTCAGCACGGACCCGCAGCCCCTCAAGGAGCAGCCCGCCC
TCAATGACTCCAGATACTGCCTGAAGATCGGAAGAGC... Consensus ends before completion of β constant region

Synthesised TCR sequences

Following are codon-optimised sequences of GeneArt α and β TCR variable fragment orders. Structure of each insert:

[Restriction site – V(D)J – Beginning of murine constant region – Restriction site]

Asterisk (*) denotes boundaries between V,D,J or plasmid backbone/murine TCR constant regions (see Figures 2.1 and 3.2b). Vertical bar (|) indicates restriction enzyme cut sites.

PADI4 Clone 1 TCRα variable

GC|GGCCGCGCCACC*ATGCTTACAGCTTCTCTGCTGAGAGCCGTGATCGCC
AGCATCTGTGTGGTGTCTAGCATGGCCCAGAAAGTGACACAGGCCCAGACCGAG
ATCAGCGTGGTGGAAAAAGAAGATGTGACCCTGGACTGCGTGTACGAGACACGG
GACACCACCTACTACCTGTTCTGGTACAAGCAGCCTCCTAGCGGCGAGCTGGTG
TTCCTGATCAGACGGAACAGCTTCGACGAGCAGAACGAGATCTCCGGCCGGTAC
AGCTGGAACTTCCAGAAGTCCACCAGCAGCTTCAACTTCACCATCACCGCCAGC
CAGGTGGTGGATAGCGCCGTGTATTTTTGCGCCCTGAGCGAGAGGC*CTTACGG
CGGAGCTACAAACAAGCTGATCTTCGGCACCGGCACACTGCTGGCTGTTCAACC
TA*ACATCCAGAACCCCGACCCCGC|GG

PADI4 Clone 1 TCRβ variable

C|C*ATGGATACCTGGCTCGTGTGCTGGGCCATCTTCAGCCTGCTGAAAGCC
GGACTGACCGAGCCTGAAGTGACCCAGACACCTAGCCACCAAGTGACACAGATG
GGCCAAGAAGTGATCCTGCGCTGCGTGCCCATCAGCAACCACCTGTACTTCTAC
TGGTACAGACAGATCCTGGGCCAGAAAGTGGAATTCCTGGTGTCCTTCTACAAC
AACGAGATCAGCGAGAAGTCCGAGATCTTCGACGACCAGTTCAGCGTGGAAAGA
CCCGACGGCAGCAACTTCACCCTGAAGATCAGAAGCACCAAGCTCGAGGACAGC
GCCATGTACTTTTGCGCCAGCAGCGAGAGAAGAACCCAGC*CTGCCTACGAGCA
GTACTTCGGCCCTGGCACAAGACTGACCGTGACAG*AGGACCTGCGGAACGTGA
CCCCCCCCAAGGTGTCCCTGTTCGAGCCCAGCAAGGCCGAGATCGCCAACAAGC

AGAAAGCCACACTGGTCTGTCTGGCTAGGGGCTTCTTCCCCGACCAC|GTG

PADI4 Clone 2 TCRα variable (1)

GC|GGCCGCGCCACC*ATGAAGTCTCTGAGAGTGCTGCTGGTCATCCTGTGG
CTGCAGCTGTCTTGGGTCTGGTCCCAGCAGAAAGAGGTGGAACAGAACAGCGGC
CCTCTGTCTGTTCCTGAAGGCGCTATCGCCAGCCTGAACTGCACCTACAGCGAT
AGAGGCAGCCAGAGCTTCTTCTGGTACAGACAGTACAGCGGCAAGAGCCCCGAG
CTGATCATGAGCATCTACAGCAACGGCGACAAAGAGGACGGCCGGTTTACAGCC
CAGCTGAACAAGGCCAGCCAGTACGTGTCCCTGCTGATCAGAGATAGCCAGCCT
AGCGACAGCGCCACCTATCTGTGTGCCGT*TAGAGGCCAGGCTGGCACAGCCCT
GATCTTTGGCAAGGGCACAACACTGAGCGTGTCCAGCA*ACATCCAGAACCCCG
ACCCCGC|GG

PADI4 Clone 2 TCRα variable (2)

GC|GGCCGCGCCACC*ATGCTTACAGCTTCTCTGCTGAGAGCCGTGATCGCC
AGCATCTGTGTGGTGTCTAGCATGGCCCAGAAAGTGACACAGGCCCAGACCGAG
ATCAGCGTGGTGGAAAAAGAAGATGTGACCCTGGACTGCGTGTACGAGACACGG
GACACCACCTACTACCTGTTCTGGTACAAGCAGCCTCCTAGCGGCGAGCTGGTG
TTCCTGATCAGACGGAACAGCTTCGACGAGCAGAACGAGATCTCCGGCCGGTAC
AGCTGGAACTTCCAGAAGTCCACCAGCAGCTTCAACTTCACCATCACCGCCAGC
CAGGTGGTGGATAGCGCCGTGTACTTTTGTGCCCTGCC*TGCCGGAAATACCCC
TCTGGTGTTTGGCAAGGGCACCAGACTGTCTGTGATCGCCA*ACATCCAGAACC
CCGACCCCGC|GG

PADI4 Clone 2 TCRβ variable

C|C*ATGGGCAGCAGACTGCTGTGTTGGGTGCTGCTGTGTCTGCTTGGAGCC
GGACCTGTGAAAGCTGGCGTGACCCAGACACCTAGATACCTGATCAAGACCAGA
GGCCAGCAAGTGACCCTGAGCTGCTCTCCTATCAGCGGCCACAGAAGCGTGTCC
TGGTATCAGCAGACACCTGGACAGGGCCTGCAGTTCCTGTTCGAGTACTTCAGC
GAGACACAGCGGAACAAGGGCAACTTCCCCGGCAGATTTTCCGGCAGACAGTTC

AGCAACAGCCGCAGCGAGATGAACGTGTCCACACTGGAACTGGGCGACAGCGCC
CTGTATCTGTGTGCCTCTTCTCT*GACCGGCGG*CAACTACGGCTACACATTTG
GCAGCGGCACCAGACTGACAGTGGTCG*AGGACCTGCGGAACGTGACCCCCCCC
AAGGTGTCCCTGTTCGAGCCCAGCAAGGCCGAGATCGCCAACAAGCAGAAAGCC
ACACTGGTCTGTCTGGCTAGGGGCTTCTTCCCCGACCAC|GTG

pMP71 γ-retroviral TCR plasmids

pMP71 CMV TCR

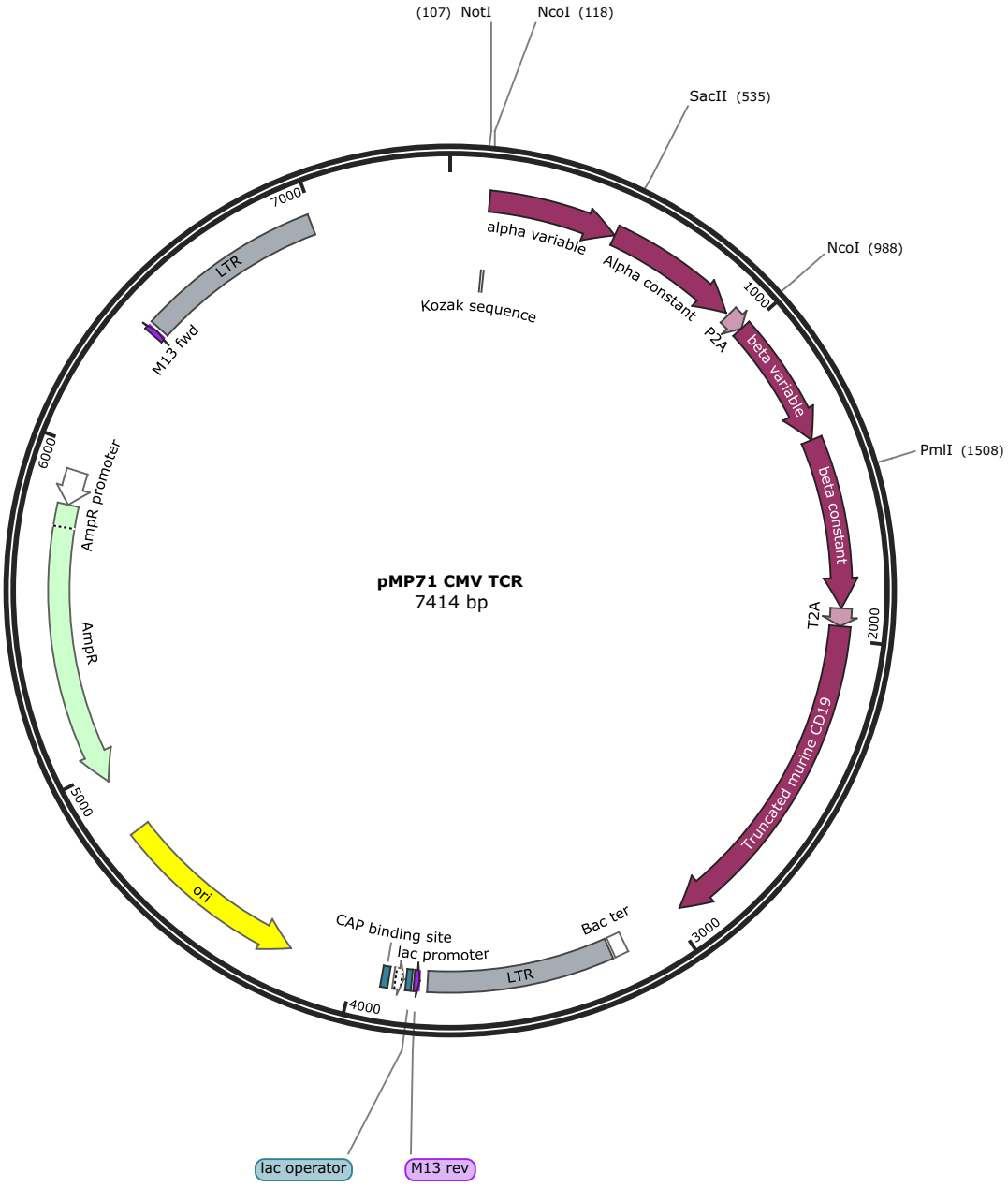

Figure 1: pMP71 CMV TCR plasmid.

GCAGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTG
AAAATTAGCTCGACAAAGTTAAGTAATAGTCCCTCTCTCCAAGCTCACTTACAG
GCGGCCGCGCCACCATGGAAAAGAACCCCCTGGCTGCACCCCTGCTGATCCTGT
GGTTCCACCTGGACTGCGTGAGCATCCTGAACGTGGAGCAGAGCCCCCAGTCTC

TGCATGTGCAGGAAGGCGACAGCACCAACTTCACCTGCAGCTTCCCCAGCAGCA
ACTTCTACGCCCTGCACTGGTACAGATGGGAGACCGCCAAGAGCCCCGAGGCCC
TGTTCGTGATGACCCTGAACGGCGACGAGAAGAAGAAGGGCCGGATCAGCGCCA
CCCTGAACACCAAAGAGGGCTACAGCTACCTGTATATCAAGGGCAGCCAGCCCG
AGGACAGCGCCACCTACCTGTGCGCCCGGAACACCGGCAACCAGTTCTACTTTG
GCACCGGCACCTCCCTGACCGTGATCCCCAACATCCAGAACCCCGACCCCGCGG
TGTACCAGCTGAAGGACCCCAGAAGCCAGGACAGCACCCTGTGCCTGTTCACCG
ACTTCGACAGCCAGATCAACGTGCCCAAGACAATGGAAAGCGGCACCTTCATCA
CCGACAAGTGCGTGCTGGACATGAAGGCTATGGACAGCAAGAGCAACGGCGCCA
TCGCCTGGTCCAACCAGACCTCCTTCACATGCCAAGACATCTTCAAAGAGACCA
ACGCCACCTACCCCAGCAGCGACGTGCCCTGCGATGCCACTCTCACCGAGAAGA
GCTTCGAGACCGACATGAACCTGAACTTCCAGAACCTGAGCGTGATGGGCCTGA
GAATCCTGCTCCTGAAAGTGGCCGGCTTCAACCTGCTGATGACCCTGCGGCTCT
GGAGTTCTGGCAGCGGCGCTACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACG
TGGAGGAAAACCCTGGCCCCATGGTGATCGGCCTGCTGTGCTGTGCCGCCCTGA
GCCTGCTGTGGGCCGGACCTGTGAACGCCGGCGTGACCCAGACCCCCAAGTTCC
AGGTGCTGAAAACCGGCCAGAGCATGACCCTGCAGTGCGCCCAGGACATGAACC
ACGAGTACATGAGCTGGTACAGGCAGGACCCCGGCATGGGCCTGCGGCTGATCC
ACTACAGCGTGGGAGCCGGCATCACCGACCAGGGCGAGGTGCCCAACGGCTACA
ACGTGAGCAGAAGCACCACCGAGGACTTCCCCCTGCGGCTGCTGTCTGCCGCCC
CTAGCCAGACCAGCGTGTACTTCTGCGCCAGCAGCTTCCAGACCGGCGCCAGCT
ACGGCTACACCTTCGGCAGCGGCACCCGGCTGACCGTGCTCGAGGACCTGCGGA
ACGTGACCCCCCCCAAGGTGTCCCTGTTCGAGCCCAGCAAGGCCGAGATCGCCA
ACAAGCAGAAAGCCACACTGGTCTGTCTGGCTAGGGGCTTCTTCCCCGACCACG
TGGAGCTGTCTTGGTGGGTCAACGGCAAAGAAGTCCATAGCGGCGTCTGCACCG
ACCCTCAGGCTTACAAAGAGAGCAACTACTCCTACTGCCTGAGCAGCCGGCTGA
GAGTGAGCGCCACCTTCTGGCACAACCCCCGGAACCACTTCCGGTGCCAGGTGC
AGTTCCACGGCCTGAGCGAAGAGGACAAGTGGCCTGAGGGCTCCCCCAAGCCCG
TGACCCAGAACATCAGCGCCGAGGCCTGGGGCAGAGCCGACTGCGGCATCACCA
GCGCCAGCTACCACCAGGGCGTGCTGTCCGCCACCATCCTGTACGAGATCCTGC

TGGGCAAGGCCACACTGTACGCCGTGCTGGTGTCCGGCCTGGTCCTGATGGCTA
TGGTGAAGAAGAAGAACAGCGAAGGCAGAGGCTCTCTGCTGACATGTGGCGACG
TCGAGGAGAATCCCGGACCGATGCCTAGCCCTCTGCCCGTGTCCTTCCTGCTGT
TCCTGACCCTCGTGGGCGGCAGACCCCAGAAAAGCCTGCTGGTGGAAGTGGAAG
AGGGCGGCAACGTGGTGCTGCCCTGCCTGCCTGATAGCAGCCCTGTGTCCTCTG
AGAAGCTGGCCTGGTACAGAGGCAACCAGAGCACCCCATTTCTGGAACTGAGCC
CCGGCTCTCCTGGCCTGGGACTGCATGTGGGATCTCTGGGCATTCTGCTCGTGA
TCGTGAACGTGTCCGACCACATGGGCGGCTTTTACCTGTGCCAGAAGCGGCCAC
CCTTCAAGGACATCTGGCAGCCTGCCTGGACCGTGAATGTGGAAGATAGCGGCG
AGATGTTCCGGTGGAACGCCAGCGACGTGCGGGACCTGGATTGCGACCTGCGGA
ATAGAAGCAGCGGCAGCCACAGATCCACCTCCGGCAGCCAGCTGTACGTGTGGG
CCAAGGACCACCCCAAAGTGTGGGGCACCAAGCCTGTGTGTGCCCCCAGAGGCA
GCAGCCTGAACCAGAGCCTGATCAACCAGGACCTGACCGTGGCCCCTGGCTCTA
CACTGTGGCTGTCTTGTGGCGTGCCCCCCGTGCCTGTGGCTAAGGGCTCTATCT
CCTGGACCCATGTGCACCCCAGACGGCCCAATGTGTCCCTGCTGTCTCTGTCTC
TGGGCGGCGAACACCCCGTGCGCGAGATGTGGGTGTGGGGCTCACTTCTGCTGC
TGCCACAGGCCACAGCCCTGGATGAGGGCACCTACTACTGCCTGCGGGGCAACC
TGACCATCGAGCGGCATGTGAAAGTGATCGCCCGCAGCGCTGTGTGGCTGTGGC
TGCTGAGAACAGGCGGATGGATCGTGCCCGTCGTGACCCTGGTGTACGTGATCT
TCTGCATGGTGTCCCTGGTGGCCTTTCTGTACTGCCAGCGGGCCTTCATCCTGC
GGCGGAAGAGAAAGAGAATGACCGACCCCGCCAGACGGTTCTTCAAAGTGACCT
GAGTCGACTGTACAAGTAAGAATTCGGATCCAAGCTTAGGCCTGCTCGCTTTCT
TGCTGTCCCATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAAC
TGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAGCGCTAAGCTT
AACACGAGCCATAGATAGAATAAAAGATTTTATTTAGTCTCCAGAAAAAGGGGG
GAATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTT
GCAAGGCATGGAAAATACATAACTGAGAATAGAGAAGTTCAGATCAAGGTTAG
GAACAGAGAGACAGCAGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTC
CTGCCCCGGCTCAGGGCCAAGAACAGTTGGAACAGCAGAATATGGGCCAAACAG
GATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCC

CCAGATGCGGTCCCGCCCTCAGCAGTTTCTAGAGAACCATCAGATGTTTCCAGG
GTGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTT
CGCTTCTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCC
ACAACCCCTCACTCGGCGCGCCAGTCCTCCGATAGACTGCGTCGCCCGGGTACC
CGTGTTCTCAATAAACCCTCTTGCAGTTGCATCCGACTCGTGGTCTCGCTGTTC
CTTGGGAGGGTCTCCTCTGAGTGATTGACTGCCCACCTCGGGGGTCTTTCATTC
TCGAGCAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTA
TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTG
GGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGC
TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGC
GGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT
AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAA
AGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA
TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG
GCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCT
CGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT
CCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTC
GGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCC
CGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACA
CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTA
TGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAG
AAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAG
AGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTT
TGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTT
GATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGAT
TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAA
AATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCA
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pMP71 PADI4 Clone 1 TCR

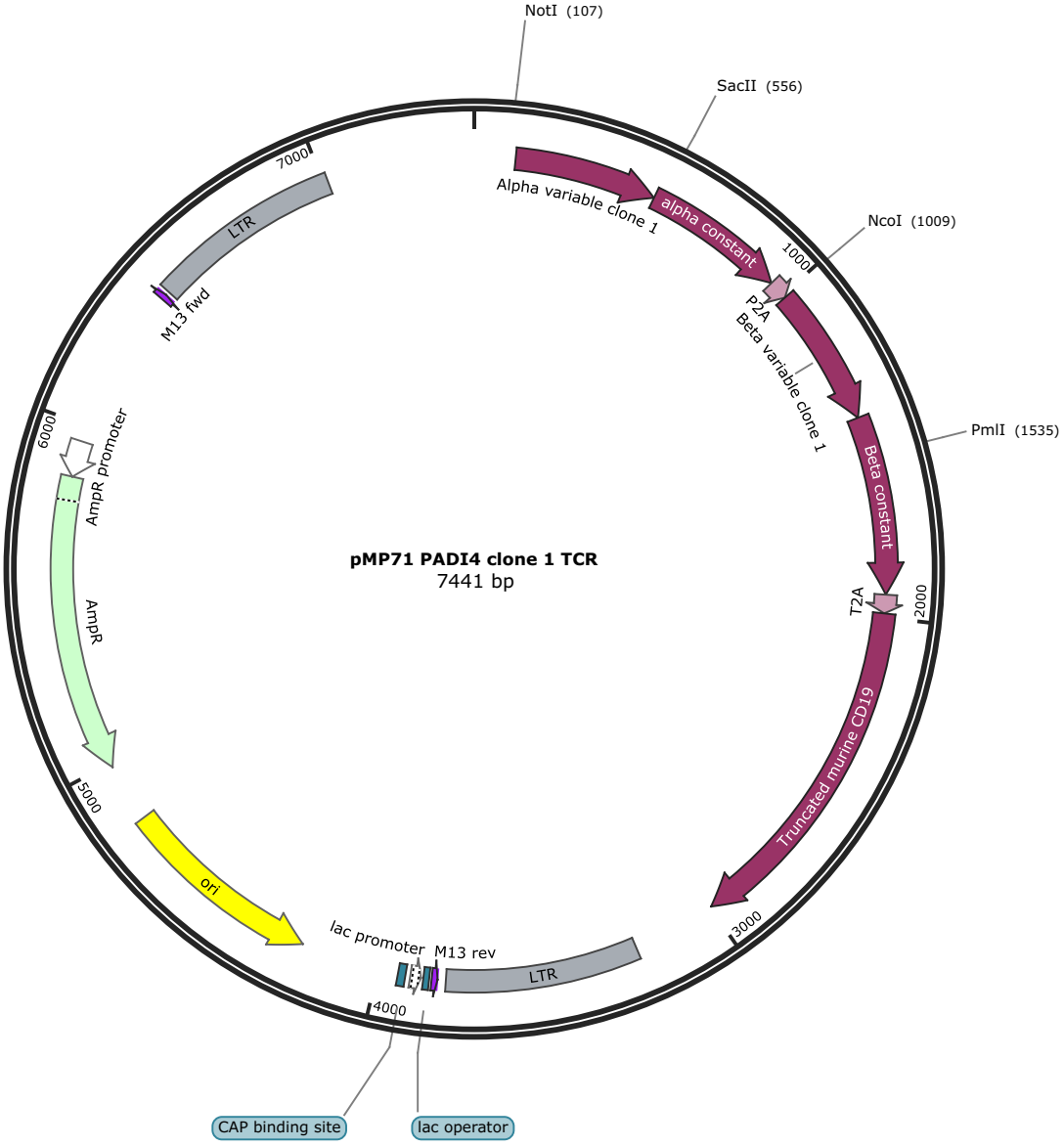

Figure 2: pMP71 PADI4 clone 1 TCR plasmid.

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pMP71 PADI4 Clone 2.1 TCR

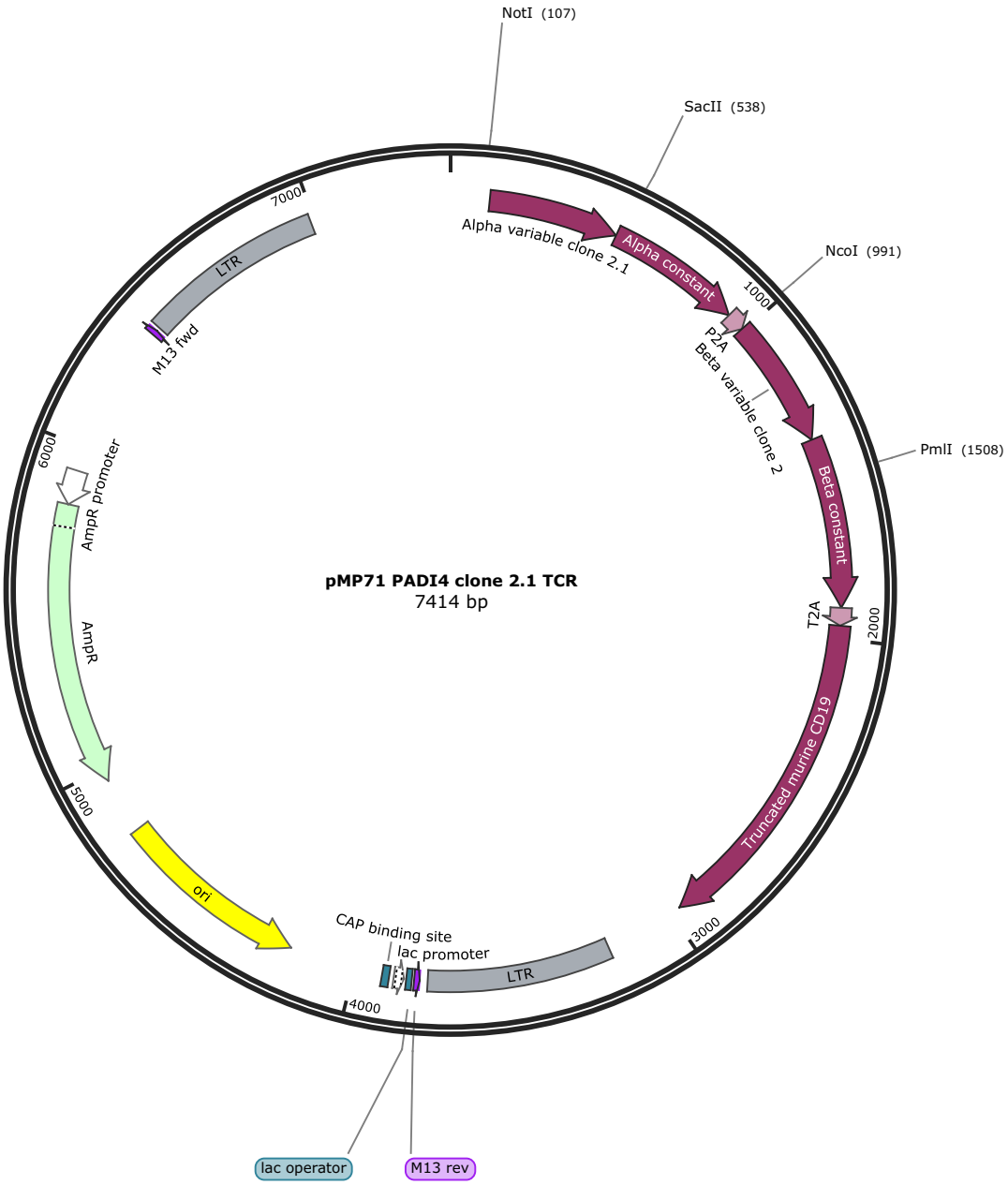

Figure 3: pMP71 PADI4 clone 2 (1) TCR plasmid. This construct contains Clone 2 TCRα (1) variable fragment and clone 2 TCRβ variable fragment.

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CGGCCAGTGAATTAGTACTCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGA
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AGCAGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCT
CAGGGCCAAGAACAGTTGGAACAGCAGAATATGGGCCAAACAGGATATCTGTG
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GACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCG
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AATAAAGCCTCTTGCTGTTTGCATCCGAATCGTGGACTCGCTGATCCTTGGGA
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TGGGAGAGGTCCCAGCGGCCTCGGGGGCCCGTTTTGTGGCCCATTCTGTATCA
GTTAACCTACCCGAGTCGGACTTTTTGGAGCTCCGCCACTGTCCGAGGGGTAC
GTGGCTTTGTTGGGGGACGAGAGACAGAGACACTTCCCGCCCCCGTCTGAATT
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pMP71 PADI4 Clone 2.2 TCR

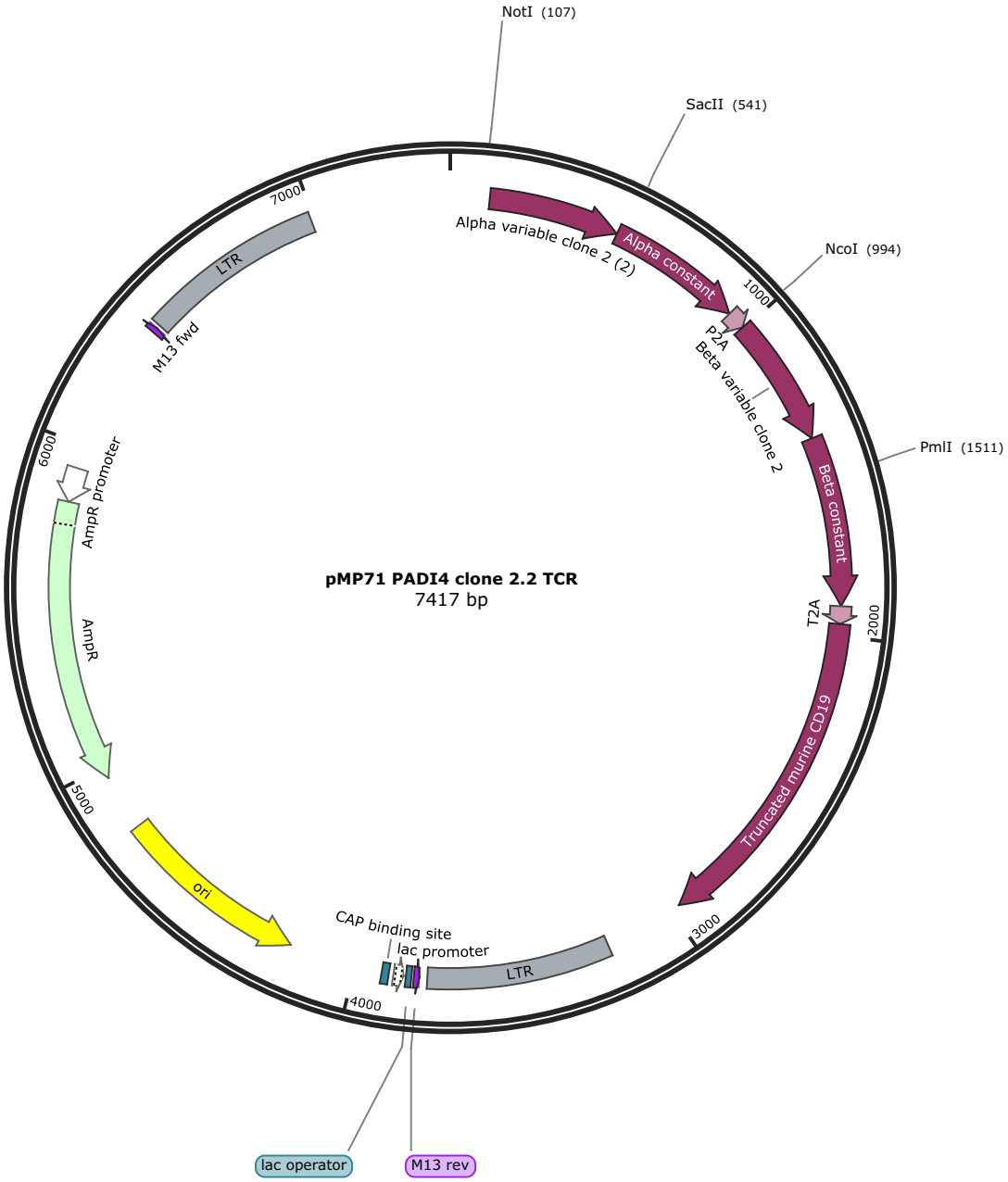

Figure 4: pMP71 PADI4 clone 2 (2) TCR plasmid. This construct contains Clone 2 TCRα (2) variable fragment and clone 2 TCRβ variable fragment.

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TCTGTGTGGTGTCTAGCATGGCCCAGAAAGTGACACAGGCCCAGACCGAGATCA

GCGTGGTGGAAAAAGAAGATGTGACCCTGGACTGCGTGTACGAGACACGGGACA
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GTTAACCTACCCGAGTCGGACTTTTTGGAGCTCCGCCACTGTCCGAGGGGTAC
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TTTGCTTTCGGTTTTACGCCGAAACCGCGCCGCGCGTCTTGTCTGCT

pHRsin lentiviral TCR plasmids

Only insert sequences are available for all pHRsin vectors. pHRsin-SFFV-1G4 plasmid insert Figure 6 shows representative TCR construct structure for all other TCR plasmids.

pHRsin-SFFV-mCherry-TCRαConstant



Figure 5: pHRsin-SFFV-mCherry-TCRαConstant.

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pHRsin-SFFV-1G4

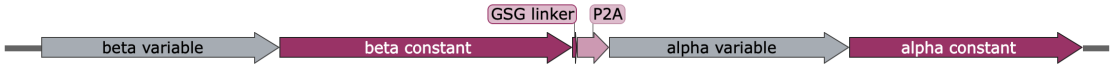

Figure 6: pHRsin-SFFV-1G4.

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pHRsin PADI4 clone 1 TCR

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pHRsin PADI4 clone 2.1 TCR

This construct contains Clone 2 TCRα (1) variable fragment and clone 2 TCRβ variable fragment.

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TGGATATGAAAGCCATGGACAGTAAGTCAAACGGCGCCATCGCTTGGAGCAAT
CAGACATCCTTCACTTGCCAGGATATCTTCAAGGAGACCAACGCAACATACCC
ATCCTCTGACGTGCCCTGTGATGCCACCCTGACAGAGAAGTCTTTCGAAACAG
ACATGAACCTGAATTTTCAGAATCTGAGCGTGATGGGCCTGAGAATCCTGCTG
CTGAAGGTCGCTGGGTTTAATCTGCTGATGACACTGCGGCTGTGGTCCTCATG
AGCGACTCTAGAGTCGACCTGCAGGCATGCAAGCTTGATATC

pHRsin PADI4 clone 2.2 TCR

This construct contains Clone 2 TCRα (2) variable fragment and clone 2 TCRβ variable fragment.

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Lentiviral PADI4 constructs

pUC19 PADI4-WT

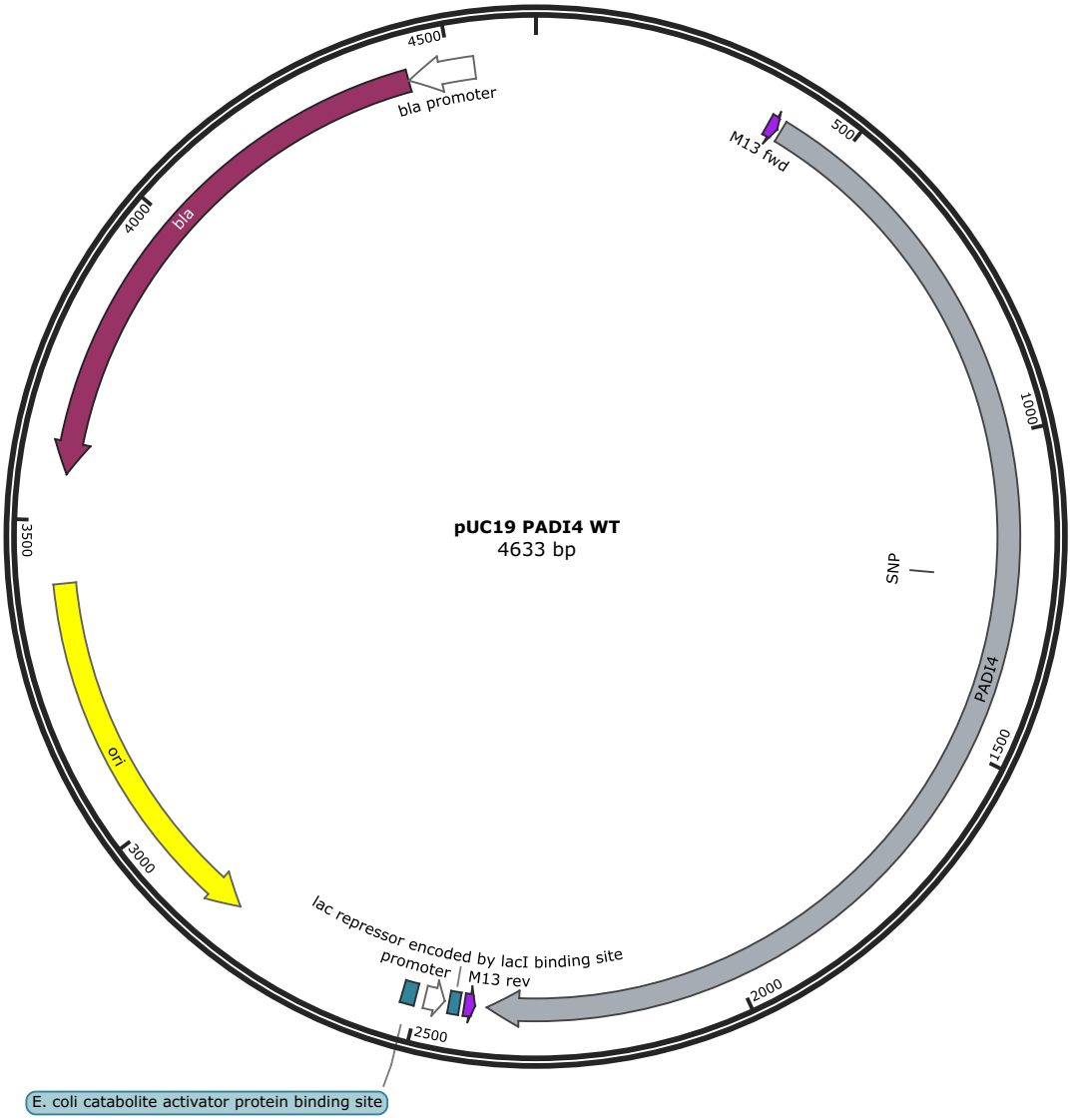

Figure 7: pUC19 PADI4-WT.

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GAGAGCTGCTGAAGAGAGAGCTGGGCCTGGCCGAGTCCGACATCATCGATATCC
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TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCA
TTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

pUC19 PADI4-Var

This plasmid is identical to pUC19 PADI4-WT except for a single nucleotide change introducing the PADI4 antigen of interest (in red):

...caccatctccctgctggacacgtTcaacctggagctgcccgaggccg...

pL-PADI4-WT.EFS.GFP

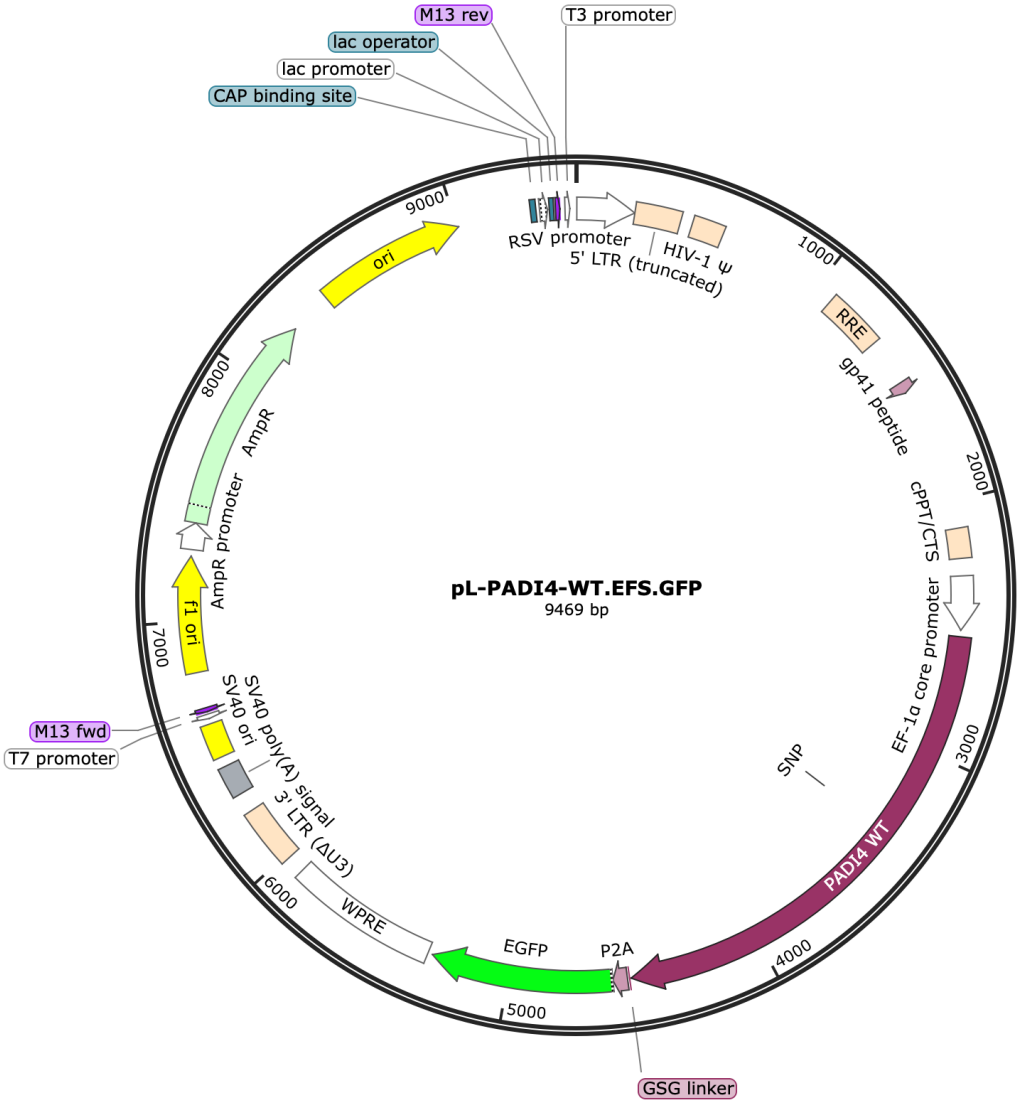

Figure 8: pL-PADI4-WT.EFS.GFP.

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CCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAA
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AATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATC
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CTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATT
AGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATT
GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAA
GCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTGCAAGC

pL-PADI4-Var.EFS.GFP

This plasmid is identical to pL-PADI4-WT.EFS.GFP except for a single nucleotide change introducing the PADI4 antigen of interest (in red):

...caccatctccctgctggacacgtTcaacctggagctgcccgaggccg...

.2 Supplemental Figures

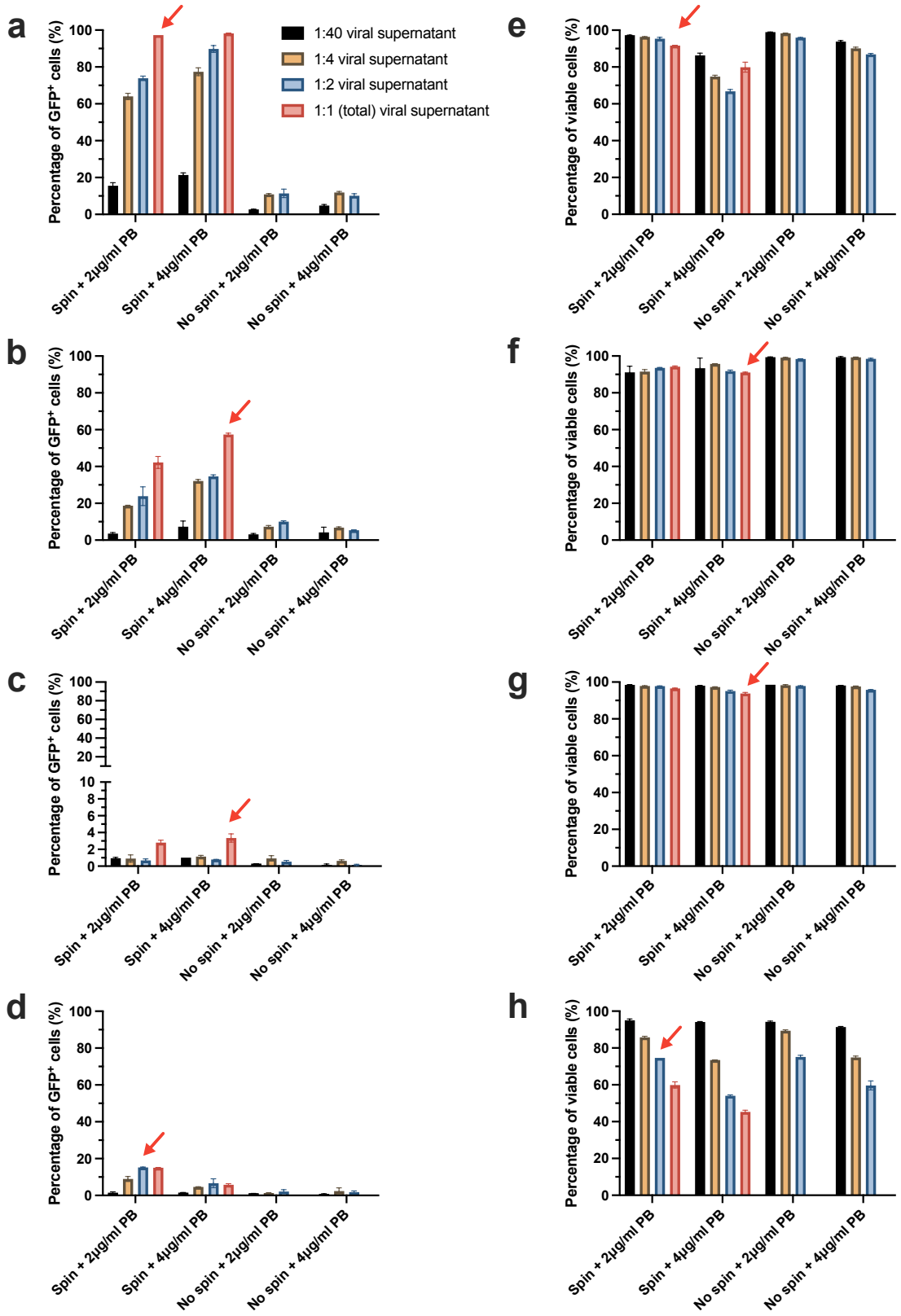
a
b
c
d
e
f
g
h
Percentage of GFP+ cells (%)
Percentage of viable cells (%)
1:40 viral supernatant
1:4 viral supernatant
1:2 viral supernatant
1:1 (total) viral supernatant
Spin + 2µg/ml PB
Spin + 4µg/ml PB
No spin + 2µg/ml PB
No spin + 4µg/ml PB

Figure 9: (Previous page) Cell line transduction optimisation using CRISPR-GFP lentivirus vector. a-d Show transduction efficiency (GFP^+) of HEL, MONOMAC1, BOLETH and SUP-B15 cell lines, respectively, 3 days post-transduction. **e-h** Show viability of HEL, MONOMAC1, BOLETH and SUP-B15 cell lines, respectively, 3 days post-transduction. Colours indicate different dilutions of added lentivirus preparation to cells. Arrows indicate most optimal transduction conditions that were taken forward. PB: polybrene. 'Spin' condition indicates spinoculation, where once PB and virus are added to cells, they are spun at 1000g for 90 minutes to increase cell proximity to virus particles.

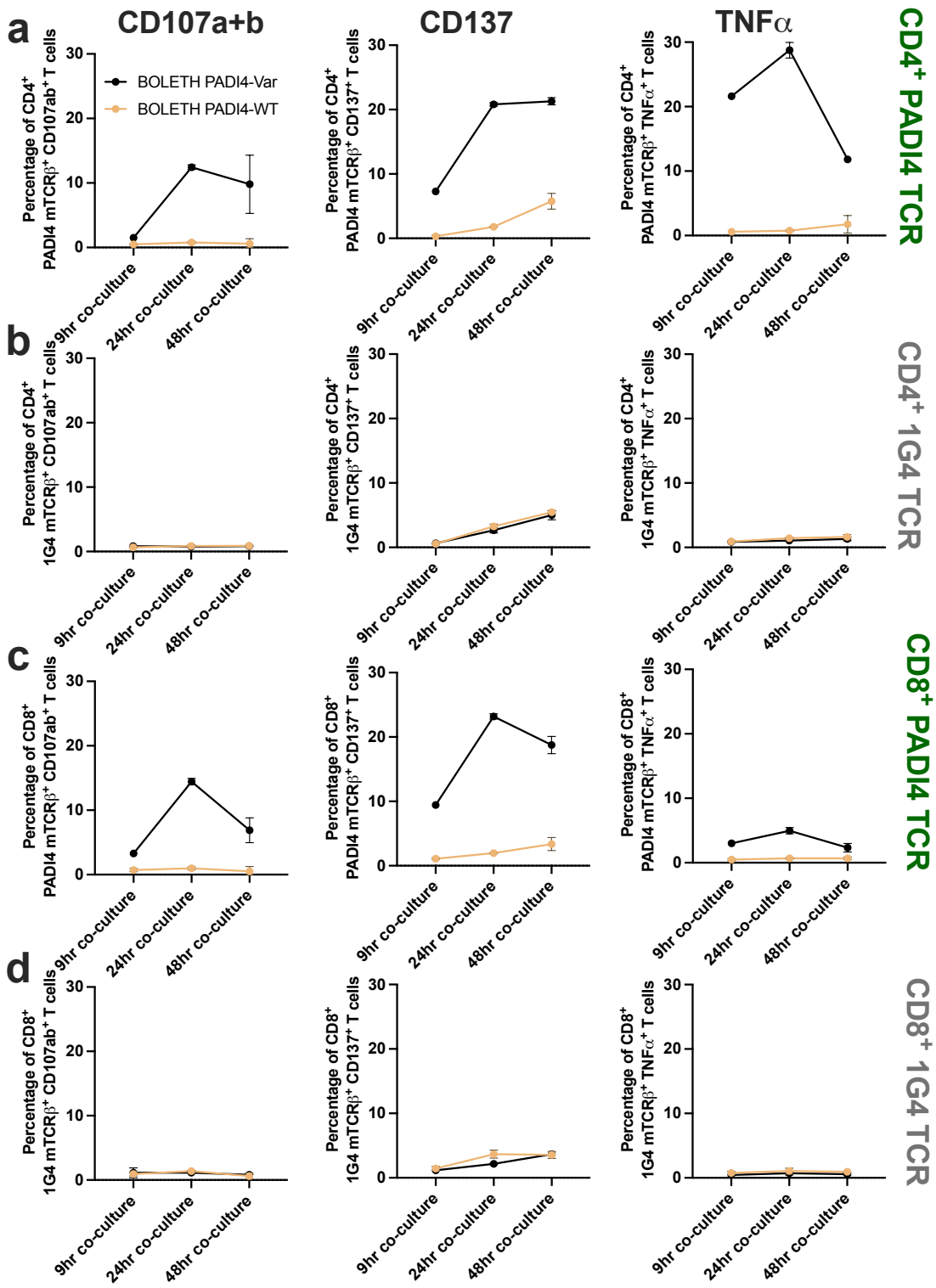

Figure 10: PADI4 TCR T cell activation to PADI4-transduced BOLETH is dependent on time of co-culture. Panels **a** and **c** show T cells transferred with PADI4 TCR, while panels **b** and **d** show T cells transferred with irrelevant 1G4 TCR. Top half of the figure shows $CD4^+$ and bottom half $CD8^+$ T cells. Columns show different activation markers: CD107a and CD107b co-stain (left), CD137 (middle) and TNFα (right). Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

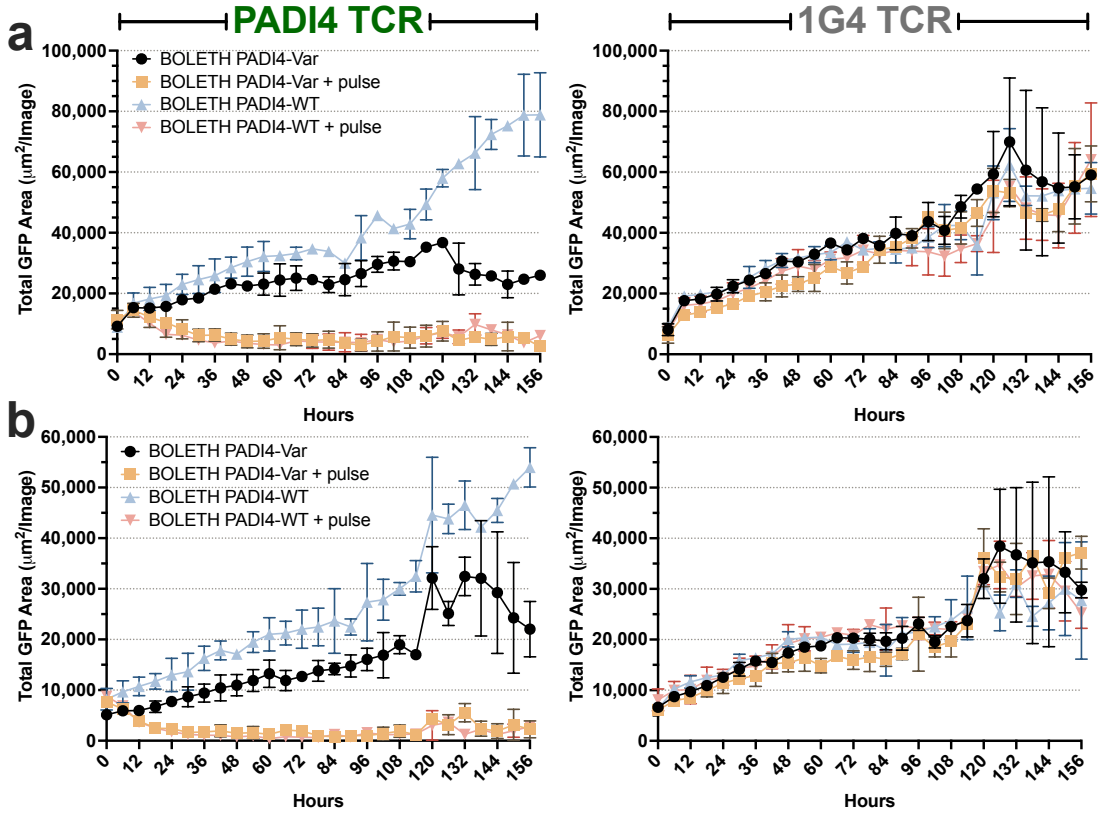

Figure 11: PADI4 T cells inhibit PADI4-transduced BOLETH growth at higher effector-to-target (E:T) ratios. Graphs show BOLETH growth over time when target cells are mixed with T cells at 5:1 (panel **a**) and 10:1 (panel **b**) E:T ratio. Growth over time is measured by calculating the total GFP+ area at the bottom of a cell culture dish using a live cell imaging system. Columns distinguish between target cell line co-culture with T cells carrying different constructs: PADI4 TCR (left) and irrelevant 1G4 TCR (right). 'Pulse' indicates that target cells were incubated with PADI4 variant peptides 21 and 22 (1μM) prior to co-culture with T cells. Graphs show one experiment and error bars represent standard deviation between technical replicates.

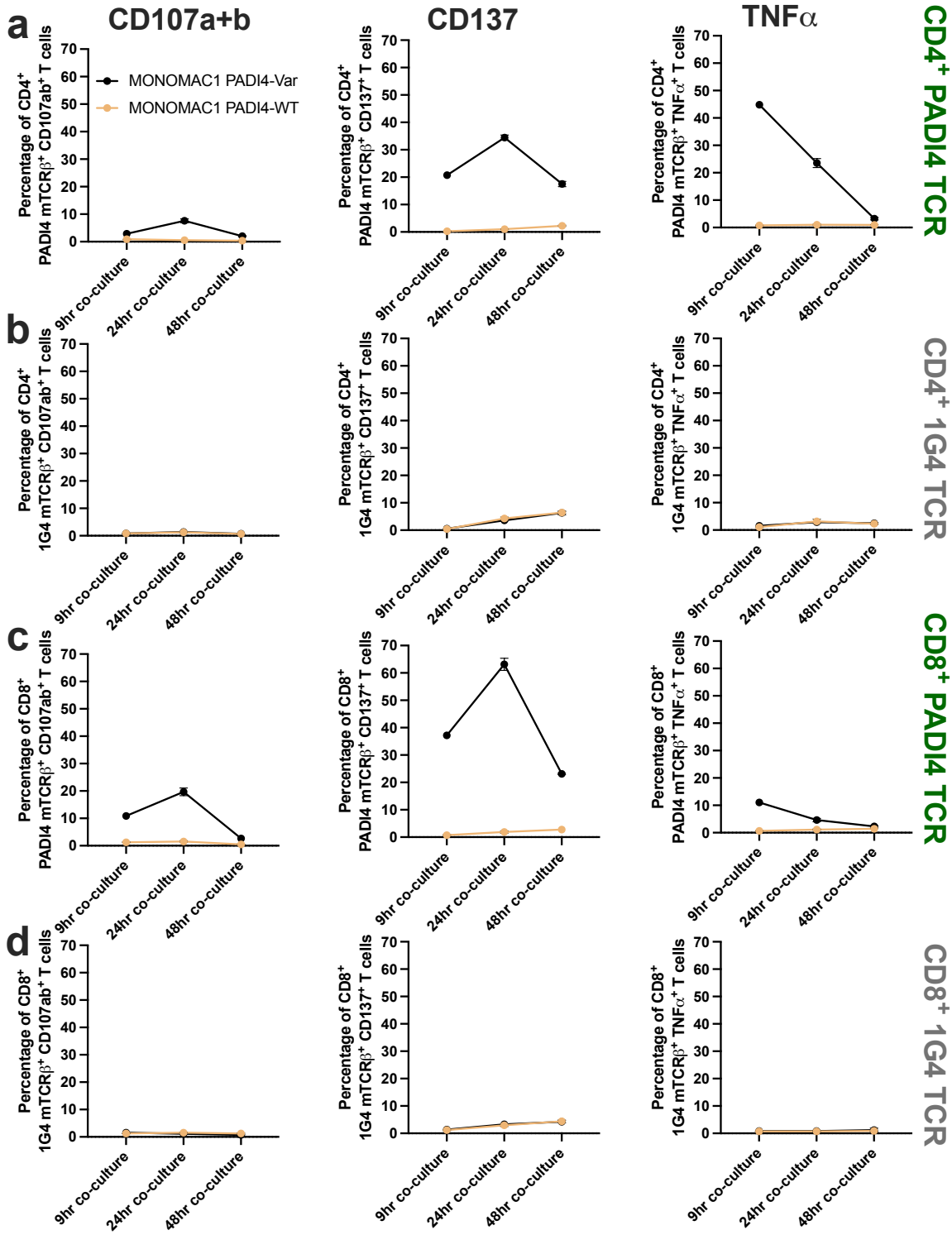

Figure 12: PADI4 TCR T cell activation to IFNγ-treated PADI4-transduced MONOMAC1 is dependent on the time of co-culture. Cell lines were treated with 50ng/ml IFNγ for 6 days prior to T cell co-culture. Panels **a** and **c** show T cells transferred with PADI4 TCR, while panels **b** and **d** show T cells transferred with irrelevant 1G4 TCR. Top half of the figure shows $CD4^+$ and bottom half $CD8^+$ T cells. Columns show different activation markers: CD107a and CD107b co-stain (left), CD137 (middle) and TNFα (right). Cells are mixed 1:2 effector:target ratio. Graphs show one experiment and error bars represent standard deviation between technical replicates.

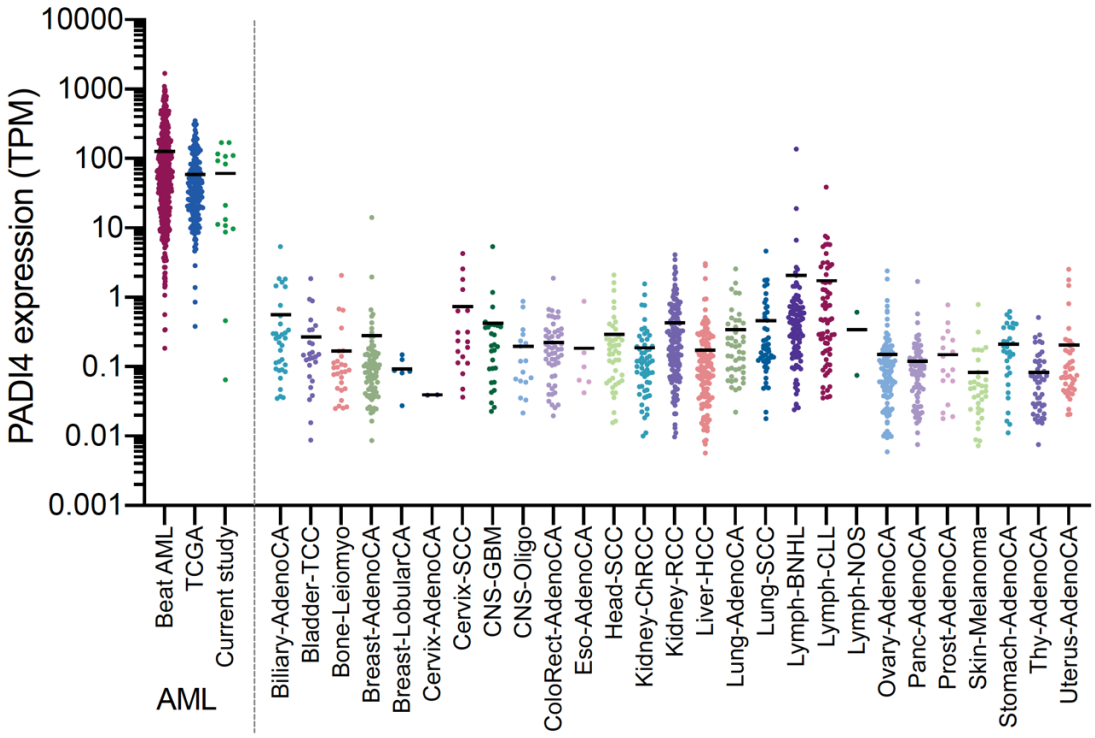

Figure 13: PADI4 expression across different cancers. Graph displays publicly available data from The Cancer Genome Atlas (TCGA) programme and Beat AML[251]. 'Current Study' refers to leukaemia samples from patients studied in the miHA discovery pipeline developed by Connor Sweeney (see Chapter 1). TPM, transcripts per million. Related to Fig. 4.1.

HLA-DP and PADI4 expression	Sample	CD3- (CD19-)	HLA-DP MFI (CD3- (CD19-))	CD34+ CD117+	HLA-DP MFI (CD34+ CD117+)	CD34+ CD117lo/-	HLA-DP MFI (CD34+ CD117lo/-)	CD34lo/- CD117+	HLA-DP MFI (CD34lo/- CD117+)	CD34lo/+ CD117lo/-	HLA-DP MFI (CD34lo/+ CD117lo/-)	CD34- CD117lo/-	HLA-DP MFI (CD34- CD117lo/-)	CD34- CD117-	HLA-DP MFI (CD34- CD117-)
Both	BIR346	82.1	5704	4.81%	1993	82.80%	6497							7.15%	1017
	CAR134	90.2	438			90.70%	435							7.72%	450
	RUH039	54.7	2526			5.53%	2758	22.20%	4817					67.30%	1593
	ABE072	64.5	881							3.94%	491	95.40%	894		
	RLH032	41.5	1597			41.00%	1484							56.50%	1682
	STB220	90.8	441			7.94%	132					90.40%	90.4		
	NOT114	3.5													
	GCH001	41													
PADI4 only	ROM103	87.7	184							1.39%	159	97.40%	177		
	STB390	73.4	331			70.30%	99.2							24%	971
HLA-DP only	STB319	9.06	3856							7.13%	5296	90.40%	3710		
	CUH018	98.9	544			77.00%	602							16.70%	331
	STB270	90.9	1059			51.60%	1646							46%	389
	ROM074	83.7	4943					64.60%	6994					28.60%	617
	BRI1442	83.7	2346							57.60%	1867			37.30%	3105
	AYL065	45.3	742							5.08%	1065			92.60%	697
	NOT110	53.2	6110							1.44%	9027			96.20%	6008
	OX862	85													
Neither	DRI001	Low viability at thaw													
	BRI2122	37.7	170			33.00%	184							61.80%	164
	BRI2348	16 million cells or fewer available													
	GEO006														

Figure 14: Summary of all available patient AML samples with restricting PADI4 and HLA-DP genotype. Table indicates the frequency of different predicted blast sub-populations and their HLA-DP mean fluorescence intensity. Related to Fig. 4.3.

Target population	Starting frequency (%)	Post-MACS frequency (%)	Related to figures
Assay 1 (PADI4 SNP-Var heterozygote HLA-DPB1*04:01 heterozygote target samples)			
CD3	54.90%	98.40%	5.8, 5.9, 5.11, 5.12
CD14	25.60%	98.30%	
CD19	11.50%	96%	
CD34	57.60%	N/A	
Assay 2 (PADI4 SNP-Var heterozygote HLA-DPB1*04:01 homozygote* target samples)			
CD3	57.50%	99.60%	
CD14	19.10%	96.70%	
CD15	95.4% (99% are granulocytes)	N/A	
CD19	6.65%	86.60%	
CD34	75.80%	N/A	
Assay 3 (PADI4 SNP-Var heterozygote HLA-DPB1*04:01 homozygote* target samples)			
CD3	45.90%	99.20%	
'Untouched' CD3		71.40%	
CD14	8.90%	97.60%	
CD15	78.6% (96% are granulocytes)	N/A	
CD19	7.92%	98.20%	
CD34 (NOC067) (killing assay only)	90.80%	N/A	
CD34 (NOC088) (activation assay only)	52.20%	N/A	
Assay 4 (PADI4 SNP-WT homozygote HLA-DPB1*04:01 heterozygote[+] target samples)			5.10
CD3	59.70%	99.40%	
'Untouched' CD3		76.90%	
CD14	9.95%	94.60%	
CD15	91.1% (98.3% are granulocytes)	N/A	
CD19	5.81%	93.00%	
CD34	85.80%	N/A	
Assay 5 (PADI4 SNP-WT homozygote HLA-DPB1*04:01 homozygote target samples)			
CD3	72.40%	98.60%	
'Untouched' CD3		83.10%	
CD14	3.05%	94.30%	
CD15	88.2% (98.40% are granulocytes)	N/A	
CD19	7.41%	99.40%	

*except for CD34 samples, which are HLA-DPB1*04:01 heterozygote

[+] except for CD34 sample, which is HLA-DPB1*04:01 homozygote

Figure 15: Normal haematopoietic target cell purity for experiments carried out in Chapter 5. In each case, CD34 target cells were derived from cryopreserved and CD34-enriched vials. Granulocytes were gated using FSC and SSC.

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