Investigating the systemic immune landscape of thymectomised paediatric transplant patients at risk of EBV-associated post-transplant lymphoproliferative disease

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Abstract

Epstein-Barr virus-associated post-transplant lymphoproliferative disease (EBV-PTLD) are rare but life-threatening lymphoid malignancies that disproportionately affect paediatric heart transplant recipients. Recent discourse has focussed on the role of early removal of the thymus gland, which is critical for normal antiviral immunity, in the aetiology of EBV-PTLD after heart transplantation. This Fellowship aimed to investigate the systemic immune landscape of thymectomised paediatric transplant patients at risk of EBV-PTLD.

Fifty-four patients awaiting transplant were recruited to the Immunology of THymectomy And childhood CArdiac transplant (ITHACA) study for immune profiling using full spectrum flow cytometry before transplant and at 3-, 6-, 12-and 24-months post-transplant. Patients were stratified into early (<6 months old) and late (≥6 months old) thymectomy for comparison to age-matched non□thymectomy controls. Induction immunosuppression with anti-thymocyte globulin was associated with diminished antiviral CD16+ monocytes compared to Basiliximab induction. Failed expansion of CD16+ monocyte subsets was notable among patients with EBV DNAemia alongside a negative correlation between EBV DNA load and their expression of activation markers. Mycophenolate Mofetil was associated with an overall reduction in NK cells and a phenotypic shift towards immunosenescence. EBV DNAemia was associated with a marked increase in CD16dim CD56dim NK cells but a failure to expand the CD56dim NKG2A+ KIR- subset required for effective EBV control.

Early thymectomy was associated with the depletion of regulatory, naïve CD4+ and CD8+ T-lymphocytes and expansion of senescent CD57+ memory T-lymphocytes. Most of these changes persisted after transplant and included the emergence of terminal effector memory subsets with increased expression of immune checkpoint proteins within the memory compartment of early thymectomy patients. Increased immunosenescence and exhaustion of EBV-specific T-lymphocytes were associated with early thymectomy and persistent EBV DNAemia. These data demonstrate that both early thymectomy and choice of immunosuppression are associated with distinct features of immunosenescence, exhaustion and impaired EBV control, all likely contributing to the risk of developing to EBV-PTLD.

Dedication

This thesis is dedicated to all the ITHACA participants and their families without whom this work would not exist and to my mother, who inspired my career choice and continues to be the touchstone for my work ethic.

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List of Abbreviations

ACM – Acquired Cardiomyopathy

ALP – Alkaline Phosphatase

APC – Antigen Presenting Cell

ATG – Anti-Thymocyte Globulin

Aza - Azathioprine

BCIP/NBT – 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium

BL – Burkitt Lymphoma

BSA – Bovine Serum Albumin

cDC – Conventional Dendritic Cell

CHD – Congenital Heart Disease

CMV – Cytomegalovirus

CNI – Calcineurin Inhibitor

CI – Confidence Interval

CTECs – Cortical Thymic Epithelial Cells

CTL – Cytotoxic T-Lymphocyte

CTLA-4 – Cytotoxic T-Lymphocyte Associated Protein 4

CVID – Common Variable Immunodeficiency

DC – Dendritic Cell

DLBCL – Diffuse Large B-Cell Lymphoma

DMSO – Dimethyl Sulfoxide

DN – Double Negative

DP – Double Positive

EBNA – Epstein-Barr Nuclear Antigen

EBV – Epstein-Barr Virus

ECMO – Extracorporeal Mechanical Oxygenation

eCRF – Electronic Case Report Form

EDTA – Ethylenediaminetetraacetic Acid

FACS – Fluorescence Activated Cell Sorting

FBS – Fetal Bovine Serum

FCCF – Flow Core Cytometry Facility

FCS – Flow Cytometry Standard

FHL – Familial Hemophagocytic Lymphohistiocytosis

FlowSOM - Flow Cytometry Self-Organising Maps

FOXP3 – Forkhead Box 3 Protein 3

FSC – Forward Scatter

GCP – Good Clinical Practice

GDPR – General Data Protection Regulation

GOSH – Great Ormond Street Hospital for Children

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HHV4 – Human Herpes Virus 4

H&I – Histocompatibility and Immunogenetics

HIV – Human Immunodeficiency Virus

HL – Hodgkin Lymphoma

HLA – Human Leukocyte Antigen

HR – Hazard Ratio

HRA – Health Research Authority

IEI – Inborn Error of Immunity

IFN – Interferon

Ig - Immunoglobulin

IL – Interleukin

ILC – Innate Lymphoid Cells

IM – Infectious Mononucleosis

IQR – Interquartile Range

IRAS – Integrated Research Application System

ITHACA – Immunology of Thymectomy and Childhood Cardiac Transplant

JAK-STAT – Janus Kinase-Signal Transducers and Activators of Transcription

KIR - Killer-cell Immunoglobulin-like Receptor

LAG-3 – Lymphocyte-Activation Gene 3

LCL – Lymphoblastoid Cell Line

LCMV – Lymphocytic Choriomeningitis Virus

LMP – Latent Membrane Protein

MAPK – Mitogen-Activated Protein Kinase

MCS – Multicolour Staining

MDSC – Myeloid-Derived Suppressor Cell

MFI – Median Fluorescent Intensity

MHC – Major Histocompatibility Complex

MMF - Mycophenolate Mofetil

MPEC – Memory Precursor Effector Cells

MTEC – Medullary Thymic Epithelial Cells

NF-κB – Nuclear Factor κB

NHSBT – National Heatlh Services Blood and Transplant

NK – Natural Killer

NKT – Natural Killer T-like Cells

NuTH – Newcastle Upon Tyne NHS Hospitals Foundation Trust

OHT – Orthotopic Heart Transplant

OMIP – Optimised Multicolour Immunofluorescence Panel

PBMC – Peripheral Blood Mononuclear Cell

PBS – Phosphate Buffer Saline

PCR – Polymerase Chain Reaction

PD-1 – Programmed Cell Death 1

pDC – Plasmacytoid Dendritic Cell

PIS – Patient Information Sheet

PPIE – Patient and Public Involvement and Engagement

PRR - Pathogen Recognition Receptor

PTLD – Posttransplant Lymphoproliferative Disease

REC – Research Ethics Committee

RIG - Retinoic acid-Inducible Gene

RTE – Recent Thymic Emigrants

sjTRECS – signal joint T-cell Receptor Excision Circles

SOP – Standard Operating Procedure

SOT – Solid Organ Transplant

SP – Single Positive

Sp-ICE – Specialist Services Integrated Clinical Environment

SRC – Single Stain Reference Control

SSC – Side Scatter

SST – Serum Separating Tube

TAP – Transporter associated with Antigen Processing

TCM – Central Memory T-lymphocyte

TCR – T-cell Receptor

TEM – Effector T-lymphocyte

TEMRA – CD45RA re-expressing Effector T-lymphocyte

TfH – Follicular T-Helper Cell

TGF – Tumour Growth Factor

TIM-3 – T-Cell Immunoglobulin Mucin 3

TLR – Toll-Like Receptor

Treg – Regulatory T-lymphocyte

UMAP – Uniform Manifold Approximation and Projection

VAD – Ventricular Assist Device

VCA – Viral Capsid Antigen

WHO – World Health Organisation

YATEC – Young Adults Thymectomised in Early Childhood

YPAGne – Young Persons Advisory Group North East

Chapter 1: Introduction

1.1 Overview

Since its landmark discovery in 1964 as the first human tumour virus, research on the Epstein-Barr virus (EBV) has helped to improve mankind's understanding of many of the molecular mechanisms that underpin oncogenesis. It has also paved a path for major advancements in the field of immuno-oncology therapy through the development of adoptive cell transfer and monoclonal antibody targets. And yet, this ubiquitous virus remains just as enigmatic as it was sixty years ago.

Few viruses are as immunologically astute as EBV. Its ability to immortalise infected B-lymphocytes through evasion of even the most robust of host T-lymphocyte immune responses serves as a harbinger of its capacity to inflict both mutational harm and lymphomagenesis. This is of particular concern for children with an underlying immunodeficiency. While paediatric patients with inborn errors of immunity (IEI) have long been known to have a higher incidence of EBV-associated lymphoid malignancies than the general population,[1] the introduction of lifelong immunosuppression therapy for childhood solid organ transplant (SOT) has given rise to a new cohort of patients with equally high risk of developing a similar type of EBV-driven neoplasm known as Post-Transplant Lymphoproliferative Disease (PTLD).

PTLD is the most common childhood cancer in paediatric recipients of a SOT. The development of this heterogenous group of life-threatening haematological malignancies is typically driven by EBV involving both lymphoid tissue and extranodal sites.[2] PTLD comprises a histological spectrum that ranges from indolent non-destructive B-cell lymphoid infiltrates through to more aggressive and destructive monoclonal B-cell lymphomas e.g., Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL), that have historically been considered as being similar to their sporadic counterparts in immunocompetent children.[3] Their incidence is allograft-dependent and can range from as low as 1-2% in renal transplant recipients up to 10-15% in heart transplant patients.[2,4]

PTLD has one of the worst clinical outcomes amongst childhood lymphomas. Its Event-Free Survival (EFS) is approximately 70% compared to 94% following a diagnosis of histologically identical mature B-cell lymphoma in immunocompetent children. [5,6] In even starker contrast is the survival outcome for PTLD in paediatric heart transplant patients, who historically have an inferior EFS of 40% compared to 75-80% associated with other non-cardiac organ transplants (**Figure 1.1**).[7] The reason

for this disparity is still poorly understood but has been ascribed in part to a higher incidence of treatment-related complications and mortality from organ rejection in this cohort.[8]

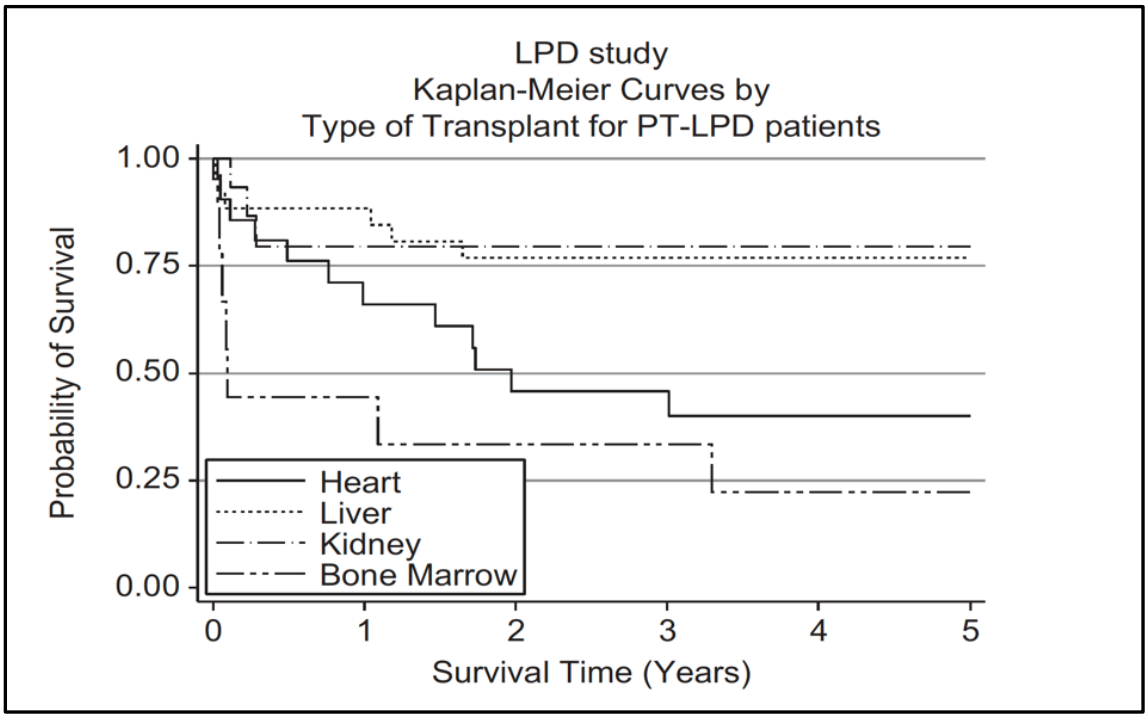


Figure 1.1: **Kaplan-Meier survival curves showing event free survival from paediatric PTLD after childhood organ transplant** (adapted from Taj M, Hadzic N, Height SE *et al*, © copyright 2012). [7] Reprinted with permission of Informa UK Limited, trading as Taylor & Taylor & Francis Group.

Untangling the complex interplay between iatrogenic immunosuppression, functional disruption of antiviral T-lymphocyte activity and the drivers of EBV-triggered lymphoproliferation is critical to our understanding of PTLD evolution. Such insight can be leveraged not only to mitigate patient risk but also to develop more effective treatment strategies. Yet how can we prevent what we can't predict? In recent years, the immunological consequence(s) of early thymectomy in paediatric heart transplant patients has gained increasing attention due to its potential role as a prognosticator for PTLD. Early childhood cardiac surgery via median sternotomy often requires routine thymectomy in order to improve the visual field to access the heart and great vessels. During fetal development and the first year of life, the thymus plays a crucial role in the development of robust cell-mediated immunity to foreign antigens (e.g. viruses) by providing a niche for precursor T-lymphocytes to proliferate and differentiate into self-tolerised mature (naïve) T-lymphocytes.[9] Whilst it has been demonstrated that neonatal thymectomy is associated with premature immunological ageing,[9] little is known about the effects of early thymectomy in immunosuppressed transplant patients. In

particular there are limited data on the effect(s) of thymectomy/transplant on EBV-specific immunity and the subsequent risk of developing PTLD.

In order to better conceptualise how early thymectomy might contribute to the risk of PTLD in paediatric heart transplant patients, it is important to first understand the processes involved in the development of a healthy T-lymphocyte immune compartment in early childhood, its response to a primary EBV infection, and what is currently known about the role of early thymectomy and long-term immunosuppression on EBV-specific immune responses. This chapter reviews the origins and development of T-lymphocyte immunity in childhood, including current concepts of competent versus dysfunctional immunity to EBV infection and PTLD pathogenesis. Furthermore, it explores the existing scientific evidence on the effects of early thymectomy and long-term immunosuppression therapy on circulating immune cell populations, EBV-specific immune responses and the risk of EBV-driven PTLD.

1.2 Ontogeny of T-lymphocyte immunity in early childhood

1.2.1 Introduction

A robust adaptive immune system forms the cornerstone of any successful immunological response to antagonistic alloantigens such as microbial pathogens. This cell-mediated activity requires the optimal formation, differentiation and activation of CD8+ Cytotoxic T-Lymphocytes (CTLs), CD4+ helper T-lymphocytes (T_H) and B-lymphocytes, with support from innate cells such as natural killer (NK) cells, Dendritic cells (DC), and monocytes. The human immune system encounters a diverse plethora of antigens during the first years of life. This formative period is characterised by the adoption of effective responses to foreign pathogens or transformed cells including tolerance to benign and self-antigens. Aberrations to this complex process can therefore lead to significant adverse outcomes. To ensure that a careful balance of T-lymphocyte selection, maturation and immune self-tolerance is maintained during early life, the thymus has evolved as a suitable microenvironment for these steps to occur.

1.2.2 Thymopoesis and establishing the T-cell receptor (TCR) repertoire

T-lymphocyte development begins *in utero* from the second trimester of fetal growth when common lymphoid progenitor stem cells that are negative for CD4 and CD8 co-receptors (i.e. double-negative) begin to migrate to the thymus from the fetal liver and bone marrow. [10] Here, they sequentially traverse the thymic subcapsular, cortical and medullary zones, first undergoing differentiation into double-positive (DP) CD4+CD8+ immature thymocytes before committing to mature single-positive (SP) naïve T_H (CD4+CD45RA+CCR7+) or CTL (CD8+CD45RA+CCR7+) lineages through a process of positive and negative selection. [11-13]

The anatomical compartments of the thymus provide distinct locations for each stage of thymopoesis to occur. Its outer cortex contains cortical thymic epithelial cells (cTECs) that interact with double-negative (DN) thymocytes to support their differentiation into immature DP thymocytes. [14, 15] The successive rearrangement of T-cell receptor (TCR) genes functions as a driver for DN thymocytes to undergo development into DP thymocytes. This process sees DN cells transition through four key stages of differentiation and proliferative expansion known as DN1 to DN4 (**Figure 1.2**). [16] Identification of these intrathymic progenitor subsets is classically based on their phenotypic advancement from CD44+ CD25- (DN1) through CD44+ CD25+ (DN2), to CD44- CD25+ (DN3) and CD44- CD25- (DN4).[16] Several cytokines (e.g., Stem Cell Factor, IL-7) and chemokines (e.g., Notch

Ligand DLL4, CXCR4, CCL25, CXCL12) that are produced by the cTECs help to support the growth, development and proliferation of these early thymocytes. [11, 17] Rearrangement of the TCR β-chain locus occurs in the DN3 stage of development. Thymocytes with successful β-chain gene rearrangement proliferate and lose their expression of CD25 to become DN4 thymocytes. β-chains go on to pair with the invariant α-chain known as pre-T cell α, thus allowing them to assemble pre-TCRs that are expressed on the cell surface as complexes coupled to CD3 signalling molecules. [18] These complexes provide the signalling components of TCRs necessary for cell proliferation, termination of further β-chain gene rearrangement and the differentiation into immature DP thymocytes through surface expression of both CD4 and CD8. [11] Once established, the DP thymocyte population, which comprises approximately 75-85% of thymic cells,[18] is subjected to one of three fates; positive selection, negative selection, or death by neglect.

In order to establish a diverse TCR repertoire that is robust enough to recognise almost any alloantigen, it is critical that the immunological milieu of the developing fetus first learn how to identify and tolerate both self-antigens and non-inherited maternal alloantigens. [19, 20] This process begins in the thymic cortex when αβ-TCRs on immature DP thymocytes are presented with complexes of self-peptides bound to major histocompatibility complex (MHC) class I and II molecules on cTECs. [21]

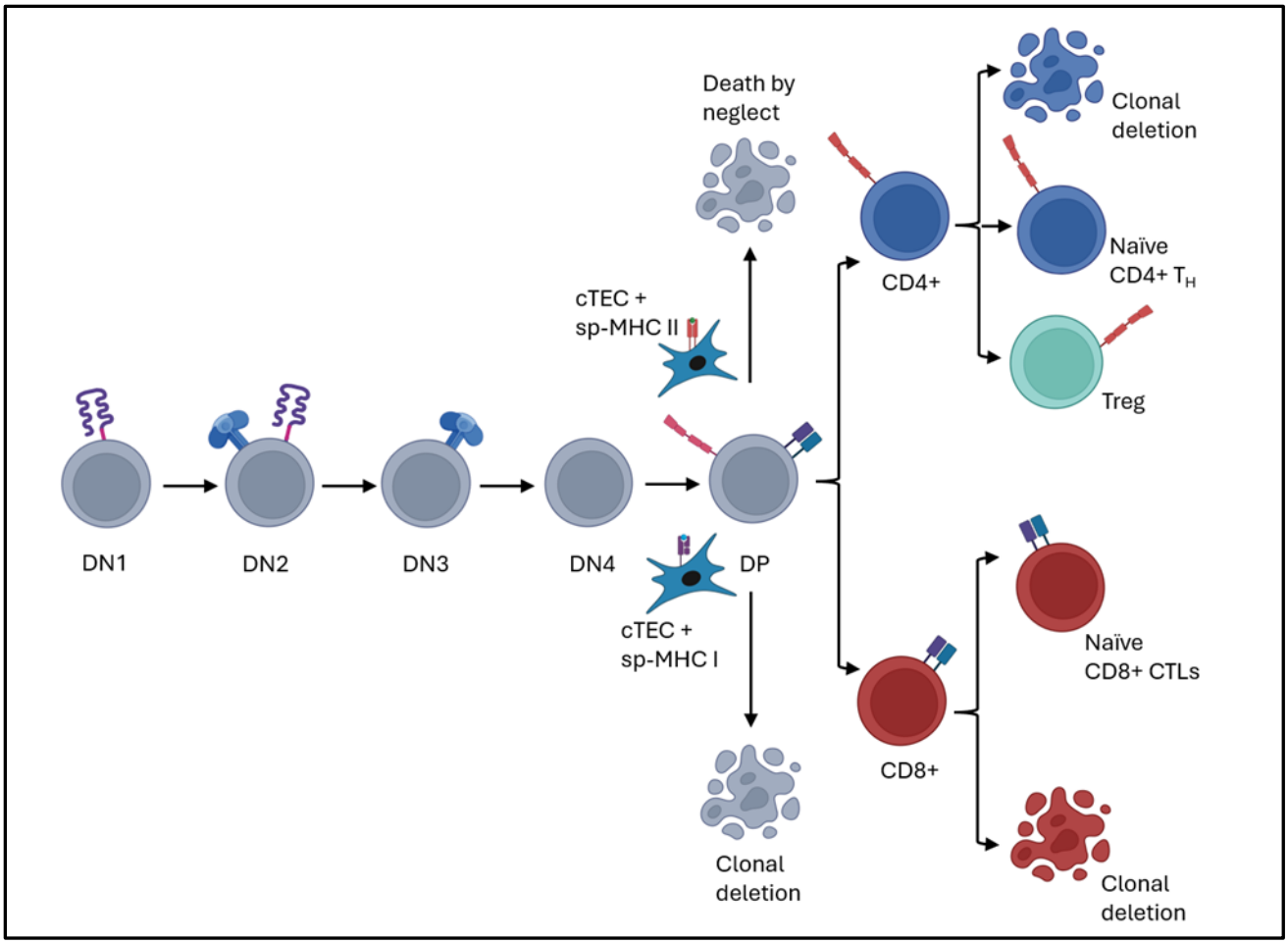


Figure 1.2: Stages of T-lymphocyte development within the thymus. The process of single positive (SP) T-lymphocyte formation begins with the trafficking of CD4-CD8- (double-negative) lymphoid progenitor stem cells from the fetal bone marrow and liver to the thymus during early *in utero* development. Engagement with the surrounding epithelial cells within the thymic cortex instigates commitment to the T-lymphocyte lineage with progressive gene rearrangement of T-cell receptors (TCR) on these cells that ensures a transition through 4 stages of differentiation (DN1 to DN4) into immature CD4+CD8+ (double-positive) thymocytes. [22] **cTEC**: Cortical thymic epithelial cells; **DN**: Double-negative; **DP:** Double-positive.

DP thymocytes that express low affinity for the self-peptide–MHC (sp-MHC) complexes subsequently undergo 'positive' selection and mature into SP T-lymphocytes. The positive selection of cells with affinity for sp-MHC class I restricted ligands will result in maturation into CD8+ CTLs, while a similar process involving sp-MHC class II-restricted ligands leads to the differentiation into CD4+ T_H lymphocytes. [14]

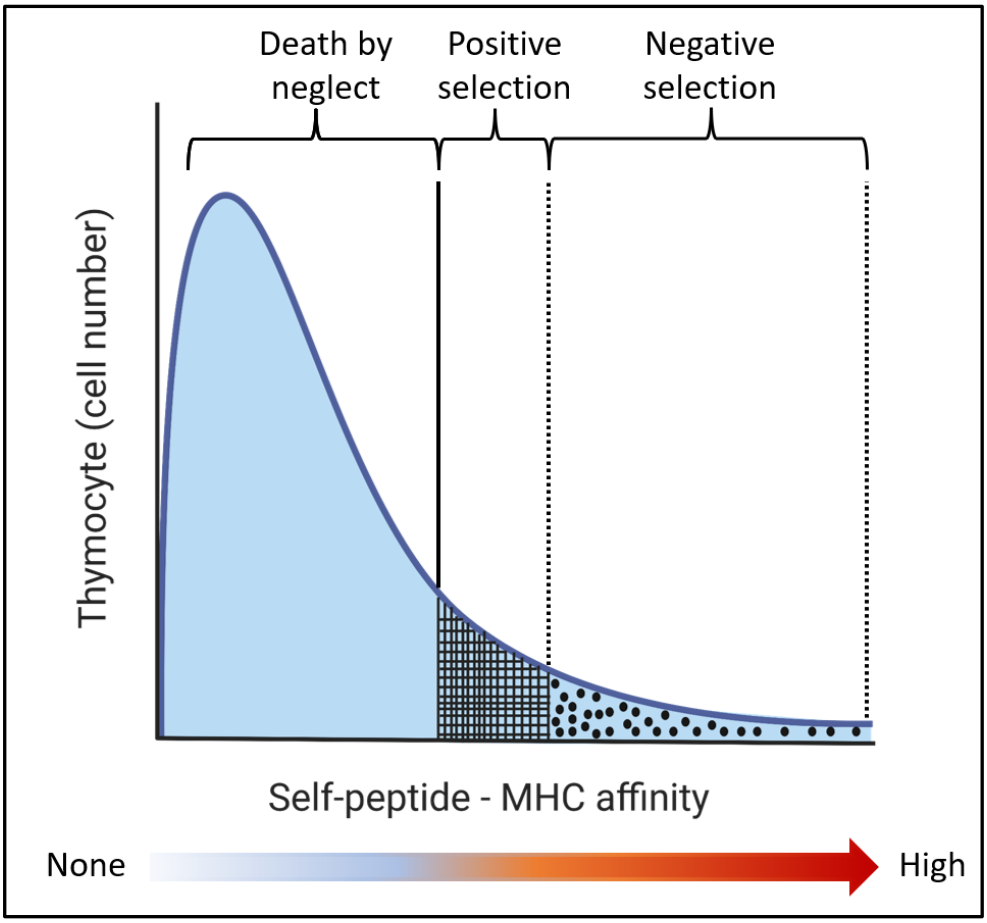


Figure 1.3: Selection fate of immature thymocytes. The diversity of the TCR repertoire is determined through three mechanisms of selection based on their affinity for sp-MHC complexes. The majority of immature thymocytes in the thymic cortex will undergo death by neglect due to a lack of response to sp-MHC complexes. The remaining cells that express sufficient affinity for sp-MHC complexes will survive and migrate to the thymic medulla where they are subjected to negative selection if they exhibit high affinity for sp-MHCs. Naïve SP T-lymphocytes that have αβ-TCRs with low affinity will undergo further positive selection in the thymic medulla before migrating into the peripheral circulation.

A substantial loss of DP thymocytes (~90%) occurs in the thymic cortex as a consequence of 'death by neglect' (**Figure 1.3**). This is programmed to occur within 3-4 days if the αβ-TCR of such cells fail to receive a survival signal due to non-engagement with a sp–MHC complex. [14, 23] Positively selected cells (~5% of immature thymocytes) are trafficked into the medullary zone of the thymus where they undergo interrogation by specialised antigen presenting cells (APCs) during the process of negative selection (clonal deletion). The thymic medulla is uniquely equipped as the principal site of negative selection due to its enrichment with an eclectic congregate of APCs. The compartment consists primarily of medullary thymic epithelial cells (mTECs), along with three subtypes of dendritic cells (DC1, DC2, plasmacytoid DC), B-lymphocytes and macrophages. [14, 24] mTECs have been demonstrated to exhibit promiscuous gene expression through the transcription of proteins that are

typically composite in peripheral tissue. This molecular mirroring of the peripheral self means that maturing thymocytes in the thymic medulla are exposed to a comprehensive library of self-antigens against which they can be sufficiently screened for adverse autoreactivity. [25-27] It is estimated that 5% of all immature thymocytes will have sufficiently high avidity for sp-MHC complexes to trigger clonal deletion through apoptosis. [28, 29] There is still some contention about whether or not negative selection of cells can also occur within the thymic cortex. [23, 30] Studies with TCR-transgenic mice have shown that thymocytes at any stage of development can undergo negative selection, therefore raising the possibility that positive and negative selection are independent rather than sequential events. [24, 31, 32] Regardless, these synergistic mechanisms offer an imperfect first-line of defence to mitigate fetal autoimmunity, and studies have demonstrated that autoreactive T-lymphocytes are still capable of escaping into the peripheral circulation after achieving maturation within the thymus. [33, 34] This necessitates an additional layer of central immune tolerance through clonal diversion of CD4+ MHC class II-restricted thymocytes to regulatory T-lymphocytes (CD4+CD25^{hi}FOXP3+) as a counter measure for targeting these rogue cells.

1.2.3 Clonal diversion and thymic regulatory T-lymphocytes

Thymic-derived regulatory T-cells (t-Tregs) make up approximately 1% of CD4+ thymocytes. [35] Transgenic mouse models support the concept that t-Treg development is instructed by cues from TCR signalling (Figure 1.4)**.** These studies have shown that while the t-Treg TCR repertoire is quite distinct from that of conventional CD4+ and CD8+ thymocytes, it is more self-reactive and markedly overlaps with the TCR repertoire of pathogenic autoreactive thymic cells. [36-38] Further evidence suggests that an intermediate affinity between TCRs on developing CD4+ thymocytes and sp-MHC complexes is a requisite stimulus for clonal diversion and cell commitment to Treg development. [39] This has led to the proposal of a two-step model for their development. The first step is characterised by the upregulated expression of CD25 in response to intermediate affinity TCR signalling. CD25 expression activates signal pathways that dampen pro-apoptotic messaging from factors such as Nur77 (nuclear receptor 4A1) and BIM (BCL-2 interacting mediator of cell death) while promoting commitment to the Treg lineage through downstream high affinity IL-2 binding. [33, 40] This, in conjunction with CD28 co-stimulation and an increased expression of Tumour Growth Factor β (TGF-β) and members of the tumour necrosis factor superfamily (TNFR2, GITR, OX40) helps to establish a population of t-Treg progenitors that lack the Forkhead box 3 protein 3 (FOXP3) transcription factor (i.e., CD25+FOXP3-). [41-43] Step 2 is TCR-independent and sees the progenitor t-Tregs undergo IL-2/IL-

15-mediated transition into mature Tregs (CD25+FOXP3+) through the activation of STAT5 and ensuing upregulation of FOXP3. [40] These mature t-Tregs migrate from the thymus to the periphery where they function as key modulators of systemic immune tolerance.

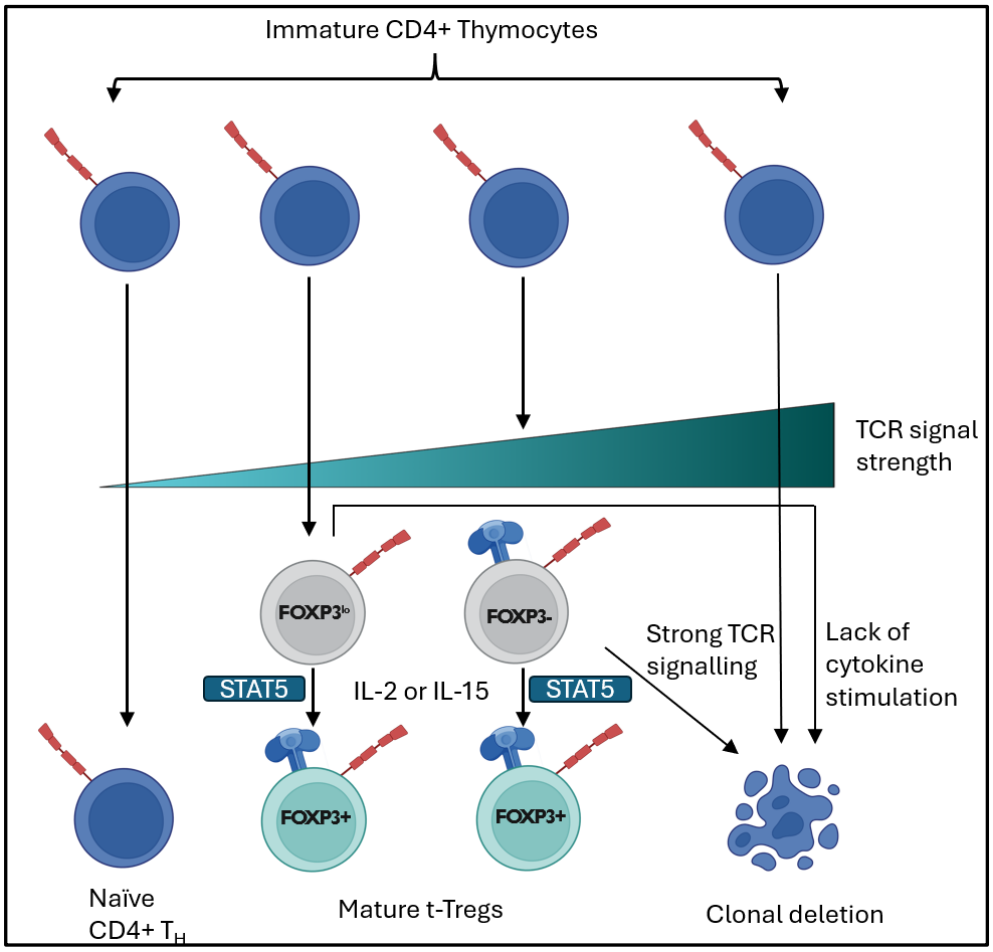


Figure 1.4: Two-step model of t-Treg development in the thymus. Clonal diversion of CD4+ thymocytes to t-Tregs is modulated by the strength of TCR signalling after stimulation by self-antigens presented by mTECs and other antigen presenting cells in the thymic medulla. The development of conventional CD4+ T_H requires weak TCR signalling while much stronger signalling is an impetus for clonal deletion. Commitment to the t-Tregs lineage is driven by intermediate TCR signal strength, with stronger signals leading to an upregulation of CD25 to establish CD25+FOXP3- progenitors while weaker signals lead to upregulation of FOXP3 to establish CD25-FOXP3^{lo} progenitors. Both progenitor populations bind to either IL-2 or IL-15 to activate the STAT5 transcription factor, which is necessary for the upregulation of FOXP3 and maturation into CD25+FOXP3+ t-Tregs.

It is of relevance that recent studies have also identified a further subset of t-Treg progenitors characterised by a low expression of FOXP3 and absence of CD25 (FOXP3^{lo}CD25-). [44-46] Both the CD25+ and FOXP3^{lo} t-Treg populations exhibit distinct TCR repertoires and RNA transcriptomes from each other. [45] This has called into question the evolutionary relevance of having two developmental pathways that lead to the same maturation endpoint. It is possible that their functions are

complementary and contribute equally to the maintenance of immune tolerance. The more classical CD25+FOXP3- t-Treg progenitors have been shown to exhibit an agonist selection profile that is reminiscent of the negative selection process used to establish conventional T-lymphocytes. [45] Mature Tregs that arise from this progenitor population possess TCRs that are skewed towards stronger affinity for thymic self-antigens. This suggests that CD25+FOXP3- t-Treg progenitors primarily contribute to the maintenance of peripheral immune tolerance and the prevention of autoimmunity. CD25-FOXP3^{lo} t-Treg progenitors on the other hand develop through a process that is similar to positive selection. This results in a broader TCR repertoire that is capable of reacting to both self-antigens and foreign agents, making them uniquely qualified to prevent autoimmunity. [45] It is important to mention that t-Tregs alone are insufficient to counteract the deleterious effects of autoimmunity and chronic inflammation. They require adequate reinforcement from peripherally induced Tregs (i-Tregs) that arise from conventional naïve CD4+ T_H, as well as input from other unconventional T-lymphocyte populations. [47]

1.2.4 Unconventional T-lymphocytes: γδ T-lymphocytes and Natural Killer T-like cells

While the vast majority of circulating T-lymphocytes consist of "conventional" populations expressing αβ TCRs, adaptive immunity is also supported by other distinct lineages of T-lymphocytes that are classed as "unconventional". These niche T-lymphocyte subpopulations are considered to be a necessary bridge between innate and adaptive immunity due to their unique effector and regulatory roles. [48] The best studied of these are the γδ T-lymphocytes and Natural Killer T-like cells (NKT).

Intrathymic development of γδ T-lymphocytes occurs at the DN3 stage when a subset of immature thymocytes undergo preferential gene rearrangement of the γ and δ TCR chains. [14] It is widely believed that the mechanism underpinning this lineage selection is driven by TCR signalling strength (i.e., instruction model), where strong γδ TCR signalling through ligand engagement mediates commitment to the γδ lineage pathway. [49-51] Once lineage commitment has occurred, γδ T-lymphocytes go on to mature and acquire their effector function within the thymus. [51]

γδ T-lymphocytes make up 0.5%-16% of CD3+ T-lymphocytes in the adult peripheral circulation and are also widely distributed across various tissues and organs. [52] Unlike their αβ counterparts, their response to antigenic stimuli is not dependent on MHC-restricted antigen processing. [52, 53] This means that they are capable of mounting an immunological response to a wide spectrum of non-

peptide antigens such as phosphoantigens, lipids and stress-induced ligands, all of which can be expressed by infectious agents and neoplastic cells. [53-55]

The γδ T-lymphocyte population is composed on 3 main subtypes, each with their own specific tissue location and functional profile. Their nomenclature (Vδ1, Vδ2, Vδ3) is defined by the TCR variable (V) gene segment that is used, with emphasis on the TCR δ chain. [56] The Vδ1 subgroup is primarily found in mucosal epithelium of the gut, skin, liver and spleen in connection with infectious organisms such as Human Immunodeficiency Virus (HIV) and Cytomegalovirus (CMV). [51, 57, 58] Clonally selected Vδ1 T-lymphocytes exhibit features of "memory-like" effector function in line with adaptive immunity. [59] Similarly, Vδ3 cells – the smallest subset of γδ T-lymphocytes – are found in the epithelium of the liver and gut, where they exhibit a functional profile that is analogous to Vδ1 cells, albeit mainly involving a response to chronic viral infections. [57, 60, 61] Little is known about the full scope of their function due to their rarity. [57] In contrast, the Vδ2 subset dominates the peripheral circulation, where it primarily pairs with the Vγ9 TCR. [51] This subset arises very early in fetal development, indicating that its function is likely to be more aligned to that of innate immunity as a protective mechanism against infections *in utero* and in infancy when the αβ lineage is still naïve. [51, 62-64] They are mainly activated by low molecular weight phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and isopentenyl pyrophosphate (IPP) that are produced by transformed and/or microbe-infected cells. [58] These molecules instigate a cascade of cytotoxic immune activities that involve the production of cytokines (interferon gamma aka INF-γ, TNF α, IL-15, IL-17a), chemokines (CCR1, RANTES) and lytic enzymes (granzyme B) including non-cytolytic responses such as antigen presentation to conventional T-lymphocytes, DC maturation, B-lymphocyte recruitment and immune tolerance. [58, 65-68]

NKT cells constitute another relevant population of unconventional T-lymphocytes. They express receptors that are capable of identifying lipid-based antigens presented by CD1d, a β2M-associated MHC class-I-like molecule. [48] NKT cells can be distinguished into 2 main groups based on their TCR arrangement and capacity to recognise the prototypic lipid antigen, α-galactosylceramide (α-GalCer). [56] Type 1 NKT cells aka invariant NKT cells (iNKT) express a CD1d-restricted semi-invariant αβ TCR comprising an invariant α-chain, Vα24Jα18, coupled to the Vβ11 chain. They are able to undergo activation and expansion when stimulated by α-GalCer. [69] iNKTs represent less than 1% of the T-lymphocyte compartment while a second subtype known as diverse NKTs (dNKT) is considered to be more abundant. [56] However, detailed examination of this subset is lacking due to their inability to

bind to α-GalCer and the absence of any specific reagents for their identification. Our current understanding of the development, maturation and function of NKT cells is therefore largely based on the analysis of iNKTs. Like all other T-lymphocyte lineages, the development of NKT cells begins in the thymus, where NKT progenitors can be identified in the fetal thymus as early as the first trimester. [70] The majority of these progenitor cells undergo positive selection at the immature DP thymocyte stage of conventional T-lymphocyte development, when invariant TCRs engage with CD1d to commit DP thymocytes to the NKT lineage. [71, 72] A much smaller contingency of NKT cells have also been noted to develop as an offshoot from the DN3 stage. [73] Unlike γδ T-lymphocytes, most NKT cells exit the thymus in an immature state. NKT cells that have recently emigrated from the thymus lack the maturation marker CD161 (NK1.1). [74] The precursor status of CD161- NKT cells has also been established by studies showing a progression of intrathymic CD161- NKT cells to the CD161+ stage (but not vice versa) upon transfer into recipient mice as well as the accumulation of CD161+ NKT cells in the peripheral circulation but not the thymus with increasing age. [75-77] The differential expression of CD4 and CD8 co-receptors can be used to subgroup iNKT cells into CD4+, CD8+ and CD4-CD8- (DN) iNKTs. [78] Temporal studies have highlighted a preponderance for CD4+ iNKTs in early life, particularly in the neonatal thymus, with CD4- iNKTs being found almost exclusively in peripheral circulation beyond 6 months of age and reaching their highest levels in adulthood. [69, 79] There appears to be a functional divergence between mature CD4+ and CD4- iNKT subsets. Activated CD4+ iNKTs are characterised by a Th0-type cytokine profile that is mediated by INF-γ and IL-4. [80] CD4- iNKTs on the other hand exhibit a Th1-biased cytokine profile consisting of INF-γ production without IL-4. [80, 81]

NKT cells act as an important bridge between innate and adaptive immunity. Not only are they capable of initiating a rapid production of several pro- and anti-inflammatory cytokines and DC maturation as part of the host's immediate response to an antigenic challenge, but they also promote antigen-specific CD8+ CTL and CD4+ T_H engagement, induce B-lymphocyte maturation and expand the memory B-lymphocyte pool. [82-85] While these effector functions can be significantly advantageous to the host, an imbalance of such responses can have equally deleterious consequences such as increased allergies and autoimmunity. [74]

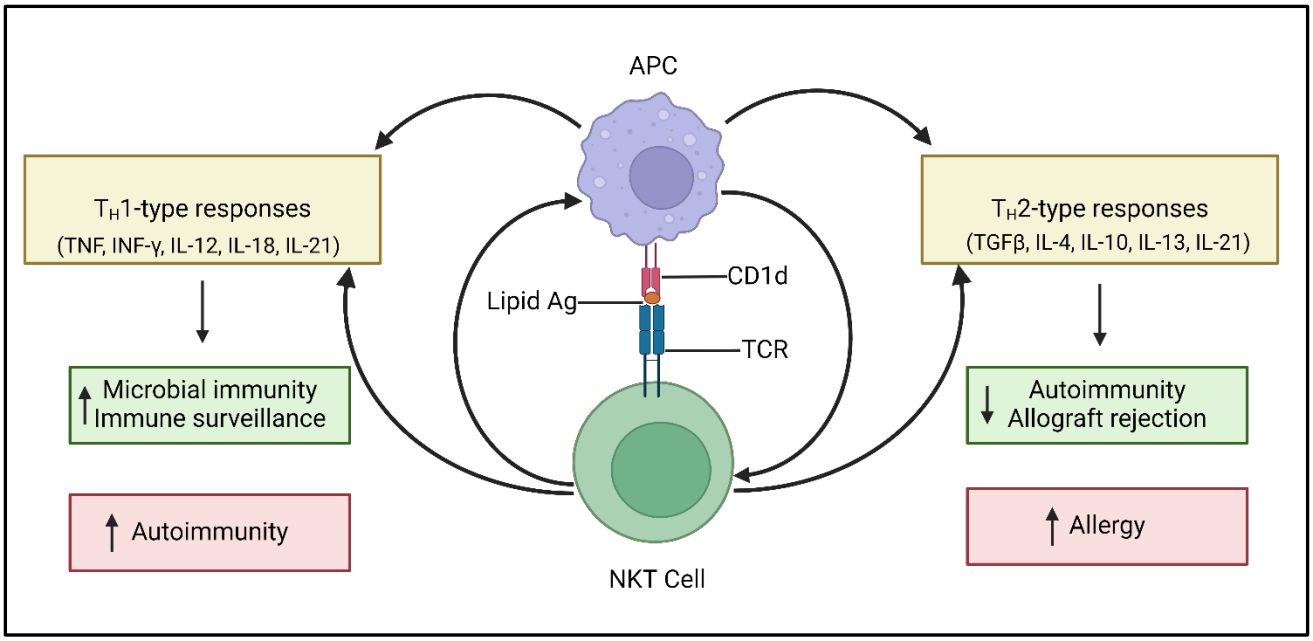


Figure 1.5: Function of NKT cells. The activation and effector function of NKT cells is mediated by the presentation of lipid-based antigens by CD1d to the NKT receptor (TCR). This leads to the activation of antigen presenting cells (APC) such as DCs, in addition to the activation, recruitment and maturation of other bystander lymphocytes. An array of pro- and anti-inflammatory cytokines are produced by following NKT cell activation as an immediate host response to an antigenic challenge. These cytokines can either be beneficial to the host (highlighted in green box) or promote significant harm (highlighted in red box).

In summary, unconventional T-lymphocytes encompass a variety of functionally distinct immune subsets that are complementary to CD8+ CTLs and CD4+ T_H. Their role in bridging innate and adaptive immunity is critically to both immune tolerance and surveillance. The functional importance of this immune compartment during childhood and the natural ageing process highlights the centrality of the thymus in establishing a fully competent immune system.

1.3 Immunological consequences of early thymectomy

1.3.1 Introduction

The complete or partial removal of the thymus via median sternotomy in infancy is a surgical procedure that is often performed during major cardiothoracic surgery for either congenital or acquired heart defects in order to improve access to the surgical field. [132] Imprecise epidemiological data poses some challenges in estimating its prevalence in this cohort with a high degree of accuracy. However, in European countries such as Sweden, approximately 250 infants (i.e., under 1 year of age) undergo a thymectomy each year while the prevalence of CHD in the UK is estimated to be 53.7 per 10,000 live births, of which a quarter have moderate-severe disease that requires major surgical intervention during infancy. [133, 134] This of course doesn't take into account the growing number of infant patients that undergo median sternotomy for non-congenital structural and/or functional cardiac pathologies. [135-137] In recent years, a growing body of evidence has begun to lay bare some of the profound immunological consequences of thymectomy in early childhood; highlighting that this procedure that was once considered to be relatively benign, may in fact be far more insidious and damaging.

1.3.2 Effect on conventional T-lymphocytes

The immunological sequelae of early childhood thymectomy have been the subject of scientific curiosity since the late 1980s when Brearley *et al* first described a significant decline in circulating T-lymphocyte counts in children who had undergone thymectomy within the first 3 months of life compared to age-matched controls. [132] Other similar studies have since gone on to corroborate some of these findings by demonstrating that thymectomy in early childhood precipitates premature immunological ageing. This is characterised by the accelerated decline in circulating RTEs, naïve CTLs and T-helper lymphocytes along with an increased expansion of follicular T-helper (TfH) and oligoclonal peripheral memory T-lymphocyte subsets.[138-143] Furthermore, an expansion of the memory Treg ($CD4^+CD25^+CD127^{lo}CD45RA^-$) compartment has been demonstrated after early thymectomy, indicating a prominence of peripheral immune tolerance. [139] The profiling of T-lymphocyte subpopulations in young adults who have undergone neonatal thymectomy (i.e. within the first 4 weeks of life) shows some indications of early onset immunosenescence through the upregulated expression of CD57 to levels that are similar to that of elderly patients (> 75 years old). [138]

Notably, while these studies have explored the impact of thymectomy on the memory T-lymphocyte compartment, the TEMRA subset and T-lymphocyte exhaustion profile have largely gone unexamined. The single study that examined TEMRAs within the CD8+ compartment noted a significant increase in the proportion of these terminally differentiated cells in children after neonatal thymectomy. [141] Furthermore, a recent systematic review of the long-term effects of early thymectomy found that T-lymphocyte subsets were not consistently decreased across studies and that this may be attributed to the substantial heterogeneity in the amount of thymic tissue removed and the age at which patients underwent thymectomy. [144] It remains unclear how much thymic tissue needs to be removed to instigate a decline in T-lymphocyte immunity as some studies have shown partial thymectomy to exert a lasting impact on circulating T-lymphocyte counts while others have contradicted this finding. [145-147] Of equal uncertainty is the age at which thymectomy is expected to be most deleterious. It stands to reason that thymic excision prior to the attainment of peak cellular density and naïve T-lymphocyte output, both of which occur in the latter half of infancy, [95-97] would be the critical window for inflicting any long-term disruption to the adaptive immune system. Indeed, thymectomy in children after 6 months of age has previously been reported not to result in overt immunodeficiency. [132] This appears to be reflected by more consistent findings of T-lymphocyte effacement among studies in children thymectomised within the first 6 months of life compared to older patients. [144, 148, 149]

1.3.3 Impact on unconventional T-lymphocytes

Very little research has been done to elucidate the effects of early thymectomy on unconventional T-lymphocyte subsets. Two studies to date have examined the impact of neonatal thymectomy on γδ T-lymphocytes. [145, 150] One study revealed a significant decline in absolute γδ T-lymphocyte counts up to 10 years after neonatal thymectomy, [145] while both studies showed evidence of a non-significant reduction in the proportion of circulating γδ T-lymphocytes in thymectomised patients compared to age-matched controls. [145, 150] In addition, Cramer *et al* demonstrated a disruption of the γδ TCR repertoire to favour an increased abundance of the fetal-derived granzyme-producing Vγ9Vδ2 subset. [150] The immunological ramifications of this altered repertoire remain uncertain. There are currently no published data on the how thymectomy influences the NKT compartment in humans. However, a study in adult mice revealed a global depletion of NKT cells across all tissues after neonatal thymectomy. [151] It still needs to be determined if such findings are replicated in humans.

1.3.4 Bystander effect on Innate and humoral immunity

The impact of thymectomy on innate and humoral immunity has also been explored. Whilst the absolute count of NK cells doesn't appear to be greatly influenced by early thymectomy, [138, 139, 143, 145, 146] there is some evidence to suggest that thymectomy in the first year of life affects the distribution pattern of circulating NK subsets, leading to a significantly increased proportion of immature CD56bright CD16- NK cells. [152] This subpopulation of cells has a notably inferior cytotoxic function compared to the mature CD56dim CD16+ NK cells that are more pervasive in healthy cohorts. [153] This thymectomy-induced impairment in NK cell differentiation is potentially linked to a deficiency in T-bet, a Th1-specific transcription factor that plays a critical role in regulating the maturation and homeostasis of NK cells. [154] However, further research is required to explore this hypothesis. Examination of humoral immunity has shown no effect on total B-lymphocyte counts. [139,141, 145] However, quite diverse results for total and specific immunoglobulin (Ig) levels as well as autoantibodies have been reported across studies with a trend toward normal serum Ig levels and variable autoreactivity. [144] Finally, there is limited evidence that shows a skewed expansion of CD14+ HLA-DR$^{lo/-}$ monocytic myeloid-derived suppressor cells (MDSCs) in adult patients thymectomised in infancy. [152] This monocyte subset is recognised as being highly immunosuppressive and has been proposed as a potential biomarker for malignancies induced by oncogenic viruses including non-Hodgkin B cell lymphomas. [155, 156]

1.3.5 Clinical consequences of early thymectomy

Although early thymectomy has been demonstrated to establish immune signatures that mirror the senescent and exhausted profile seen in elderly populations, some studies have suggested that early thymectomy is not associated with a significant clinical consequence, specifically in relation to the risk of opportunistic infections. [157-159] Some studies have reported an increased incidence of atopic disorders such as asthma but no associated increase in hospital stay, severity/frequency of infections or admission to intensive care. [160 – 163] An adequate T-lymphocyte response to vaccinations has also been reported in this cohort. [163] However, the relationship between early thymectomy and the risk of viral oncogenesis, particularly EBV-driven neoplasms, has mostly been overlooked. Only one study to date has explored the functional attributes of EBV-specific T-lymphocytes (EBV-TLs) in patients a few decades after they underwent neonatal thymectomy. [138] No phenotypic difference in EBV-TLs was observed between young adults with a history of neonatal thymectomy and their age-matched controls. Similarly, functional profiling of these cells showed that patients were still capable of

producing an adequate anti-EBV response when stimulated with viral epitopes and that their capacity for degranulation and cytokine production did not differ from those of controls. [138] It is important to note that these findings are based on a very restricted sample size (n =12) and did not involve any patients who subsequently underwent a SOT. It is possible that any overt disruption of anti-EBV immunity in thymectomised patients requires a "second hit" process, such as long-term immunosuppression therapy after SOT, to manifest itself. In fact, this is a possible explanation for why paediatric heart transplant patients are more likely to experience complications of chronic EBV carriage and a higher risk of EBV-driven PTLD compared to other SOT cohorts. [2, 164, 165] Another model that is worth further exploration is the role of a CMV co-infection, a virus known to significantly alter T-lymphocyte activity and cause major morbidity in transplant patients. [166] To gain better insight into the mechanisms deployed by EBV to exploit the perturbance in T-lymphocyte surveillance after early thymectomy, it is important to first explore how the 'intact' immune system responds to a primary EBV infection.

1.3.6 Peripheral T-lymphocyte composition and thymic involution

At birth, a full complement of naïve CD4+ and CD8+ T-lymphocyte subsets exists within the lymphoid and peripheral compartments, waiting to encounter an appropriate cognate antigen stimulus. These cells differ functionally in their programmed response to activation signals compared to adults. [86] They exhibit innate-like defence mechanisms that are less dependent on TCR recognition and geared more towards a rapid response to inflammation and danger signals e.g., production of INF-γ through the stimulation of Toll-like receptors on naïve CTLs. [87] Next-generation sequencing of neonatal T-lymphocytes has also revealed that these cells predominantly exist in an effector-primed state. [88, 89] While this is arguably counterintuitive to the need for plasticity in their response to anodyne foreign and self-antigens, it is theorised that this lower threshold for activation enables them to differentiate into short-lived effector cells more rapidly (in the presence of inflammatory cytokines and co-stimulators) or become self-tolerant (if primed by innocuous antigens presented to TCRs), depending on what is most beneficial to the host. [86] This has been confirmed by mouse models and human studies showing that neonatal naïve CTLs preferentially undergo terminal differentiation in the presence of intracellular pathogens at the expense of expanding the long-lived memory T-lymphocyte pool. [90, 91] Whether this is due to an immature priming pathway or intrinsic cellular defects remains unclear. However, it is possible that this response could be an evolutionary strategy that confers a survival advantage to the host by prioritising a prompt elimination of pathogens during

the early developmental stage of the naïve TCR repertoire. Beyond the neonatal period, naïve T-lymphocytes continue to dominate the periphery and across various tissue sites during childhood. There is however a gradual expansion of the memory compartment, particularly in local mucosal sites such as the lungs and intestine but less notably in the peripheral circulation and lymphoid tissue compared to adults. [92] Recent system-level analyses suggest that while B-lymphocytes, NK cells and DCs develop adult-like phenotypic and functional profiles within the first 3 months of life, T-lymphocytes fail to reach this state until much later. [93] Invariably, the immune state in early childhood is skewed towards rapid defence mechanisms (e.g., greater IL-6, IL-10, IL-23 production) that target potentially fatal extracellular bacterial pathogens at the expense of Th1 pro-inflammatory cytokine reactions (e.g., lower IFN-γ, IL-12) needed to control intracellular pathogens such as viruses. [94] The tolerogenic immune microenvironment of early childhood is further supported by a higher absolute number and proportion of circulating and tissue-resident Tregs during infancy. [10, 92]

Thymopoesis is the key driver for maintaining a high output of new naïve T-lymphocytes during the first years of life. During this period, the thymus reaches its peak cellular density at 9 months with its optimal volume and T-lymphocyte export being achieved by 1 year of age. [95-97] Freshly emigrated naïve T-lymphocytes from the thymus express the surface molecule CD31 (PECAM-1) in addition to significantly higher content of signal joint TCR excision circles (sjTRECs). [98] CD31 has been shown to be a useful measure of thymic function as it helps to discriminate between naïve CD4+ T-lymphocytes that are recent thymic emigrants (RTE) and those that have propagated through peripheral homeostasis. [99] Due to the thymopoesis-driven expansion of RTEs and naïve T-lymphocytes in early childhood, the total number of circulating cells increases dramatically from birth until the first months of life. This is followed by a gradual decline over time to reach adult levels as the fall in thymic output persists in association with age-related thymic involution. [10, 100]

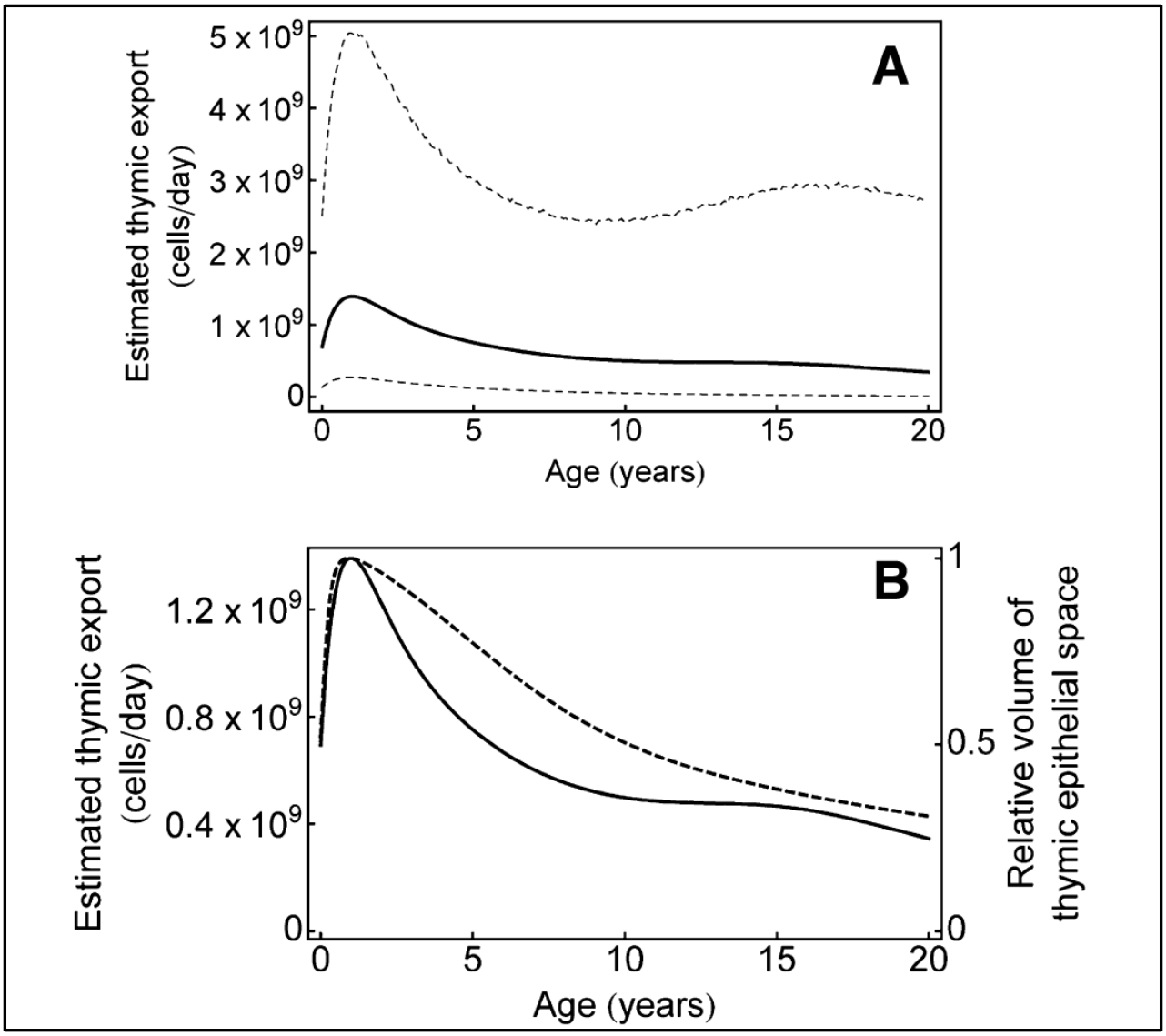


Figure 1.6: Estimated daily thymic export of CD4+ T_H during early life. (A) Mathematical modelling predicts that the average thymus exports up to 690 million CD4+ T_H lymphocytes per day at birth. This peaks of roughly 1.4 billion cells per day by 1 year of age. Thymic involution sees a rapid decline of naïve T-lymphocyte export during the first decade of life, followed by a more gradual fall in thymic output into adulthood (mean cell number is represented by the solid line and interquartile range by the dashed lines). (B) The age-related decline in volume of the thymic epithelial space (dashed lined) is predictive of the daily thymic export of CD4+ T_H (solid line). (adapted from Bains I, Thiébaut R, Yates AJ *et al*, © copyright 2009). [96] Reprinted with permission of AAI publications.

Functional atrophy is the inescapable fate of the thymus. This hardwired decline in cellular composition and activity begins in late infancy and appears to follow a biphasic pattern into adulthood. [97] It is characterised by a rapid contraction in thymic volume and number of epithelial cells between 1-8 years of age (~12% per year), followed by a relatively slower rate of involution (~3-4% per year) that involves an ongoing loss of thymic epithelial cells in concert with an expanding adiposity within the perivascular space. [10, 96, 101]

For decades immunologists have pondered the evolutionary advantage of thymic involution without reaching a clear consensus. While the exact reason for this process still remains uncertain, there are suggestions that it could be linked to a survival benefit. The long-term maintenance of a competent immune system requires a T-lymphocyte compartment that is enriched with antigen-specific memory cells that can be recalled swiftly when re-challenged by environmental pathogens. This is potentially at the expense of generating new naïve T-lymphocytes in order to conserve energy expenditure. [102] This shift from a 'learning' phase to a 'reproductive' phase could allow for more successful effector immune responses that are optimised for fighting infections and negating self-reactivity. [103] A more recent hypothesis is that thymic involution is essential for the peripheral selection of clones of naïve T-lymphocytes with TCRs that are optimised for the detection of non-self. It proposes that thymic involution is needed to help increase the homeostatic proliferation of a select repertoire of naïve T-lymphocyte clones that have been optimised for their TCR affinity, diversity and precursor frequency by early adulthood. [104] This mechanism however becomes detrimental in old age when the diversity of naïve TCR repertoire becomes restricted to just a small number of large clones. [104] This age-related decline in robustness of the immune system could be considered an unfortunate collateral damage of the selective process. It is important to mention that many of the proposed explanations are based on theoretical modelling alone and are yet to undergo vigorous scientific validation by *in vitro* and/or *ex vivo* testing. What is known with certainty is that thymic involution ultimately leads to an immunological perturbance that is typified by T-lymphocytes lacking in both proliferative capacity (immune senescence) and functional activity (cellular exhaustion).

1.3.7 Ageing, T-lymphocyte senescence and cellular exhaustion

The natural process of human ageing involves major phenotypic changes within the T-lymphocyte compartment that are associated with a progressive decline in the proliferation and functionality of immune cells. The gradual age-related remodelling of adaptive immunity is imposed by both intrinsic (genetics, thymic involution) and extrinsic (environmental factors, chronic antigen stimulation) events with deleterious effects. [105] This cellular state of functional effacement, referred to as immunosenescence, is characterised by the accumulation of terminally differentiated T-lymphocytes that exhibit telomere attrition, accumulated DNA damage, apoptosis resistance and low proliferative capacity. [105, 106]

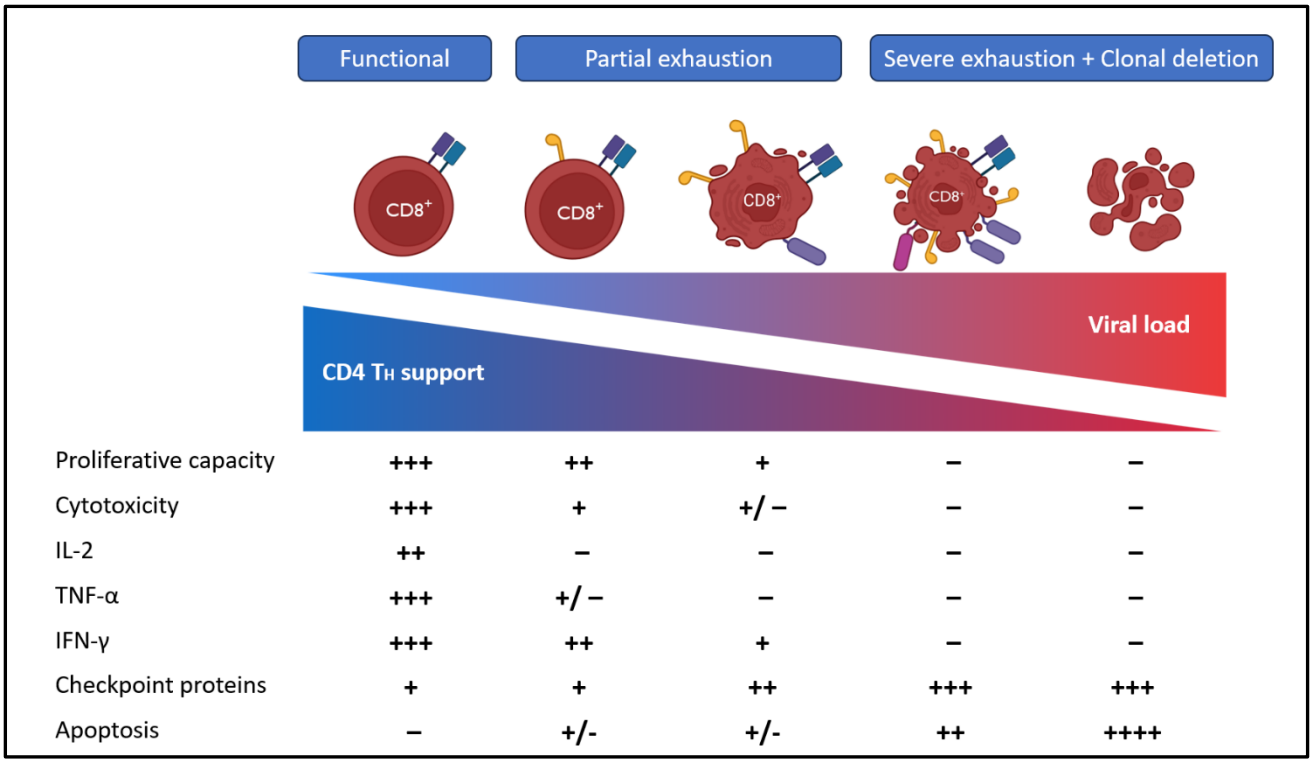
Thymic involution is a major player in age-related immunosenescence particularly when it comes to CTLs. The CD8+ T-lymphocyte compartment is much less capable than CD4+ T_H cells in using homeostatic proliferation of circulating TCR clones to compensate for the contraction in thymic output of naïve T-lymphocytes. [107] This initiates a decline in the clonal diversity of TCRs while simultaneously expanding the memory T-lymphocyte subset and population of dysfunctional terminally differentiated cells. [108] Although dogma has long held that the enrichment of the memory T-lymphocyte pool with ageing is mediated by lifelong antigen stimulation, recent evidence shows that aged antigen-inexperienced CD8+ CTLs are more prone to differentiation into 'virtual memory T-lymphocytes' upon activation by cytokines such as IL-4 and IL-15. [109, 110] These virtual memory T-lymphocytes are phenotypically similar to their conventional antigen-experienced counterparts but exhibit higher affinity for self-antigens along with features of cellular senescence. [110, 111]

The phenotypic markers of immunosenescent T-lymphocytes are reflected by their terminal differentiation status. They classically have reduced gene and surface expression of the co-stimulatory molecules CD27 and CD28, which are essential for interacting with B-lymphocytes and APCs to generate immunoglobulins and are involved in the activation of T-lymphocytes to produce cytolytic agents. [112, 113] In addition, they exhibit an upregulation of CD57, a glycoepitope that has been repeatedly demonstrated to be the best marker of replicative senescence in T-lymphocytes. [114, 115] Terminally differentiated CD57+ T-lymphocytes are known to accumulate with normal ageing and chronic antigen exposure, although CD57+ T_H do so at a much lower rate that CD57+ CTLs. [114, 116] Such CD57+ terminal effector cells also re-express the CD45RA surface marker and are designated as TEMRA T-lymphocytes. CD57+ TEMRAs are considered the hallmark of immunosenescence and thrive in a pro-inflammatory milieu that supports adverse events such as autoimmunity and oncogenesis. [116, 117] These cells also express lower levels of CD62L (L-selectin), CCR7 and CXCR4 along with the upregulation of CX3CR1, thus indicating an impaired migration of virus-specific T-lymphocytes to lymph nodes and a permissiveness of poorly regulated viral replication. [118] TEMRAs have been shown to display a high degree of cytotoxicity through the secretion of cytokines (IFN-γ, TNF-α) and cytolytic factors (granzyme B, perforin). However, it is important to note that their capacity to produce antiviral cytokines such as IFN-γ diminishes over time as TEMRAs become functionally exhausted due to persistent TCR stimulation. [106, 117] Additionally, previous studies may have over-estimated their cytotoxic potential by failing to concurrently evaluate the intracellular expression of cytotoxic molecules and the surface expression of CD107a, a degranulation marker that

allows for direct quantitation of cytotoxic activity. [118] Regardless of the functional prowess of CD57+ TEMRAs, it is clear that the repeated stimulation of TCRs by their cognate antigens and/or inflammatory cytokine signals eventually leads to a loss of T-lymphocyte effector function in a stepwise manner. Proliferative capacity and IL-2 production of cells are the first functions to be lost, followed by the secretion of TNF-α and eventually IFN-γ. [119, 120] This state of functional hyporesponsiveness is referred to as cellular exhaustion.

T-lymphocyte exhaustion is defined by poor effector function, sustained expression of inhibitory checkpoint receptors and a transcriptional state that is distinct from that of functional effector or memory T-lymphocytes. [119] A central feature of cellular exhaustion is the increased surface expression of immune checkpoint proteins such as programmed cell death 1 (PD-1), Lymphocyte-activation gene 3 (LAG-3), T-cell Immunoglobulin Mucin 3 (TIM-3) and Cytotoxic T-lymphocyte associated protein 4 (CTLA-4). [120] These inhibitory receptors mediate T-lymphocyte exhaustion through various signalling pathways that impair T-lymphocyte function such as the upregulation of basic leucine zipper transcription factor (BATF) and by working synergistically to supress effector cytokine production after TCR stimulation. [121, 122]

Much of our current understanding of T-lymphocyte exhaustion has been gleamed from immunological studies of chronic viral infections in mice and humans. Exhausted T-lymphocytes were first identified in the late twentieth century as antigen-specific CD8+ CTLs that failed to produce effector cytokines during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice. [120] Similar dysfunctional states have since been identified in humans with Hepatitis B (HBV), Hepatitis C (HCV), and HIV. [123, 124] Such studies have also demonstrated that higher viral load and prolonged duration of infection both contribute to a more severe exhausted state. [125] This endpoint of cell exhaustion is characterised by low levels of CD4+ T_H, an increased surface expression of multiple inhibitory immune checkpoint receptors and the eventual clonal deletion of virus-specific T-lymphocytes (Figure 1.7). [106, 126]



Proliferative capacity	+++	++	+	–	–
Cytotoxicity	+++	+	+/–	–	–
IL-2	++	–	–	–	–
TNF-α	+++	+/–	–	–	–
IFN-γ	+++	++	+	–	–
Checkpoint proteins	+	+	++	+++	+++
Apoptosis	–	+/-	+/-	++	++++

Figure 1.7: Hierarchical evolution of CD8+ T-lymphocyte exhaustion during chronic viral infection. A progressive loss of effector T-lymphocyte function is observed alongside an increased surface expression of multiple immune checkpoint proteins (e.g. PD-1, TIM-3, LAG-3 and CTLA-4). The virus-specific CTLs eventually become severely hyporesponsive and undergo clonal deletion from the host's T-lymphocyte compartment.

It remains unclear if senescent cells are more likely to become exhausted. Although immunosenescence and cellular exhaustion share overlapping characteristics, epigenetic and transcriptomic analyses have demonstrated that these two dysfunctional cell states are distinct phenomena that can nevertheless co-exist in the same cell. [127-129] In addition, while the expansion of senescent and exhausted T-lymphocytes is largely associated with advanced ageing and the elderly, their prevalence has also been described in young individuals subjected to persistent antigenic TCR stimulation in chronic disease states. [130, 131] Of particular interest is their accelerated appearance in children who have undergone iatrogenic thymectomy early in life and how this perturbation of their systemic immune landscape could have long term sequalae when it comes to oncogenesis.

1.4 EBV and the immune system

1.4.1 Life cycle of a ubiquitous virus

EBV or Human Herpesvirus 4 (HHV4) is a gammaherpes virus whose infection is well known for its pervasiveness in the general population, reaching a prevalence of over 95% by adulthood. [167] Primary viral infection is usually acquired asymptomatically in early childhood. By contrast, infection in adolescence or early adulthood tends to result in an immunopathological response that is associated with an acute self-limiting illness known as infectious mononucleosis (IM) or glandular fever. [168] This illness is characterised by the triad of fever, pharyngitis and cervical lymphadenopathy along with haematological evidence of (transient) exaggerated lymphocytosis. [168] There is strong evidence to suggest that a history of IM is a risk factor for the later development of lymphoid malignancies such as Hodgkin lymphoma (HL) and autoimmune disorders like multiple sclerosis. [169, 170] Similarly, there is an established aetiological link between EBV infection and lymphomagenesis in both immunocompetent and immune dysregulated children. [171-173]

The structure of EBV consists of a double stranded DNA encased in an icosahedral viral capsid. The capsid is itself surrounded by a host cell membrane-derived envelope which separates it from a protein tegument. The envelope is embedded with several glycoproteins (e.g., gp350, gp42, gp110, gH, gL) that are all involved in various stages of cell recognition and tropism (Figure 1.8).

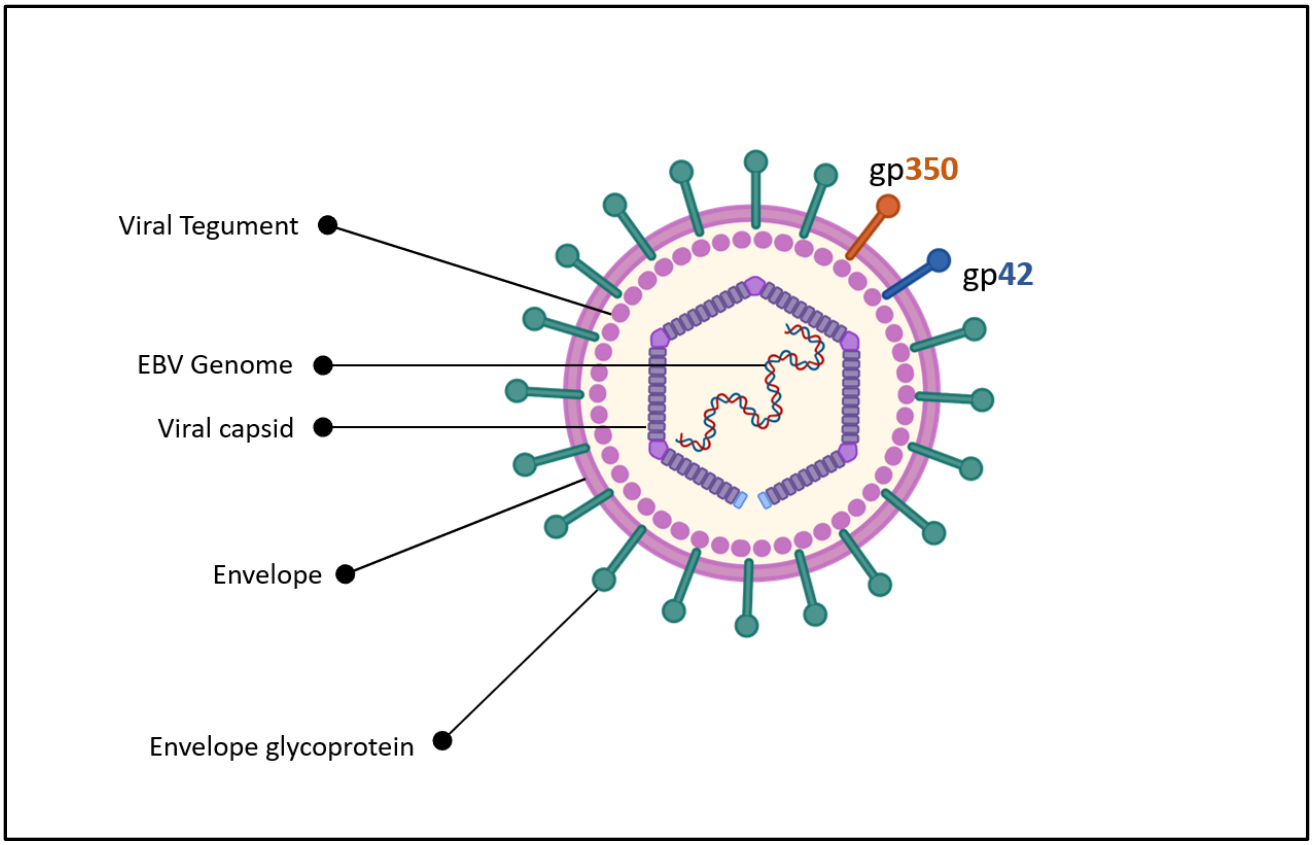


Figure 1.8: **Structure of Epstein-Barr Virus (EBV)**. EBV consists of a linear double-stranded DNA that is surrounded by an icosahedral viral capsid. The viral tegument is a non-solid structure made up of viral and cellular proteins which occupy the space between the viral capsid and a lipid bilayer envelope. Several viral glycoproteins project outwards from the envelope to interact with the target B-lymphocytes. This includes glycoproteins such as gp350, which attaches the virus to target cells by binding to the complement receptor CD21 expressed on the surface of B-lymphocytes. Other glycoproteins such as gp42 are involved in viral entry through binding of HLA class II molecules that function as co-receptors.

Primary EBV transmission principally occurs through saliva exchange in the oropharynx, where initial infiltration and viral replication occurs in mucosal epithelial cells and naïve B-lymphocytes. [168] However, alternative routes of primary EBV include transfusion of blood products (rarely) and solid organ transplantation between a positive donor and an EBV naïve recipient. [174, 175] EBV binds to its target cells either through gH (used for attachment to epithelial cells) or through gp350 which couples to the CD21 receptor on the B-lymphocyte surface. [167] This in turn facilitates viral entry into host cells either through an interaction between gp42 and HLA class II molecules on B-lymphocytes or via gH-gL complexes for epithelial cells. [176] Active replication of the virus begins once the EBV genome has been inserted into the infected cell's nucleus.

Like all herpesvirus infections, EBV replication is composed of both a lytic and a latent phase of infection. The lytic phase is characterised by a high level of virus shedding instigated by virion production in epithelial cells of the oropharynx and locally infiltrating B-lymphocytes. [168, 177] This process is mediated by several viral proteins that are integral to the virus's survival and replication. Their expression occurs in a distinct time-regulated cascade that can be used to classify them as immediate-early (IE), early (E) and late (L) lytic proteins. [178] A critical step in the lytic cascade involves the IE proteins BZLF-1 and BRLF-1, both of which are transactivators involved in the downregulation of pro-inflammatory factors. [179] They also work synergistically to amplify the production of early viral proteins such as BNFL2a that are required for viral replication, metabolism and the blockade of antigen processing. [180] Once replication of the EBV genome has been established within the virion-infected host cells, late lytic proteins such as gp350 and BFRF3 which are integral to the viral structure and function are encoded. [181]

During the latent phase of infection, EBV persistence is established through the immortalisation of infected circulating memory B-lymphocytes. [171] EBV-mediated proliferation of infected cells is sustained during this phase through various mechanisms deployed to evade immune surveillance by the host. This involves the utilisation of distinct latency patterns that are characterised by varied expression of key viral nuclear proteins (Table 1.1). These include Epstein-Barr Nuclear proteins (EBNAs: EBNA-1, EBNA-2, EBNA-3A/B/C, EBNA-LP), latent membrane proteins (LMPs: LMP-1, LMP-2A/B), non-encoding RNAs (EBERs) and the anti-apoptotic protein BHRF1. [173, 182] There still remains some uncertainty about how EBV gets into memory B-lymphocytes. It has been proposed that this is either through the direct infection of memory B-lymphocytes or involves a 'germinal centre' model which begins with viral infiltration of naïve B-lymphocytes resulting in the expression of all latent viral proteins (latency III pattern). [171, 183] This expression patten establishes the aberrant proliferation of infected cells through anti-apoptotic and growth transforming signalling pathways. [184] After an initial clonal expansion, the infected B-lymphocytes migrate to the germinal centre of secondary lymphoid tissue where they establish a latency II pattern that is characterised by the suppression of EBNA-2, EBNA-3 and EBNA-LP. [171, 183, 184] These infected naïve B-lymphocytes undergo differentiation into resting memory B-lymphocytes within the germinal centre and further restrict the expression of latent viral proteins to only EBNA-1 (i.e., latency I pattern) or no viral proteins (latency 0 pattern). [171] EBNA-1 is integral for the homeostatic proliferation of the viral genome, which makes it integral for sustaining a viral reservoir that permits lifelong infection. [185]

Latency pattern	Viral protein(s) expressed
0	-
I	EBNA-1
II	EBNA-1, LMP-1, LMP-2A/2B
III	EBNA-1, EBNA-2, EBNA-3A/3B/3C, LMP-1, LMP-2A/2B, EBNA-LP

Table 1.1: **EBV latency patterns.** Latency III is characterised by the expression of all latent viral proteins including the immunodominant EBNA-3 antigens. The latency II pattern is restricted to EBNA-1 and the 3 LMPs making it less immunogenic than latency III. Latency I and 0 are both considered to be quiescent patterns of viral protein expression.

The reactivation of EBV infection exploits the normal differentiation pathway of B-lymphocytes by periodically upregulating its expression of lytic phase proteins when memory B-lymphocytes undergo differentiation into plasma cells. [186] The intermittent switching on of the lytic phase helps to produce new virions that are capable of infecting new naïve B-lymphocytes. This establishes a lifelong persistence within the B-lymphocyte compartment by continuously replenishing the pool of virus-infected cells.

1.5 Immunological response to primary EBV infection

Much of our current understanding of the host's cellular responses to a primary EBV infection has been gleaned from human and *ex vivo* studies of the temporal immune kinetics involved in acute IM in older children, adolescents and young adults. These works elucidate the various concerted efforts used by the innate and adaptive immune systems to restrict EBV proliferation (Figure 1.9). As a caveat, it is important to highlight that the immunopathological underpinnings of acute IM indicate that some of the immune changes observed in these studies may actually represent dysregulated rather than successful host responses to EBV and that these possibly differ from responses evoked by an asymptomatic primary infection. Regardless, they provide precious insight to how these processes can be overcome by intricate measures employed by EBV to evade antiviral immunity.

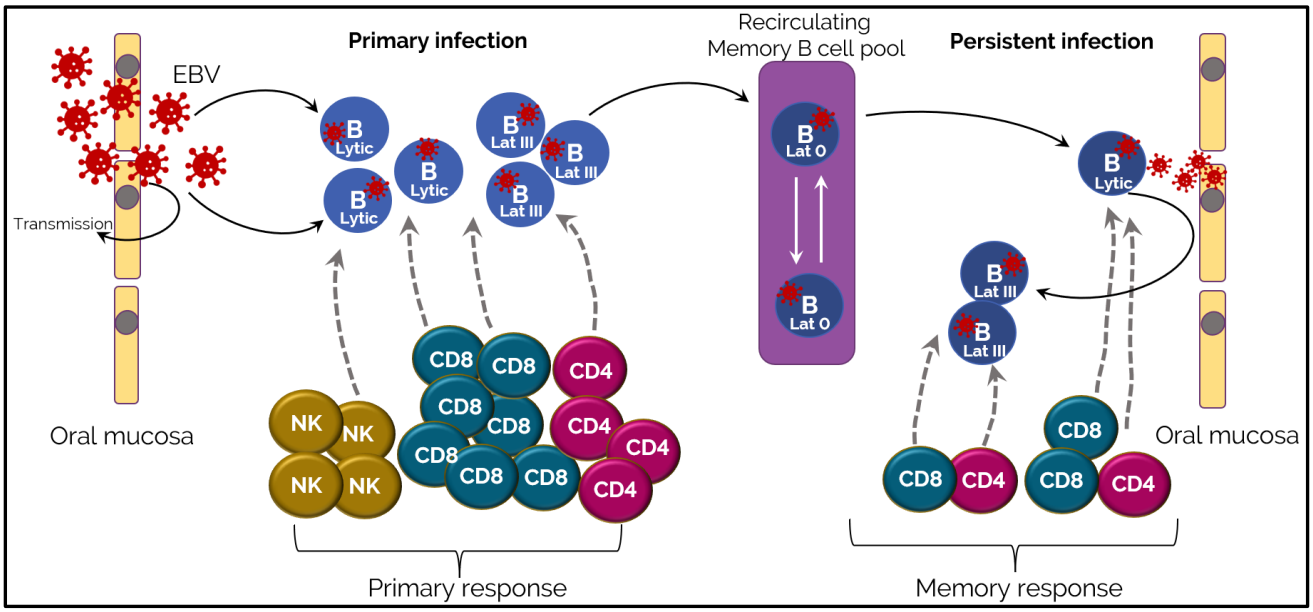


Figure 1.9: Overview of primary EBV infection in immunocompetent human hosts. A primary infection is acquired through saliva. The virus directly infects mucosal cells and resting naive B-lymphocytes in the oropharynx before undergoing a "lytic" phase of infection in the surrounding lymphoid tissue. This phase of infection is defined by high viral replication along with the expression of lytic viral proteins and the full complement of latent viral proteins (Latency III pattern). Immune response is mediated to a large extent by NK cells and CD8+ CTLs, which mostly target the lytic viral proteins. CD4+ T-helper lymphocytes also have an important, albeit lesser, role in mitigating viral proliferation. The subsequent downregulation of latent proteins (Latency 0 pattern) permits viral immune evasion and immortalisation as a chronic infection in memory B-lymphocytes ("latent" phase of infection). These latently infected cells travel between the peripheral circulation and lymphoid tissue in the oropharynx, intermittently undergoing reactivation into the lytic phase to infect new B-lymphocytes and maintain a viral reservoir. This reactivation process in kept in check by EBV-specific memory T-lymphocytes.

1.5.1 Innate immune response to primary EBV

The innate immune system is the bedrock of the host's first-line response to an EBV infection. Its activation is crucial for early viral recognition and the subsequent instigation of adaptive antiviral immunity. An important aspect of its response to EBV infection involves immune sensors known as pathogen recognition receptors (PRRs) that are present on APCs (e.g., monocytes, DCs and macrophages). [187] PRRs that include Toll-like receptors (TLRs) such as TLR3 and TLR9, Retinoic acid-inducible gene-I-like (RIG-I-like) receptors, and the DNA sensors cGAS-STING, are able to initiate a cascade of pro-inflammatory cytokine and chemokine release through the identification of pathogen-associated molecular patterns (PAMPs) on EBV such as viral lipids and components of its genome. [167, 188] The anti-EBV activities of innate immune cells are vast and highly interconnected. This section will focus on the key responses that are mediated by NK cells, unconventional T-lymphocytes and DCs.

1.5.1.1 NK cells

NK cells account for up to 15% of lymphocytes and play a major role in the early immune response to a primary EBV infection. [189] The NK cell compartment is composed of diverse differentiation stages that are defined by the expression of surface markers such as CD16, CD56 and two types of inhibitory receptors known as NKG2A (CD194a) and killer-cell immunoglobulin-like receptors (KIRs). [189]. NK cells exhibit an inherent antiviral cytotoxic capacity that is exerted through the release of IFN-γ and TNF-α during the lytic phase of EBV infection. [190, 191] A significant rise in the frequency and absolute count of NK cells has been observed during IM. [192-194] Interestingly, these same studies present contradictory evidence on how this cell expansion relates to circulating viral DNA and symptom severity. While one study found an inverse correlation between NK cell expansion, viral DNA load and the severity of symptoms, [192] a much larger study has reported a positive correlation instead. [194] More recent works have explored the impact of primary EBV infection on NK cell subsets and found that while there is a contraction of the CD56bright compartment, [195, 196] the overall expansion of NK cells is characterised by a preferential proliferation of the early-differentiated CD56dim NKG2A+ KIR- subset which exhibit highly effective cytotoxic and cytolytic function. [195, 197] Unlike the total NK cell expansion that appears to decline shortly after the onset of infection, the CD56dim NKG2A+ KIR- subset remains elevated for much longer and is still prominently expanded at up to six months post-acute infection. [195, 197] It is pertinent to mention that there is a recognised age-dependent decline in early-differentiated NK cells, with a lower frequency and number being

found in adolescents compared to younger children. [197] It is possible that this reflects an age-related transition of NK cell subsets towards antigen-induced terminal differentiation. This progressive loss of the early-differentiated CD56dim NKG2A+ KIR− NK cell subset could hypothetically serve as a catalyst for the symptomatic presentation of acute IM in older patients due to the exaggerated expansion of CD8+ CTLs in this age group and their greater reliance on adaptive immune responses. [198]

1.5.1.2 NKT cells

The role of NKT cells in EBV immune recognition and response is uniquely understudied. A sub-analysis of 11 patients in one study examined the response of NKT cells during primary EBV infection and found no evidence of their expansion. [192] However, there is evidence that NKT cells are capable of controlling EBV-mediated B-lymphocyte transformation in lymphoblastoid cell lines (LCLs). [199] It has also been noted that the expression of CD1d on infected B-lymphocytes becomes heavily downregulated during the transformation process, eventually leading to a failure of NKT cells to recognise infected LCLs. [199] This process of CD1d downregulation has been reported in infections with other herpesviruses and suggests a mechanism by which EBV is able to circumvent NKT immunity quite early in the infective period. [200, 201]

1.5.1.3 γδ T-lymphocytes

Similar to NKT-like cells, little work has been done to fully examine the role of γδ T-lymphocytes in primary EBV infection. Small scale studies have identified an increase in the frequency and absolute number of circulating γδ T-lymphocytes during acute IM. [202-204] Likewise, transcriptional analysis has shown that both the Vδ1 and Vδ2 subsets are increased in IM. [205] A similar study of a subset of EBV-positive children noted a marked expansion of Vγ9Vδ2 T-lymphocytes in 4/17 IM patients and 6/17 EBV positive healthy children in excess of 5% of total lymphocytes. [206] It remains unclear why this Vγ9Vδ2 T-lymphocytes expansion was only achieved by a handful of patients. It is interesting that Vγ9Vδ2 T-lymphocytes appear to preferentially recognise EBV-infected cell lines that express the latency I pattern (i.e., only EBNA-1). [206] This might indicate that it is the accumulation of latently infected memory B-lymphocytes in the peripheral circulation rather than the lytic phase of EBV infection that functions as a driver for γδ T-lymphocytes activation and expansion. Further experimental analysis will be required to explore this hypothesis.

1.5.1.4 Dendritic Cells

There are few human studies on the temporal immune kinetics of DCs in relation to a primary EBV infection. Two studies have demonstrated a significant loss of plasmacytoid DCs (pDCs) during acute IM which is associated with more severe symptoms and a loss of type I interferon activity. [195, 207] The pDC compartment appears to gradually increase during convalescence, with long-term post-IM values similar to those seen in healthy virus carriers. [207] However, it is unclear if the loss of pDCs is due to EBV-induced apoptosis or sequestration in tissue sites. Humanised mouse models have provided some insight to how DCs contribute to the control of EBV. They show that DCs, particularly pDCs, can mediate EBV infection directly through the potent production of type I interferons which are important for restricting EBV-induced B-lymphocyte transformation in the first 24 hours of infection, [208, 209] and by using PRRs such as TLR9 to recognise the virion DNA. [210] Furthermore, DCs are key primers of NK cell and EBV-specific T-lymphocyte function. They are involved in the activation of NK cells through the surface presentation of IL-12, IL-15 and IFN-γ. [211, 212] *In vitro* assays have demonstrated that the transformation of EBV-infected B-lymphocytes is tempered via this DC-NK cell activation pathway. [212, 213] The priming of adaptive immunity by DCs involves the stimulation of EBV-specific T-lymphocyte responses either through MHC presentation of EBV antigens by phagocytic conventional DCs (cDCs) or by pDC-mediated trogocytosis of EBV epitope-presenting MHC complexes. [214, 215]

1.5.2 Adaptive Immune response to primary EBV

Broadly speaking, adaptive immunity is defined by the antigen-specificity of its responses and the ability to establish long-lived immunological memory. T- and B-lymphocytes are component cell subsets of the adaptive immune niche, both expressing high-order specificity and memory potential within their clonally diverse receptor repertoires. The functional profile of adaptive anti-EBV immunity is principally mediated by specific T-lymphocytes against EBV lytic and latent proteins along with EBV-specific antibody support by plasma cells. Similar to studies on the innate immune responses to primary EBV infection, most of the investigations on anti-EBV T- and B-lymphocyte activity have been inferred from symptomatic acute IM. It is unclear how/if these immune mechanisms differ from those employed during asymptomatic primary infection. However, they still offer valuable insight to how EBV naïve hosts respond to primary viral exposure.

1.5.2.1 CD8+ T-lymphocytes

Longitudinal studies of IM patients have helped to demonstrate the acute phase responses of EBV-specific CD8+ CTLs (EBV-CTLs) to lytic and latent viral proteins and their evolution over time during convalescence and long-term EBV carriage. The acute phase of symptomatic infection is dominated by a significant polyclonal expansion of CD8+ CTLs. [195] Despite this apparent polyclonal expansion, there is convincing evidence of a marked oligoclonal restriction in the usage of TCRs dominated by specificity to epitopes of IE, E, late lytic EBV proteins in decreasing hierarchy. [216-219] These EBV lytic epitope-specific CTLs can account for up to 40% of the total circulating CD8+ population. [220] CD8+ EBV-CTLs that are specific for latent viral proteins are also expanded albeit to a lesser degree, accounting for 0.1-5% of the total CD8 population. [221,222] These are primarily in response to the EBNA-3s along with LMP-2. [221, 222]

Immunophenotypic analysis of CTLs from children with symptomatic EBV infection shows that they exhibit an immune signature dominated by an expansion of CD8+ TCM and TEM compartments (TEM > TCM) alongside a contraction of the naïve and TEMRA subsets. [223] This likely represents the rapid differentiation of naïve CTLs into functional CTL subsets that are capable of providing immediate effector and memory responses to stimulation by EBV antigens. Polyclonally expanded CD8+ cells and EBV-specific CTLs both exhibit an activated profile characterised by an increased surface expression of CD27, CD38, HLA-DR and CD69. [220, 223, 224] EBV-CTLs also exhibit a high propensity for apoptosis. This has been inferred from their low expression of the anti-apoptotic protein Bcl-2. [225]
A recent study examined the expression of inhibitory immune checkpoint proteins on CTLs in a small cohort of paediatric IM patients. An expansion of PD-1+ CTLs along with higher expression of other inhibitory receptors (TIM-3, 2B4 and KLRG1) was observed both in the overall CD8+ population compared to healthy EBV seropositive controls. [226] Interestingly, this cell subset retained their proliferative and cytotoxic capacity. Furthermore, the frequency of PD-1+ EBV-CTLs against BMLF-1 and LMP-2 were similarly raised in EBV-infected mice reconstituted with human immune system components (huNSG). [226] Other viruses have been shown to induce CTL expression of inhibitory receptors, particularly PD-1, during the acute phase of infection. [227] Although its role in T-lymphocyte exhaustion and immune dysregulation during chronic viraemia has been clearly established, [228, 229] the exact function of PD-1 during acute viral infections is still under exploration. Its expression on activated T-lymphocytes probably indicates a self-tolerance mechanism by the host

to limit collateral tissue damage during acute infections through modulation of the length and magnitude of effector/memory responses. [230, 231]

1.5.2.2 CD4+ T-lymphocytes

CD4+ T_H lymphocytes do not appear to undergo the same extensive expansion seen in CD8+ CTLs during acute IM. [223, 232] In addition, the magnitude of their response to EBV antigens is significantly less than that of CD8+ CTLs, accounting for approximately 1.5% of the total CD4+ population. [233] However, compared to EBV-CTLs, they react to a broader range of viral epitopes but are dominated by responses to latent rather lytic viral proteins. [233, 234] Kinetic studies show that while CD4+ T_H responses to IE lytic proteins such as BZLF-1 peak rapidly in the acute phase of IM before declining during convalescence, [235, 236] the frequency of EBV-specific CD4+ T_H to latent proteins such as EBNA-3B continues to increase over time. [237] Curiously, the response of CD4+ T_H to EBNA-1 is delayed for several months in patients with acute IM and appears to be in line with the eventual appearance of EBNA-1 IgG antibodies as a terminal feature of seroconversion. [233, 238, 239]

Immunophenotyping of CD4+ subsets patients with acute IM indicates a similar trend towards the establishment of an effector immune signature that is characterised by a decreased proportion of naïve CD4+ T_H and an increased frequency of CD4+ TEMs. [223] EBV-specific CD4+ lymphocytes within the effector compartment express multiple markers of activation and are capable of producing cytolytic molecules (e.g., Granzyme B, perforin) and multiple pro-inflammatory cytokines in response to *in vitro* stimulation with EBV-infected cells. [237, 240, 241] A significant contraction of the Treg subset can be seen during the acute phase of IM, [195, 242] even though LMP-1 has been demonstrated to induce Treg activity. [243, 244] It is possible that Tregs play an important role in controlling primary EBV infection to a subclinical level in most cases and that IM represents a failure of this protective mechanism. [242] An altered frequency of circulating TfH (CD4+ CXCR5+) has also been reported in acute IM patients compared to healthy controls, with IM patients exhibiting a significantly lower proportion of this CD4+ T_H subset. [245] TfH lymphocytes are important for the activation and differentiation of B-lymphocytes in germinal centres of secondary lymphoid tissue. [246] Interestingly, the ICOS+PD1+ TfH subset has been found to be significantly increased in the IM patients with a negative correlation to the absolute count of naïve B-lymphocytes but positive correlation with memory B-lymphocyte and plasma cell counts as well as EBV DNA load. [245] These cell populations changed significantly after antiviral therapy with an increase in overall TfH alongside a contraction of the ICOS+PD1+ TfH subset. These results suggest that a dysregulation of TfH likely contributes to the

pathogenesis of IM through inefficient humoral responses. *In vitro* experiments of ICOS+PD1+TfH function will be required to validate this proposed immune mechanism.

1.5.2.3 Humoral immunity

A lot of scientific emphasis has been placed on the dynamic changes within the T-lymphocyte compartment during primary EBV infection at the expense of B-lymphocytes. Published evidence on the humoral response to EBV has coalesced around the evolution of EBV-specific antibody production during a primary infection without much insight to how or if B-lymphocyte subsets are altered by EBV and vice versa.

The few studies that have tangentially investigated this relationship have identified a significantly lower frequency and/or absolute count of B-lymphocytes during acute IM. [223, 245, 247-249] One study looked at the frequency of the B-lymphocyte subsets during the acute phase of infection and noted that while the percentage of naïve B-lymphocytes (CD19+IgD+CD27-) was significantly higher, those of the unswitched (CD19+IgD+CD27+) and class-switched memory (CD19+IgD-CD27+) compartments were significantly lower compared to healthy controls. [223] These findings likely reflect the sequestered proliferation of acutely infected naïve B-lymphocytes in concert with overarching lysis initiated by active viral replication and the host's immune responses. However, due to the lack of longitudinal data, it is unclear if these changes in the B-lymphocyte compartment evolve as the host transitions from the acute phase of infection to convalescence and subsequent long-term viral carriage or in fact how this relates to the appearance of anti-EBV antibodies.

A lot more is known about the timing and sequence of EBV-specific antibody responses during symptomatic primary infection. Shortly after the onset of symptoms, both anti-VCA IgM and anti-VCA IgG can be detected in circulation either simultaneously or with the former being antecedent. [250] The detection of anti-VCA IgM with or without anti-VCA IgG is considered to be the serological hallmark of an acute EBV infection. [251] A caveat is that anti-VCA IgM immunoassays are known to exhibit cross-reactivity with other primary viral infections and false-positive results have been reported especially with CMV infection. [252] Additionally, not all patients with a primary EBV infection exhibit an anti-VCA IgM response. [253, 254] As the infection transitions from the acute phase to a latent persistent infection, the anti-VCA IgM begins to wane to undetectable levels within 3 months alongside a rising titre of anti-VCA IgG which peaks during this period before subsequently persisting at a stable lower level. [251] The Anti-EBNA-1 IgG level also begins to rise slowly but usually isn't

detectable until 3-6 months or more after the onset of illness. [194, 250] Therefore, these three EBV-specific antibodies are typically used in diagnosing EBV infection and to determine if it is a recent/acute or past infection. An anti-VCA IgM+, anti-VCA IgG+, anti-EBNA-1 IgG− serological picture is classically diagnostic of a primary or recent EBV infection, whereas a fully resolved primary infection is characterized as anti-VCA IgM−, anti-VCA IgG+, anti-EBNA-1 IgG+. [177] However, similar to the caveat for anti-VCA IgM in acute EBV, not all patients go on to develop an anti-EBNA-1 IgG response as marker of seroconversion. [194] This makes anti-VCA IgG a more reliable marker of past EBV infection in the absence of detectable anti-VCA IgM and viral DNA in the peripheral circulation. In addition, EBV reactivation and chronic active viraemia can both evoke detectable responses from all three antibodies. [255] Any interpretation of EBV-specific antibody responses must therefore be done judiciously in conjunction with an assessment of the patient's viral DNA load and clinical presentation.

1.6 Asymptomatic primary infection

The longitudinal tracking of EBV seronegative patients has provided some insight on the trajectory of cellular immune responses in individuals as they acquire an asymptomatic primary EBV infection. Studies of young children in low/low-middle income countries, where EBV infection is more prevalent at a younger age, have found that similar to acute IM, asymptomatic patients present with high levels of activated EBV-specific CD8+ CTLs and circulating/tonsillar viral DNA albeit without a congruent expansion of their total CD8+ compartment. [256, 257] These findings are replicated in prospective immunological studies of asymptomatic young adults, which have additionally found no disturbance of the NK cell compartment. [258, 259] The results from these studies collectively indicate that asymptomatic individuals are able to control their primary infection through focussed immune responses by EBV-specific CD8+ CTLs. More detailed work is needed to determine what happens to the other cell populations that are involved in a host's immune response to primary EBV.

1.7 EBV and B-cell lymphomagenesis

EBV has been consistently implicated in the aetiology of the three major types of high-grade mature B-cell lymphomas (HL, BL and DLBCL), [260] including lymphoproliferative diseases that arise secondary to IEIs and post-SOT immunosuppression. [1, 2] The process of malignant B-lymphocyte transformation is artfully modulated by EBV's lytic and latent proteins, each of which contribute effectively to lymphomagenesis by engaging in signalling and transcriptional pathways that are involved in immune evasion, genomic instability and the growth transformation of infected B-lymphocytes. [260, 261]

1.7.1 Immune evasion by EBV – The first step in viral oncogenesis

During the lytic phase of infection, the IE viral protein BRLF-1 has been shown to support the evasion of antiviral immunity through the inhibition of PRRs such as RIG-I, an inflammasome that is required for the cytokine-mediated activation of T-lymphocytes and NK cells. [262, 263] Early lytic proteins such as BNLF-2a are also capable of protecting EBV-infected cells from elimination by CD8+ EBV-CTLs through the inhibition of the transporter associated with antigen presentation. [264] Other lytic phase proteins are similarly able to exert immunomodulatory activities that support lymphomagenesis by dysregulating MHC I and II molecules, thus impairing T-lymphocyte function. [265 -267] In addition, viral IL-10 (vIL-10), a homologue of human IL-10 that is produced by BCRF-1, has been shown to interfere with the cytolytic activities of NK cells and CD4+ T_H. [264]

Latent viral proteins also display similar immunomodulatory activities. Chief of these is EBNA-1, a viral nuclear protein that is critical for maintaining and replicating the viral genome in latently infected cells. Not only does EBNA-1 attenuate the expression of NKG2D by NK cells, thus impairing their recognition of EBV-infected cells, [268] but it's glycine-alanine repeat domain (GAr) also displays an immune evasion function that prevents MHC-I restricted presentation of the viral epitope to CTLs. [269] Another viral nuclear protein with a recognised immunomodulatory role is EBNA-2. EBNA-2 is capable of initiating the expression of IL-18R in B-lymphocytes which can form a complex with IL-37 to suppress the release of pro-inflammatory cytokines. [270, 271] This helps to create an anti-inflammatory milieu that sustains EBV immune evasion and proliferation. The latent membrane proteins contribute to immune evasion not only through an LMP-1 mediated downregulation of the RIG-I inflammasome signalling pathway, [272] but also involves the LMP-2 induced dysregulation of interferon production and MHC class II expression, both of which interferes with T-lymphocyte anti-viral functions. [273, 274] A similar effect of EBNA-3A/C reducing the production of type I interferon by

pDCs has been noted as well. [275] These immune evasion mechanisms all act in concert with the growth transforming activities of EBV viral proteins to promote lymphomagenesis.

1.7.2 The role of EBV viral proteins in the malignant growth transformation of B-lymphocytes

A key system by which EBV evokes its growth transforming potential is by hijacking the apoptosis pathway of infected B-lymphocytes. This is established early in the infective process when EBV encodes BHRF-1 and BALF-1, two early lytic proteins that are viral homologues of the cellular pro-survival Bcl-2 proteins. [276] Both of these viral proteins are capable of inhibiting DNA-damage-induced apoptosis of infected cells through antagonistic effects on a variety of pro-apoptotic proteins such as BIM, Bcl-2 homologous antagonist killer (BAK) and p53 upregulated modulator of apoptosis (PUMA). [277-279] Although the lytic viral proteins contribute intimately to the evolution of high-grade B-cell lymphomas, much of EBV's growth transforming capability is actually mediated by its latent viral proteins. The expression of latent EBV proteins in growth transformed B-lymphocytes is restricted to varying latency patterns that typically differ by the type of lymphoma (**Table 1.2**).

Lymphoma type	Subtype	% EBV positive	Latency pattern	Ref
Hodgkin Lymphoma	Classic	20-50	II	280, 281
Diffuse Large B-cell Lymphoma	EBV+ DLBCL NOS	5-15	II/III	282
Burkitt Lymphoma	Endemic	90-100	I[b]	283
	Sporadic	15-30		284, 285
	IDA	25-40[a]		286
Post-transplant Lymphoproliferative Disease	Early onset	90	III	289
	Late onset	60-80		289

Table 1.2: Incidence of EBV positivity and associated latency pattern in the major types of mature high-grade B-cell lymphomas. DLBCL NOS – Diffuse Large B-cell Lymphoma Not Otherwise Specified; IDA – Immunodeficiency-associated. [a]This figure is based on data from an adult demographic. Smaller case-control studies estimate an EBV prevalence of ~5% in children. [287] [b]10-15% of endemic Burkitt lymphoma patients express EBNA-1, EBNA3s, EBNA-LP and BHRF-1 (Wp/BHRF-1 restricted) [288]

All six latent nuclear proteins are complicit in the dysregulation of apoptotic pathways. EBNA-1 upregulates the expression of survivin, an anti-apoptotic protein that promotes the survival of EBV-infected cells through the inhibition of caspase pathways. [290] It is also capable of inducing genomic

instability by increasing the production of reactive oxygen species through the activation of a catalytic subunit of leukocyte NADPH oxidase known as NOX2/gp91^{phox}. [291] EBNA-2 exhibits a similar anti-apoptotic verve through super-enhancer transactivation of multiple cellular genes such as the *c-myc* oncogene. [292, 293] This EBV-driven MYC enhancer activation might be a contributing factor in the genesis and localisation of MYC-Immunoglobulin translocation breakpoints in BL. [293] Of note, a recent European study demonstrated that EBNA-2 positive DLBCL has a poorer prognosis in patients older than 50 years compared to EBNA-2 negative tumours. [294] It remains to be seen if similar prognostication is exhibited in a paediatric setting. EBNA-LP evokes its oncogenic potential through its role as a transcriptional co-activator for EBNA-2. [295] The EBNA-3 family of nuclear proteins are predominantly implicated in lymphomas that arise in the setting of immunodeficiency or immunosuppression. Although all three EBNA-3s are significant contributors to the viability of transformed B-lymphocytes through their regulation of viral and host cell transcription, [296] only EBNA-3A and EBNA-3C are actually essential for the transformation of infected cells. [296-298] Epigenetic silencing of the pro-apoptotic BIM protein and tumour suppressors p16INK4a and p14ARF, including the attenuation of p53 activity are just some of the mechanisms used by EBNA-3A/C to promote lymphomagenesis. [299-301] The EBNA-3s are estimated to interact with more than 7,000 sites on the host cellular genome. [302] Further studies are important to unravel the full spectrum of cell survival genes that they regulated.

The latent membrane proteins are also important contributors to the pathogenesis of mature B-cell lymphomas (with the exception of BL). LMP-1 is considered to be a key growth transforming EBV oncoprotein. [303] Not only does it simulate the function of CD40 *in vivo* to promote B-lymphocyte proliferation, [304] but it also initiates abnormal cell signalling through the activation of several transcription pathways such as NF-κB (Nuclear Factor κB), MAPK (mitogen-activated protein kinase) and JAK-STAT (Janus kinase-signal transducers and activators of transcription), all of which contribute to the transformation and survival of infected cells. [302] LMP-2A on the other hand contributes to the lymphomagenesis process through anti-inflammatory and pro-survival pathways mediated by the upregulation of IL-10 via PI3K. [305] It is also capable of promoting cell cycle progression and hyperproliferation of infected B-lymphocytes by downregulating cyclin-dependent kinase inhibitor p27 (kip1) in a proteasome-dependent manner. [306] Finally, it is able to facilitate the bypassing of p53 inactivation in *c-myc*-induced lymphomagenesis models. [307] It is important to mention that while LMP-1 and LMP-2A promote early lymphomagenesis, neither of them is essential to the process.

Murine models have shown that the simultaneous knock-out of both viral proteins doesn't inhibit lymphomagenesis but instead has a significant effect on the incidence and speed at which cell transformation occurs. [308]

In spite of the extensive mechanisms by which EBV's viral proteins are able to induce lymphomagenesis, most of the general population with a latent EBV infection don't go on to develop a B-cell neoplasm. However, the burden of evidence shows that EBV's capacity to inflict maximum oncogenic malfeasance is felt most acutely in the setting of an underlying immune dysregulation. This is demonstrated by the fact that lymphomas in patients with primary and acquired immune disorders have a significantly higher risk of developing EBV-driven lymphomas compared to immunocompetent patients. [1,2] For children living in a high-income country, this most often occurs in the setting of iatrogenic immune dysregulation following a SOT, particularly a heart transplant. It is therefore important to consider what predisposing factor(s) might act as contributors to this 'high risk' profile. One such factor that is unique to paediatric heart transplant patients is their early age at thymectomy. The existing evidence that supports a need for a more robust study of this field of research is reviewed in the next section of this chapter.

1.8 The intersection of thymectomy, heart transplantation and the risk of EBV-driven PTLD

Paediatric heart transplantation requires lifelong immunosuppression therapy in order to prevent graft rejection by the host. This inadvertently establishes a tolerogenic immune microenvironment where pathogens such as EBV can easily proliferate unchecked to leverage their growth transforming potential and support lymphomagenesis in the form of EBV-PTLD. [2, 309]

1.8.1 Classification of PTLD

Six histopathological subtypes of PTLD are currently recognised by the World Health Organisation (WHO). [3] These subtypes are designated as either "non-destructive" or "destructive" PTLD depending on the degree of pathological effacement within the lymphoid tissue architecture.

1.8.1.1 Non-destructive PTLD

Previously described as "early lesions", these subtypes of PTLD exhibit polyclonal proliferation of EBV positive B-cells while retaining a semblance of normal underlying tissue architecture. [310] They are sub-classified further as either florid follicular hyperplasia, infectious mononucleosis-like PTLD, or plasmacytic hyperplasia. [3] These lesions tend to have a low risk of morbidity and often present clinically with acute self-limiting symptoms similar to infectious mononucleosis. [311]

1.8.1.2 Destructive PTLD

These comprise both polymorphic and monomorphic lesions. Polymorphic PTLD consists of polyclonal or monoclonal lymphoid infiltrates with evidence of nuclear atypia, necrosis, and loss of architectural integrity of the lymphoid tissue. [310] Monomorphic PTLD typically involves the monoclonal proliferation of EBV-infected B lymphocytes with effacement of the underlying lymphoid tissue architecture. They bear histological resemblance to high-grade mature B-cell lymphomas seen in immunocompetent patients (e.g., BL, DLBCL). [3, 310, 311] Other rarer subtypes of monomorphic PTLD exist such as classical Hodgkin-like PTLD, EBV-positive MALT lymphoma, and T-cell lymphomas with(out) EBV infection. [310] Destructive PLTD are clinically aggressive and life threatening. They require active treatment ranging from first-line reduction in immunosuppression (RIS) to Rituximab immunotherapy (monoclonal anti-CD20 antibody) with(out) low dose multiagent chemotherapy. [310]

1.8.2 Risk factors for EBV-PTLD after paediatric heart transplant

1.8.2.1 Age, pre-transplant EBV status and immunosuppression therapy

Young age at transplantation (< 10 years old), pre-transplant EBV naïvety and donor-recipient EBV mismatch (D+/R-) have long been established as significant independent contributors to the risk of PLTD development. [8, 312] In contrast, though the use of immunosuppression therapy is considered to be a natural impetus for EBV-PTLD, its exact role in the lymphomagenesis pathway is much less clearly determined. While there is some evidence that using two or more doses of T-lymphocyte depleting induction agents such as Anti-thymocyte globulin (ATG) in paediatric heart transplant patients is associated with significantly higher EBV viral load and incidence of EBV-PTLD, [313] several large observational studies in this cohort have found no link between EBV-PTLD and induction immunosuppression with either ATG or Basiliximab, a non-depleting CD25 antagonist.[314-318] Similar studies have also examined the effects of long-term maintenance immunosuppression with calcineurin inhibitors (CNIs) and found no consistent evidence that shows a higher risk of EBV-PTLD with either ciclosporin or tacrolimus. [318, 319] It could be postulated that any association between long-term immunosuppression and EBV-PTLD risk is likely to be more dependent on the intensity of therapy rather than the actual immunosuppressive agent of choice. [320] This would partially explain the higher incidence of PTLD in paediatric heart transplant recipients, whose immunosuppression regimen tends to be more intensive compared to other paediatric SOT patients. In support of this theory is the fact that heart transplant patients often remain on multiple immunosuppressants in the long-term. [321] This is usually made up of a CNI backbone in addition to a steroid and/or a purine synthesis inhibitor. [321] There is growing evidence that purine synthesis inhibitors such as azathioprine and mycophenolate mofetil (MMF) can exert a pro-PTLD effect through multiple pathways such as promoting persistently higher EBV viraemia in paediatric heart transplant patients, the selective depletion of CD56dim CD16+ NK cells and IFN-γ (crucial for successful EBV control), and the reactivation of latent herpesvirus infections. [313, 322]

1.8.2.2 Does early thymectomy have a role in the risk of EBV-PTLD?

The identification of modifiable contributors to the pathogenesis of EBV-PTLD has become a key area of scientific interest in recent years due to the significant improvement in survival outcomes for paediatric SOTs. One such factor that has emerged as a viable candidate in the last decade is the influence of early thymectomy on EBV-PTLD amongst paediatric heart transplant recipients. In 2017, a Swedish study observed that children transplanted for a pre-existing congenital heart disease (CHD)

had a significantly higher risk of developing EBV-PTLD compared to those transplanted for an underlying cardiomyopathy and that all CHD patients with EBV-PTLD had also undergone a sternotomy during infancy for palliative cardiac surgery. [323] In fact, the impact of CHD on the risk of EBV-PTLD was first alluded to in 2006 by Mendoza *et al*, but had been dismissed by the authors as being "likely a result of random occurrence or statistical chance." [324] The first attempt to directly link early thymectomy to the risk of EBV-PTLD was my study of risk factors for EBV-PTLD in the largest UK cohort of paediatric heart transplant patients to date. [4] This work identified that the risk of EBV-PTLD was significantly higher in children with CHD and those undergoing an early thymectomy at ≤ 1 years old (**Figure 1.10**).

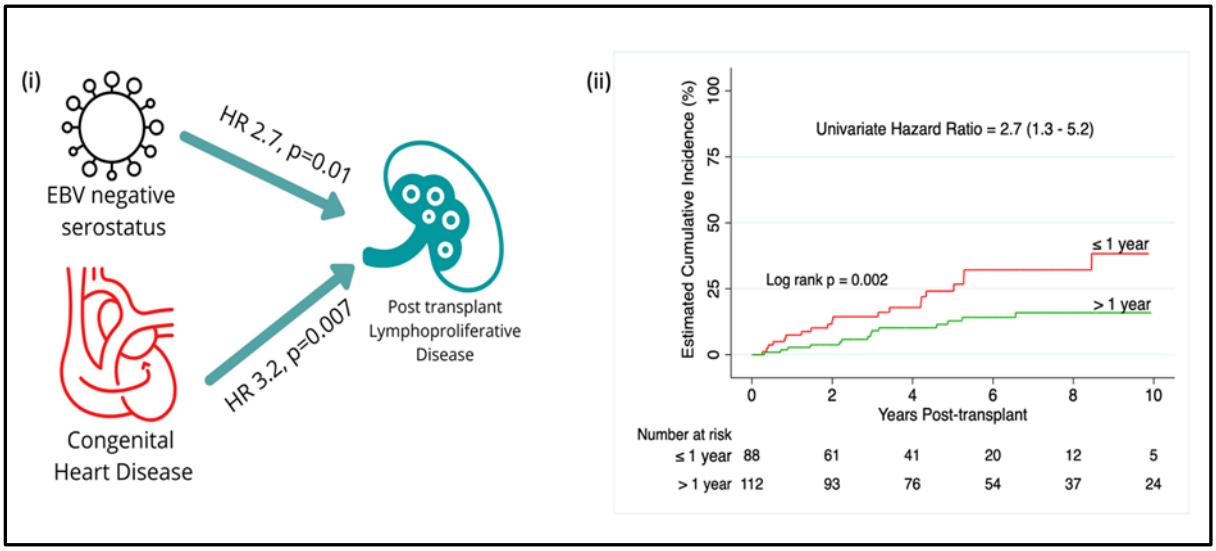


Figure 1.10: Risk factors for EBV-PTLD in paediatric heart transplant patients (i) Illustration showing the independent risk factors associated with EBV-PTLD in paediatric heart transplant patients; (ii) Kaplan-Meier curves depicting a higher incidence of EBV-PTLD after early thymectomy at ≤1 year old. [4]
HR – Hazard Ratio

Furthermore, our study highlighted a long-term disruption of the T-lymphocyte compartment in children with CHD, whose CD4+ T_H and CD8+ T-lymphocyte absolute counts remained persistently lower at 5-years follow up compared to those transplanted for an acquired cardiomyopathy (Figure 1.11).

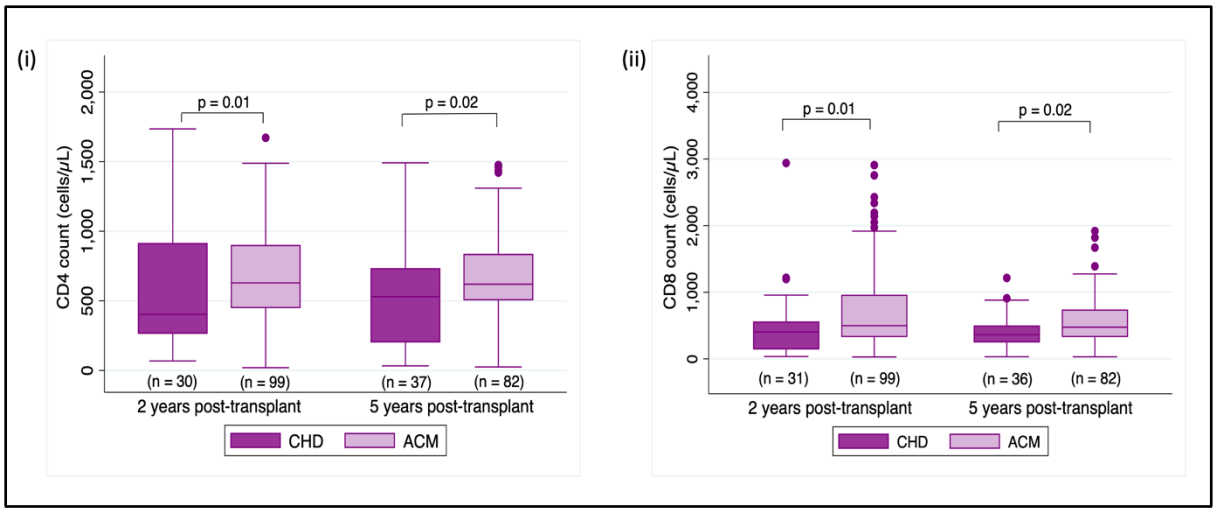


Figure 1.11: Box plots illustrating the significant and sustained differences in post-transplant T-lymphocytes according to cardiac diagnosis **(i)** CD4+ T_H and **(ii)** CD8+ T-lymphocytes absolute counts in paediatric heart transplant patients. [4] **ACM**: Acquired Cardiomyopathy; **CHD**: Congenital Heart Disease

Limited evidence from studies that have examined the post-transplant composition of T-lymphocytes in paediatric heart transplant recipients provide some context of how early thymectomy could prime an immune microenvironment that supports a higher risk of EBV immune escape and the growth transformation of infected B-lymphocytes compared to other SOT groups. For example, heart transplant patients were found to have lower absolute counts of total and naïve CD4+ T_H, CD8+ CTLs and B-lymphocytes compared to kidney transplant patients and healthy controls with a higher proportion of TfH.[325] Such perturbation of the T- and B-lymphocyte compartments, both of which are necessary components of a healthy anti-EBV response, might explain why heart transplant patients have a much higher risk of EBV-PTLD than kidney transplant recipients. Furthermore, recent murine experiments have shown that the risk of EBV-driven lymphoproliferative disease in SCID mice was highest in those inoculated with cells from healthy EBV positive donors who had significantly higher baseline TfH and was delayed/prevented when this cell subset was depleted. [326] Furthermore, both the incidence of chronic EBV viraemia and its progression to EBV-PTLD are noted to be significantly higher in paediatric heart transplant patients compared to other SOT groups. [327, 328] Finally, single cell analysis of mononuclear cells from paediatric heart, liver and kidney transplant patients was used recently to identify an immune signature unique to heart transplant recipients with asymptomatic high EBV load. It showed that this subgroup of patients display distinct CD8+ CTLs with (1) decreased naive phenotype and altered memory differentiation, (2) accumulation of terminally exhausted cells with a decrease in functional precursors of effector subsets. [329] In addition, CD4+ T-lymphocytes exhibited similar changes in naive and memory subsets, including

elevated Th1 cells and plasma IL-21, suggesting an alternative inflammatory mechanism that governs antiviral T-lymphocyte responses in heart transplant recipients. [329]

These findings collectively demonstrate a growing evidence-base that suggests major adverse immunological sequalae in heart transplant patients as a consequence of early thymectomy. This warrants longitudinal analysis to clearly delineate how these factors interact with each another to facilitate EBV-PTLD development. To date, no study has prospectively examined the systemic immune landscape of thymectomised children undergoing SOT with the explicit aim of exploring their immune trajectory prior to, during, and after the acquisition of a primary EBV infection and its relationship to the risk of EBV-PTLD. This PhD project has therefore been developed to address this prominent gap in the evidence base.

1.9 Hypothesis and project aims

1.9.1 Hypothesis

The central hypothesis for this project is that early childhood thymectomy, compounded by long-term iatrogenic immunosuppression, establishes a dysregulated systemic immune profile consisting of senescent and exhausted adaptive immune cells with an impaired response to primary EBV infection. This subsequently leads to the poor immunological control of EBV-infected B-lymphocytes and neoplastic transformation into EBV-PTLD.

1.9.2 Project aims

This project aims to employ an integrative approach using clinical data, high dimensional flow cytometry and T-lymphocyte functional analysis to identify key immunological factors associated with poor EBV control and an increased risk of PTLD. This will involve defining the impact of age at thymectomy on the temporal trajectory of adaptive/innate immunity and EBV-specific responses after childhood cardiac transplant by comparing peripheral immune cell signatures in early thymectomy and late-/non-thymectomy patients within this cohort.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Laboratory Equipment

Equipment	Developer
5-laser Aurora Full Spectrum Flow Cytometer	Cytek™
ImmunoSpot reader	Cellular Technology Limited
Benchtop Centrifuge 5804 R	Eppendorf
Microcentrifuge 5424	Eppendorf
Neubauer cell counting chamber	Weber Scientific International
Cell Culture Microscope CKX53	Olympus
37°C CO_2 Incubator	Panasonic

2.1.2 Software

Spectral flow cytometry	Developer
SpectroFlo v3.3.0	Cytek™
FCS Express v7	De Novo Software
OMIQ Data Science Platform	Dotmatics

ELISpot	Developer
ImmunoSpot Single-Color ELISPOT Enzymatic	Cellular Technology Limited

Statistical Analysis	Developer
GraphPad Prism v10	GraphPad Software Inc
Excel 16.8	Microsoft

2.1.3 Tissue Culture Media, Reagents and Experimental kits

Tissue Culture Media and Reagents	Supplier
Roswell-Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 2mM L-glutamine	Sigma-Aldrich
Phosphate-Buffered Saline (PBS)	Sigma-Aldrich

Lymphoprep®	StemCell Technologies
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Trypan Blue	Sigma-Aldrich
Heat-inactivated Fetal Bovine Serum (FBS)	Gibco
Universal Nuclease for Cell lysis	Fisher Scientific
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Penicillin-Streptomycin (P/S)	Gibco
Sterile Human Serum	Sigma Aldrich
Brilliant Stain Buffer	BD Biosciences
Human Fc Block	BD Biosciences
Compensation beads	BioLegend
FluoroFix™ Buffer	BioLegend

PBMC Freezing medium:

- 90% FBS + 10% (v/v) DMSO

PBMC Thawing medium:

- 2% (v/v) FBS + PBS (Thawing medium A)
- RPMI-1640 + 8% (v/v) FBS + 1% (v/v) P/S (ELISpot medium)

Fluorescence Activated Cell Sorting (FACS) Staining Buffer:

- 0.5% (w/v) BSA + PBS

Experimental kits

ELISpot Pro: Human IFN-γ (ALP)	MABTECH
PepMix™ EBV EBNA-1 (mix of 158 peptides)	JPT Peptide Technologies
PepMix™ EBV LMP-2 (mix of 122 peptides)	JPT Peptide Technologies
PepMix™ EBV BZLF-1 (mix of 59 peptides)	JPT Peptide Technologies

2.1.4 PepMix™ sequences

I. EBV EBNA-1

MSDEGPGTGP GNGLGEKGDT SGPEGSGGSG PQRRGGDNHG RGRGRGRGRG
GGRPGAPGGS GSGPRHRDGV RRPQKRPSCI GCKGTHGGTG AGAGAGGAGA
GGAGAGGGAG AGGGAGGAGG AGGAGAGGGA GAGGGAGGAG GAGAGGGAGA
GGGAGGAGAG GGAGGAGGAG AGGGAGAGGG AGGAGAGGGA GGAGGAGAGG
GAGAGGAGGA GGAGAGGAGA GGGAGGAGGA GAGGAGAGGA GAGGAGAGGA
GGAGAGGAGG AGAGGAGGAG AGGGAGGAGA GGGAGGAGAG GAGGAGAGGA
GGAGAGGAGG AGAGGGAGAG GAGAGGGGRG RGGSGGRGRG GSGGRGRGGS
GGRRGRGRER ARGGSRERAR GRGRGRGEKR PRSPSSQSSS SGSPPRRPPP
GRRPFFHPVG EADYFEYHQE GGPDGEPDVP PGAIEQGPAD DPGEGPSTGP
RGQGDGGRRK KGGWFGKHRG QGGSNPKFEN IAEGLRALLA RSHVERTTDE
GTWVAGVFVY GGSKTSLYNL RRGTALAIPQ CRLTPLSRLP FGMAPGPGPQ
PGPLRESIVC YFMVFLQTHI FAEVLKDAIK DLVMTKPAPT CNIRVTVCSF
DDGVDLPPWF PPMVEGAAAE GDDGDDGDEG GDGDEGEEGQ E

II. EBV LMP-2

MGSLEMVPMG AGPPSPGGDP DGYDGGNNSQ YPSASGSSGN TPTPPNDEER
ESNEEPPPPY EDPYWGNGDR HSDYQPLGTQ DQSLYLGLQH DGNDGLPPPP
YSPRDDSSQH IYEEAGRGSM NPVCLPVIVA PYLFWLAAIA ASCFTASVST
VVTATGLALS LLLLAAVASS YAAAQRKLLT PVTVLTAVVT FFAICLTWRI
EDPPFNSLLF ALLAAAGGLQ GIYVLVMLVL LILAYRRRWR RLTVCGGIMF
LACVLVLIVD AVLQLSPLLG AVTVVSMTLL LLAFVLWLSS PGGLGTLGAA
LLTLAAALAL LASLILGTLN LTTMFLLMLL WTLVVLLICS SCSSCPLSKI
LLARLFLYAL ALLLLASALI AGGSILQTNF KSLSSTEFIP NLFCMLLLIV
AGILFILAIL TEWGSGNRTY GPVFMCLGGL LTMVAGAVWL TVMSNTLLSA
WILTAGFLIF LIGFALFGVI RCCRYCCYYC LTLESEERPP TPYRNTV

III. EBV BZLF-1

MMDPNSTSED VKFTPDPYQV PFVQAFDQAT RVYQDLGGPS QAPLPCVLWP
VLPEPLPQGQ LTAYHVSTAP TGSWFSAPQP APENAYQAYA APQLFPVSDI
TQNQQTNQAG GEAPQPGDNS TVQTAAAVVF ACPGANQGQQ LADIGVPQPA
PVAAPARRTR KPQQPESLEE CDSELEIKRY KNRVASRKCR AKFKQLLQHY
REVAAAKSSE NDRLRLLLKQ MCPSLDVDSI IPRTPDVLHE DLLNF

2.1.5 HLA-restricted EBV epitope sequences

All EBV epitope sequences were purchased from GenScript and directly shipped to the National Institute of Health (NIH) Tetramer Core Facility, Atlanta, Georgia, USA, for tetramerisation and fluorochrome labelling. Tetramers of lytic and latent EBV antigens were labelled with APC and PE respectively. The tetramer pool was selected based on published immunodominant EBV epitopes [330], while simultaneously maximising the coverage of HLA genotypes in the study population.

HLA Restriction	Epitope sequence	EBV Antigen	Fluorochrome
A*02:01	YVLDHLIVV	BZLF-1	APC
A*02:01	GLCTLVAML	BMLF-1	APC
A*02:01	CLGGLLTMV	LMP-2	PE
A*11:01	AVFDRKSDAK	EBNA-3B	PE
A*11:01	ATIGTAMYK	BRLF-1	APC
A*24:02	TYGPVFMCL	LMP-2	PE
B*07:02	RPQKRPSCI	EBNA-1	PE
B*07:02	RPQGGSRPEFVKL	BMRF-1	APC
B*08:01	RAKFKQLL	BZLF-1	APC
B*08:01	QAKWRLQTL	EBNA-3A	PE
B*35:01	HPVGEADYFEY	EBNA-1	PE
B*35:01	EPLPQGQLAY	BZLF-1	APC
DRB1*03:01	PAQPPPGVINDQQHHLPSG	EBNA-2	PE
DRB1*03:01	VKLTMEYDDKVSKSH	BMRF-1	APC
DRB1*04:01	GQTYHLIVDTDSLGNPSLSV	EBNA-2	PE
DRB1*04:01	PYYVVDLSVRGM	BHRF-1	APC
DRB1*07:01	SRDELLHTRAASLLY	BaRF-1	APC
DRB1*07:01	PRSPTVFYNIPPMPLPPSQL	EBNA-2	PE
DRB3*02:02	LTAYHVSTAPTGSWF	BZLF-1	APC
DRB3*02:02	PRSPTVFYNIPPMPLPPSQL	EBNA-2	PE

2.1.6 Flow Cytometry Antibodies

Specificity	Fluorochrome	Clone	Supplier	Titre (ng/100ul test)
CD45	Spark YG 593	HI03	BioLegend	125
CD45RA	BUV 395	5H9	BD Biosciences	125
CD3	BV 510	SK7	BioLegend	200
CD4	PerCP	SK3	BD Biosciences	50
CD8	BUV 805	SK1	BD Biosciences	62.5
TCRγd	PerCP-eFluor 710	B1.1	Thermo Fisher	250
CD11c	PE-Cy7	B-ly6	BD Biosciences	1000
CD14	Spark Blue 550	63D3	BioLegend	100
CD16	BUV 496	3G8	BD Biosciences	250
CD19	Spark NIR 685	HIB19	BioLegend	125
CD20	BV 786	2H7	BD Biosciences	62.5
CD21	PE-Cy5	B-ly4	BD Biosciences	31.3
CD24	PE-AF 610	SN3	Thermo Fisher	500
CD25	PE-AF700	CD25-3G10	Thermo Fisher	250
CD27	APC-H7	M-T271	BD Biosciences	125
CD28	BV650	CD28.2	BioLegend	125
CD31	BV711	WM59	BioLegend	100
CD38	APC-Fire 810	HIT2	BioLegend	250
CD39	BUV661	TU66	BD Biosciences	500
CD56	BUV 737	NCAM16.2	BD Biosciences	625
CD57	Pacific Blue	NK-1	BioLegend	62.5
CD62L	BUV 496	SK-11	BD Biosciences	1000
CD69	AF 647	FN50	Abcam	320
CD95	PE-Cy5	DX2	BioLegend	62.5
CD123	Super Bright 436	6H6	Thermo Fisher	125
CD127	APC-R700	HIL-7R-M21	BD Biosciences	250
KIR (CD158)	BUV 605	DX27	BD Biosciences	250
NKG2A (CD159a)	APC	REA110	Miltenyi	150
NKG2C (CD159c)	PE	REA205	Miltenyi	200
IgD	BV 480	IA6-2	BD Biosciences	125

IgM	BV 570	MHM-88	BioLegend	312.5
HLA-DR	PE-Fire 810	L243	BioLegend	125
CCR4	BB 700	1G1	BD Biosciences	250
CCR5	BUV 563	2D7/CCR5	BD Biosciences	250
CCR6	BV 480	11A9	BD Biosciences	1000
CCR7	BV 421	G043H7	BioLegend	350
CXCR3	AF 488	GH025H7	BD Biosciences	1000
CXCR5	BV 750	RF8B2	BD Biosciences	125
PD-1	BV 785	EH12.2H7	BioLegend	250
LAG-3	PE-Cy7	3DS223H	Thermo Fisher	30
TIM-3	BV 605	F38-2E2	BioLegend	250
CTLA4	PE-CF594	BNI3	BD Biosciences	250
Viability	Live/Dead Blue	-	Thermo Fisher	5µl of 1:40 stock dilution

2.1.7 Ethics approval for using patient-derived and healthy control blood samples

Whole blood samples were collected from patients recruited to the Immunology of THymectomy and childhood CArdiac transplant (ITHACA) study between March 2022 and June 2024. Written informed consent (+/- assent) was obtained from patients and/or legal guardians according to study protocols approved by the North of Scotland Research Ethics Committee (REC reference number: 21/NS/0142, IRAS: 298986). Anonymised biobanked peripheral blood mononuclear cells (PBMC) and serum specimens from five (5) healthy children collected for the Coronavirus Immunological Analysis study (approved by North West Preston Research Ethics Committee, REC reference number: 20/NW/0204, IRAS: 282164), were obtained from the University of Birmingham's Institute of Immunology and Immunotherapy. Similar biobanked specimens were included for a further thirteen (13) healthy children who were recruited to the Troubled Immune System (TrIm) study approved by the North East - Newcastle & North Tyneside 1 Research Ethics Committee (REC reference number: 20/NE/0044, IRAS: 251734). These healthy children were usually attending hospital for routine surgical procedures. All healthy children were screened for underlying conditions prior to recruitment and were not known to have any underlying immune or thymic disorder. University ethics approval was used to collect peripheral blood samples from five (5) healthy adult volunteer donors working within Newcastle University and two (2) working at the University of Birmingham.

2.2 Methods

2.2.1 Sample collection

Whole blood samples for PBMC isolation were collected from patients and healthy donors (where applicable) in 5ml vacutainer tubes containing Ethylenediaminetetraacetic acid (EDTA). Additional blood samples for EBV viral load and antibody quantification were simultaneously collected in 1ml EDTA and Serum Separating (SST) microtainer tubes respectively. Blood volumes for patients recruited to the ITHACA study were taken in adherence to the WHO's guidance for blood sampling in child health research.[331] The guidance suggests that there is minimal adverse risk of sampling up to 5% of a child's total blood volume. This can be reduced to 3% in the presence of serious illness.[331] A safe sampling range of 5-20mls per single venipuncture draw was implemented for all recruited patients. This safe range was calculated based on the estimate that a child's circulating blood volume is 80ml/kg (this is age dependent and is generally taken to vary from 80-90ml/kg for children). The safe upper limit of 3% of the total blood volume is 2.4ml/kg, which for a child of 2.5kg = 6mls. Since a minimum of 5ml blood was determined to be appropriate for this satisfactory PBMC retrieval, we only include children above 2.5kg in this study. All samples were transported at room temperature and delivered to Newcastle University within 48 hours of collection. This included the use of Royal Mail Safeboxes™ for the shipment of samples taken outside the Newcastle area.

2.2.2 EBV serology and viral DNA assay

Blood samples for viral serology were tested centrally in the Newcastle upon Tyne NHS Hospitals Foundation Trust (NuTH) virology laboratories. EBV and CMV viral DNA were measured from either whole blood or plasma using polymerase chain reaction (PCR) assays according to established clinical laboratory protocols. Plasma samples were tested for antibody response to key EBV antigens including IgM and IgG for Viral Capsid Antigen (VCA) and IgG for EBV Nuclear Antigen 1 (EBNA-1). Results for the viral DNA assay were reported as total titres (IU/ml) while antibody testing were reported as a binary "detected/not detected" based on NuTH laboratories reference cut-off values.

The EBV status of patients was classified as either "positive" or "negative" based on the clinical report provided by the NuTH virology laboratory in conjunction with consensus guidance set out by the International Paediatric Transplant Association (IPTA). This is illustrated in the flow chart (Figure 2.1):

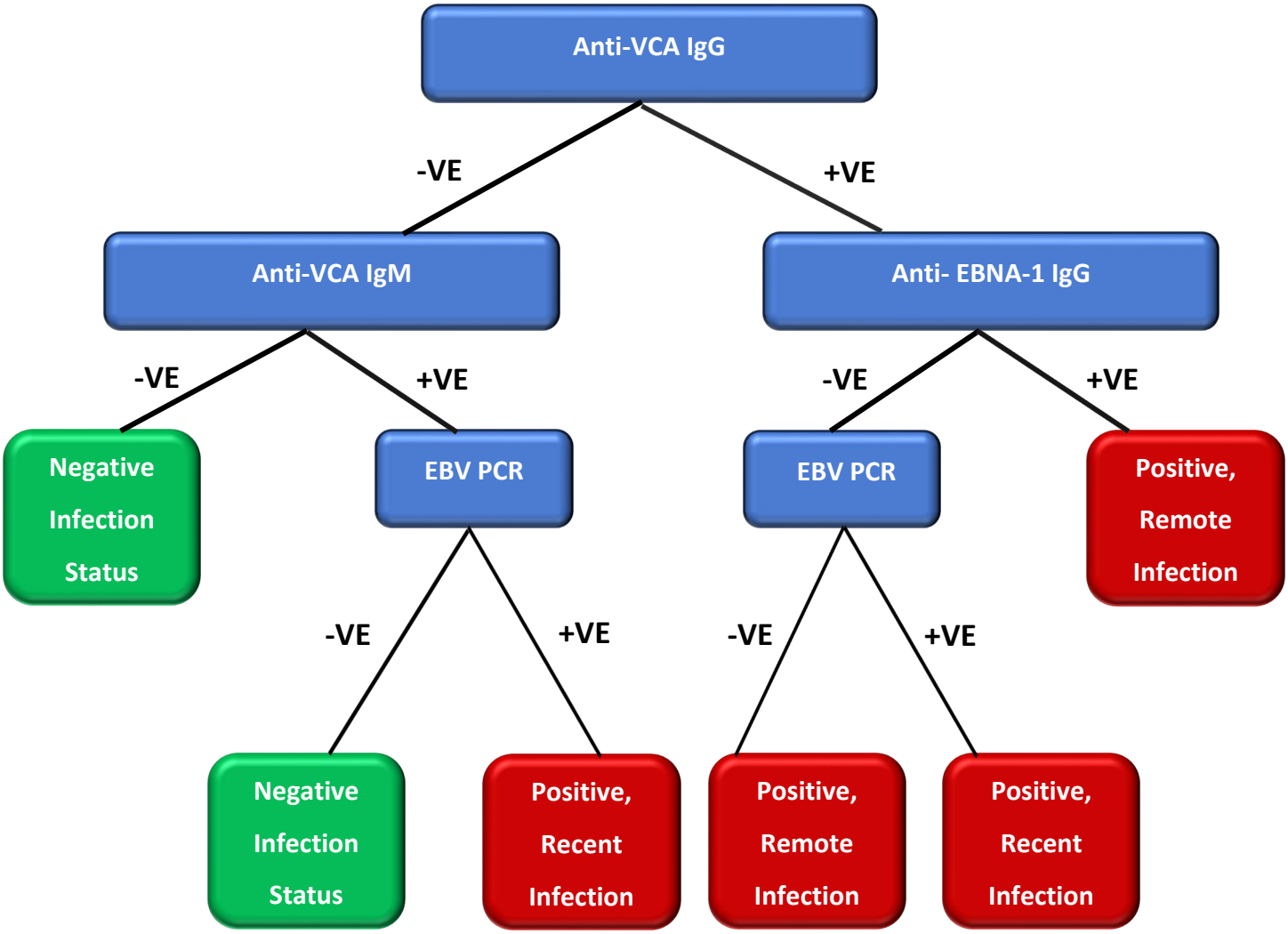


Figure 2.1: Interpretation schema for EBV serology. The flow chart was adapted from the IPTA consensus guidelines for EBV load and other biomarker monitoring after solid organ transplantation. [332] Additionally, both Anti-VCA IgM and Anti-VCA IgG may also be positive in recent EBV infection. A serology result that is Anti-VCA IgG negative, but Anit-EBNA-1 IgG positive is considered to be biologically improbable.

2.2.3 HLA typing of transplant patients and subset of healthy adult donors

The HLA typing for all transplant patients was carried out by National Health Service Blood and Transplant (NHSBT) as part of their pre-transplant clinical work up. The evaluation of patient-specific class I and II HLA expression was determined by either Next Generation Sequencing (NGS) or PCR sequence-specific oligonucleotide (PCR-SSO) analysis using DNA isolated from peripheral whole blood, according to established clinical laboratory protocols.

Histocompatibility and Immunogenetics (H&I) reports on HLA type for locally recruited study participants were accessed through the NHSBT Specialist Services Integrated Clinical Environment (Sp-ICE) web-based reporting system. Similar reports for patients recruited from Great Ormond Street Hospital for Children (GOSH) were provided to the study by their clinical team using a semi-anonymous approach. This typically involved the provision of an electronic HLA report that was linked to the relevant participant's study number after redacting relevant demographic identifiers from the reports.

Details of HLA serological phenotype were included in H&I reports for all patients with the exception of samples analysed by NGS. Due to the relevance of HLA serological specificity in selecting appropriate immunodominant EBV epitopes for tetramerisation, the 2024 WHO nomenclature for factors of the HLA system was used to assign the serological equivalent of such cases through cross-referencing with their HLA genotype [Table 2.1]. [333] This was mostly necessary for the DRB family of class II HLAs included in the study's tetramer pool. As per guidance from NHSBT, such conversions were avoided with alleles for which the phenotype has not been unequivocally defined. [334]

HLA allele	Serological specificity
DRB1*03:01	DR17
DRB3*02:02	DR52b
DRB1*04.01	DR4
DRB1*07:01	DR7

Table 2.1: Serological specificity of the DRB family of class II HLAs. The sequenced HLA genotype was obtained for each patient from the NHSBT H&I report and used to designate their corresponding serological specificity (adapted from the 2010 WHO nomenclature for factors of the HLA system). This process was used to simplify the downstream matching of patients to the relevant EBV tetramer based on their HLA restriction.

2.2.4 Preparation of Peripheral Blood Mononuclear Cells

2.2.4.1 Peripheral Blood Mononuclear Cell Isolation and Cryopreservation

PBMCs were separated from other mixed cell populations in whole blood samples using density gradient centrifugation. Whole blood was diluted 1:1 with phosphate buffered saline (PBS) and carefully layered onto Lymphoprep® density gradient medium (2:1) in either 15ml or 50ml polypropylene falcon tubes.

The layered blood samples were subsequently centrifuged at 350 x *g* for 40 minutes at ambient temperature with the brakes off. Plasma (1-3mls) was carefully removed from the top layer of the cell separation using a sterile Pasteur pipette and transferred into a 20ml universal container for further processing and storage. PBMCs were then isolated from the density medium – plasma interface using a sterile Pasteur pipette and transferred into a new sterile 15ml polypropylene falcon tube. The retrieved cells were washed in 10ml of PBS by centrifuging at 400 x *g* for 10 minutes at ambient temperature with the brakes on. After centrifugation, the supernatant was carefully aspirated to avoid disturbing the cell pellet. The cell pellet was then resuspended in 1ml of PBS and an estimation of the cell concentration was made using an improved Neubauer counting chamber. Cells in suspension were diluted 1:5 (i.e. dilution factor of 5) with trypan blue and 10µl placed into the counting chamber. Total viable cells, identified as those that remained unstained by the trypan blue, were visualised with an inverted phase contrast microscope, and counted within each quadrant of the counting chamber using a 2-key tally counter. The cell concentration (cells/ml) was calculated as:

$$10^6 \text{ cells/ml} = (C_T/QC) \times DF \times 10^4$$

Where: C_T = Total Cells Counted; QC = Quadrant Counted (each Quadrant = $1mm^2$); DF = Dilution Factor.

Separated PBMCs were washed again in PBS and cell pellets were resuspended in ice-cold freezing medium (90% FBS with 10% DMSO) as $1x10^6$ – $4x10^6$ cells/ml. Cell suspensions were aliquoted into 1.8ml Nunc CyroTube® vials (Thermo Fisher Scientific) and stored within CoolCell® freezing containers (Corning Inc) in a -80°C freezer for at least 24 hours prior to being transferred to a -150°C freezer for long-term storage. After PBMC processing was complete, the plasma that had been

decanted earlier was diluted 1:1 with PBS and aliquoted into 1.8ml Nunc CyroTube® vials (Thermo Fisher Scientific) for storage in a -80°C freezer.

2.2.4.2 Thawing of Peripheral Blood Mononuclear Cells

a. PBMC samples for flow cytometry

Cell thawing medium A (2% FBS with PBS) was prepared and warmed in a water bath at 37°C. Two separate aliquots of the medium containing universal nuclease for cell lysis (Thermo Fisher) were prepared by adding 1µl nuclease (stock 250U/µl) to 50ml media (5U/ml) and 1µl nuclease (stock 250U/µl) to 5ml media (50U/ml) respectively. Stored PBMC samples were retrieved from the -150°C freezer and thawed rapidly in a water bath at 37°C. Thawed cells were transferred into 15ml polypropylene falcon tubes after which 9ml of thawing medium A containing 5U/ml nuclease (1µl in 50ml) was added in a drop-wise manner. Cell suspensions were centrifuged at 500 x *g* for 5 minutes and the supernatant discarded. Cell pellets were resuspended in 1ml of thawing medium A containing 50U/ml nuclease (1µl in 5ml) and transferred to a 37°C humidified CO_2 incubator for 5 minutes. This was followed by the addition of 4ml thawing medium A before centrifuging the cells again at 500 x *g* for 5 minutes. Cells were resuspended in 1-5ml of thawing medium A (without nuclease) and cell counting performed. The desired cell number for single stain reference controls (~300,000 cells) and multicolour stained samples (~$1x10^6$ cells) were transferred in suspension into appropriately labelled Fluorescence Activated Cell Sorting (FACS) tubes for downstream antibody staining and cytometric analysis.

b. PBMC samples for ELISpot

ELISpot medium (RPMI-1640 with 8% FBS and 1% P/S) was prepared in 500ml batches under sterile conditions and filtered through a 0.2µm vacuum filter/storage bottle system (Corning Inc). Each batch of media was stored at 4°C for up to one month. Stored PBMC samples were retrieved from the -150°C freezer and prepared in an identical step-wise manner as illustrated in section 2.2.4.2(a) using ELISpot medium that had been warmed in a water bath at 37°C. Once washing of PBMC samples was complete, the cell pellets were resuspended in 10ml of ELISpot medium and transferred into a sterile labelled 50ml polypropylene falcon tube. Cells were placed in a 37°C humidified CO_2 incubator to rest at an angle for 2 hours prior to ELISpot assay in order to optimise the removal of unwanted cell debris.

2.2.5 ELISpot assay

I. Overview

The ELISpot^{PRO} human interferon gamma (IFN-γ) kit (MABTECH) was used to establish the functional status of EBV-specific T-lymphocytes in an HLA-unbiased manner for patients with confirmed EBV positive status on PCR and/or serological assessment. The production of IFN-γ (effector cytokine) was measured following the stimulation of PBMCs with pools of overlapping EBV peptides (JPT PepMix™) that represented the full epitope sequences of latent (EBNA-1 and LMP-2) and lytic (BZLF-1) phase viral proteins. The kit comprised of:

a. Strip plates containing wells with a polyvinylidene fluoride (PVDF) membrane base pre-coated with human IFN-γ capture antibody.
b. Anti-CD3 monoclonal antibody (for staining of positive controls).
c. DMSO solution (for staining of negative controls).
d. Alkaline Phosphatase (ALP) conjugated monoclonal antibody (7-B6-ALP) for detection of human IFN-γ.
e. 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium (BCIP/NBT-plus) substrate solution for colorimetric detection of ALP activity.

II. Recovery of cryopreserved cells and blocking of ELISpot plates

All samples were kept sterile by working within a class 2 biological safety cabinet in addition to using sterile materials and aseptic techniques. Cross-contamination of samples was avoided through handling with separate polypropylene and eppendorf tubes, pasteur pipettes and tips. Cryopreserved PBMC samples were thawed and prepared for assay in warm ELISpot medium as previously outlined in section 2.2.4. The required number of ELISpot strips was assembled in the plate frame and washed four times using 200µl PBS per well. After the final PBS wash, the supernatant was discarded and the wells in each strip were conditioned with 200µl of ELISpot medium. They were then incubated at room temperature for at least 30 minutes.

After the PBMCs had rested for 2 hours, they were centrifuged at 1600rpm for 5 minutes with the brakes on. The supernatant was carefully discarded, and cells resuspend in 5ml of ELISpot media by gently flicking the bottom of the tube. This was followed by cell counting. Samples were returned to the 37^{o}C humified $C0_2$ incubator afterwards while awaiting downstream analysis.

The volume of media required for resuspension of the cell pellet to achieve 200,000 - 250,000 cells per well (i.e. 200,000 - 250,000 cells per 50µl) was calculated as:

$$Volume\ of\ media\ required\ (\mu l) = Maximum\ number\ of\ wells\ x\ 50\mu l$$

$$where\ maximum\ number\ of\ wells = \frac{Total\ number\ of\ viable\ cells}{250{,}000}$$

The calculated maximum number of wells was used to inform the order of priority for adding cells to the plate. Where total viable cell counts permitted, the wells for PepMixes™ and positive/negative controls were prepared as triplicates. When the estimated viable cell counts resulted in the maximum number of wells being less than the required number, consideration was given to the following steps in sequential order:

a. Reducing the number of cells per well (not below 200,000 cells per well)
b. Testing the CD3 control in duplicate or as a single well
c. Testing PepMixes™ in duplicate or as single wells
d. Excluding some PepMixes™ (BZLF-1 > LMP-2 > EBNA-1)

III. Preparation of PepMixes™ and anti-CD3 dilution

Each EBV PepMix™ was supplied as a lyophilised mixture of overlapping peptides (25µg/vial). The PepMixes™ stock solutions were prepared by dissolving the mixture with 125µl of DMSO to a concentration of 0.2mg/ml. This was then aliquoted into tubes each containing 10µl to minimise freeze thawing. PepMix™ aliquots were stored at -20^{o}C and the date of preparation was recorded. PepMix™ dilutions (1:20) for each assay were prepared from their stock solution by adding 0.5µl of each stock PepMix™ (0.2mg/ml) to 9.5µl of ELISpot medium per well. The anit-CD3 monoclonal antibody included in the ELISpot^{PRO} kit was then prepared by adding 1µl of the antibody solution to 99µl of ELISpot media per 10 wells.

IV. ELISpot assay procedure

The ELISpot medium was discarded from the conditioned plates. This was followed immediately by the addition of 40µl of fresh ELISpot medium per well. The strips, plate and plate lid of each ELISpot plate were clearly labelled with details of the sample ID number, PepMixes™ and positive/negative controls to allow unambiguous identification. Next, 10µl of PepMix™, DMSO and anti-CD3 antibody was added to their respective wells as required. Care was taken to avoid contaminating any wells and

damaging the membrane at the bottom of the plate.

The suspended cells were then removed from the incubator and centrifuged at 1600rpm for 5 minutes with the brake on. Cell pellets were gently resuspended in the appropriate volume of ELISpot media required and 50µl immediately added to each of the prepared wells in the plate. The cells were first added to the negative control wells (DMSO) before adding cells to the other wells containing each PepMix™ being tested. This process was repeated for all patient sample being tested. The plates were subsequently wrapped in aluminium foil and placed into the 37ºC humidified CO_2 incubator for 18-24 hours. All efforts were made to avoid disturbing or moving the plates once they had been placed into the incubator.

V. ELISpot plate development

The ELISpot plates were removed from the incubator (after ~18-hours incubation) and the content of each well was flicked off into a bucket containing 1 % (w/v) Virkon. The plates were washed six (6) times using 200µl PBS per well. After the final wash, the wells were refilled with 200µl of PBS per well to prevent them from drying out. A 1:200 dilution of the ALP detection antibody (7-B6-ALP) was prepared using filtered PBS supplemented with 0.5% FBS. The amount of diluted 7-B6-ALP required per assay was calculated as:

Total volume of diluted detection solution required = (Number of wells x 100µl) + 1000µl

$$\therefore \textit{Amount of 7-B6-ALP stock solution required} = \frac{Total\ volume\ of\ detection\ solution\ required}{200}$$

The appropriate quantity of 0.5% FBS in PBS was then added to the 7-B6-ALP stock solution and filtered into a new 50ml tube through a 0.2µm syringe filter. After flicking out the last PBS from each ELISpot plate, 100µl of the diluted 7-B6-ALP antibody solution was added per well. The plate lid was then placed onto each plate and left to incubate at room temperature for 2 hours.

Once the incubation was complete, the contents of each ELISpot plate was flicked off and each plate washed six times with 200µl of PBS. After the final wash, the wells were refilled again with 200µl PBS to prevent the plates from drying out. The final step involved development of the ELISpot plates with the ALP-detection substrate (BCIP/NBT-plus). The volume of BCIP/NBT-plus solution required to develop each assay was calculated as:

Total volume of BCIP/NBT-plus required = (Number of wells x 100µl) + 1000µl

The required volume of BCIP/NBT-plus solution was first pipetted into a 50ml tube before being filtered into a new tube using a 0.45µm syringe filter. After discarding the last PBS wash solution from each plate, 100µl well of BCIP/NBT solution was added per well to initiate colour development. This was followed by incubation of the plates for 10-30 minutes at room temperature protected from light (covered with aluminium foil). Colour development was stopped by gently rinsing the plate several times under a cold-water tap. The plates were blotted dry and stored overnight in a dark cupboard at room temperature until data acquisition on the automated ELISpot reader.

VI. Reading the ELISpot plate and data analysis

ELISpot plates were scanned and analysed with the ImmunoSpot (ImmunoSpot Single-Color ELISPOT Enzymatic, Cellular Immune Technologies, Germany). The IFNγ-specific response for each sample was calculated by subtracting the mean number of spots in the negative control wells from the test wells. The results were expressed as spot-forming units (SFU)/10^5 PBMCs.

2.2.6 Full spectrum flow cytometry staining procedure

I. Preparation of multicolour stain samples

Thawed PBMCs from each patient sample were prepared for multicolour staining (MCS) and pipetted into labelled FACS tubes as ~1x10^6 cells per tube. Similarly prepared PBMCs from a healthy adult donor were used alongside compensation beads (BioLegend) to prepare high-quality single stain reference controls (SRC) and unstained controls needed for downstream spectral unmixing. Patient samples were stained in batches by the same operator to minimise inter-assay variation and batch effects. An anchor/bridging sample was also included with each MCS batch to help Identify and correct any batch effects.

MCS samples were first washed in 3ml of FACS staining buffer and centrifuged at 500 x g for 5 minutes. The supernatant was carefully discarded and the cell pellet resuspended by gently flicking the bottom of the tube. Cell suspensions and the viability reference control were stained with 5µl of a 1:40 stock dilution of Live/Dead blue dye, vortexed and incubated in the dark for 15 minutes at room temperature. After 15 minutes, the samples were washed and the supernatant discarded. Each MCS sample was then incubated in 5µl of Human Fc block (BD Biosciences) for 10 minutes to minimise non-specific binding of antibodies to Fc receptors. This was followed by the addition of 10µl of Brilliant Stain Buffer Plus to each MCS tube.

A master-mix of antibodies representing cell surface markers of interest in each MCS flow panel was prepared for immune phenotyping of samples. Aggregates were removed from antibody stocks by centrifuging at 10,000 x *g* for 5 minutes. Relevant pre-tested and titrated antibodies were added to FACS staining buffer to make up a staining volume of 100µl per test. All samples were first stained with only anti-TCRγδ for 10 minutes in the dark at room temperature. The rest of the antibody master-mix was then added with further incubation in the dark for 30 minutes. PBMC samples stained with the T-lymphocyte flow panel had an additional sequential staining step implemented for anti-CXCR5 and anti-CCR5. These samples were first stained with anti-TCRγδ alone for 10 minutes in the dark at room temperature, followed by staining with both anti-CXCR5 and anti-CCR5 for 10 minutes and subsequent incubation with the remaining antibody master-mix for 30 minutes.

After staining, PBMCs were washed twice with 1ml of FACS staining buffer. Cell pellets were then resuspended in 250µl of FluoroFix™ buffer and incubated for at least 1 hour in the dark at room temperature. PBMC samples were again washed twice with 1ml of FACS staining buffer, resuspended

in 400µl of the staining buffer and stored at 2-8°C until acquisition on the Cytek® Aurora 5-laser full spectrum cytometer.

II. Preparation of single stain reference controls

SRCs were acquired as either single-stain PBMCs or single-stain compensation beads (BioLegend) with optimised titrations of each fluorochrome-conjugated antibody in a staining volume of 100µl per test. For SRCs using PBMCs, approximately 300,000 cells were stained for each antibody in the relevant flow panel. The preparation of SRCs with compensation beads involved the mixing of one (1) drop each of positive and negative compensation beads with the relevant fluorochrome-conjugated antibody titration. All reference controls underwent identical staining procedures as MCS samples, including washes, buffers, and fixation steps. Reference controls were acquired once every 2-3 months for spectral unmixing of MCS batches that were acquired within the same time-period. New reference controls were acquired sooner in the event of a lot number changed for any of the antibodies in the flow panels.

III. Staining protocol for fluorescently labelled HLA-restricted tetramers

An optimised panel of HLA-restricted peptide-Major Histocompatibility (pMHC) class I/II tetramers relevant to patient HLA genotype was used to evaluate EBV-specific CD8 and CD4 T-lymphocyte immunity (see **section 2.1.5**).

Tetramer HLA-restriction	Epitope specificity	Fluorochrome	Volume (µl)/test	Stock Dilution	MHC Class
A*02:01	YVLDHLIVV	APC	10	1:100	Class I
A*02:01	GLCTLVAML	APC	20	1:100	Class I
A*02:01	CLGGLLTMV	PE	5	1:100	Class I
A*11:01	AVFDRKSDAK	PE	0.5	1:100	Class I
A*11:01	ATIGTAMYK	APC	0.5	1:100	Class I
A*24:02	TYGPVFMCL	PE	10	1:100	Class I
B*07:02	RPQKRPSCI	PE	2	1:100	Class I

B*07:02	RPQGGSRPEFVKL	APC	2	1:100	Class I
B*08:01	RAKFKQLL	APC	5	1:100	Class I
B*08:01	QAKWRLQTL	PE	5	1:100	Class I
B*35:01	HPVGEADYFEY	PE	2	1:100	Class I
B*35:01	EPLPQGQLAY	APC	2	1:100	Class I
DRB1*03:01 (DR17)	PAQPPPGVINDQQHHLPSG	PE	25	1:100	Class II
DRB1*03:01 (DR17)	VKLTMEYDDKVSKSH	APC	25	1:100	Class II
DRB1*04:01 (DR4)	GQTYHLIVDTDSLGNPSLSV	PE	25	1:100	Class II
DRB1*04:01 (DR4)	PYYVVDLSVRGM	APC	15	1:100	Class II
DRB1*07:01 (DR7)	SRDELLHTRAASLLY	APC	11	1:100	Class II
DRB1*07:01 (DR7)	PRSPTVFYNIPPMPLPPSQL	PE	12	1:100	Class II
DRB3*02:02 (DR52b)	LTAYHVSTAPTGSWF	APC	10	1:100	Class II
DRB3*02:02 (DR52b)	PRSPTVFYNIPPMPLPPSQL	PE	25	1:100	Class II

Table 2.2*:* Epitope sequence of EBV tetramers and their corresponding MHC class I and II restriction. Tetramers of EBV epitopes involved in the lytic phase of viral infection were labelled with APC while the viral epitopes involved in the latent phase of infection were labelled with PE. Individual mastermixes of relevant tetramers were prepared for each patient

Analysis of PBMCs using appropriately matched pools of lytic and latent tetramers was included as a component of the staining procedure for the 30 colour T-lymphocyte flow panel. Cryopreserved PBMCs were recovered and prepared as per protocol for multicolour staining. Pools of both lytic and latent HLA-matched tetramers were only included for the MCS samples from patients confirmed to be EBV positive at the time of their sample collection (see **section 2.2.2**). Stock dilutions using 1µl of each tetramer were made with FACS buffer as 1:100 dilutions based on titrations that had been optimised during flow panel development (Table 2.2). This was done to improve the accuracy of pipetting.

After MCS samples were stained with viability dye and human Fc block, they were washed in 200µl of batch-tested human serum (Gibco) by centrifuging at 500 x *g* for 5 minutes. Optimal volumes of relevant tetramers to the appropriate MCS sample tube and vortexed. Each tube was place in the 37^{o}C incubator for 1 hour and flicked every 10-15 minutes to resuspend the cells. Tetramer-stained samples were then washed by adding 2ml of FACS buffer to each tube and centrifuging at 500 x *g* for 5 minutes. This was followed by staining with all the antibody mastermix for the T-lymphocyte panel. Where adequate viable cell count permitted, fluorescent minus one (FMO) samples were prepared alongside tetramer-stained MCS tubes.

2.2.7 Spectral flow cytometry analysis pipeline

I. Instrument setting and data acquisition

Daily quality control (QC) of the spectral flow cytometer was performed by trained members of the flow cytometry core facility (FCCF) prior to data acquisition. The results of the daily QC were used to optimise the experimental setting.

Acquisition parameters were set up for the experiment template. These included setting of the stopping gate (P1), storage gate (All events), and number of events to record for each sample tube. The number of events recorded varied depending on if the sample was a SRC (60,000 – 100,000 events) or MCS tube ($1x10^6$ – $1.5x10^6$ events). Appropriate assay settings, including adjustment of gains for forward scatter (FSC) and side scatter (SSC) were implemented to ensure that all samples were visualised on scale. The same gains setting was used for the acquisition of all study samples. A review of the signals for all detector channels was performed to ensure that they didn't exceed 10^6 (i.e. not saturated). The FSC and SSC threshold was set to exclude as much debris as possible without involving the cell population of interest. During the acquisition of SRCs, tubes stained with cells were acquired before tubes stained with compensation beads. This enabled the adjustment of acquisition gates and gains as/if needed. Once data acquisition was completed, the Flow Cytometry Standard (FCS) files that were generated from each experiment underwent spectral unmixing with SpectroFlo software v3.3.0 (Cytek®).

II. Spectral unmixing of multicolour stained samples

Accurate spectral unmixing of raw FCS files for MCS samples was a critical step in the data analysis pipeline. Within the experiment template in SpectroFlo, histograms were used to identify and gate over the negative and positive fluorescence peaks of the SRCs (Figure 2.2). This was followed by an assessment of the spectral signature for each SRC to ensure that they matched the known spectral profile of their corresponding fluorochrome (Figure 2.3). [335] The spectral signature of an unstained control sample was used for autofluorescence extraction from MCS samples if necessary. Unmixing of the MCS samples was only performed after all gates and spectra had been fully assessed. The accuracy of the unmixing process was evaluated by plotting every feasible permutation of N x N bivariate dot plots for all markers in the flow panel. These plots were used to determine if any unmixing errors had occurred by ensuring that both positive and negative populations for each marker in the bivariate plots were well aligned (Figure 2.4). If any unmixing errors were identified

even after accurate and optimal unmixing, then manual adjustment of the compensation matrix by ≤ 3% was permissible. [336] The unmixed FCS files were then exported from SpectroFlo and imported into the OMIQ data science platform (Dotmatics) for downstream analysis.

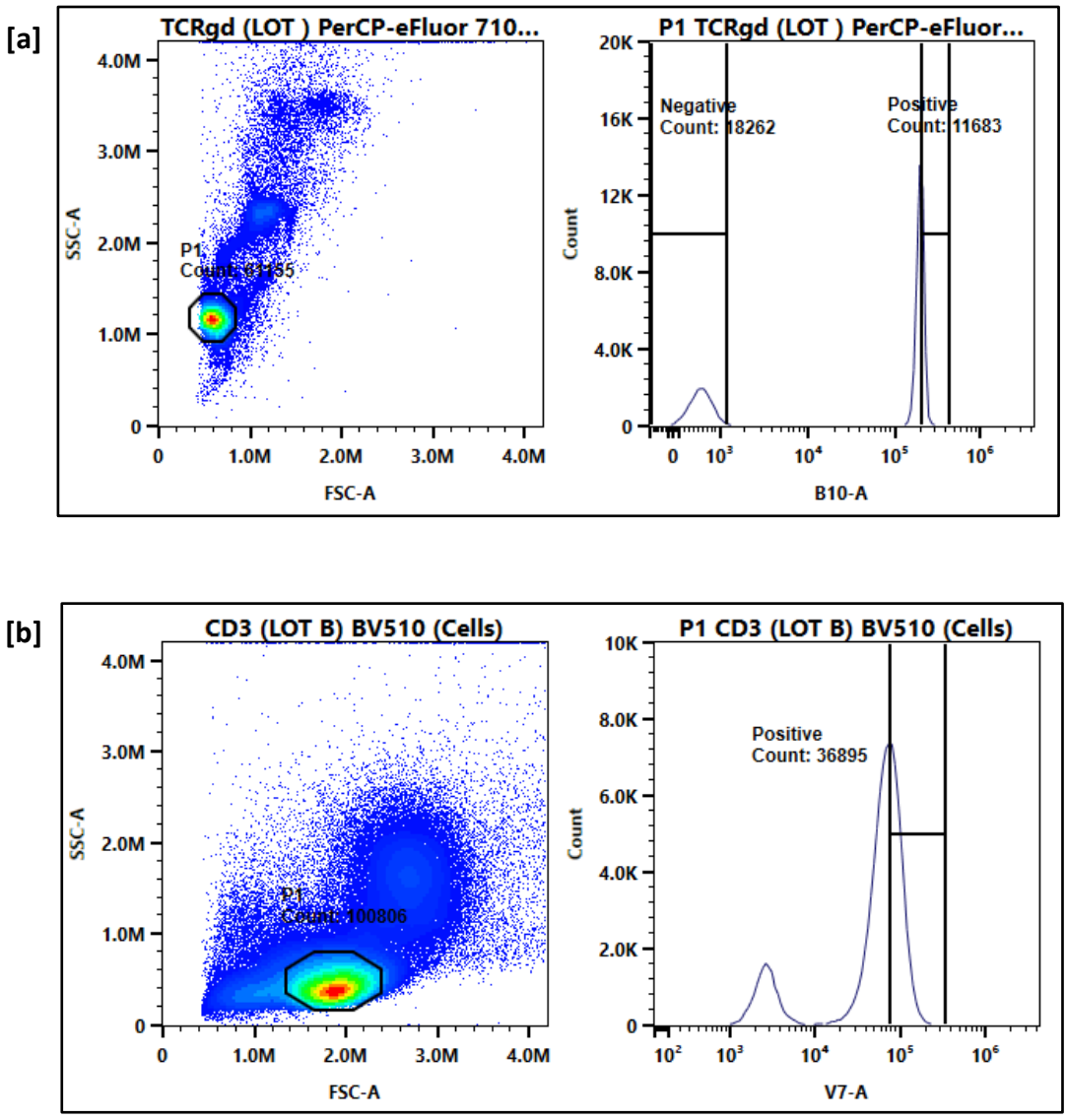


Figure 2.2: Gating single stain reference controls for spectral unmixing. Clear identification of positive and negative cell populations for **[a]** TCRγd PerCP eFluor 710 (Beads) and **[b]** CD3 BV510 (PBMC) single stain reference controls (SRCs*)*. Gating on at least 300 events within the positive cell population is required for accurate spectral unmixing. Gates for the positive cell population were placed at the highest peak of the histogram to record the clearest positive signal.

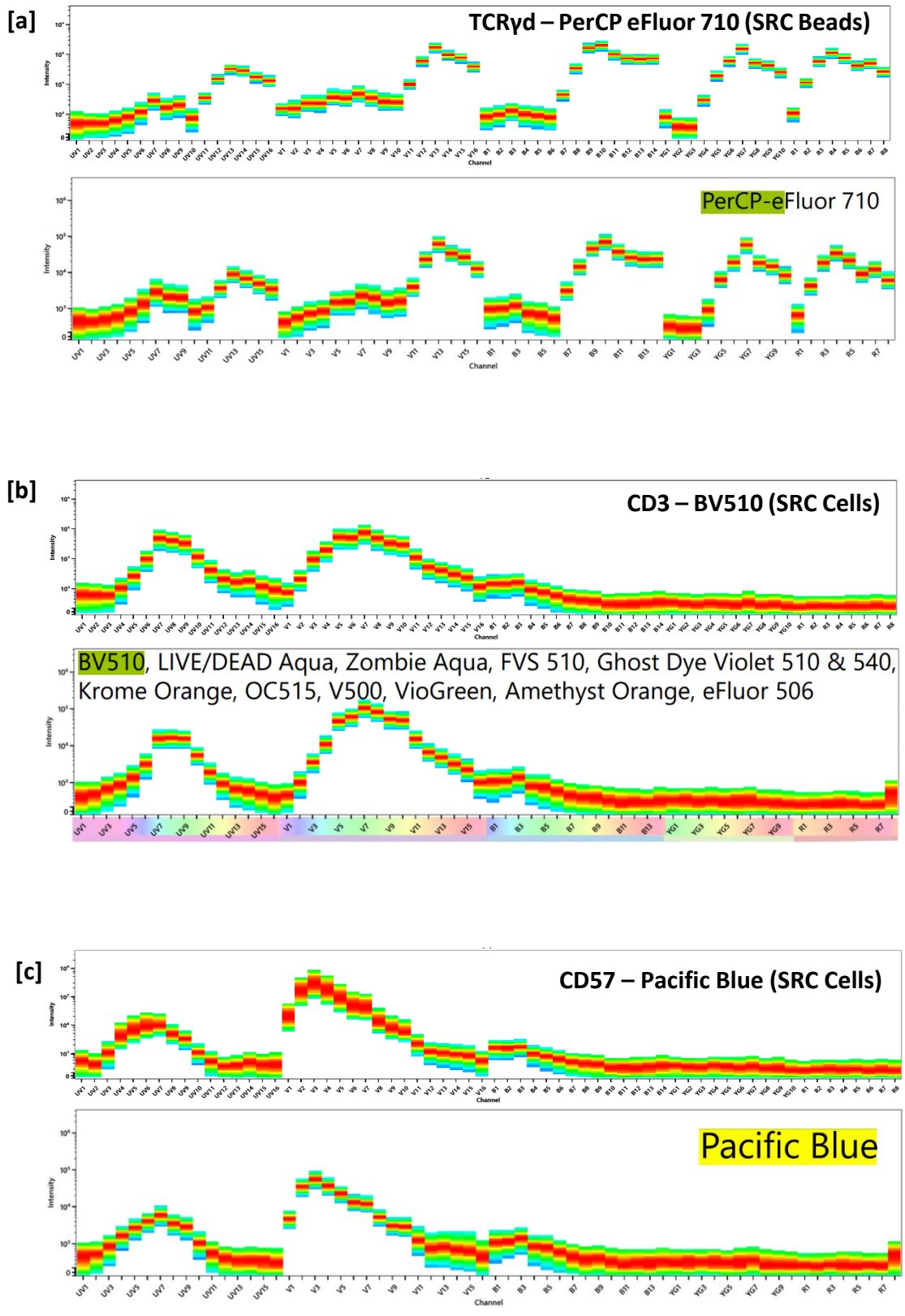


Figure 2.3: **Quality control assessment of spectral signatures.** Visualisation of the spectral signatures for [a] TCRγd PerCP eFluor 710 (Beads), [b] CD3 BV510 (Cells) and [c] CD57 Pacific Blue (Cells) SRCs. Identical matching of peaks across all 64 detector channels of the Cytek® Aurora flow cytometer was observed with their corresponding fluorochrome profiles published by Cytek. The fluorescent signals were also compared to ensure that they were all on scale (MFI < 10^6).

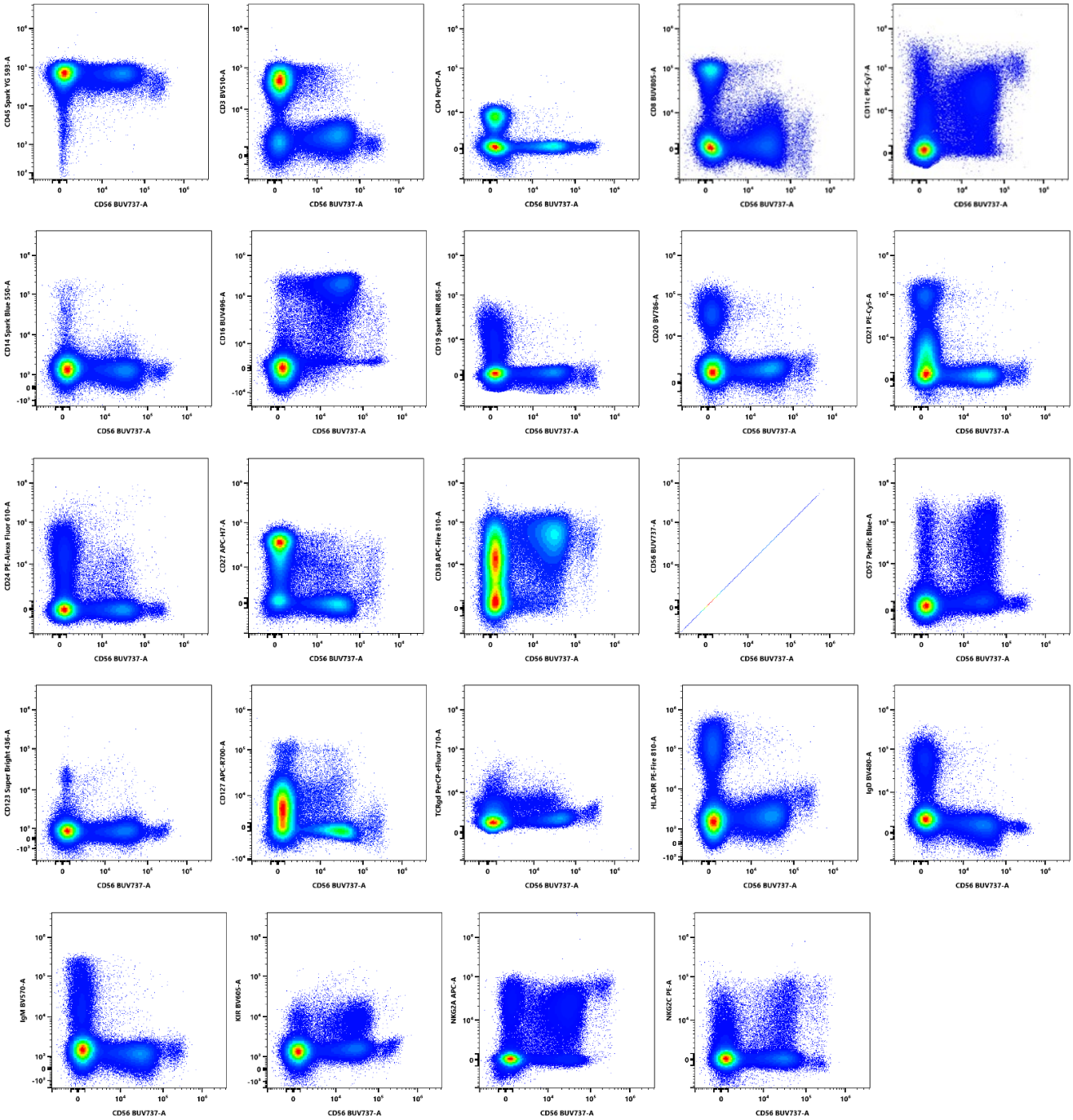


Figure 2.4: Quality control assessment of unmixing accuracy of MCS sample. Representative example of successful unmixing of a multicolour stained sample demonstrated with bivariate plots of CD56 BUV737. Presented data were gated on singlets, scatter and viable/live cells. Each bivariate plot shows CD56 BUV737 plotted on the x-axis against all other fluorochromes in the panel plotted on the y-axis. A perpendicular alignment of the positive cell population in both axes with the negative cell population indicates accurate spectral unmixing. Similar visual inspection of bivariate plots was performed for each fluorochrome included in the broad innate/adaptive immune panel (24x24 plots per fluorochrome) and T-lymphocyte panel (30x30 plots per fluorochrome).

Chapter 3. Flow Panel Development and Optimisation

3.1 Selection of cell surface markers

3.1.1 Introduction

A total of 42 cell surface markers (see section 2.5) were deployed for the comprehensive immunophenotyping of circulating immune cells in the study cohort. These surface markers were selected with the aim of capturing the major cell lineages (T-lymphocytes, B-lymphocytes, Natural Killer [NK] cells, monocytes and dendritic cells) in addition to the cellular subsets that are implicated in antiviral immunity. The immune cell populations of interest were determined *a priori* from the literature review outlined in chapter 1.

For this purpose, two high dimensional full spectrum flow cytometry panels were developed and validated on the Cytek® Aurora equipped with 5 lasers (355, 405, 488, 561, 640 nm) and 64 detectors (16UV-16V-14B-10YG-8R). The panels consisted of a 24-colour flow panel designed to probe the broad innate and adaptive immune landscape of the study cohort, complemented by a targeted 30-colour flow panel for deep immunophenotyping of the T-lymphocyte compartment. Details of the cell surface antigen composition of each panel and the rationale for their inclusion are outlined in the next section of this chapter.

3.1.2 Broad innate/adaptive immunity flow cytometry panel

This panel was designed to explore the frequencies of the T-lymphocyte population (including γδ T-lymphocytes), NK T-like cells, NK cells, B-lymphocytes, Innate lymphoid cells (ILCs), Monocytes and Dendritic cells (DC). Additional surface markers allowed for the characterisation of relevant immune subsets within the non-T lymphocyte compartment. The antigens used to delineate the indicated cell types are outlined in Table 3.1.

CD45 was used to identify all leukocytes while CD19 and CD20 were employed as key surface antigens of B-lymphocytes. The differentiation of B-lymphocytes was characterised by the inclusion of several antigen markers such as IgD, IgM, CD27, CD21, CD24 and CD38. IgD and CD27 are recognised makers of naïve (IgD+CD27−), marginal zone-like (IgD+CD27+), and memory (IgD−CD27+) B-lymphocytes.[337]

Cell surface antigen	Fluorochrome	Antibody clone	Antigen class	Purpose
CD45	Spark YG 593	HI03	Primary	Leukocytes
TCRγδ	PerCP-eFluor 710	B1.1	Primary	γδ T-lymphocyte
CD3	BV 510	SK7	Primary	Pan T-lymphocyte, NK T-like cells
CD4	PerCP	SK3	Primary	CD4 T helper lymphocytes
CD8	BUV 805	SK1	Primary	CD8 cytotoxic T-lymphocytes
CD11c	PE-Cy7	B-ly6	Secondary	Dendritic cell differentiation
CD14	Spark Blue 550	63D3	Primary	Monocyte differentiation
CD16	BUV 496	3G8	Primary	Monocyte, NK cell, and Dendritic cell differentiation
CD19	Spark NIR 685	HIB19	Primary	B-lymphocytes
CD20	BV 786	2H7	Secondary	B-lymphocytes
CD21	PE-Cy5	B-ly4	Secondary	B-lymphocyte differentiation
CD24	PE-Alexa Fluor 610	SN3	Tertiary	B-lymphocyte differentiation
CD27	APC-H7	M-T271	Secondary	T/B-lymphocyte differentiation
CD38	APC-Fire 810	HIT2	Secondary	Monocyte, Dendritic cell, T/B-lymphocyte activation and differentiation
CD56	BUV 737	NCAM16.2	Secondary	NK cells
CD57	Pacific Blue	NK-1	Secondary	NK cell and CD8 T-lymphocyte immune senescence
CD123	Super Bright 436	6H6	Tertiary	Plasmacytoid dendritic cells
CD127	APC-R700	HIL-7R-M21	Tertiary	Cytokine receptor; T-lymphocyte differentiation
KIR (CD158)	BV 605	DX27	Tertiary	NK cell differentiation
NKG2A (CD159a)	APC	REA110	Tertiary	NK cell differentiation
NKG2C (CD159c)	PE	REA205	Tertiary	NK cell differentiation
IgD	BV 480	IA6-2	Secondary	B-lymphocyte differentiation
IgM	BV 570	MHM-88	Secondary	B-lymphocyte differentiation
HLA-DR	PE-Fire 810	L243	Secondary	Monocyte activation, dendritic cell lineage, NK cell lineage discrimination,
Viability	Live/Dead Blue	-	-	Live cells

Table 3.1: Cell surface antigens used in the Broad innate/adaptive immunity flow cytometry panel.

CD21 and IgM were used to further categorise and refine class-switched and non-switched B-lymphocytes. [338, 339] The additional combination of CD24 and CD38 were helpful in classifying memory B-lymphocytes (CD24+ CD38lo/neg) that have been implicated in immune senescence as well as the identification of regulatory B-lymphocytes (CD24hiCD38hi), which have been shown to exhibit several immunosuppressive functions in chronic viral infections.[340-343] Plasmablasts were identified based on the expression of CD19, CD20, CD27, and CD38.[344]

NK cells and their subsets were defined by the surface expression of CD56 and CD16.[345] The inhibitory receptor NKG2A (CD159a) and CD158 killer immunoglobulin-like receptors (KIR) were included as antigen markers to identify the early differentiated NK subset ($CD56^{dim}$NKG2A+KIR-). This cellular subset has a well-established role in the early immune response to EBV infection and virus-mediated inhibition of its function has been associated with the pathogenesis of EBV+ lymphomas. [346, 347] Similarly, the activation receptor NKG2C (CD159c), which is known to expand during viral infections (particularly in CMV infection) was added as a marker of memory-like differentiation.[348, 349] CD57 was included as a maturation marker for NK cells, helping to identify immunosenescent cells that exhibit potent cytotoxicity and impaired replicative capacity.[350] CD56 was further used for the identification of NKT-like cells (CD3+CD56+).

The identification of monocyte subsets was facilitated by the inclusion of CD14 and CD16. These two antigen markers permitted the classification of this cell population into classical (CD14hiCD16-), intermediate (CD14hiCD16+) and non-classical (CD14+CD16hi) monocytes.[351] The main DC subsets were established with CD11c, HLA-DR and CD123. Conventional DCs were defined as CD11c+ HLA-DR+ lineage negative [Lin -] (CD3- CD19- CD14- CD56-) while plasmacytoid DCs were defined as CD123+ CD11c- HLA-DR+. [339] The total ILC population was identified as CD45+CD127+Lin-.[352]

The inclusion of T-lymphocyte lineage markers in the broad innate/adaptive immunity flow panel allowed for a limited overview of the T-lymphocyte population in relation to other circulating immune cells. CD3, CD4, CD8 and pan-γδ TCR were used to classify helper, cytotoxic and γδ T-lymphocytes respectively. Further in-depth interrogation of the T-lymphocyte compartment was conducted with the 30-colour flow cytometry panel.

3.1.3 T-lymphocyte flow cytometry panel

Table 3.2 illustrates the 30 cell surface antigens used for the in-depth immunophenotyping of the T-lymphocyte compartment.

Cell surface antigen	Fluorochrome	Antibody clone	Antigen class	Purpose
CD45	Spark YG 593	HI03	Primary	Leukocytes
CD45RA	BUV 395	5H9	Secondary	T-lymphocyte differentiation
CD3	BV510	SK7	Primary	Pan T-lymphocyte
CD4	PerCP	SK3	Primary	CD4 T helper lymphocytes
CD8	BUV 805	SK1	Primary	CD8 cytotoxic T-lymphocytes
TCRγδ	PerCP-eFluor 710	B1.1	Primary	γδ T-lymphocytes
CD25	PE-Alex Fluor 700	CD25-3G10	Tertiary	Regulatory T-lymphocytes
CD27	APC-H7	M-T271	Secondary	T -lymphocyte differentiation
CD28	BV650	CD28.2	Secondary	T-lymphocyte differentiation
CD31	BV711	WM59	Secondary	Recent thymic emigrants
CD38	APC-Fire 810	HIT2	Secondary	T-lymphocyte activation/differentiation
CD39	BUV 661	TU66	Tertiary	Regulatory T-lymphocytes
CD57	Pacific Blue	NK-1	Secondary	T-lymphocyte immune senescence
CD62L	BUV 496	SK-11	Secondary	T-lymphocyte differentiation
CD69	Alexa Fluor 647	FN50	Secondary	T-lymphocyte activation
CD95	PE-Cy5	DX2	Secondary	T-lymphocyte activation/differentiation
CD127	APC-R700	HIL-7R-M21	Tertiary	Chemokine receptor; T-lymphocyte differentiation
HLA-DR	PE-Fire 810	L243	Secondary	T-lymphocyte activation
CCR4	BB 700	1G1	Tertiary	Chemokine receptor; T-lymphocyte differentiation
CCR5	BUV 563	2D7/CCR5	Tertiary	Chemokine receptor; T-lymphocyte differentiation
CCR6	BV 480	11A9	Tertiary	Chemokine receptor; T-lymphocyte differentiation
CCR7	BV 421	G043H7	Secondary	T-lymphocyte differentiation

CXCR3	Alexa Fluor 488	GH025H7	Tertiary	Chemokine receptor; T-lymphocyte differentiation
CXCR5	BV 750	RF8B2	Tertiary	Chemokine receptor; T-lymphocyte differentiation
PD-1	BV 785	EH12.2H7	Tertiary	Co-inhibitory receptor; T-lymphocyte exhaustion
LAG-3	PE-Cy7	3DS223H	Tertiary	Co-inhibitory receptor; T-lymphocyte exhaustion
TIM-3	BV 605	F38-2E2	Tertiary	Co-inhibitory receptor; T-lymphocyte exhaustion
CTLA4	PE-CF594	BNI3	Tertiary	Co-inhibitory receptor; T-lymphocyte exhaustion
Tetramer (lytic)	APC	-	Tertiary	EBV-specific T-lymphocytes
Tetramer (latent)	PE	-	Tertiary	EBV-specific T-lymphocytes
Viability	Live/Dead Blue	-	-	Live cells

Table 3.2: Cell surface markers used in the T-lymphocyte immunity flow cytometry panel.

All T-lymphocytes were defined by their CD45+ and CD3+ expression. Pan-γδ TCR was used to separate unconventional γδ T-lymphocytes from substantive αβ CD4+ and CD8+ T-lymphocytes.
The γδ T-lymphocyte subset is uniquely equipped with both innate and adaptive immune functions due to its independence from the MHC-mediated antigen presentation pathway.[353] Its functional capabilities are of major consequence in various disease states such as viral infections, autoimmune disorders, organ transplantation and lymphoid malignancies.[353-355] Importantly, the diversity of its repertoire can be shaped by primary EBV infection as well as reactivation of CMV and EBV in transplanted patients.[356-358]

Principal CD4 and CD8 T-lymphocytes subsets were characterised with CD45RA, CCR7, CD27, CD28 and CD31. These markers of memory and differentiation status were used to define naïve (CD45RA+CCR7+), central memory (T_{CM}: CD45RA-CCR7+), effector memory (T_{EM}: CD45RA-CCR7-) and terminal effector memory (T_{TEMRA}: CD45RA+CCR7-) cell states.[359, 360] CD31 (PECAM-1) expression within the naïve CD4-T-lymphocyte subset was used to monitor thymic output as a measure of recent thymic emigrants (RTEs). This surface antigen is preferentially expressed by naïve CD4+ T-lymphocytes that are rich in signal joint T receptor excision circles (sjTRECs), thus indicating their

developmental proximity to emergence from the thymus.[361-363] The inclusion of CD27 and CD28 permitted further distilling of the effector memory compartment into early-like effector, intermediate effector, terminal effector and RA-terminal effector subsets.[339, 359, 364]

Several antigen markers were also incorporated to assess the activation and functional status of T-lymphocytes. CD38 and HLA-DR were chosen as markers of cellular activation. Their surface expression on T-lymphocytes is known to increase and remain elevated during infection/inflammation, thus enabling the temporal monitoring of immunological responses to (neo)antigens.[356, 360, 365] CD69 was included as an established antigen marker of early activation status on all T-lymphocyte subtypes while CD95 (Fas) was added due to its importance as a mediator of T-lymphocyte apoptosis during acute EBV infection.[366, 367] CD95 was also used in combination with CD62L to identify stem cell-like memory T-lymphocytes (T_{SCM}: CD45RA+CD62L+CD95+), a rare subset of cells with multipotent capacity to reconstitute the entire T_{CM} and T_{EM} compartments, which could be of potential relevance after early thymectomy.[368]

T-lymphocyte dysfunction was evaluated using multiple antigen markers of cellular immunosenescence and exhaustion including CD57, Programmed Death 1 (PD-1), T cell immunoglobulin and mucin-containing protein 3 (TIM-3), Lymphocyte activated gene 3 (LAG-3) and Cytotoxic T-lymphocyte associated factor 4 (CTLA4). Expression of immune checkpoint proteins (either singly or in combination) is significantly upregulated on T-lymphocytes that have undergone chronic antigen stimulation.[369, 370] This immunosuppressive milieu is often characterised by impaired cell proliferation, early apoptosis and poor cytokine production, all of which enable successful immune evasion by viruses such as EBV and CMV.[371, 372] Similar studies have shown that T-lymphocytes expressing CD57 exhibit severely impaired proliferative capacity, suggesting that it is the best antigen marker of replicative senescence.[373,374]

Regulatory T-lymphocytes (T_{regs}) were defined by their expression of CD25 (IL2Rα) and CD127 (IL7Rα). Multiple studies have validated CD4+CD25hiCD127lo/- cells as a reliable correlate of circulating T_{regs} in the absence of intracellular staining for FoxP3. [339, 375-377] This T-lymphocyte subset is characterised by their overt immunosuppressive function and has been implicated in EBV-driven lymphomagenesis. [378, 379] CD39 was included in this panel as a marker of highly active and suppressive T_{regs} (CD39+).[380]

The expression of chemokine receptors was used to examine the various CD4+ helper T-lymphocyte subsets and the homing properties of CD8+ cytotoxic T-lymphocytes and T_{regs}. CXCR3 and CCR4 were used to distinguish between Th1 (CXCR3+ CCR4-) and Th2 (CXCR3-CCR4+) subsets.[381, 382] Th1 cells produce pro-inflammatory cytokines such as IFN-γ, Tumour Necrosis Factor Beta (TNF-β) and Interleukin 2 (IL2) in response to intracellular pathogens.[383] The Th2 subset is primarily involved in anti-inflammatory responses through the release of IL-4, IL-5 and IL-13.[383] The inclusion of CCR6 permitted the categorisation of Th1/Th17 (CCR6+CXCR3+CCR4-) and Th17 (CCR6+CXCR3-CCR4+) phenotypes.[381] T follicular helper cells (TfH) were denoted by the expression of CXCR5 (CD4+ CXCR5+). CXCR5 communicates with CXCL13 to initiate T-lymphocyte trafficking to the B-lymphocyte follicles and germinal centres of lymphoid tissue. These are critical sites for the production of high-affinity antibody responses.[384] Finally, the details of all tetramers that were incorporated into this panel along with the justification for their inclusion have already been elucidated in chapter 2 (see **section 2.1.5**).

3.2 Selection of Fluorochromes

3.2.1 Introduction

While spectral flow cytometry allows for much greater plasticity in the breadth of fluorochromes that can be combined in a single multiparameter panel, the inherent complexity of such large panels requires a careful consideration of fluorochrome compatibility in the same way that is employed in the design of conventional flow cytometry panels. Selecting an appropriate combination of fluorochromes was a crucial step in developing the high dimensional flow cytometry panels used for this study. The key aim was to exploit the Cytek® Aurora's capacity to accurately distinguish between fluorochromes that have highly similar emission maxima but different off-peak spectral signatures without compromising the resolution of surface antigen markers or identification of cell populations of interest.[385] Another critical factor was to ensure that each fluorochrome was appropriately matched with a surface antigen based on its brightness and the expected level of antigen expression. For this reason, validated selection criteria that had been applied in the workflows of seminal publications of optimised multicolour immunofluorescence panels (OMIPs) for spectral flow cytometry were adapted for use in the early stage of each panel's design.

3.2.2 Establishing a fluorochrome reference library

At the time of designing the flow panels for this study, a total of 83 OMIPs had been published in Cytometry Part A. This peer-reviewed journal specialises in the reporting of newly designed and optimised multicolour panels for flow cytometry, fluorescence microscopy, image cytometry, and other polychromatic fluorescence-based methods. [386, 387] One of the principle aims of OMIPs is to mitigate the development time for researchers who need the same or highly similar panels.[388]

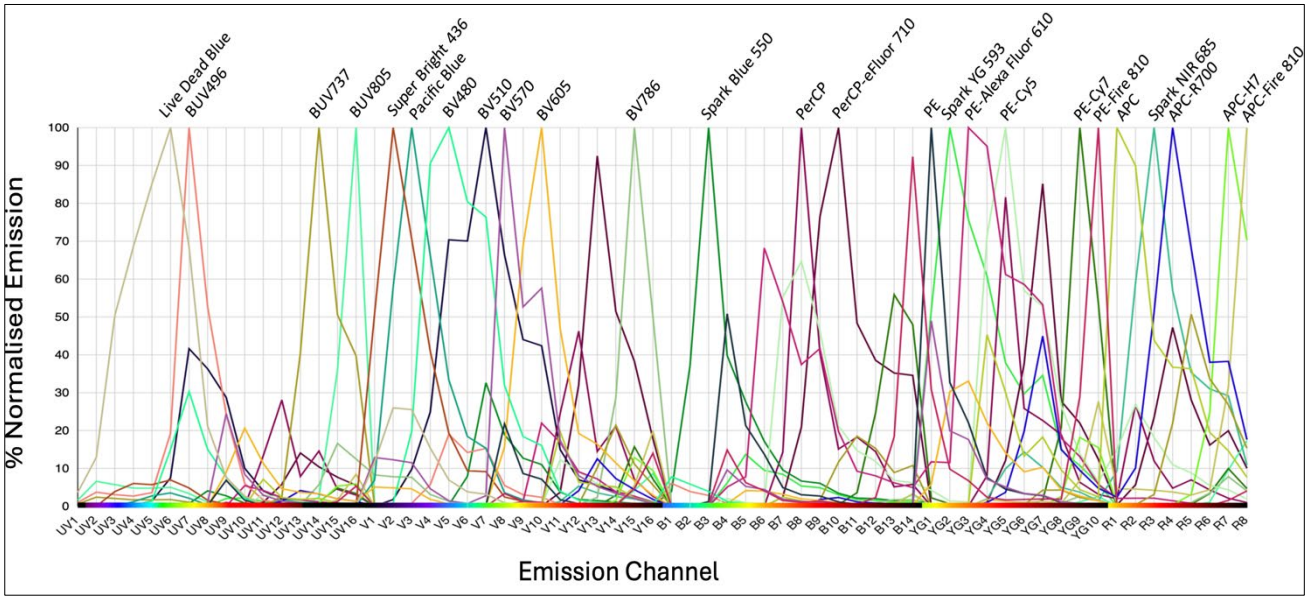
Relevant OMIPs were reviewed to identify fluorochrome-conjugated monoclonal antibodies that could be used to detect the immune cell populations of interest. A comprehensive library of all viable options, including fluorochrome-conjugated antibodies not previously used in a published OMIP (identified through catalogue searches of the major commercial vendors), was created as a resource for building each of the multiparameter flow panels.

3.2.3 Strategy for fluorochromes selection and assignment to surface antigen markers

The methodology used in OMIP-069 was influential in developing the strategy used to select and allocate fluorochromes to surface markers in this study. OMIP-069 is a 40-colour full spectrum flow cytometry panel that was designed for use in studies aimed at characterising the immune response in the context of infectious or autoimmune diseases, monitoring cancer patients on immuno- or chemotherapy and the discovery of unique and targetable biomarkers.[339] Due to the significant overlap between the surface antigens used in OMIP-069 and those used for this study's flow panels, the former served as a framework for selecting a majority of the fluorochrome-conjugated antibodies that made up the final broad innate/adaptive immunity panel (17/24 surface markers) and the T-lymphocyte panel (16/30 surface markers).

Next, compatible fluorochrome-conjugated antibodies were chosen from the reference library for the remaining surface antigen markers in both panels. These were specifically selected to ensure that all fluorochromes had their peak emissions occurring in different channels (Figure 3.1)

[a]



[b]

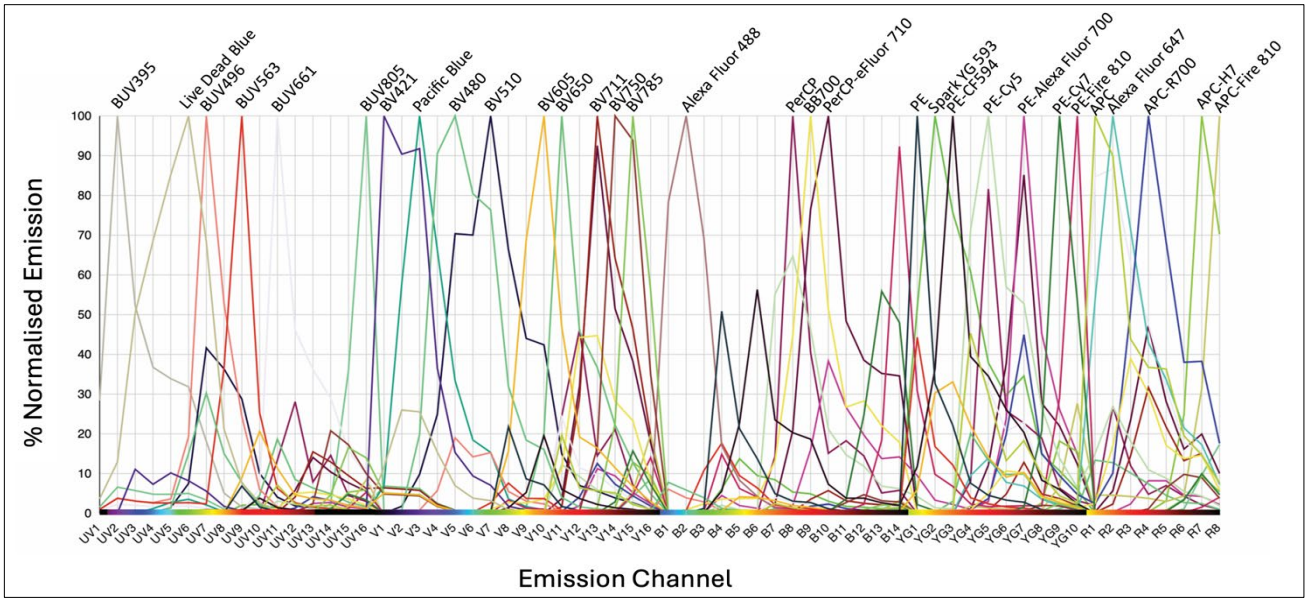


Figure 3.1: Full spectrum evaluation of fluorochromes. **[a]** Shows the spectral signatures of the 25 fluorochromes (viability dye inclusive) included in the broad innate/adaptive immune panel while **[b]** shows the signatures of the 31 fluorochromes (viability dye inclusive) included in the T-lymphocyte panel. Spectral signatures were visualised with the Cytek full spectrum viewer with all 64 detector channels of the 5-laser Cytek Aurora being represented. Spectral signatures have been normalised to peak channels to allow for direct comparison between fluorochromes.

The same stepwise strategy from OMIP-069 was used to assign fluorochromes to all outstanding surface antigen markers selected from the reference library:

I. **Choosing a viability dye**: Live/Dead™ fixable blue (Thermo Fisher) was used to assess cell viability due to the uniqueness of its spectral signature which doesn't interfere with other ultraviolet (UV) excitable dyes in the panels.

II. **Assigning fluorochromes to surface antigens with limited antibody conjugate availability:** Top priority was given to the allocation of rare/low expressed surface antigens that had limited options for commercially available fluorochrome conjugates e.g. PE-Cy7 assigned to LAG-3 in the T-lymphocyte panel and BV605 assigned to KIR in the broad innate/adaptive immunity panel.

III. **Assigning fluorochromes to primary cell surface antigens**: The dimmest fluorochromes were assigned to highly expressed cell lineage surface antigens that were known to have high levels of co-expression with other antigen markers in each panel. This was to help optimise the resolution of important cell subsets (e.g., CD4 assigned to PerCP due to its co-expression with all T helper lymphocyte markers). Particular care was taken to ensure that the fluorochromes

assigned to primary cell lineage surface antigens that overlapped between the two panels could be used in both panels without any impact on resolution.

IV. **Assigning fluorochromes to tertiary cell surface antigens**: Tertiary cell surface antigens were defined as any critical marker with known or expected low density of expression.[389] These antigen markers were assigned to the brightest available fluorochromes that were most compatible with the rest of the panel e.g. CTLA4 in PE-CF594, CCR4 in BB700, and CD31 in BV711 have all been deployed successfully in other OMIPs.[390-392] All reasonable attempts were made to pair them with fluorochromes whose spectra were not severely impacted by spread from multiple fluorochromes. APC and PE were reserved for in the T-lymphocyte panel for the EBV-specific tetramers.

V. **Assigning fluorochromes to all remaining cell surface antigens**: These mostly consisted of secondary surface antigens whose expression was anticipated to be in higher densities across a continuum.[389] Significant care was taken to minimise the risk of spread and spillover that could impact the resolution of cell populations with dimly expressed co-receptors.

The distribution of primary laser excitation and peak emission wavelengths for all fluorochrome-conjugated antibodies in each panel was appraised in tabular form (Table 3.3) in keeping with best practice recommendations. [339, 385] This was done to interrogate any potential risk(s) of spread induced by co-expressed antigen markers. As a general rule, the closer the emission peaks of two fluorochromes are, the higher the risk of spreading error and spillover being observed between them.[385] Allocations were assessed to curtail situations where: a) Primary cell surface antigens were placed in adjacent cells on the same column (indicating primary excitation laser) as co-expressed secondary/tertiary cell surface antigens; (b) Primary cell surface antigens were placed in the same cell on the same row (indicating similar emission wavelength) as co-expressed secondary/tertiary cell surface antigens. Furthermore, it was ensured that fluorochromes which contributed the most to spreading error had been assigned to markers with the lowest antigen density.

[a]

Approx emission wavelength (nm)	UV	Violet	Blue	Yellow Green	Red
395	**CD45RA** BUV395 (UV2)				
420		**CCR7** BV421 (V1)			
440					
450	Live/Dead Blue (UV6)	**CD57** Pacific Blue (V3)			
480		**CCR6** BV480 (V5)	**CXCR3** Alexa Fluor 488 (B2)		
500	**CD62L** BUV496 (UV7)				
520		**CD3** BV510 (V7)			
550					
570	**CCR5** BUV563 (UV9)				
580				**EBV Latent Tetramers** PE (YG1)	
600		**TIM3** BV605 (V10)		**CD45** Spark YG 593 (YG2) / **CTLA4** PE-CF594 (YG3)	
660	**CD39** BUV661 (UV11)	**CD28** BV650 (V11)			**EBV Lytic Tetramers** APC (R1)
680			**CD4** PerCP (B8)	**CD95** PE-Cy5 (YG5)	**CD69** Alexa Fluor 647 (R2)
690					
700		**CD31** BV711 (V13)	**TCRγδ** PerCP-eFluor 710 (B10) / **CCR4** BB700 (B9)	**CD25** PE-Alex Fluor 700 (YG7)	**CD127** APC-R700 (R4)
730					
750		**CXCR5** BV750 (V14)			
780		**PD-1** BV785 (V15)		**LAG3** PE-Cy7 (YG9)	**CD27** APC-H7 (R7)
800	**CD8** BUV805 (UV16)			**HLA-DR** PE-Fire 810 (YG10)	**CD38** APC-Fire 810 (R8)

[b]

Approx emission wavelength (nm)	UV	Violet	Blue	Yellow Green	Red
395					
420					
440		**CD123** Super Bright 436 (V2)			
450	Live/Dead Blue (UV6)	**CD57** Pacific Blue (V3)			
480		**IgD** BV480 (V5)			
500	**CD16** BUV496 (UV7)				
520		**CD3** BV510 (V7)			
550			**CD14** Spark Blue 550 (B3)		
570		**IgM** BV570 (V8)		**NKG2C** PE (YG1)	
580					
600		**KIR** BV605 (V10)		**CD45** Spark YG 593 (YG2)	
660				**CD24** PE-Alexa Fluor 610 (YG4)	**NKG2A** APC (R1)
680			**CD4** PerCP (B8)	**CD21** PE-Cy5 (YG5)	
690					**CD19** Spark NIR 685 (R3)
700			**TCRγδ** PerCP-eFluor 710 (B10)		**CD127** APC-R700 (R4)
730	**CD56** BUV737 (UV14)				
750					
780		**CD20** BV786 (V15)		**CD11c** PE-Cy7 (YG9)	**CD27** APC-H7 (R7)
800	**CD8** BUV805 (UV16)			**HLA-DR** PE-Fire 810 (YG10)	**CD38** APC-Fire 810 (R8)

Table 3.3: Optical layout of fluorochromes. The approximate emission wavelengths across the 5 lasers of the Cytek® Aurora are represented for each fluorochrome-conjugated antibodies in the broad innate/adaptive immunity panel (**3.3a**) and T-lymphocyte panel (**3.3b**). Peak detector channels are indicated in parentheses. Each antigen-fluorochrome combination was assigned to the row corresponding to the emission peak of the fluorochrome. CD45 in Spark YG 593 and CTLA4 in PE-CF594 shared the same emission block as did TCRγδ in PE-eFluor 710 and CCR4 in BB700. None of these conjugates shared the same peak detector channel with each other and so were still expected to have satisfactory single cell resolution.

3.3 Reviewing the theoretical integrity of the flow panels

Once all surface antigen markers had been allocated to a fluorochrome, the robustness of each high dimensional flow panel was evaluated further before proceeding to practical testing on biological samples. This involved an assessment of the spectral uniqueness for each fluorochrome across all 64 detectors of the Cytek® Aurora. The Cytek® full spectrum viewer™ was used for this purpose.

The full spectrum viewer is an online software that is freely available through the Cytek® website (spectrum.cytekbio.com). Its key function is to visually assess the uniqueness and compatibility of fluorochrome wavelength signatures in full-spectrum panels. In addition, it generates two key metrics called the similarity index and complexity index. The similarity index ranges from 0 – 1. Fluorochromes with highly overlapping signatures have a similarity index close to 1 while those with distinct signatures have a value closer to 0. [385] The 5-laser Cytek® Aurora flow cytometer is capable of unmixing fluorochromes with similarity indices up to 0.98. [339] The complexity index on the other hand evaluates the overall spectral uniqueness of the panel by accounting for the similarity index of each fluorochrome in the panel. The lower the complexity index, the higher the likelihood that the given combination of fluorochromes will yield optimal results.

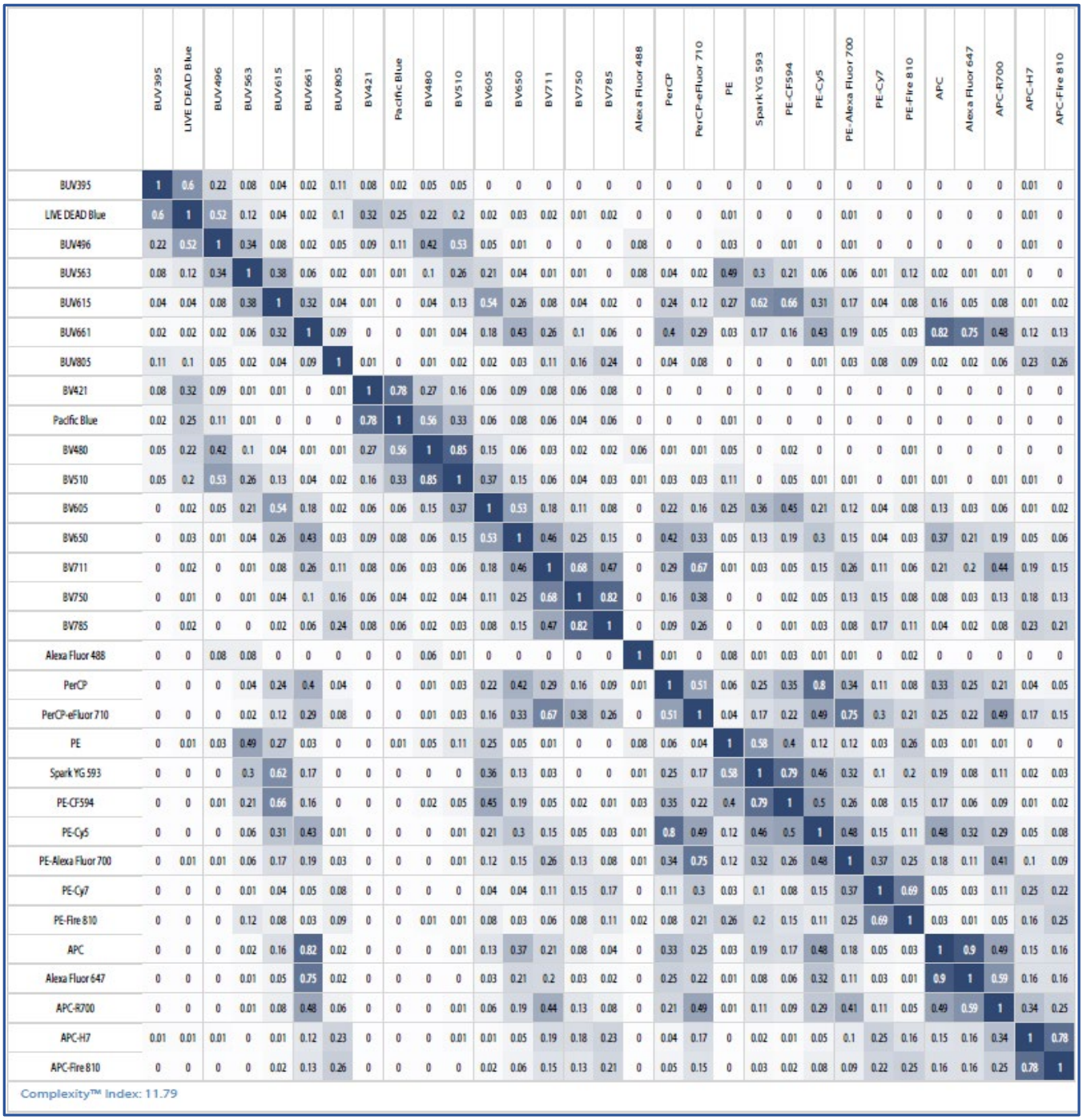
Expert recommendation from the Newcastle University Flow Core Cytometry Facility (FCCF) suggested that a 20-30 parameter spectral flow panel should have a complexity index of ≤ 20. This approach has been validated in other similar sized spectral flow cytometry panels. [393-395] Figure 3.2 illustrates the similarity index matrix for both spectral flow panels used in the study along with their corresponding complexity index. Both panels met the *a priori* complexity index criteria. Of note, no similarity index was found to be greater than 0.9 in either panel.

[a]

	LIVE DEAD Blue	BUV496	BUV737	BUV805	Super Bright 436	Pacific Blue	BV480	BV510	BV570	BV605	BV786	Spark Blue 550	PerCP	PerCP-eFluor 710	PE	Spark YG 593	PE-Alexa Fluor 610	PE-Cy5	PE-Cy7	PE-Fire 810	APC	Spark NIR 685	APC-R700	APC-H7	APC-Fire 810
LIVE DEAD Blue	1	0.52	0.03	0.1	0.32	0.25	0.22	0.2	0.05	0.02	0.03	0.02	0	0	0.01	0	0	0	0	0	0	0	0	0.01	0
BUV496	0.52	1	0.01	0.05	0.1	0.11	0.42	0.53	0.1	0.05	0.01	0.11	0	0	0.03	0	0.01	0	0	0	0	0	0	0.01	0
BUV737	0.03	0.01	1	0.41	0	0	0	0.02	0.02	0.06	0.25	0.01	0.19	0.37	0	0.02	0.05	0.1	0.15	0.09	0.19	0.27	0.42	0.29	0.21
BUV805	0.1	0.05	0.41	1	0.01	0	0.01	0.02	0.01	0.02	0.22	0	0.04	0.08	0	0	0.01	0.01	0.08	0.09	0.02	0.04	0.06	0.23	0.26
Super Bright 436	0.32	0.1	0	0.01	1	0.86	0.34	0.21	0.16	0.08	0.11	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0
Pacific Blue	0.25	0.11	0	0	0.86	1	0.56	0.33	0.15	0.06	0.09	0.04	0	0	0.01	0	0	0	0	0	0	0	0	0	0
BV480	0.22	0.42	0	0.01	0.34	0.56	1	0.85	0.24	0.15	0.04	0.21	0.01	0.01	0.05	0	0.01	0	0	0.01	0	0	0	0	0
BV510	0.2	0.53	0.02	0.02	0.21	0.33	0.85	1	0.5	0.37	0.04	0.29	0.03	0.03	0.11	0	0.04	0.01	0	0.01	0.01	0	0.01	0.01	0
BV570	0.05	0.1	0.02	0.01	0.16	0.15	0.24	0.5	1	0.68	0.04	0.23	0.08	0.05	0.56	0.3	0.22	0.09	0.02	0.12	0.04	0.01	0.02	0	0.01
BV605	0.02	0.05	0.06	0.02	0.08	0.06	0.15	0.37	0.68	1	0.08	0.15	0.22	0.16	0.25	0.36	0.4	0.21	0.04	0.08	0.13	0.04	0.06	0.01	0.02
BV786	0.03	0.01	0.25	0.22	0.11	0.09	0.04	0.04	0.04	0.08	1	0.01	0.09	0.27	0	0	0.02	0.03	0.17	0.1	0.04	0.05	0.08	0.21	0.18
Spark Blue 550	0.02	0.11	0.01	0	0.03	0.04	0.21	0.29	0.23	0.15	0.01	1	0.08	0.05	0.24	0.05	0.12	0.08	0.02	0.08	0.01	0	0	0	0
PerCP	0	0	0.19	0.04	0	0	0.01	0.03	0.08	0.22	0.09	0.08	1	0.51	0.06	0.25	0.51	0.8	0.11	0.08	0.33	0.24	0.21	0.04	0.05
PerCP-eFluor 710	0	0	0.37	0.08	0	0	0.01	0.03	0.05	0.16	0.27	0.05	0.51	1	0.04	0.17	0.37	0.49	0.3	0.21	0.25	0.31	0.49	0.17	0.15
PE	0.01	0.03	0	0	0	0.01	0.05	0.11	0.56	0.25	0	0.24	0.06	0.04	1	0.58	0.29	0.12	0.03	0.26	0.03	0.01	0.01	0	0
Spark YG 593	0	0	0.02	0	0	0	0	0	0.3	0.36	0	0.05	0.25	0.17	0.58	1	0.69	0.46	0.1	0.2	0.19	0.06	0.11	0.02	0.03
PE-Alexa Fluor 610	0	0.01	0.05	0.01	0	0	0.01	0.04	0.22	0.4	0.02	0.12	0.51	0.37	0.29	0.69	1	0.75	0.11	0.13	0.29	0.1	0.16	0.02	0.03
PE-Cy5	0	0	0.1	0.01	0	0	0	0.01	0.09	0.21	0.03	0.08	0.8	0.49	0.12	0.46	0.75	1	0.15	0.11	0.48	0.29	0.29	0.05	0.08
PE-Cy7	0	0	0.15	0.08	0	0	0	0	0.02	0.04	0.17	0.02	0.11	0.3	0.03	0.1	0.11	0.15	1	0.69	0.05	0.05	0.11	0.25	0.22
PE-Fire 810	0	0	0.09	0.09	0	0	0.01	0.01	0.12	0.08	0.1	0.08	0.08	0.21	0.26	0.2	0.13	0.11	0.69	1	0.03	0.02	0.05	0.16	0.25
APC	0	0	0.19	0.02	0	0	0	0.01	0.04	0.13	0.04	0.01	0.33	0.25	0.03	0.19	0.29	0.48	0.05	0.03	1	0.68	0.49	0.15	0.16
Spark NIR 685	0	0	0.27	0.04	0	0	0	0	0.01	0.04	0.05	0	0.24	0.31	0.01	0.06	0.1	0.29	0.05	0.02	0.68	1	0.79	0.26	0.21
APC-R700	0	0	0.42	0.06	0	0	0	0.01	0.02	0.06	0.08	0	0.21	0.49	0.01	0.11	0.16	0.29	0.11	0.05	0.49	0.79	1	0.34	0.25
APC-H7	0.01	0.01	0.29	0.23	0	0	0	0.01	0	0.01	0.21	0	0.04	0.17	0	0.02	0.02	0.05	0.25	0.16	0.15	0.26	0.34	1	0.78
APC-Fire 810	0	0	0.21	0.26	0	0	0	0	0.01	0.02	0.18	0	0.05	0.15	0	0.03	0.03	0.08	0.22	0.25	0.16	0.21	0.25	0.78	1

Complexity™ Index: 10.11

[b]



	BUV395	LIVE DEAD Blue	BUV496	BUV563	BUV615	BUV661	BUV805	BV421	Pacific Blue	BV480	BV510	BV605	BV650	BV711	BV750	BV785	Alexa Fluor 488	PerCP	PerCP-eFluor 710	PE	Spark YG 593	PE-CF594	PE-Cy5	PE-Alexa Fluor 700	PE-Cy7	PE-Fire 810	APC	Alexa Fluor 647	APC-R700	APC-H7	APC-Fire 810
BUV395	1	0.6	0.22	0.08	0.04	0.02	0.11	0.08	0.02	0.05	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0
LIVE DEAD Blue	0.6	1	0.52	0.12	0.04	0.02	0.1	0.32	0.25	0.22	0.2	0.02	0.03	0.02	0.01	0.02	0	0	0	0.01	0	0	0	0.01	0	0	0	0	0.01	0
BUV496	0.22	0.52	1	0.34	0.08	0.02	0.05	0.09	0.11	0.42	0.53	0.05	0.01	0	0	0	0.08	0	0	0.03	0	0.01	0	0.01	0	0	0	0	0.01	0
BUV563	0.08	0.12	0.34	1	0.38	0.06	0.02	0.01	0.01	0.1	0.26	0.21	0.04	0.01	0.01	0	0.08	0.04	0.02	0.49	0.3	0.21	0.06	0.06	0.01	0.12	0.02	0.01	0.01	0	0
BUV615	0.04	0.04	0.08	0.38	1	0.32	0.04	0.01	0	0.04	0.13	0.54	0.26	0.08	0.04	0.02	0	0.24	0.12	0.27	0.62	0.66	0.31	0.17	0.04	0.08	0.16	0.05	0.08	0.01	0.02
BUV661	0.02	0.02	0.02	0.06	0.32	1	0.09	0	0	0.01	0.04	0.18	0.43	0.26	0.1	0.06	0	0.4	0.29	0.03	0.17	0.16	0.43	0.19	0.05	0.03	0.82	0.75	0.48	0.12	0.13
BUV805	0.11	0.1	0.05	0.02	0.04	0.09	1	0.01	0	0.01	0.02	0.02	0.03	0.11	0.16	0.24	0	0.04	0.08	0	0	0	0.01	0.03	0.08	0.09	0.02	0.02	0.06	0.23	0.26
BV421	0.08	0.32	0.09	0.01	0.01	0	0.01	1	0.78	0.27	0.16	0.06	0.09	0.08	0.06	0.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pacific Blue	0.02	0.25	0.11	0.01	0	0	0	0.78	1	0.56	0.33	0.06	0.08	0.06	0.04	0.06	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0
BV480	0.05	0.22	0.42	0.1	0.04	0.01	0.01	0.27	0.56	1	0.85	0.15	0.06	0.03	0.02	0.02	0.06	0.01	0.01	0.05	0	0.02	0	0	0	0.01	0	0	0	0	0
BV510	0.05	0.2	0.53	0.26	0.13	0.04	0.02	0.16	0.33	0.85	1	0.37	0.15	0.06	0.04	0.03	0.01	0.03	0.03	0.11	0	0.05	0.01	0.01	0	0.01	0.01	0	0.01	0.01	0
BV605	0	0.02	0.05	0.21	0.54	0.18	0.02	0.06	0.06	0.15	0.37	1	0.53	0.18	0.11	0.08	0	0.22	0.16	0.25	0.36	0.45	0.21	0.12	0.04	0.08	0.13	0.03	0.06	0.01	0.02
BV650	0	0.03	0.01	0.04	0.26	0.43	0.03	0.09	0.08	0.06	0.15	0.53	1	0.46	0.25	0.15	0	0.42	0.33	0.05	0.13	0.19	0.3	0.15	0.04	0.03	0.37	0.21	0.19	0.05	0.06
BV711	0	0.02	0	0.01	0.08	0.26	0.11	0.08	0.06	0.03	0.06	0.18	0.46	1	0.68	0.47	0	0.29	0.67	0.01	0.03	0.05	0.15	0.26	0.11	0.06	0.21	0.2	0.44	0.19	0.15
BV750	0	0.01	0	0.01	0.04	0.1	0.16	0.06	0.04	0.02	0.04	0.11	0.25	0.68	1	0.82	0	0.16	0.38	0	0	0.02	0.05	0.13	0.15	0.08	0.08	0.03	0.13	0.18	0.13
BV785	0	0.02	0	0	0.02	0.06	0.24	0.08	0.06	0.02	0.03	0.08	0.15	0.47	0.82	1	0	0.09	0.26	0	0	0.01	0.03	0.08	0.17	0.11	0.04	0.02	0.08	0.23	0.21
Alexa Fluor 488	0	0	0.08	0.08	0	0	0	0	0	0.06	0.01	0	0	0	0	0	1	0.01	0	0.08	0.01	0.03	0.01	0.01	0	0.02	0	0	0	0	0
PerCP	0	0	0	0.04	0.24	0.4	0.04	0	0	0.01	0.03	0.22	0.42	0.29	0.16	0.09	0.01	1	0.51	0.06	0.25	0.35	0.8	0.34	0.11	0.08	0.33	0.25	0.21	0.04	0.05
PerCP-eFluor 710	0	0	0	0.02	0.12	0.29	0.08	0	0	0.01	0.03	0.16	0.33	0.67	0.38	0.26	0	0.51	1	0.04	0.17	0.22	0.49	0.75	0.3	0.21	0.25	0.22	0.49	0.17	0.15
PE	0	0.01	0.03	0.49	0.27	0.03	0	0	0.01	0.05	0.11	0.25	0.05	0.01	0	0	0.08	0.06	0.04	1	0.58	0.4	0.12	0.12	0.03	0.26	0.03	0.01	0.01	0	0
Spark YG 593	0	0	0	0.3	0.62	0.17	0	0	0	0	0	0.36	0.13	0.03	0	0	0.01	0.25	0.17	0.58	1	0.79	0.46	0.32	0.1	0.2	0.19	0.08	0.11	0.02	0.03
PE-CF594	0	0	0.01	0.21	0.66	0.16	0	0	0	0.02	0.05	0.45	0.19	0.05	0.02	0.01	0.03	0.35	0.22	0.4	0.79	1	0.5	0.26	0.08	0.15	0.17	0.06	0.09	0.01	0.02
PE-Cy5	0	0	0	0.06	0.31	0.43	0.01	0	0	0	0.01	0.21	0.3	0.15	0.05	0.03	0.01	0.8	0.49	0.12	0.46	0.5	1	0.48	0.15	0.11	0.48	0.32	0.29	0.05	0.08
PE-Alexa Fluor 700	0	0.01	0.01	0.06	0.17	0.19	0.03	0	0	0	0.01	0.12	0.15	0.26	0.13	0.08	0.01	0.34	0.75	0.12	0.32	0.26	0.48	1	0.37	0.25	0.18	0.11	0.41	0.1	0.09
PE-Cy7	0	0	0	0.01	0.04	0.05	0.08	0	0	0	0	0.04	0.04	0.11	0.15	0.17	0	0.11	0.3	0.03	0.1	0.08	0.15	0.37	1	0.69	0.05	0.03	0.11	0.25	0.22
PE-Fire 810	0	0	0	0.12	0.08	0.03	0.09	0	0	0.01	0.01	0.08	0.03	0.06	0.08	0.11	0.02	0.08	0.21	0.26	0.2	0.15	0.11	0.25	0.69	1	0.03	0.01	0.05	0.16	0.25
APC	0	0	0	0.02	0.16	0.82	0.02	0	0	0	0.01	0.13	0.37	0.21	0.08	0.04	0	0.33	0.25	0.03	0.19	0.17	0.48	0.18	0.05	0.03	1	0.9	0.49	0.15	0.16
Alexa Fluor 647	0	0	0	0.01	0.05	0.75	0.02	0	0	0	0	0.03	0.21	0.2	0.03	0.02	0	0.25	0.22	0.01	0.08	0.06	0.32	0.11	0.03	0.01	0.9	1	0.59	0.16	0.16
APC-R700	0	0	0	0.01	0.08	0.48	0.06	0	0	0	0.01	0.06	0.19	0.44	0.13	0.08	0	0.21	0.49	0.01	0.11	0.09	0.29	0.41	0.11	0.05	0.49	0.59	1	0.34	0.25
APC-H7	0.01	0.01	0.01	0	0.01	0.12	0.23	0	0	0	0.01	0.01	0.05	0.19	0.18	0.23	0	0.04	0.17	0	0.02	0.01	0.05	0.1	0.25	0.16	0.15	0.16	0.34	1	0.78
APC-Fire 810	0	0	0	0	0.02	0.13	0.26	0	0	0	0	0.02	0.06	0.15	0.13	0.21	0	0.05	0.15	0	0.03	0.02	0.08	0.09	0.22	0.25	0.16	0.16	0.25	0.78	1

Complexity™ Index: 11.79

<u>Figure 3.2</u>: Results of the similarity index for each pair of fluorochromes in **[a]** the Broad innate/adaptive immune panel and **[b]** the T-lymphocyte panel. A value of "1" indicates that the spectral signatures of 2 fluorochromes are virtually identical, while a value of "0" indicates that they are completely unique. The 5-laser Cytek® Aurora flow cytometer is capable of unmixing fluorochromes with similarity indices up to 0.98. The overall complexity index of each panel is displayed at the bottom of the matrix.

3.4 Panel Testing

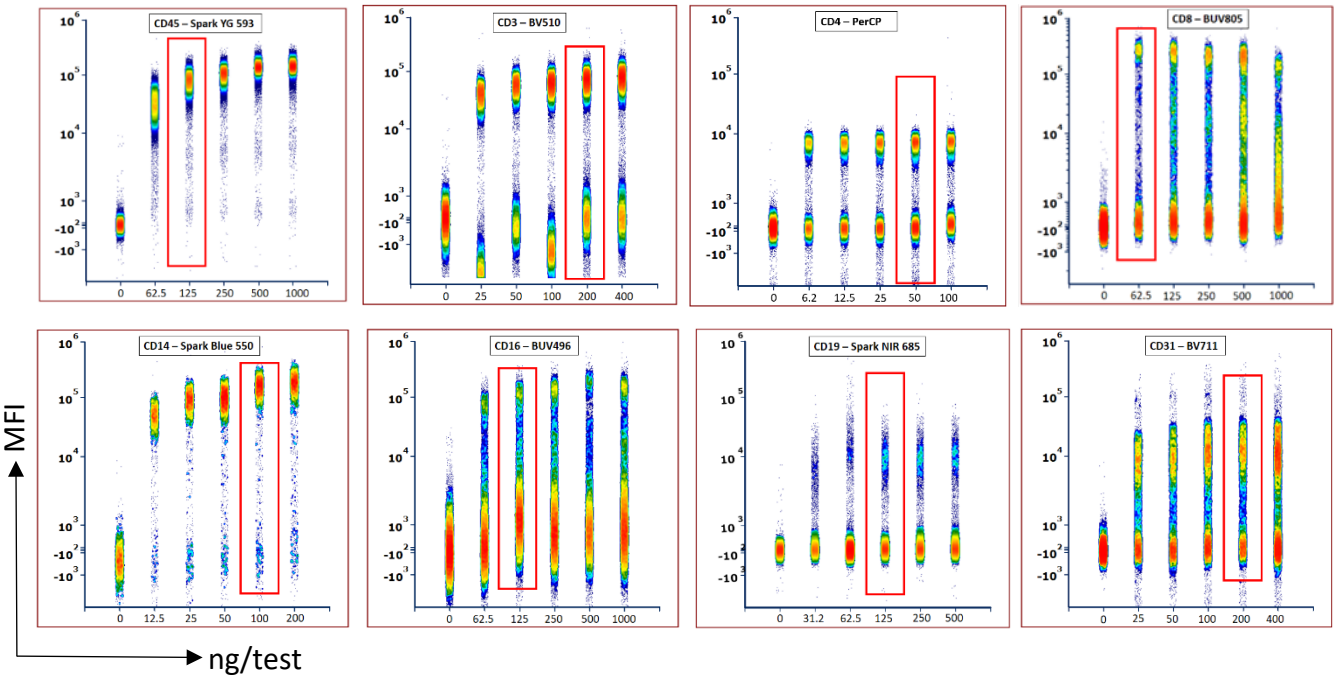
3.4.1 Antibody titration

Cryopreserved PBMCs from healthy adult donors were used to titrate the antibody reagents. Cells were resuspended in 100µl of FACS staining buffer to mirror the final staining volume of the MCS samples. The antibodies were tested in decreasing concentration across 5 serial two-fold dilutions (1:20 to 1:320), starting with the manufacturer's recommended antibody concentration per test. The same staining protocol and spectral flow cytometry QC'ing described in Chapter 2 was used.

After spectral unmixing, FCS files were exported to FCS Express version 7 (De Novo software) for concatenation and downstream analysis. The median fluorescent intensities (MFIs) of the positive and negative cell populations were used to estimate the stain index based on the following formula:

$$Stain\ Index = \frac{MFI\ of\ positive\ cell\ population - MFI\ of\ negative\ cell\ population}{(Standard\ deviation\ of\ negative\ cell\ population)^2}$$

The optimal concentration for each antibody was then selected based on either the ideal stain index or on the best possible resolution between the positive and negative cell populations while minimizing spreading error (Figure 3.3).



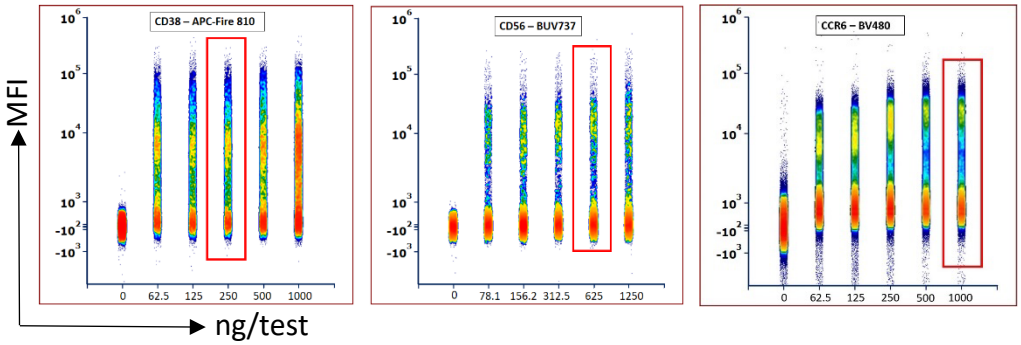


Figure 3.3: Representative illustration of the serial two-fold antibody titrations performed for the surface antigen markers used in the panels (1:20 to 1:320). Antibodies were tested at five different concentrations starting with the manufacturer's recommended antibody concentration per test. FCS express version 7 (De Novo Software) was used to concatenate the FCS files and for estimation of the stain index. All files were pre-gated on live singlet cells. The final titration results are reported as ng/test. Red squares depict the chosen antibody concentration.

3.4.2 Evaluation and optimisation of single stain reference controls

Great emphasis was placed on identifying high quality SRCs that would express similar or higher MFI compared to their counterparts in the MCS samples. This was essential for the accurate spectral unmixing of MCS samples. It is well recognised that slight differences can occur in the spectral signatures emitted from fluorochrome-conjugated antibodies when they are bound to beads versus PBMCs. [396] For this reason, both compensation beads and PBMCs were tested in tandem to determine which SRC materials could be used to produce spectra that matched the MCS samples. To account for donor variability in the density of secondary and tertiary cell surface antigens, SRCs for these markers were evaluated with samples obtained from different healthy donors (Figure 3.4).

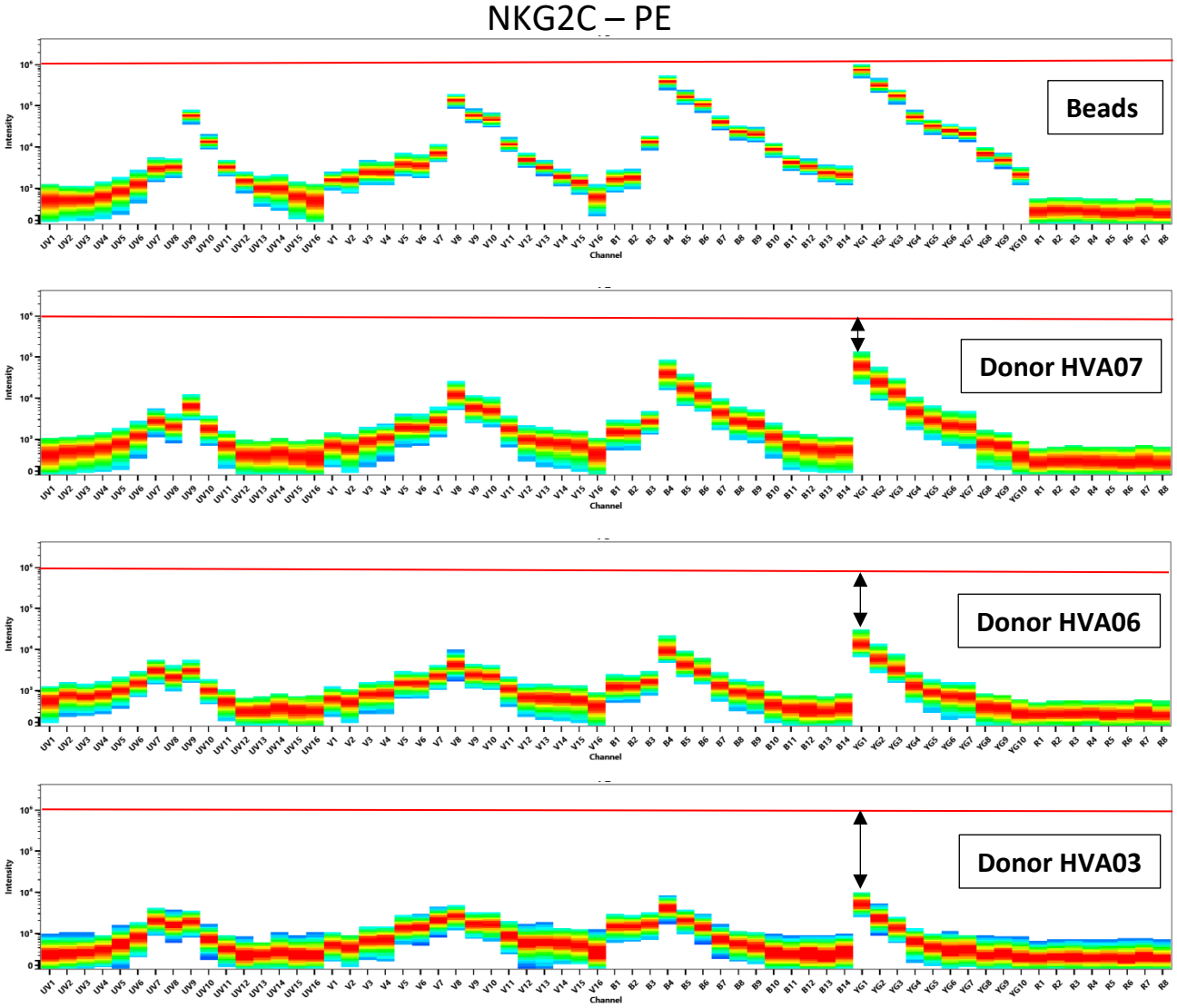


Figure 3.4: Spectral signature of NKG2C PE for a range of single stain reference controls bound to either beads or cells obtained from three healthy donors. The red line indicates the brightest signal intensity emitted by NKG2C – PE when bound to beads. As expected, donor variation in the fluorescent signal intensity was observed for NKG2C – PE when bound to cells. This was particularly noticeable in the peak detector channel for PE (YG1). Donor HVA07 had the least difference in fluorescent signal intensity compared to beads (indicated by the black arrow). Beads and cryopreserved cells from donor HVA07 were taken forward as SRC candidates for NKG2C – PE and tested in tandem to evaluate their accuracy in unmixing single stain samples.

Candidates with the brightest antigen expression were selected and their accuracy in unmixing single stain (SS) samples was compared against SRCs bound to beads. For some of the markers/fluorochromes, the use of a bead SRC led to significant unmixing inaccuracies (Figure 3.5).

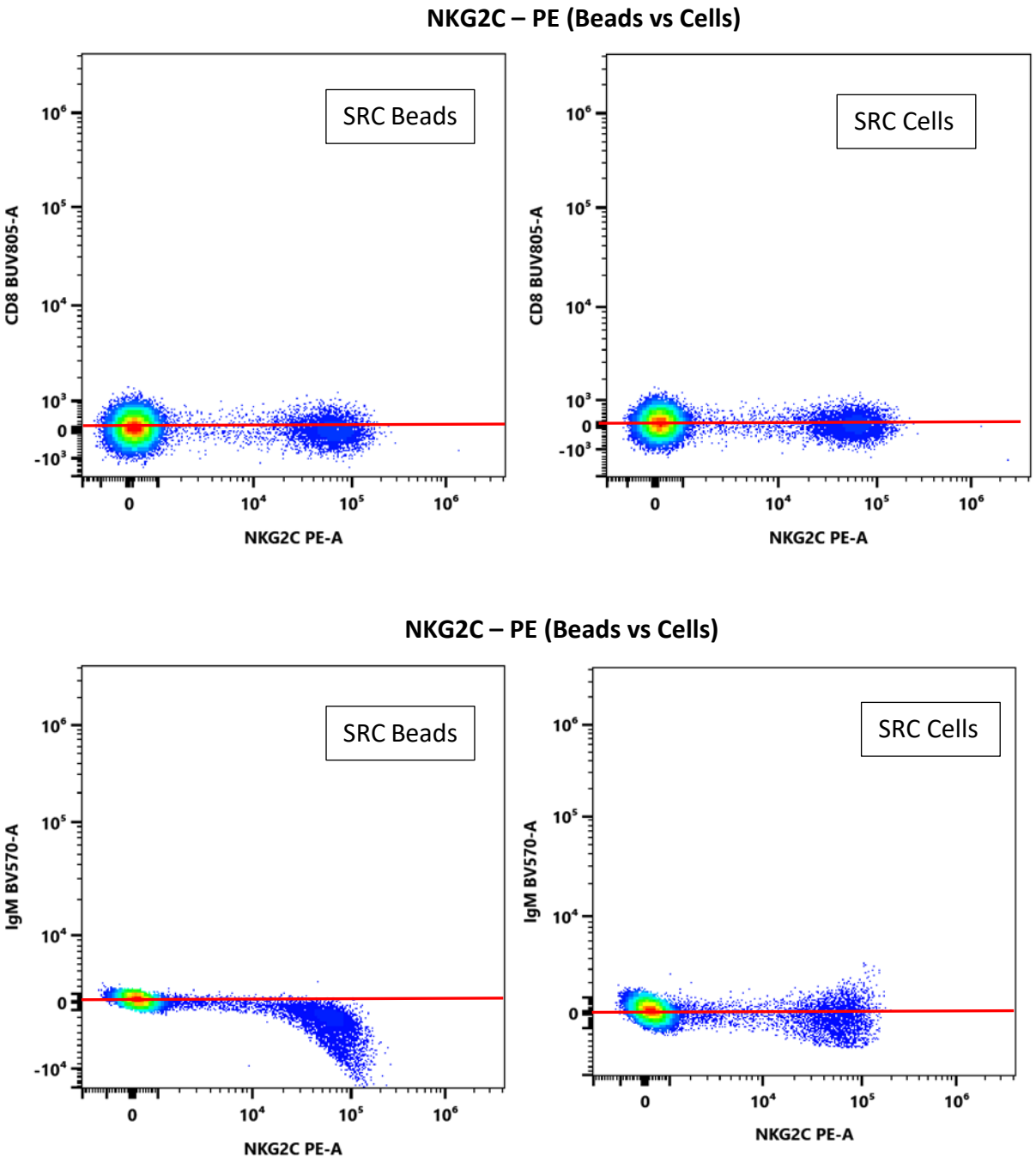


Figure 3.5: Representative bivariate plots of a single stain sample unmixed with either NKG2C – PE bound to compensation beads or cells. The horizontal red line demonstrates the degree of alignment between the negative and positive cell populations in each bivariate plot. There was accurate unmixing in all parameters e.g. CD8 – BUV805 (top plots) except for the IgM BV570 parameter, where an unmixing error was present with beads but not identified when the samples were unmixed with cells (bottom plots). Cells were therefore selected as the SRC material for NKG2C – PE.

This iterative process was followed for both panels until a final consensus was reached on the optimal SRC material needed for each fluorochrome (beads or cells). Cells were the preferred choice for SRCs wherever possible. However, a small number of SRCs in both panels required beads (Tables 3.4 and 3.5). This was indicated when no suitable donor with good fluorescent signal intensity could be identified for a secondary/tertiary surface antigen marker in the T-lymphocyte panel (CD45RA, TCRγδ, CD31, CD127, PD-1, LAG-3, CTLA4 and TIM-3) and/or the broad innate/adaptive immune panel (CD123, CD127 and TCRγδ). No significant unmixing errors were observed with these SRCs.

[Table 3.4]

1	Spark YG53 **CD45**	Cells	9	Spark NIR 685 **CD19**	Cells	17	APC **NKG2A**	Cells
2	BV510 **CD3**	Cells	10	BV786 **CD20**	Cells	18	PE **NKG2C**	Cells
3	PerCP **CD4**	Cells	11	PE-Cy5 **CD21**	Cells	19	BUV605 **KIR**	Cells
4	BUV805 **CD8**	Cells	12	PE-Alexa Fluor 610 **CD24**	Cells	20	PE-Cy7 **CD123**	Beads
5	PerCP-eFluor 710 **TCRγδ**	Beads	13	APC-H7 **CD27**	Cells	21	APC-R700 **CD127**	Beads
6	PE-Cy7 **CD11c**	Cells	14	APC-Fire 810 C**D38**	Cells	22	BV480 **IgD**	Cells
7	Spark Blue 550 **CD14**	Cells	15	BUV737 **CD56**	Cells	23	BV570 **IgM**	Cells
8	BUV496 **CD16**	Cells	16	Pacific Blue **CD57**	Cells	24	PE-Fire 810 **HLA-DR**	Cells

[Table 3.5]

1	Spark YG53 **CD45**	Cells	11	APC-Fire 810 C**D38**	Cells	21	BV421 **CCR7**	Cells
2	BUV395 **CD45RA**	Beads	12	BUV661 **CD39**	Cells	22	Alexa Fluor 488 **CXCR3**	Cells
3	BV510 **CD3**	Cells	13	Pacific Blue **CD57**	Cells	23	BV750 **CXCR5**	Cells
4	PerCP **CD4**	Cells	14	BUV496 **CD62L**	Cells	24	BV785 **PD1**	Beads
5	BUV805 **CD8**	Cells	15	Alexa Fluor 647 **CD69**	Cells	25	BV605 **TIM-3**	Beads
6	PerCP-eFluor 710 **TCRγδ**	Beads	16	PE-Cy5 **CD95**	Cells	26	PE-Cy7 **LAG-3**	Beads
7	PE-Alexa Fluor 700 **CD25**	Cells	17	APC-R700 **CD127**	Beads	27	PE-CF594 **CTLA4**	Beads
8	APC-H7 **CD27**	Cells	18	BUV615 **CCR4**	Cells	28	PE-Fire 810 **HLA-DR**	Cells
9	BV650 **CD28**	Cells	19	BUV563 **CCR5**	Cells	29	Lytic Tetramers **APC**	Cells
10	BV711 **CD31**	Beads	20	BV480 **CCR6**	Cells	30	Latent Tetramer **PE**	Cells

Tables 3.4 and 3.5*:* Final selection of SRC material for the broad innate/adaptive immune panel (Table 3.4) and T-lymphocyte panel (Table 3.5)

3.4.3 Evaluation of surface antigen marker resolution after multicolour staining

The resolution of each surface antigen marker in fully stained MCS samples was evaluated after all SRCs had been optimised for unmixing. This was achieved by visually inspecting overlay histograms of each marker in a single stain sample and the multicolour sample to determine if there was spread of the negative cell population and/or reduced fluorescent signal intensity of the positive cell population in the MCS sample compared to the SS sample. Two scenarios were noted:

1) Identical resolution of surface antigen markers:

The resolution of most surface antigen markers in the MCS samples was identical to the SS samples and did not require any further action.

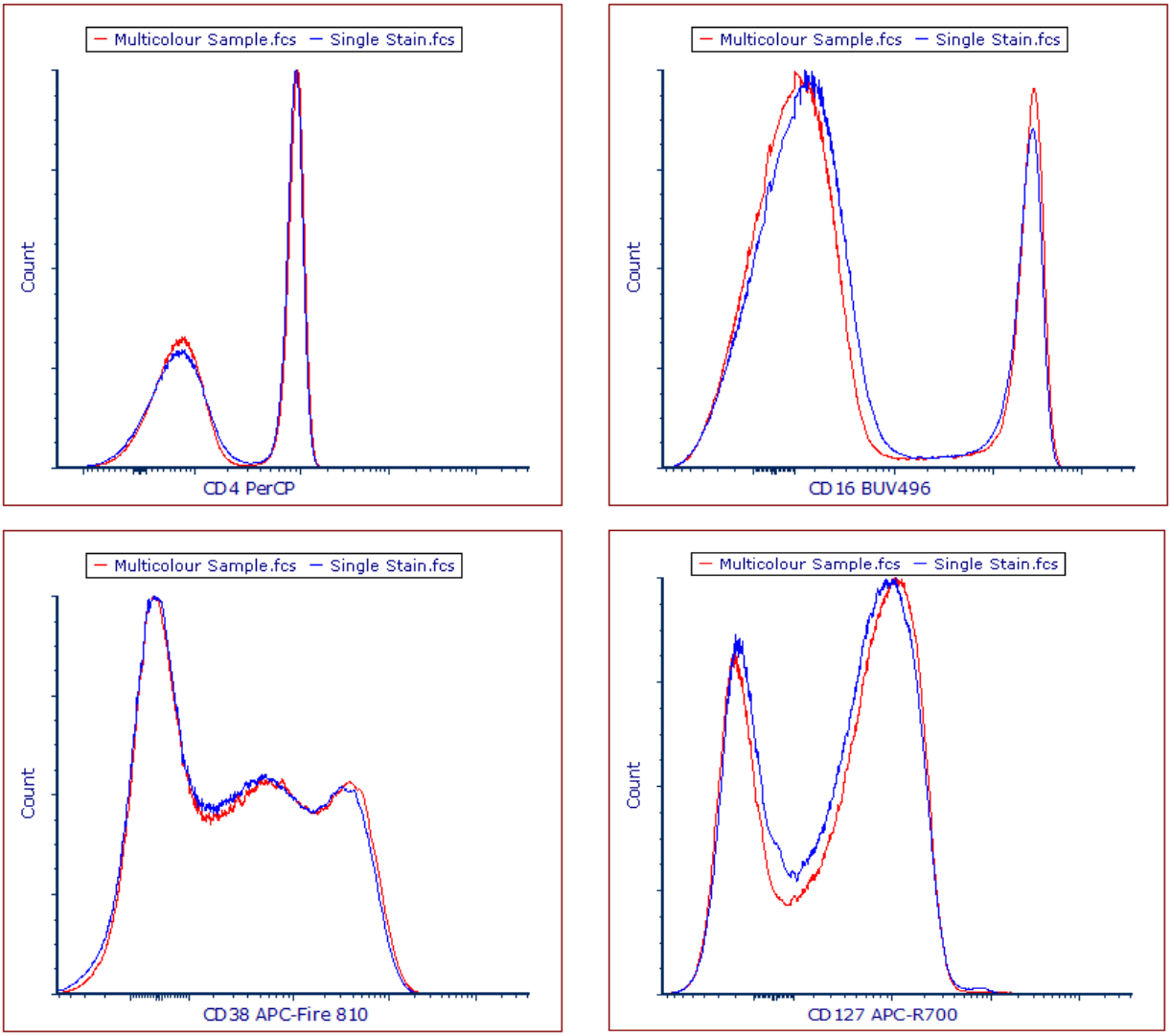


Figure 3.6: Surface antigen marker resolution (single stain vs multicolour sample). Representative overlay histograms showing identical resolution of surface antigen markers in the single stain sample and multicolour sample. All plots have been pre-gated on singlets, scatter and viable/live cells.

2) Dimmer positive cell population in the multicolour sample:

A minority of the surface antigen markers were observed to have a slightly reduced fluorescent signal intensity in the MCS sample compared to the SS sample (Figure 3.7a). The reduction in MFI was mostly negligible and had no negative impact on the ability to resolve the positive cell population of interest (Figure 3.7b). However, in the case of CD57 – Pacific Blue and CXCR3 – Alexa Fluor 488, a doubling of the antibody concentration was warranted to achieve better resolution. This increase did not adversely affect the spread of the negative cell population (Figure 3.8).

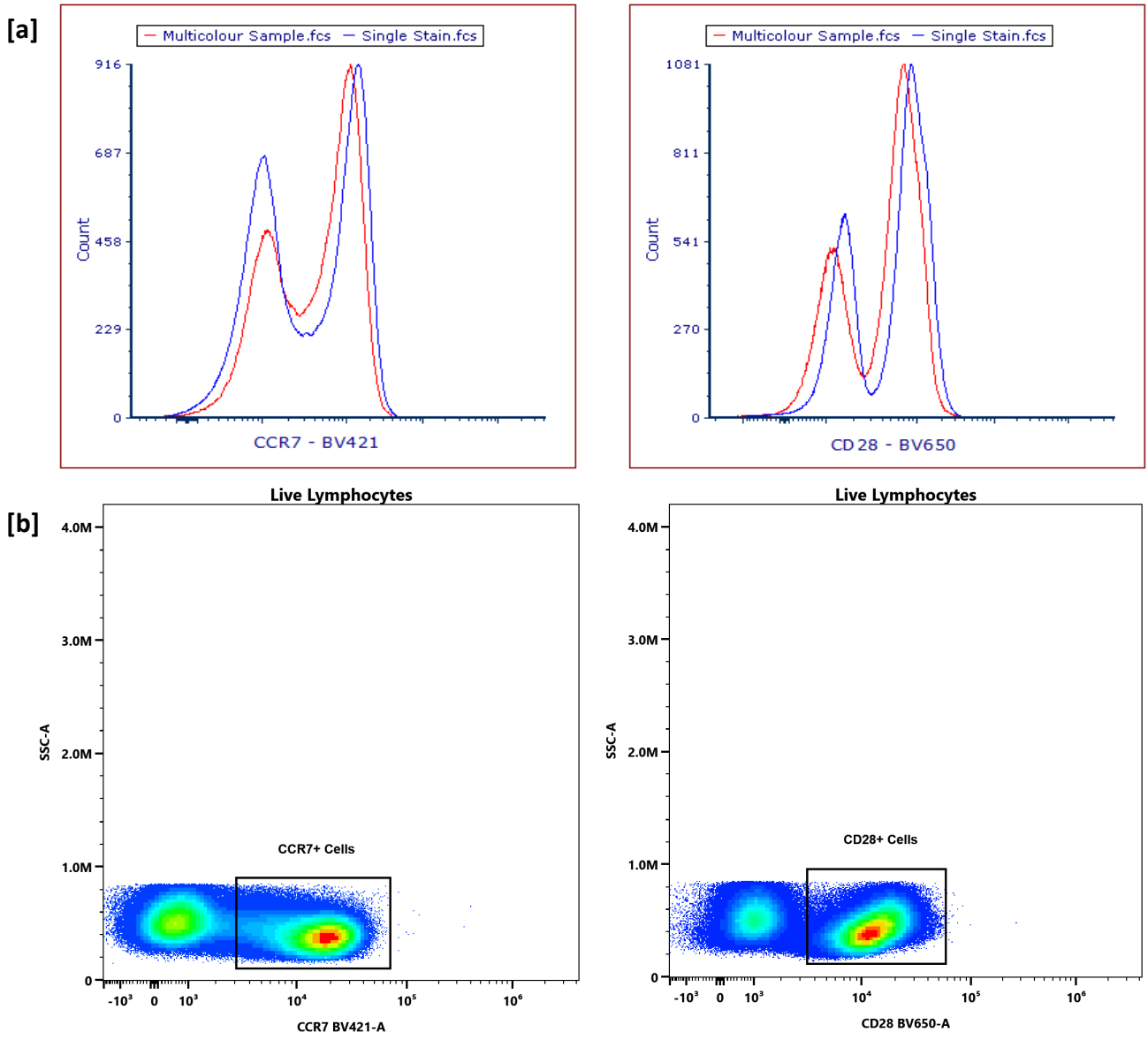


Figure 3.7: Single stain vs Multicolour resolution of CCR7+ and CD28+ cells. The overlay histograms **[a]** show a marginal reduction in the MFI of the positive cell population on multicolour staining. This did not affect their resolution in the flow panel as demonstrated by their clear delineation in the density dot plots. **[b]** All plots were gated on singlets, scatter, and viable/live cells.

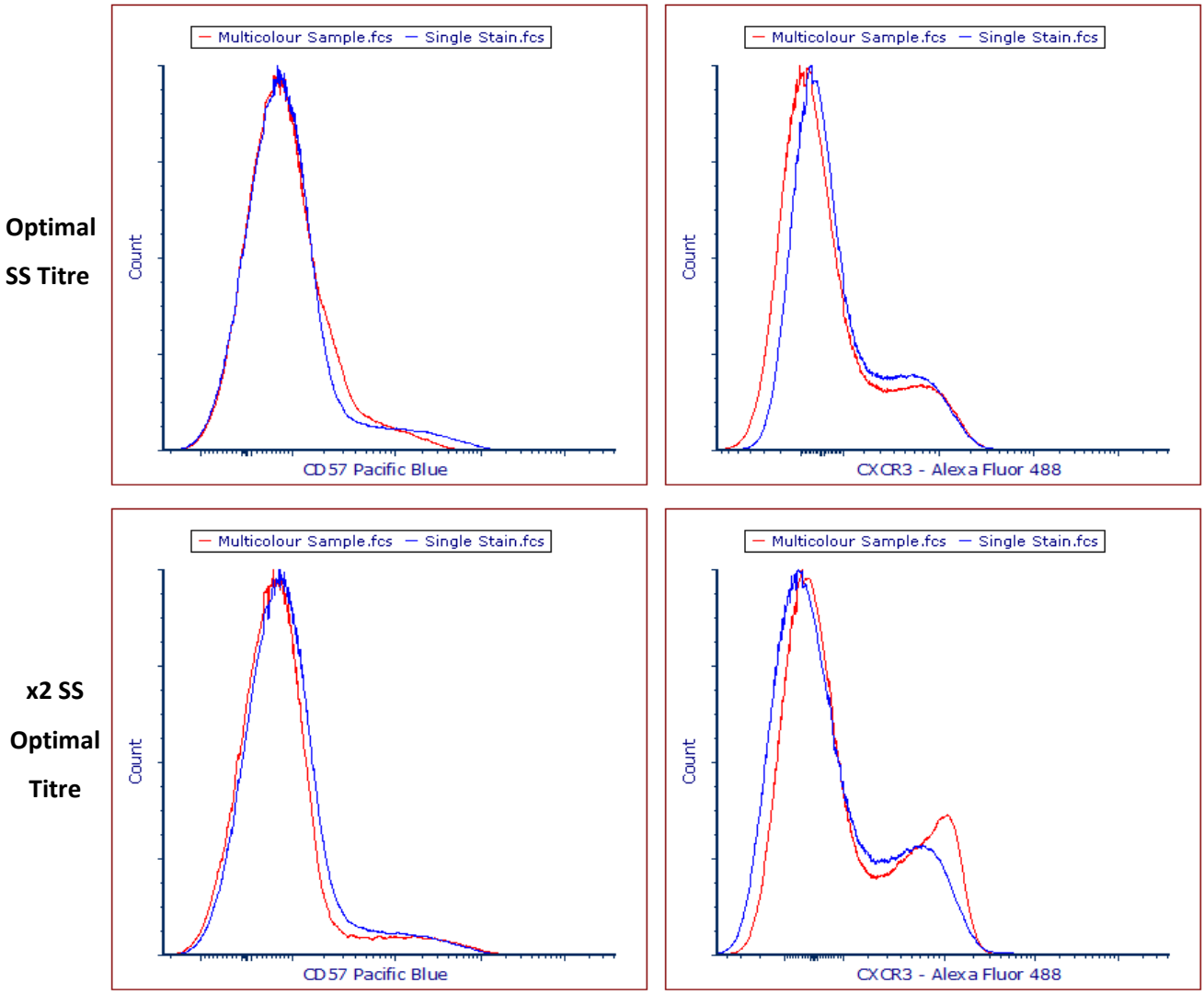
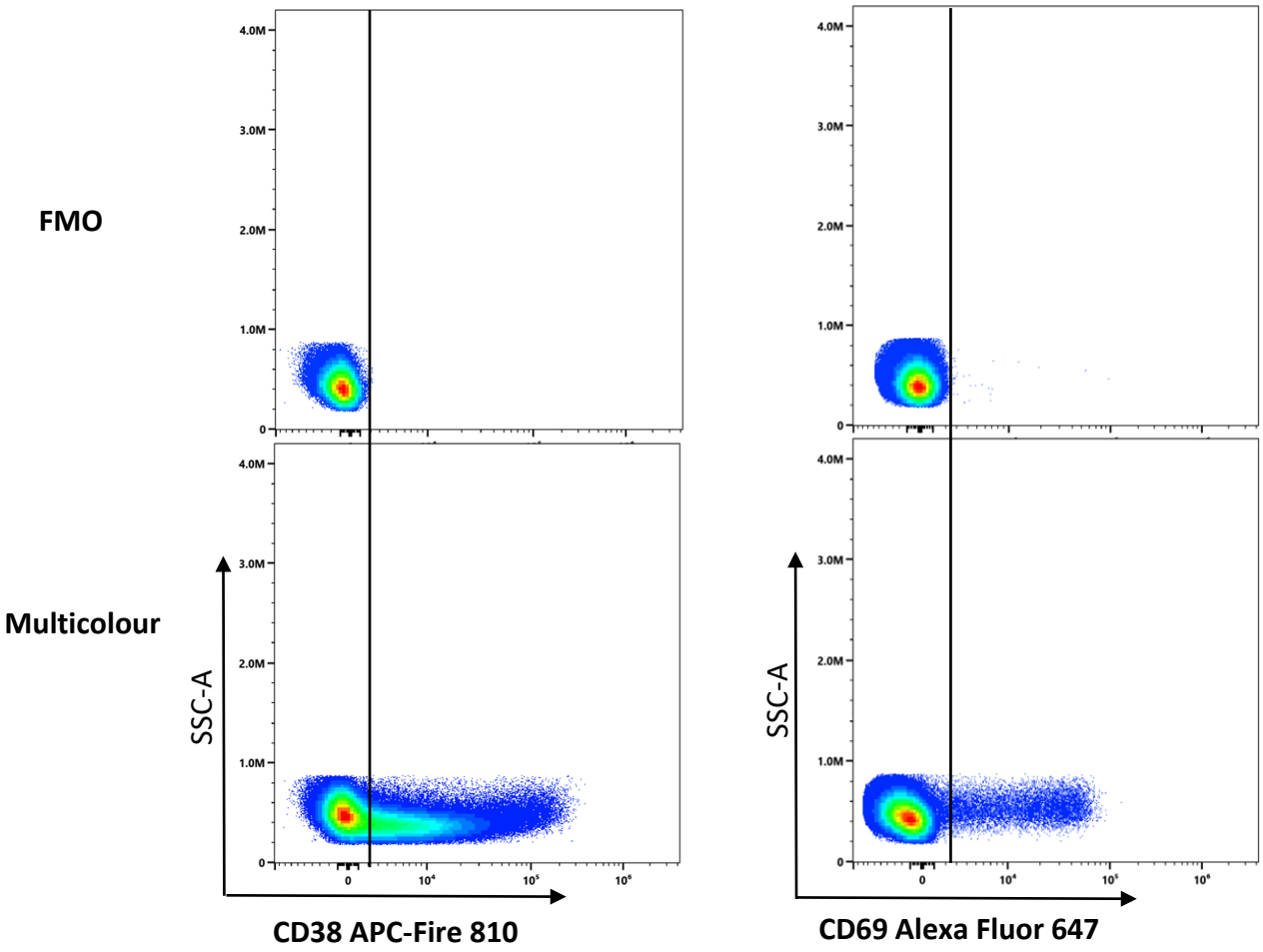
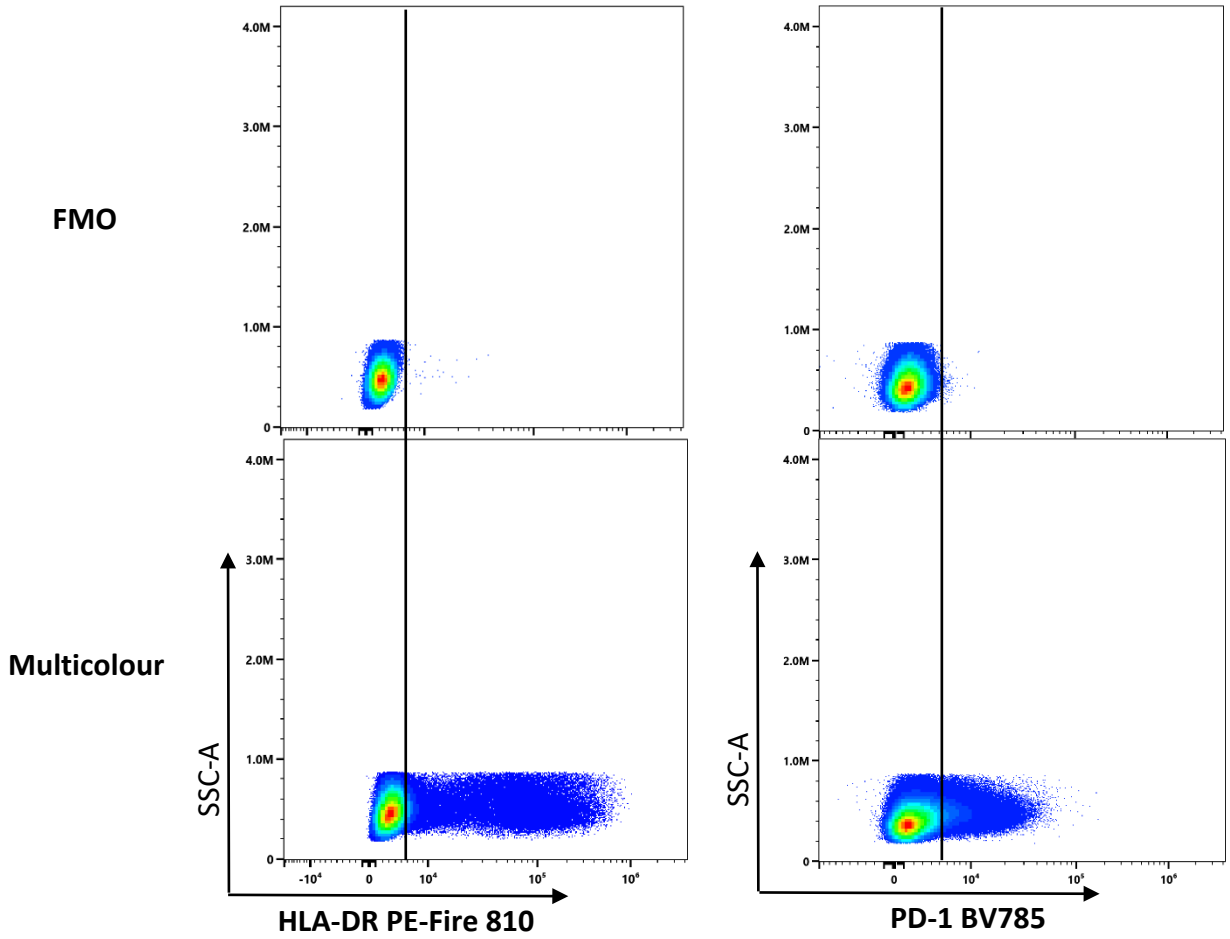


Figure 3.8: Improved resolution of CD57 – Pacific Blue and CXCR3 – Alexa Fluor 488 in the multistain sample. The optimal single stain (SS) antibody titres provided reasonable resolution of both markers (top row). However, their titres were doubled in the MCS samples (bottom row) to further optimise the signal intensity of the positive cell population due to the relevance of these markers in detecting important cell subsets of cellular senescence (CD57) and Type 1 T helper lymphocytes (CXCR3) that were central to the study hypothesis. This helped to increase the brightness of the positive cell population without a significant impact on the negative cell population (no increased spread).

In addition, full stain minus one (FMO) controls (Figure 3.9) were used as a quality control measure to help delineate the positive cell population for surface antigen markers of cellular exhaustion (PD1, LAG-3, TIM-3, CTLA4) and activation (CD38, CD69, HLA-DR). This was necessary due to their expression being dependent on antigenic stimulation.


FMO
Multicolour
SSC-A
CD38 APC-Fire 810
CD69 Alexa Fluor 647


FMO
Multicolour
SSC-A
HLA-DR PE-Fire 810
PD-1 BV785

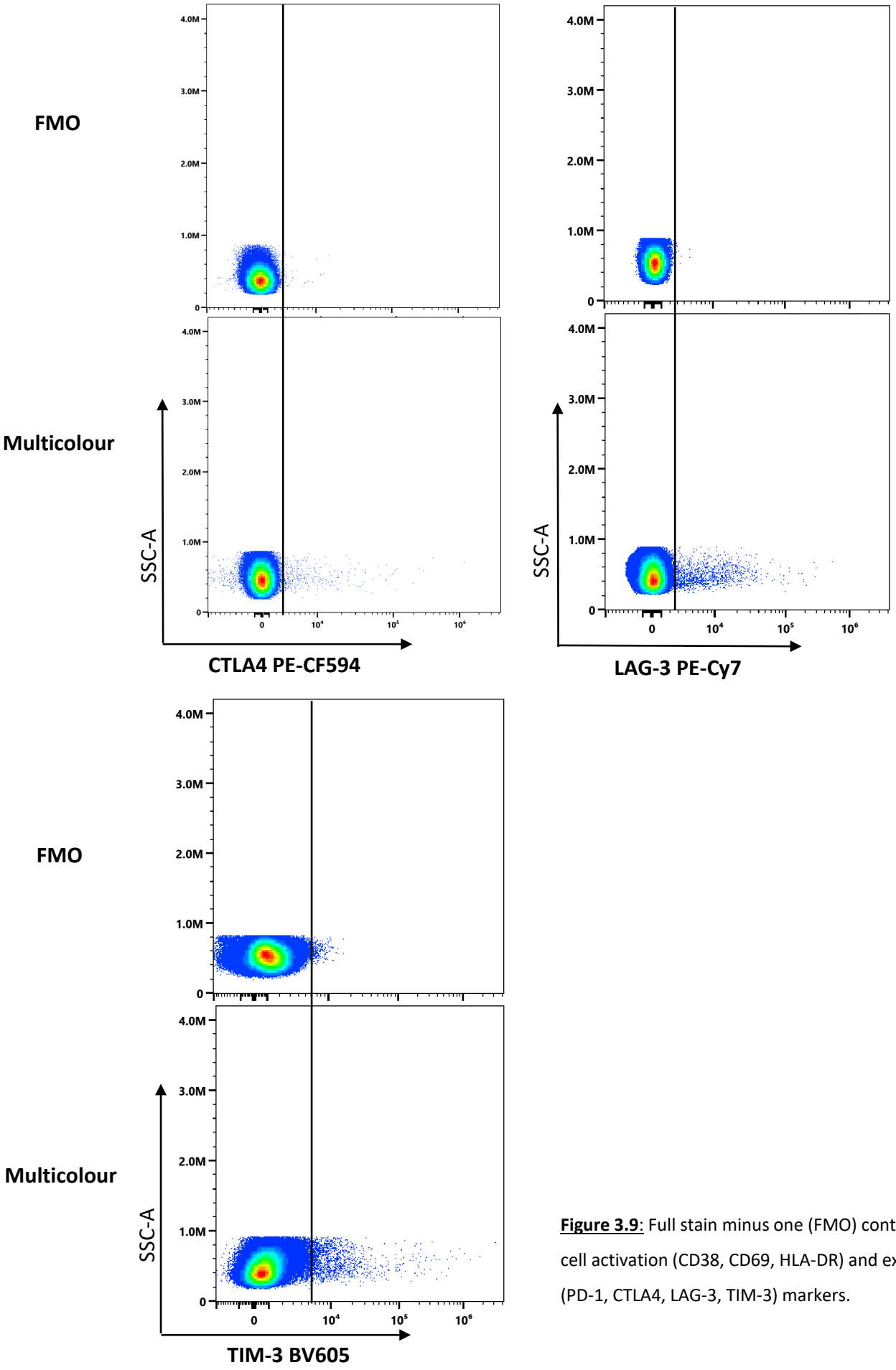


Figure 3.9: Full stain minus one (FMO) controls for cell activation (CD38, CD69, HLA-DR) and exhaustion (PD-1, CTLA4, LAG-3, TIM-3) markers.

3.4.4 *Tetramer titration*

All tetramer titrations were performed independently by the Long group at the Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom. Serial two-fold dilutions of the tetramer stock solutions (1µl to 0.005µl) were made for staining HLA-matched PBMCs obtained from EBV-exposed healthy adult volunteers using a procedure similar to the tetramer staining of MCS patient samples (see section 2.2.6).

In addition, samples were stained with APC-Cy7 (dumping channel for dead cells and non-T lymphocytes i.e. CD14, CD16, CD19 and CD56), BV510 (CD3), BV421 (CD4) and FITC (CD8). Samples were acquired on a BD LSR II flow cytometer. FCS files were exported to FCS Express (version 7) for data cleaning and downstream analysis. The titres of MHC class I tetramers were gated on live CD8+ T-lymphocytes while MHC class II tetramers were gated on live CD4+ T-lymphocytes.

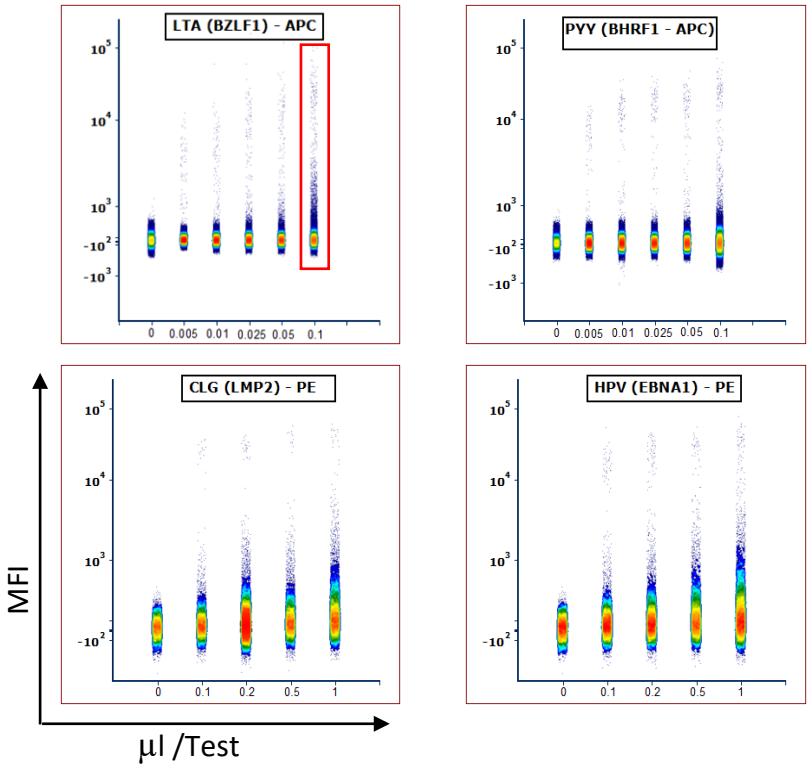


Figure 3.10: **Illustrative examples of the serial two-fold antibody titrations performed for EBV tetramers in the T-lymphocyte panel**. The EBV epitope is represented by the first 3 letters in its sequence while the associated viral protein is shown in parentheses. Lytic phase EBV tetramers were labelled with APC (top row) and latent phase EBV tetramers with PE (bottom row). Optimal titres were selected based on the antibody concentration that gave the highest MFI with the lowest background staining. The red square depicts the chosen tetramer titre. Some of the tetramers were deemed to have optimal titres that lay just below the lowest value (e.g. CLG and HPV) or above the highest concentration that had been titrated (e.g. PYY). In such cases, the optimal tetramer titre was selected as the next lowest or highest titre respectively.

3.4.5 Manual gating strategy

A gating strategy was developed to identify the major cell lineages of interest and their relevant subsets. This approach was guided by the canonical cell classifications outlined in Table 3.6.

Immune cell population	Cell surface antigen marker(s)	Surface
Leukocytes	Live/Dead Blue$^-$ CD45$^+$	
CD3$^+$ T-lymphocytes	Live/Dead Blue$^-$ CD3$^+$	
TCR γδ T-lymphocytes	CD3$^+$ TCRγδ$^+$	
CD4$^+$ T-lymphocytes	CD3$^+$ CD4$^+$ CD8$^-$	
CD8$^+$ T-lymphocytes	CD3$^+$ CD8$^+$ CD4$^-$	
Double negative T-lymphocytes	CD3$^+$ CD4$^-$ CD8$^-$	
Naïve CD4$^+$ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^+$ CCR7$^+$	
TCM CD4$^+$ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^+$	
TEM CD4$^+$ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^-$	
TEMRA CD4$^+$ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^+$ CCR7$^-$	
Early-like TEM CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^-$ CD27$^-$ CD28$^+$	
Early TEM CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^-$ CD27$^+$ CD28$^+$	
Intermediate TEM CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^-$ CD27$^+$ CD28$^-$	
Terminal TEM CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD45RA$^-$ CCR7$^-$ CD27$^-$ CD28$^-$	
Naïve CD8$^+$ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^+$ CCR7$^+$	
TCM CD8$^+$ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^+$	
TEM CD8$^+$ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^-$	
TEMRA CD8$^+$ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^+$ CCR7$^-$	
Early-like TEM CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^-$ CD27$^-$ CD28$^+$	
Early TEM CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^-$ CD27$^+$ CD28$^+$	
Intermediate TEM CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^-$ CD27$^+$ CD28$^-$	
Terminal TEM CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD45RA$^-$ CCR7$^-$ CD27$^-$ CD28$^-$	
Activated CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD38$^+$ HLA-DR$^+$	
Activated CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD38$^+$ HLA-DR$^+$	
Senescent CD4+ T-lymphocytes	CD3$^+$ CD4$^+$ CD57$^+$	
Senescent CD8+ T-lymphocytes	CD3$^+$ CD8$^+$ CD57$^+$	

Exhausted CD4+ T-lymphocytes	$CD3^{+}$ $CD4^{+}$ $PD1^{+}$ $LAG\text{-}3^{+/-}$ $TIM\text{-}3^{+/-}$ $CTLA4^{+/-}$
Exhausted CD8+ T-lymphocytes	$CD3^{+}$ $CD8^{+}$ $PD1^{+}$ $LAG\text{-}3^{+/-}$ $TIM\text{-}3^{+/-}$ $CTLA4^{+/-}$
Tregs	$CD3^{+}$ $CD4^{+}$ $CD25^{+}$ $CD127^{lo}$
Naïve Tregs	$CD3^{+}$ $CD4^{+}$ $CD25^{+}$ $CD127^{lo}$ $CD45RA^{+}$
Memory Tregs	$CD3^{+}$ $CD4^{+}$ $CD25^{+}$ $CD127^{lo}$ $CD45RA^{-}$
RTE	$CD3^{+}$ $CD4^{+}$ $CD45RA^{+}$ $CCR7^{+}$ $CD31^{+}$
TfH cells	$CD3^{+}$ $CD4^{+}$ $CXCR5^{+}$
Th1 cells	$CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $CXCR5^{-}$ $CXCR3^{+}$ $CCR6^{-}$
Th2 cells	$CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $CXCR5^{-}$ $CXCR3^{-}$ $CCR4^{+}$ $CCR6^{-}$
Th1/Th17 cells	$CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $CXCR5^{-}$ $CXCR3^{+}$ $CCR4^{-}$ $CCR6^{+}$
Th17 cells	$CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $CXCR5^{-}$ $CXCR3^{-}$ $CCR4^{+}$ $CCR6^{+}$
Total B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$
Unswitched B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ IgD^{+} IgM^{+}
Class-switched B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ IgD^{-} IgM^{-}
Naïve B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ IgD^{+} $CD27^{-}$
Marginal Zone B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ IgD^{+} $CD27^{+}$
Memory B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ IgD^{-} $CD27^{+}$
Regulatory B-lymphocytes	$CD3^{-}$ $CD19^{+}$ $CD20^{+}$ $CD24^{++}$ $CD38^{++}$
Plasmablasts	$CD3^{-}$ $CD19^{+}$ $CD20^{-}$ $CD24^{-}$ $CD38^{++}$
NKT-like cells	$CD3^{+}$ $CD8^{+/-}$ $CD56^{+}$
Total NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{+}$
Early NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD16^{-}CD56^{bright}$
Mature NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD16^{+}CD56^{dim}$
Terminally differentiated NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD16^{+}CD56^{dim}$ $CD57^{+}$
$CD16^{-}$ $CD56^{dim}$ NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD16^{-}$ $CD56^{dim}$
$CD16^{dim}CD56^{dim}$ NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{dim}$ $CD16^{dim}$
$CD16^{+}$ $CD56^{bright}$ NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD16^{+}$ $CD56^{bright}$
$NKG2A^{+}$ KIR^{-} Mature NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{dim}$ $NKG2A^{+}$ KIR^{-}
$NKG2C^{+}$ KIR^{+} Mature NK cells	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{dim}$ $NKG2C^{+}$ KIR^{+}
Classical monocytes	$CD14^{+}$ $CD16^{-}$

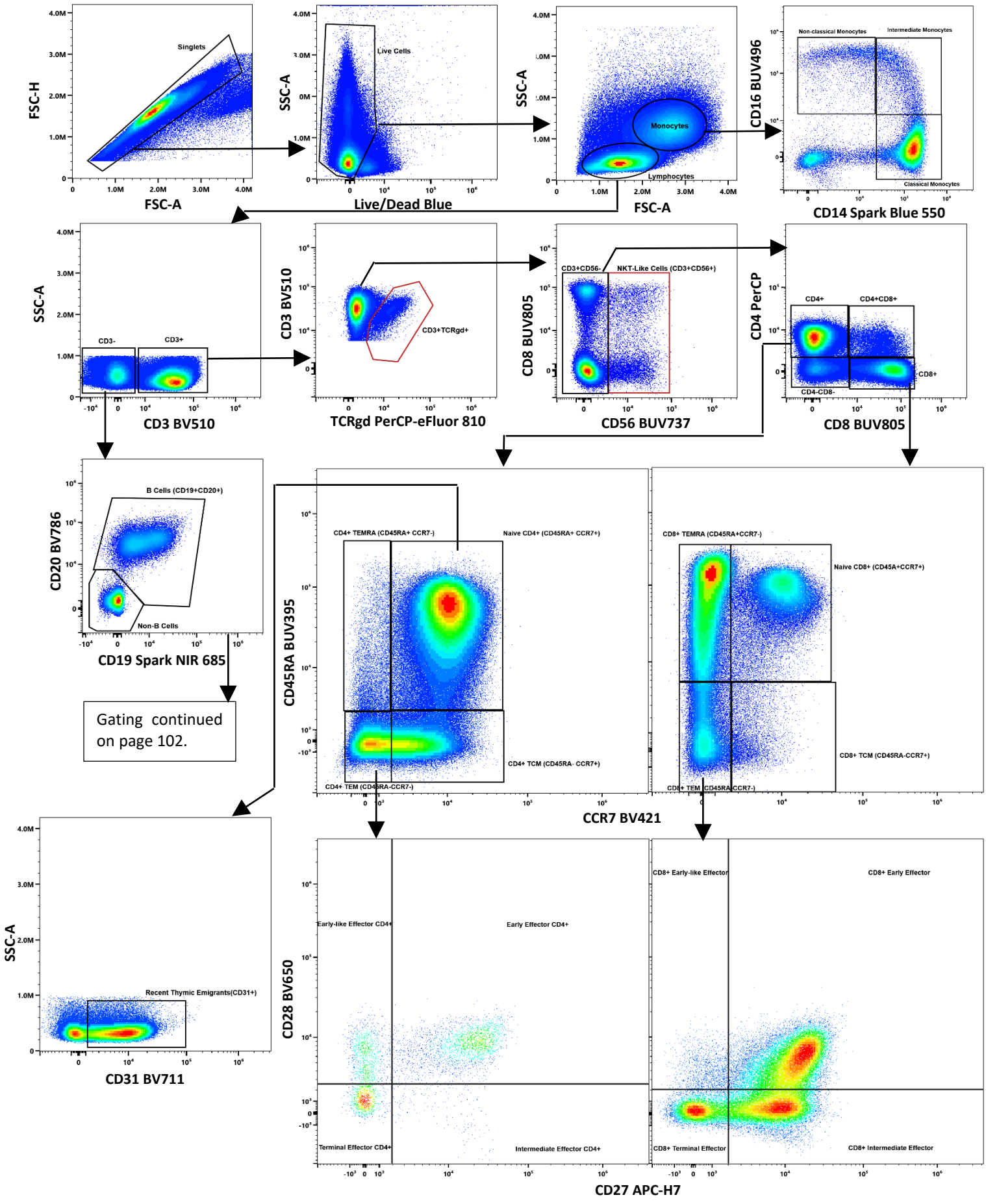
Intermediate monocytes	$CD14^{+}$ $CD16^{+}$
Non-classical monocytes	$CD14^{dim}$ $CD16^{+}$
Conventional dendritic cells (cDCs)	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{-}$ $CD14^{-}$ $CD11c^{+}$ HLA-DR^{+}
Plasmacytoid dendritic cells (pDCs)	$CD3^{-}$ $CD19^{-}$ $CD20^{-}$ $CD56^{-}$ $CD14^{-}$ $CD11c^{-}$ $CD123^{+}$ HLA-DR^{+}
Total ILCs	Lin^{-} $CD127^{+}$

Table 3.6: **Cell surface antigen profiles for immune cells of interest**. **Lin** – Lineage; **NK** – Natural Killer; **RTE** – Recent Thymic Emigrants; **TCM** – Central Memory; **TEM** – Effector Memory; **TEMRA** – CD45RA+ Effector Memory; **Th** – T-helper Lymphocytes; **Tregs** – Regulatory T-Lymphocytes; **TfH** – Follicular helper T-lymphocytes.

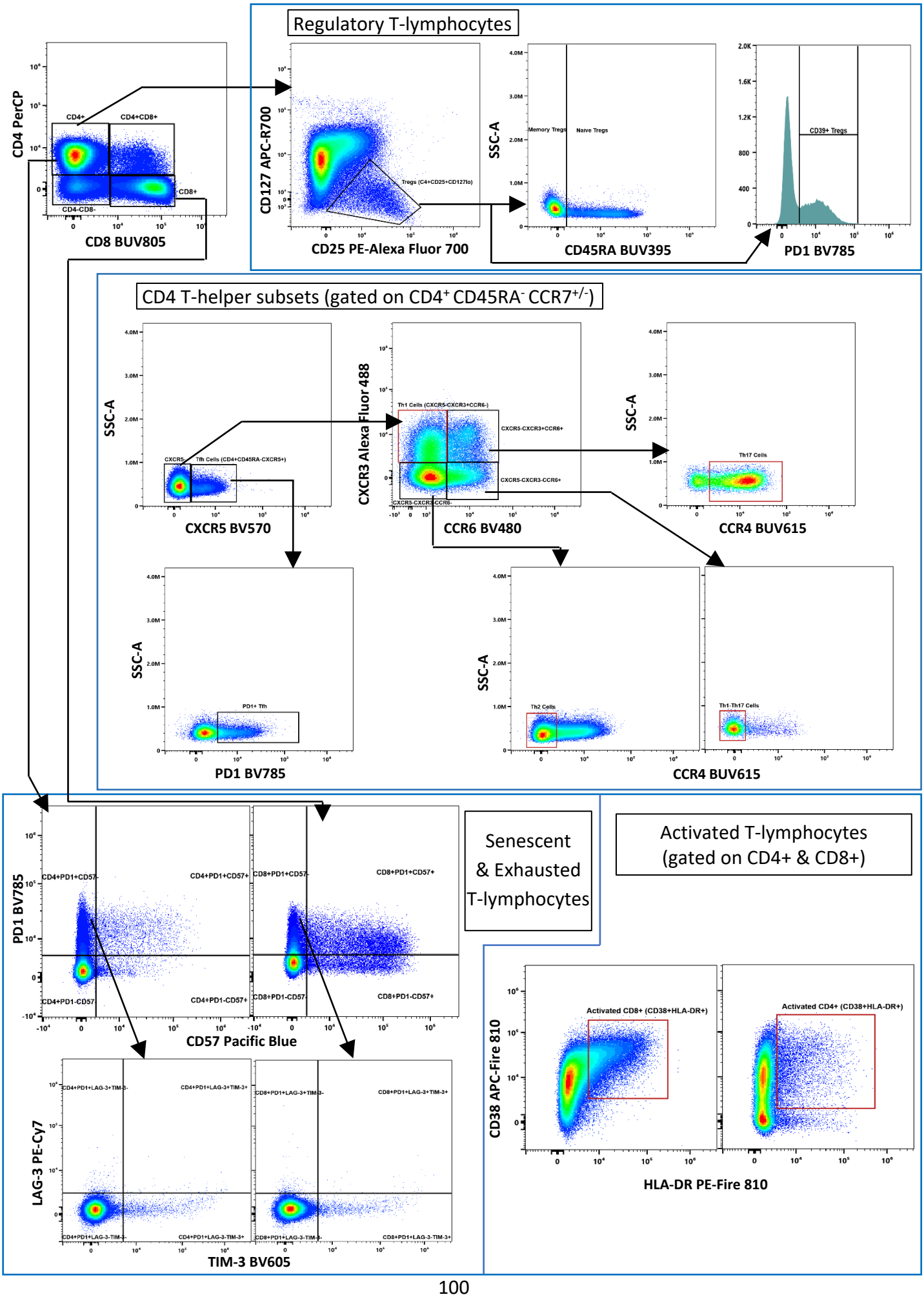
After excluding doublets and dead cells, live lymphocytes and monocytes were identified based on their forward and side scatter properties. T-/B-lymphocytes, NK cells, NKT-like cells, dendritic cells, ILCs and monocytes were then categorised into their relevant subsets based on their surface antigen marker expression as outlined in Table 3.5.

The supervised gating of fully stained healthy adult donor samples was used to validate the robustness of both flow panels in delineating the immune cell subsets considered as central to the study's hypothesis. An unbiased automated analysis pipeline was also deployed within the OMIQ platform to further interrogate the high dimensional data. This consisted of dimensional reduction and data visualisation with uniform manifold approximation and projection (UMAP) in addition to data clustering with Flow cytometry self-organising maps (FlowSOM) to enable statistical analysis. The computational analytic approach and configurations that were used in this study will be discussed in greater detail in chapters 6 and 7.

The broad innate/adaptive immune panel and T-lymphocyte panel were used to analyse cryopreserved PBMCs obtained from patients recruited to the ITHACA study only after the reproducibility of the manual gating strategy had been rigorously tested.



Gating continued on page 102.


Regulatory T-lymphocytes
CD4 PerCP
CD4+
CD4+CD8+
CD8+
CD4-CD8-
CD8 BUV805
CD127 APC-R700
Tregs (C4+CD25+CD127lo)
CD25 PE-Alexa Fluor 700
SSC-A
Memory Tregs
Naive Tregs
CD45RA BUV395
CD39+ Tregs
PD1 BV785
CD4 T-helper subsets (gated on CD4+ CD45RA- CCR7+/-)
SSC-A
CXCR5+
Tfh Cells (CD4+CD45RA-CXCR5+)
CXCR5 BV570
CXCR3 Alexa Fluor 488
Th1 Cells (CXCR5-CXCR3+CCR6-)
CXCR5-CXCR3+CCR6+
CXCR5-CXCR3-CCR6+
CXCR5-CXCR3-CCR6-
CCR6 BV480
SSC-A
Th17 Cells
CCR4 BUV615
SSC-A
PD1+ Tfh
PD1 BV785
SSC-A
Th2 Cells
Th1-Th17 Cells
CCR4 BUV615
Senescent & Exhausted T-lymphocytes
Activated T-lymphocytes (gated on CD4+ & CD8+)
PD1 BV785
CD4+PD1+CD57-
CD4+PD1+CD57+
CD8+PD1+CD57-
CD8+PD1+CD57+
CD4+PD1-CD57-
CD4+PD1-CD57+
CD8+PD1-CD57-
CD8+PD1-CD57+
CD57 Pacific Blue
LAG-3 PE-Cy7
CD4+PD1+LAG-3+TIM-3-
CD4+PD1+LAG-3+TIM-3+
CD8+PD1+LAG-3+TIM-3-
CD8+PD1+LAG-3+TIM-3+
CD4+PD1+LAG-3-TIM-3-
CD4+PD1+LAG-3-TIM-3+
CD8+PD1+LAG-3-TIM-3-
CD8+PD1+LAG-3-TIM-3+
TIM-3 BV605
CD38 APC-Fire 810
Activated CD8+ (CD38+HLA-DR+)
Activated CD4+ (CD38+HLA-DR+)
HLA-DR PE-Fire 810

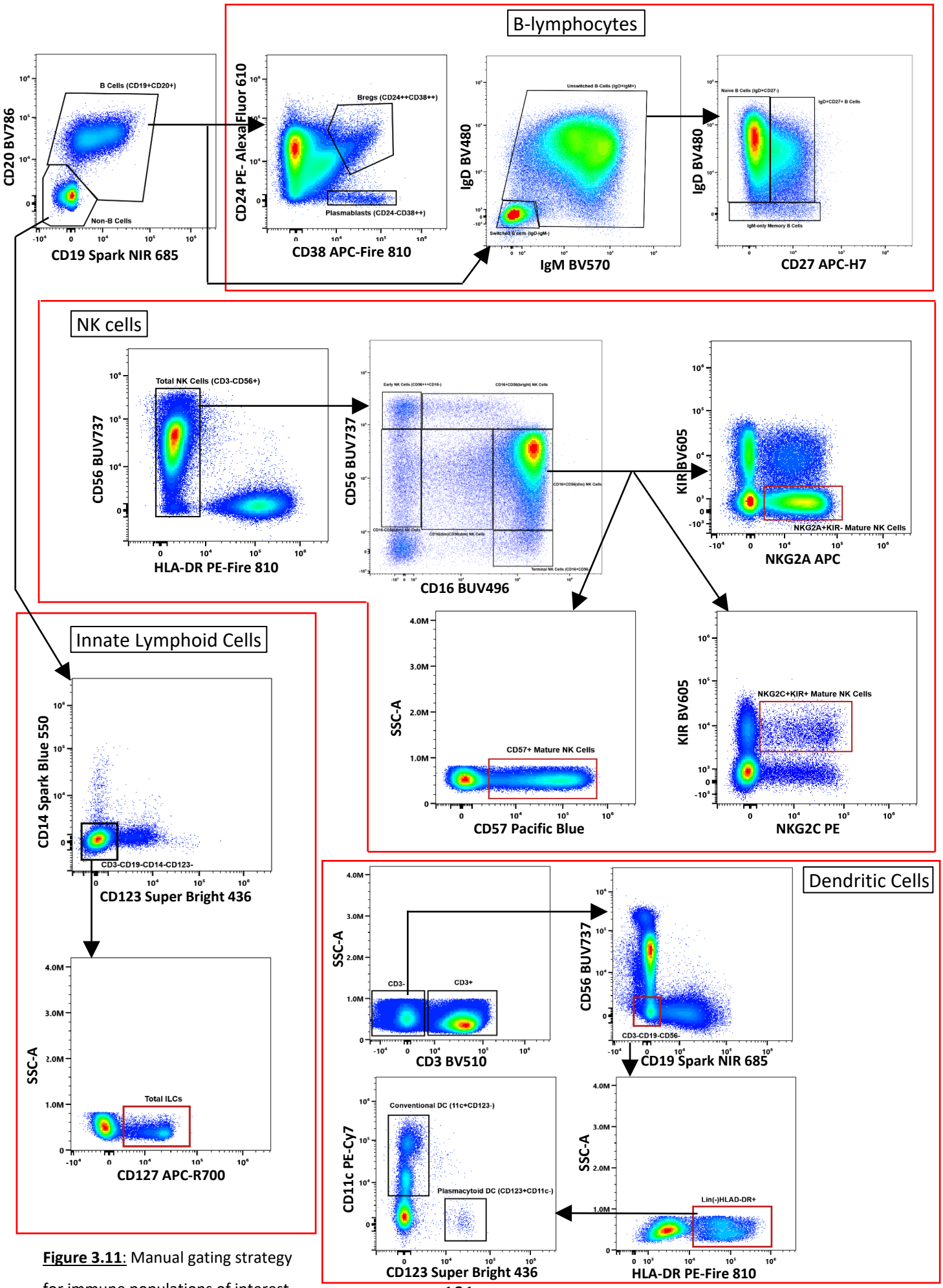


Figure 3.11: Manual gating strategy for immune populations of interest

Chapter 4. Development and implementation of the Immunology of THymectomy And childhood CArdiac transplant (ITHACA) study

4.1 Introduction

A major challenge in addressing the central hypothesis of this project was the limited availability of appropriate biological specimens and relevant clinical data for monitoring EBV immune responses during the peri-transplant period. The ITHACA study, a prospective nationwide cohort study, was developed to circumvent this barrier. The study's key objective was to recruit eligible heart transplant patients and an age-matched non-thymectomy cohort in order to provide an in-depth understanding of the complex interplay between thymectomy, iatrogenic immunosuppression and impaired EBV-specific immunity, and how these factors support an immune microenvironment that drives PTLD development. This chapter elucidates the integral stages of the study's design, its conduct and the necessary amendments made during the study period to achieve the project's aims and objectives.

4.2 Study development and initiation phase

4.2.1 Study aim and objectives

The first step in designing the ITHACA study was to establish its aim and objectives. This was needed to ensure that the central hypothesis of the project would be addressed in full.

- **Study aim**

I. To investigate the systemic immune profile and EBV-specific immunity of early versus late/non-thymectomised transplant patients.

- **Primary study objectives**

I. To dissect the peri-transplant innate and adaptive immune cell populations and their temporal responses to EBV infection in early versus late/non-thymectomised patients.

II. To define the peri-transplant EBV-specific immunity in early versus late/non-thymectomised patients.

- **Secondary study objectives**

I. To detect peri-transplant immune signatures that either promote or inhibit PTLD development.

II. To identify potential biomarkers that can be used for patient risk stratification in future clinical trials of patients with lymphoproliferative disorders.

4.2.2 Study design

The ITHACA study was designed as a non-interventional multicentre longitudinal cohort study. Peripheral blood samples from study recruits were collected pre-transplant and at 3, 6, 12 and 24-months post-transplant (Figure 4.1). In the case of a prolonged interval between the pre-transplant blood sample and subsequent organ transplant (e.g. >6 months) additional blood samples were specifically requested from the patient/parent and verbal consent recorded in the patient's medical record.

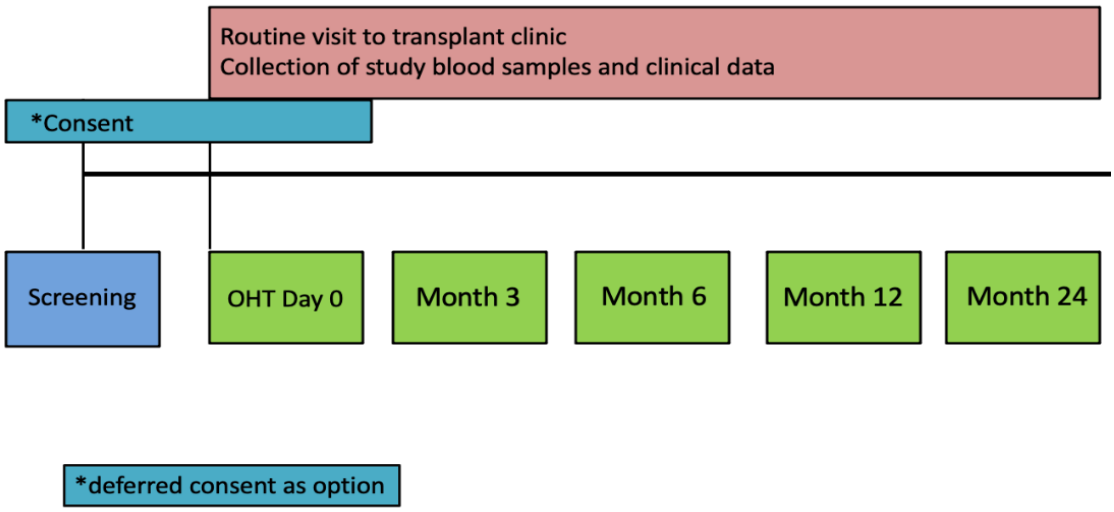


Figure 4.1: ITHACA study flow chart. **OHT** – Orthotopic heart transplant

Procedures for blood sample collection and evaluation of EBV serology were followed as outlined in chapter 2 (sections 2.2.1 and 2.2.2). Clinical data were collected by local study teams using a purpose-built electronic case report form (eCRF). Immunosuppressive therapy was adjusted according to clinical needs based on standard local protocols. Any deviations from the protocol were documented in the eCRF. All medical therapies were applied as clinically indicated and as per the local post-transplantation protocols. No additional interventions were proposed by the ITHACA study protocol.

4.2.3 Defining study participants

Paediatric heart transplant patients constituted the main target population for study recruitment. In order to investigate the interplay between thymectomy and immunosuppression on EBV immunity, the scope of study participants was broadened to include a small cohort of similar-aged non-thymectomised transplant patients. Paediatric renal transplant patients were selected to comprise the non-thymectomy transplant group due to the local availability of these patients and published evidence that renal transplantation is associated with the lowest incidence of PTLD after childhood solid organ transplant.[397] The eligibility criteria for the ITHACA study was established once study participants had been defined.

Inclusion Criteria

- Aged 0 – 18 years.
- Actively listed on the NHSBT waiting list for a primary organ transplant OR awaiting transplant with a living related donor kidney OR recently transplanted with pre-transplant blood samples available.
- Written informed consent.

Exclusion Criteria

The participant may not enter the study if ANY of the following apply:

- Has a pre-existing diagnosis of an inherited or acquired immunodeficiency.
- Has an underlying thymic disorder.
- Has previously received a bone marrow or organ transplant.
- Has had a previous cancer diagnosis.
- Withheld consent.
- Weight under 2.5kg.

Withdrawal Criteria

Due to the observational nature of the study and negligible risk to the health of study participants, it was decided that study withdrawal would occur only upon patient and/or parental request.

4.2.4 Identification of study participants

Eligible participants were identified when they presented to the paediatric transplant services at NuTH or GOSH as listed candidates for urgent cardiac or renal transplantation on the NHSBT waiting list. Of relevance, NuTH and GOSH are the only centres commissioned to provide paediatric heart transplant services in the UK. The involvement of both centres in the study was therefore crucial for the optimisation of participant recruitment. All patients who met the study inclusion criteria were deemed eligible and approached regarding participation in the study by members of either the local transplant or study teams.

4.2.5 Participant enrolment and informed consent

All members of the ITHACA study team were required to have up-to-date training in Good Clinical Practice (GCP) prior to inclusion on the study's delegation log. Each member was assigned to specific study-related tasks (e.g. patient screening/recruitment, obtaining informed consent, data collection, etc). All members of the study team were responsible for explaining the purpose of the study to patients/carers and providing opportunity for questions and further discussion as necessary. Key points that were reiterate to the patient/carer included:

- Participation would not affect the child's treatment and would not benefit them/their child during the study but could benefit other children in the future.
- Participation in the study was voluntary and the volunteer could withdraw at any time without any explanation being necessary.

The patients/parents/guardian were given patient information sheets (PIS) along with clear guidance on how to contact the research team. They were also informed that if consent was obtained, any leftover blood sample would be stored in the laboratory and added to the Newcastle Biobank at the end of the study. These samples could then potentially be used for future ethically approved research. Informed consent was obtained from the parents/legal guardians of children aged < 16 years or directly from young people aged 16-18 years. Children aged < 16 years were given the opportunity to provide assent if they wished.

The capacity to use a deferred consent approach was included for eligible study participants who attend a research site's transplant service for transplantation in a critical/life threatening clinical situation. This involved the collection of baseline study samples from eligible participants at the point of pre-surgical workup without written informed consent being received. Discussion about the study,

the giving of PIS and receiving of written informed consent/assent were offered at a more appropriate time before further follow-up blood tests were taken or clinical data collected. Such cases were at the discretion of the local transplant team if they considered obtaining informed consent prior to transplant to be inappropriate. This was documented in the patient's clinical notes. Study samples collected under such circumstances were processed for storage but not analysed until written informed consent was obtained. Allowance was made for any patient who had study samples collected by deferred consent but subsequently declined enrolment in the study to have their samples destroyed in a timely manner according to local laboratory standard operating procedures (SOP).

Every effort was made to maintain participant privacy. Collation of clinical information was restricted to delegated members of the study team who had NHS privileges. Only minimal patient identifiable information was collected about study participants to allow validation of data across study time points. Access to data across all study participants was restricted to Dr Ugo Offor (co-investigator) and Dr Simon Bomken (chief investigator). Once data collection had been completed and audited to ensure accuracy, it was fully anonymised and exported for ongoing analysis. Patient samples were identified using only the study identification number (prefix of 'NUTH' for Newcastle recruits and 'GOSH' for London recruits). No patient identifiers were made available to non-NHS staff or in research documentation. Similarly, only semi-anonymised patient information for GOSH recruits was made available to the co-investigator and chief investigator (CI). A key linking patient identifiers and study ID numbers for NuTH recruits is currently held by Dr Ugo Offor and the CI on a password protected, secure NHS server. Similar data protection measures were also implemented by the local CI at GOSH (Dr Jacob Simmonds).

4.2.6 Study sample size

A sample size calculation wasn't required to power this study due to the descriptive nature of its objectives. Instead, the sample size was selected to ensure that the study was feasible and large enough to conduct comprehensive analyses. An initial sample size of 40 children was chosen to consist of 34 prospective cardiac transplant patients and 6 renal transplant patients who would be recruited over a 2-year period. This sample size was based on the national average of 30 childhood cardiac transplants per year, a local average of 5 childhood renal transplants per year and a 60% recruitment rate.[398, 399] The study sample size was eventually amended to consist of 55 prospective

transplant patients due to factors identified during the study's recruitment phase. The reasons for this amendment are discussed more extensively in section 4.3 of this chapter.

4.2.7 Study setup and initiation

A timeline of ITHACA's development and setup process is illustrated in **Table 4.1**.

	2020	2021							2022					2023
Objectives	Nov	Feb	May	Jul	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	Dec	Feb
Patient & Public Involvement and Engagement (PPIE)														
Study funding awarded														
Writing study protocol, PIS, consent/assent forms														
Study sponsorship agreement with NuTH														
IRAS submission														
Development of eCRFs														
REC approval														
HRA approval														
Agreement for microbiology lab support from NuTH														
Capacity and Capability approval from NuTH														
ITHACA open to patient recruitment (NuTH site)														
ITHACA open to patient recruitment (GOSH site)														

Table 4.1: Timeline of ITHACA study development and setup. **eCRF** – Electronic case report form; **GOSH** – Great Ormond Street Hospital for Children; **HRA** – Health Research Authority; **IRAS** – Integrated Research Application System; **NuTH** – Newcastle upon Tyne Hospitals NHS Foundation Trust; **PIS** – Patient Information Sheet; **REC** – Research Ethics Committee.

4.2.7.1 Patient and Public Involvement and Engagement (PPIE)

An important aspect of the study design was ensuring that the voice of young people was embedded early in the study's development and delivery phase. For this reason, the ITHACA study was developed in close collaboration with the Young Person's Advisory Group – North England (YPAGne). YPAGne is a patient advisory group consisting of children and young people aged 10-18 years, who help plan and shape research projects to ensure that they are relevant to a paediatric population.

The first meeting with YPAGne was held on 12th November 2020. This was a virtual online meeting due to the national lockdown during the COVID-19 pandemic. A presentation summarising the study

rationale, aim and objectives and proposed study procedures was delivered to a YPAGne focus group. Details of the questions asked to the group and their recommendations can be found in appendix A. Appropriate and acceptable study procedures were then defined based on feedback received during the focus group discussions. These included:

a) Support for a deferred consent process to be used for critically unwell patients. This process is explained in greater detail in section 4.2.5.
b) Recommendation that study-related investigations coincide with routine patient visits to transplant clinic in order to mitigate excessive blood sampling and patient discomfort.

In addition, age-appropriate lay summaries of the study were developed as a component of the patient information sheets (PIS). A further meeting with YPAGne was held on 8th December 2022 in which a presentation summarising progress with study recruitment and proposed amendments to the consenting process was given. The advice provided by YPAGne was used to further refine the study's methodology.

4.2.7.2 Writing of study protocol, PIS and consent/assent forms

The ITHACA study protocol was written by Dr Ugo Offor and Dr Simon Bomken between October 2020 and July 2021 with further substantial amendments made in February 2022, August 2022, March 2023 and February 2024 (details of these amendments can be found in section 4.3). The study protocol served as an important guide that described the study's rationale, objectives, methodology, investigative procedures and ethical considerations in comprehensive detail. This was done in accordance with GCP recommendations to ensure harmonisation of study-related practices across the NUTH and GOSH research sites. The study protocol was peer-reviewed and published as an open-access journal article in line with the aforementioned recommendations (appendix B).

All PIS and consent/assent forms were formulated based on guidance from the Health Research Authority (HRA) on research involving children and young people. As highlighted in the previous section, these documents were written with significant PPIE input from YPAGne. The PIS documents were created for the following age groups: (6-10 years; 11-15 years; 16-18 years; Parents/Guardians, reflecting the expected chronological progression in cognitive development and capacity to participate in discussions about voluntary participation in clinical research. The final version of these documents can be found in appendix C. Recommendations from the HRA and YPAGne that were implemented in the design of PIS documents include:

I. **Separation of the PIS into two parts:** Part 1 of the PIS consisted of a lay summary of the study and an invitation to the child/young person to read part 2 of the PIS if they were interested in taking part. Part 2 provided comprehensive details of the study using the principle of the 5 W's (What, Where, When, Why, Who). This mitigated the risk of overloading patients and their families with study-related information while also giving them more autonomy during the recruitment process.

II. **Use of bright colour schemes and pictorials:** This was particularly emphasised for the 6-10 years age group. Cartoons and illustrations were used to provided context to study methods and procedures that had been outlined in the PIS. PPIE feedback from YPAGne was instrumental in selecting an appropriate colour scheme that would be acceptable to most children.

III. **Enhancement of PIS readability:** The layout of PIS documents for the 6-10 years and 11-15 years age groups was formatted to convey study-related information in bitesize portions. This avoided long unbroken sections of text, which in turn helped to enhance the readability of the PIS. This was also done to improve patient engagement with the study material.

IV. **Adoption of an age-specific approach to PIS content:** The inclusion of study participants aged 0-18 years underscored the importance of developing PIS documents whose contents were tailored to the expected intellectual capacity for the patient's age. Not only was this reflected in the variance of lay terminologies used to explain study procedures (e.g. the use of "small amount of extra blood" versus "extra 3 teaspoons of blood" in describing the volume of study samples to the 6-10 years and 11-15 years age groups respectively), but also in the actual content of the information provided. Of note was the inclusion of information pertaining to General Data Protection Regulation (GDPR) and contact details for the study sponsor's data controller in only the 16-18 years and parents/guardians PIS. The HRA have only recently developed age-appropriate literature on GDPR that can be included in PIS documents for children under 16 years.[400] The generic content that was available on their website at the time of developing this study's PIS documents was considered by YPAGne to be too difficult for young children to comprehend. While explicit details on data handling were omitted from the under-16 PIS documents, key concepts of patient privacy and confidentiality were highlighted. Further discussions about data protection were conducted at the discretion of the patient's family.

4.2.7.3 Development of Electronic Case Report Forms

Electronic Case Report Forms (eCRFs) for data capture were developed between September 2021 and January 2022. The REDCap database, which is licensed through the Newcastle Joint Research Office (NJRO), was used for this purpose. REDCap is a secure web-based platform that is purpose-built to support data acquisition for research studies. It is hosted on servers with security exceeding NHS specifications. [401] The use of this platform not only permitted the protection of patient identifiable information but also provided an audit trail feature for tracking data handling and export. The eCRFs were designed to collate relevant clinical data obtained during the study-related visits outlined in Figure 4.1 (section 4.2.2). All clinical variables that were collected are summarised in Table 4.2. The template for the eCRF can be found in appendix D.

	CATEGORY	CLINICAL DATA
I.	**BASELINE PATIENT DETAILS**	Age at transplant, Age at thymectomy, Cardiothoracic procedure at time of thymectomy, Sex at birth, Ethnicity, Weight, Height, Comorbidities prior to transplantation, Concomitant medication at time of transplantation
II.	**TRANSPLANT-RELATED**	Cardiac/Renal diagnosis, Indication for transplant, Blood group compatibility
III.	**INFECTION-RELATED**	EBV and CMV (where available) serostatus of donor & recipient at transplant
IV.	**IMMUNOSUPPRESSION**	Induction therapy, maintenance immunosuppression drugs (dose and trough levels at each follow up visit), changes to maintenance immunosuppression drugs and indication for changes
V.	**COMPLICATIONS**	Opportunistic infections, Graft failure/rejection, PTLD, Mortality, documentation of "other" complications
VI.	**FOLLOW UP**	Weight, Height, Additional medication, Relevant additional comments (also see categories IV & V)

Table 4.2: Clinical information accrued for all study participants at each study timepoint.

4.2.7.4 Study funding and sponsorship

External funding for the study setup, study-related investigations, digital database for data collection, and data storage was successfully obtained through national competitive application for a Cancer Research UK Clinical Research Fellowship (obtained on 15th February 2021) and an early-career researcher grant from the Lymphoma Research Trust (obtained on 18th May 2021). A study sponsorship agreement with NuTH was ratified on 28th July 2021.

4.2.7.5 Research Ethics Committee and Health Research Authority approvals and other study-related agreements

The ITHACA study protocol and other study-related documents were submitted for ethical review via the Integrated Research Applications System (IRAS) portal on 13th October 2021 (IRAS project ID: 298986). Research Ethics Committee (REC) approval (REC 21/NS/0142) was obtained from the North of Scotland REC on 15th November 2021. HRA approval was obtained on 24th November 2021. Agreement from the integrated laboratory medicine directorate of NuTH was obtained on 10th December 2021 for microbiology support with processing viral serology samples. Final study capacity and capability approval was obtained from NuTH on 25th February 2022.

4.2.7.6 Capability and capacity planning

Several strategic meetings were held during the initiation phase of the ITHACA study to ensure that adequate support for its successful delivery was available to the clinical and laboratory teams. These sessions continued across the lifetime of the study's recruitment phase. Planning sessions were held with the transplant teams at NuTH and GOSH, as well as the virology and paediatric intensive care unit (PICU) team at NuTH. These meetings were used to raise awareness of the study among members of the clinical teams, adapt study processes to fit local pathways for patient identification/recruitment, study procedures and follow up, and to identify potential pitfalls that required mitigation. This process helped to guide the development of study packs, study-related lab forms and a study flow chart poster that was put up in clinical areas (appendix E). In addition, a monthly catch-up meeting with the transplant teams was held on the first Friday of every month to monitor patient recruitment and identify study-related issues. The outcome of these meetings informed the substantial amendments that were implemented to the study methodology during its recruitment phase as discussed in the next section of this chapter.

4.3 Study amendments

4.3.1 Substantial amendments

Four substantial amendments were made to the ITHACA study between February 2022 and February 2024. All amendments were discussed with the study sponsor and obtained favourable REC and HRA approvals prior to their implementation.

III. **Substantial Amendment 1**: This consisted of procedural changes that allowed PIS documents to be sent out by post/email to potential study participants in addition to face-to-face discussions about the study. It mirrored the increased use of tele-communication between the transplant teams and out of area patients as a consequence of the COVID-19 pandemic. In addition, the study inclusion criteria were broadened to include renal transplant patients who were listed to receive an organ from a living related donor. This subgroup made up approximately 50% of renal transplant patients and did not meet the original inclusion criterion of being listed on the NHSBT register. The amendment was submitted for REC and HRA approval on 19th January 2022 and obtained a favourable opinion on 7th February 2022.

IV. **Substantial Amendment 2**: This amendment sought to improve the consenting process to reflect the significant clinical urgency encountered at the point of a patient's admission for transplantation. The initial pathway for obtaining informed consent had consisted of a single nodal point during which patients and their families were approached for consent at the time of hospital admission for transplantation. The approach was reviewed during the early stages of the recruitment phase and identified as a potential barrier to participant enrolment. This was based on feedback from the transplant teams, who highlighted the difficulty in co-ordinating an additional consenting process during a period when the patient and their family were also being approached by multiple clinical teams (transplant co-ordinators, cardiology team, surgeons, anaesthetists, PICU, etc) for various discussions and consent. To this effect, a two-stage consenting procedure was established. This consisted of an initial "screening consent" that would be taken from eligible patients prior to their admission for transplant. The initial study samples would be taken and stored at the time of admission followed by "confirmatory consent" that would be taken post-transplant to permit analysis of the samples. Withheld consent at this point would result in existing blood samples being destroyed. This amendment was submitted on 8th July 2022 and received the necessary favourable approvals on 2nd August 2022.

V. **Substantial Amendment 3**: The aim of this amendment was to withdraw the screening consent form and revert back to a single step for obtaining informed consent. Following the introduction of the screening consent process, pre-transplant study samples had been collected for a number of patients who were still awaiting an organ transplant. This highlighted the significant scientific value of the pre-transplant blood samples within the study for those children who wouldn't proceed to transplant during the study period. The study protocol, PIS and consent forms were amended to remove the two-step consent process and allow the use of pre-transplant study samples and clinical data in all eligible patients followed by the collection of post-transplant samples and data in those who proceed to transplant. The substantial amendment was submitted on 16th March 2023 and given favourable REC and HRA opinions on 21st March 2023.

VI. **Substantial Amendment 4**: The final substantial study amendment sought to increase the study's sample size to a maximum of 55 patients with a focus on expanding the cohort of study recruits with congenital heart disease/early thymectomy. This was done to ensure a better balance with the proportion of study recruits who had acquired cardiomyopathy (ACM)/late thymectomy and permit more robust patient stratification required to achieve the study's objectives. The amendment was submitted on 19th February 2024 and received favourable REC and HRA approvals on 15th March 2024.

4.3.2 Minor amendments

Two minor amendments were made to the study between December 2022 and September 2023. These were discussed with the study sponsor and didn't require REC or HRA approval prior to their implementation. Both research bodies were however informed about these study changes in keeping with statutory requirements.

I. **Minor Amendment 1:** This updated the study's inclusion criteria to allow the recruitment of non-UK patients. It reflected the fact that paediatric cardiac transplant services within the UK is accessible to patients who are normally resident in the Republic of Ireland under the Health Service Executive's Treatment Abroad Scheme. This amendment was made on 22nd December 2022.

II. **Minor Amendment 2:** This amendment was made on 19th September 2023. It extended the end date for study recruitment from 31st December 2023 to 30th June 2024 and increased the study's sample size from 40 participants to a total of 50 children. This was required in response to the slow rate of recruitment at the start of the study and a need to ensure that a sufficient number of patients were recruited to address the primary objectives of the study.

4.4 Quality assurance of study samples

Due to the longitudinal design of the study and its methodological requirement for batch analysis of cryopreserved samples, it was imperative that the effects of sample transport time and duration of cryopreservation on PBMC viability and maintenance of major immune cell proportions were evaluated. This was of particular importance for study samples taken from patients at the GOSH study site as these samples required overnight shipment to Newcastle University in Royal Mail Safeboxes™ for processing prior to downstream analysis. Up to 60ml of whole blood was taken at a single venepuncture from 3 healthy adult volunteers and immediately transferred into 6ml EDTA vacutainer blood collection tubes. Samples were split into aliquots of 5mls to mimic the expected small volumes of study samples from a paediatric cohort. Three aliquots of each sample (i.e. triplicate analysis) were either processed within 24 hours of sample collection or after being stored at room temperature without agitation for 25–36 hours, 37–47 hours and 48 hours or more post-venesection. This was done to represent sample transport time and potential delays that might be encountered in the processing of samples shipped from GOSH. PBMC isolation and cryopreservation were performed using study methods described in chapter 2 (see section 2.2.4). Aliquots of PBMC samples from one healthy adult volunteer were thawed for similar quality assessment including the estimation of immune cell lineage proportions after 3-, 6-, 9-, and 12-months of cryopreservation at -150^{o}C. The procedures described below were used to define a threshold for “high quality” that all samples were required to meet for inclusion in any downstream cytometric and functional analysis.

4.4.1 Evaluating the effects of transport time on sample processing and PBMC viability

The effect of sample transport time on cell separation by density gradient centrifugation was conspicuous across the samples. While a clear PBMC layer could always be visualised for samples processed within 24 hours of venesection, there was a slight but noticeable contamination of this layer with haemolysed red cells in samples processed between 25-47 hours post-venesection and very prominent contamination if processed 48 hours or more after sample collection (Figure 4.2).

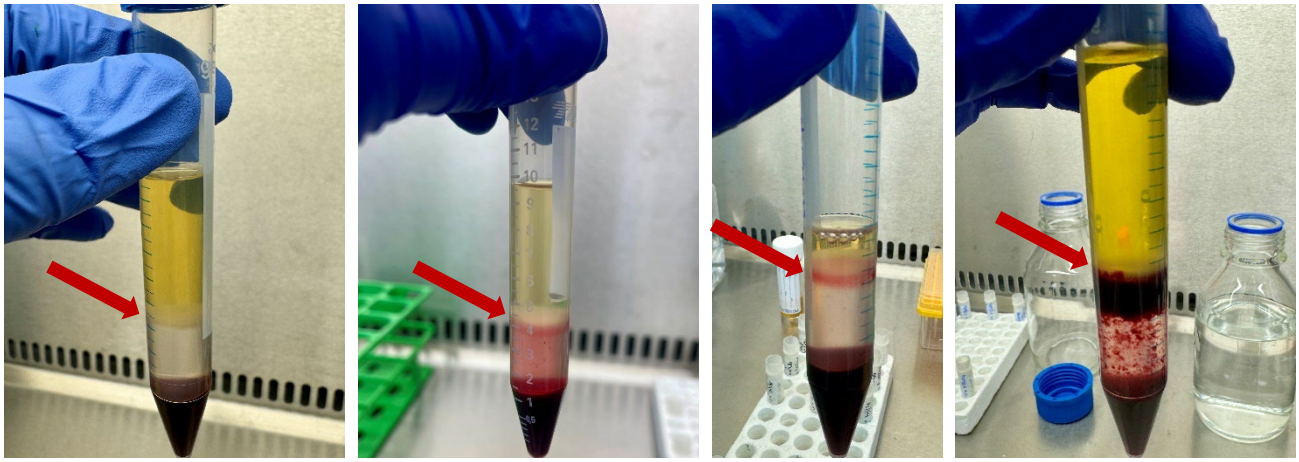


Figure 4.2: Illustration of the effects of sample storage time on cell separation by density gradient centrifugation. The red arrow highlights the clear PBMC layer seen in samples processed within 24 hours of venesection and the progressive increase in contamination of this layer with haemolysed red cells over time.

Sample transport time was also found to have an effect on cell viability. As expected, samples that were processed within 24 hours of collection had the highest recovery of viable cells while those that were processed at 25-36 hours and 37-47 hours post-venesection had comparable albeit slightly lower percentages of viable cells. Notably, there was a significant decline in cell viability for all samples that were processed 48 hours or more after collection. This is illustrated in Figure 4.3.

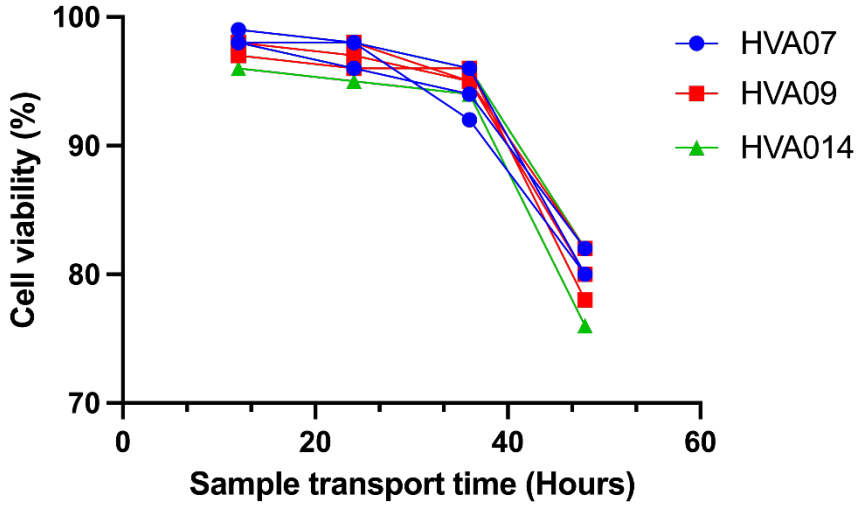
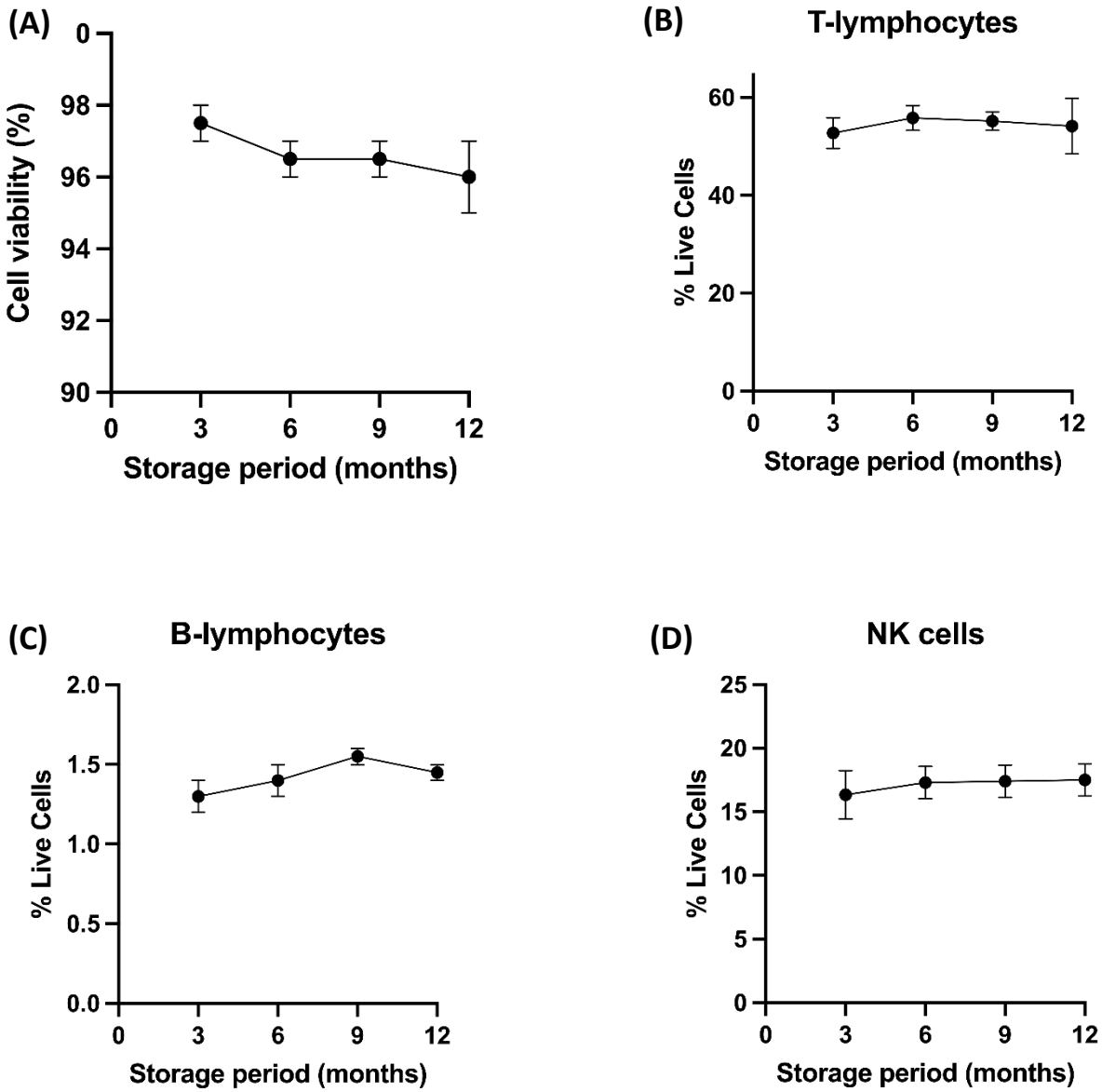


Figure 4.3: Effects of sample transport time on the retrieval of viable cells. Analysis of 3 healthy adult volunteer samples in triplicates shows a relatively robust recovery of viable PBMCs (>90%) from samples processed less than 48 hours after sample collection. Cell recovery from samples processed after this time point was much less effective.

4.4.2 Evaluating the effects of sample storage time on PBMC viability and the proportion of major immune cell lineages

The next step in establishing quality assurance for study samples was to determine the impact of sample storage time on cell viability and the overall proportion of T-/B-lymphocytes, NK cells, monocytes and dendritic cells. For this purpose, aliquots of cryopreserved samples from a single healthy adult volunteer (HVA014) that had processed less than 48 hours after collection and stored for up to 12 months at -150^{o}C were analysed in triplicate. Cell viability remained relatively stable for up to 12 months after being cryopreserved (Figure 4.4). Similarly, there was minimal effect on the proportions of major immune cell lineages across the various storage time points (Figure 4.4).



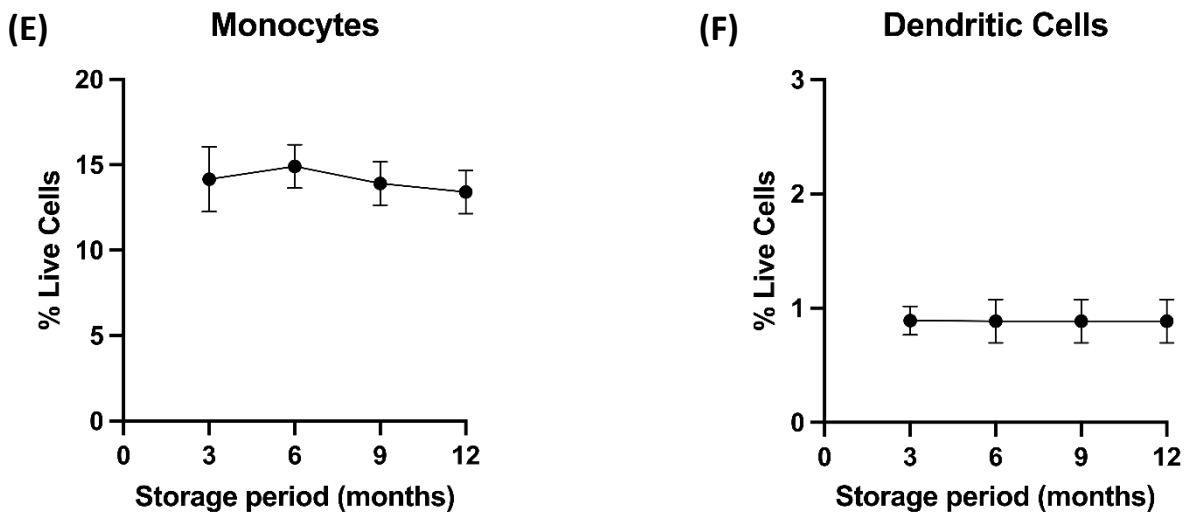


Figure 4.4: Effects of storage time on PBMCs. (**A**) Triplicate analysis of PBMCs isolated from a healthy adult volunteer (HVA014) shows good preservation of cell viability (>90%) when thawed up to 12-months after cryopreservation. (**B – F**) A similar stability in the proportions of all major immune cell lineages was observed. Each line graph shows mean percentages with error bars representing standard deviation (SD).

4.5 Discussion

The ITHACA study was designed as a novel scientific approach to examine and sequentially monitor innate and adaptive immune responses to EBV infection within a group of patients at high risk of PTLD, while simultaneously exploring how this contributes to the disease process. The early engagement of YPAGne as a PPIE partner ensured that all study objectives, methods, and procedures were appropriate and acceptable to the target population. The positive impact of PPIE is reflected by the near universal uptake of the ITHACA study by eligible patients who were approached about participation in the study. The outcome of participant recruitment/enrolment is discussed in further detail in chapter 5 of this thesis.

The comprehensive assessment of EBV immunity in children with a heart transplant presents a unique opportunity to fully understand the health impact of routine early thymectomy during major childhood heart surgery, a procedure whose consequence has never been explored within the transplant population. This study also addresses clearly unmet needs of childhood cancer research by providing highly detailed immunological profiles of paediatric heart transplant patients, identifying systemic peri-transplant immune patterns associated with the risk of developing PTLD and potential mechanisms underpinning the pathogenesis of PTLD. Furthermore, it offers an in-depth understanding of the complex interplay between thymectomy, iatrogenic immunosuppression and impaired EBV-specific immunity and how these factors may support an immune microenvironment that drives PTLD development. This helps to illuminate key differences between EBV-related PTLD in immunosuppressed patients and other EBV driven B-cell lymphomas in immune competent patients, thus leading to a better understanding of viral oncogenesis. The outcomes of this study are also vital in facilitating future patient risk stratification as well as the development of more effective immune-based treatments for PTLD.

Prospective clinical research that involves the shipment of biological specimen from multiple study sites for centralised processing can often encounter logistical practicalities that result in delays between sample collection and processing. This can have important consequences for the sufficient recovery of viable cells that are needed for downstream analysis. While previous studies have proposed a peak window of 8-12 hours between venesection and the processing of PBMCs from whole blood samples, [402, 403] this is impractical for multicentre clinical studies that employ centralised processing of patient samples and for translational research where the timing of sample collection can't always be predicted (e.g. during hospital admission for critical illness or

urgent/emergency surgical procedures). Much of the literature to date has explored the effect of delayed sample processing by up to 24 hours on the viability of PBMCs. These works have all shown that PBMC viability remains stable during this period, particularly if stored at room temperature. [402, 404-406] Complementary studies have provided robust evidence that the period of cell viability can be extended further by optimising sample handling, storage temperature and the choice of anticoagulated specimen tube for shipment. For example, no significant decline in cell viability was observed after delays in sample processing of up to 48 hours when samples are stored at room temperature without agitation.[407] Furthermore, the use of EDTA anticoagulated specimen tubes has been shown to help preserve cell viability.[407, 408] One problematic consequence of prolonged delays in the processing of samples involves the increasing contamination of the PBMC layer with red cell and granulocytes, particularly if this is delayed for up to 24-48 hours or more.[406, 409, 410] This can sometimes pose difficulties with cell retrieval and the accuracy of cell counting.

There is contradictory evidence on the combined impact of sample processing time and cryopreservation on the composition and functionality of immune cells. Studies using high dimensional cytometry have shown that the proportion and functional status of major cell lineages and their relevant subsets aren't significantly altered in cryopreserved specimen that have been stored at room temperature for 24-48 hours. [408, 410] T-lymphocytes have been reported as being even more resilient to delays in sample processing of up to 72 hours. [410] Similarly, IFN-γ production by T-lymphocytes has been shown to remain mostly unaffected by delays of up to 48 hours in sample processing.[409] This is in contrast to other studies that demonstrate a progressive change in cell composition (e.g. increased CD3+ T-lymphocytes, reduced B-lymphocytes/NK cells/monocytes/DCs) and impaired cytokine production by T-lymphocytes as early as 6-12 hours after sample collection. [402, 411] Crucially, it must be noted that these studies utilised vastly different anticoagulated specimen tubes, storage states (agitated versus resting), density gradient media, PBMC isolation techniques, freezing media and thawing processes, all of which are known to have an impact on PBMC stability and could explain the inconsistencies in their findings.

The methodological considerations that were undertaken for quality assurance in this project demonstrate the preservation of cell viability in whole blood samples when stored in EDTA - containing specimen tubes without agitation and at ambient room temperature for less than 48 hours. Similarly, the composition of major immune cell lineages in cryopreserved samples remained stable after an extended period of storage. The outcomes of this evaluation indicate that the study's

design and optimised methods for the collection, processing, storage and downstream experimental analyses are suitable for the study of high-quality patient samples collected across this multicentre trial.

4.6 Conclusion

The ITHACA study was developed and conducted with strict adherence to all statutory requirements proposed by the regulatory bodies responsible for the ethical conduct of non-interventional clinical research in children and young people. Study procedures were refined based on valuable recommendations from YPAGne, which contributed to the high study uptake among eligible patients. The quality assurance measures that were undertaken for this study are highly indicative of the scientific robustness and reliability of experimental techniques used in the downstream analysis of patient samples processed within 48 hours of collection and cryopreserved for a prolonged period of time.

Chapter 5. Characteristics of the ITHACA study cohort and control group

5.1 Materials and Methods

A total of 55 children were recruited to the ITHACA study between 1st March 2022 and 30th June 2024. One patient (GOSH013) was subsequently excluded from the study due to the unavailability of samples for both pre-transplant EBV serology and immune profiling. A total of 44/54 (81.5%) patients recruited to the ITHACA study had undergone a solid organ transplant by 30th June 2024.

Pre-transplant data were analysed independent to post-transplant study timepoints to account for ITHACA recruits who hadn't proceeded to transplantation at the time of data analysis. This intuitive approach also permitted a better distinction between the impact of thymectomy alone versus the combined effect(s) of thymectomy and post-transplant immunosuppression on the immunological landscape of study participants. The analysis of post-transplant data has primarily focussed on the 3- and 6-month study timepoints (i.e. < 12-months post-transplant) for the purpose of this chapter. This early post-transplant period is considered to be the immunological nadir in SOT and is associated with the highest risk of infectious diseases and the development of EBV-PTLD. [412-415] Furthermore, this was implemented to mitigate the risk of any false (non)significant findings from later study timepoints as only 40% of patients were ≥12 months post-transplant at the time of analysis. Thymectomy status was used to assign patients either to the early thymectomy, late thymectomy or non-thymectomy group. Any non-thymectomised patient who subsequently underwent a thymectomy at the point of transplantation was re-classified as early or late thymectomy for the purpose of post-transplant data analysis. The extent of thymectomy (i.e. partial or total) was classified as "unknown" if this wasn't documented clearly in the patient's surgical notes.

5.2 Statistical analysis

Demographic and clinical data of ITHACA participants were extracted from the REDCap database in an anonymised format as .xlsx files and imported to STATA (version 18.0) for downstream analysis. Fisher's exact test was used to compare categorical events by patient thymectomy status. Categorical variables were summarised as proportions and/or percentages while continuous variables were represented as medians and interquartile ranges (IQRs). Wilcoxon rank sum and Kruskal-Wallis tests were used for the comparison of medians between 2 or >2 continuous variables respectively. The p-value significance was set at <0.05 with post hoc adjustment when appropriate for multiple pairwise comparison by thymectomy status using the Benjamini-Hochberg method. [416] Cox proportional regression was used to calculate hazard ratios (HRs) with 95% Confidence Intervals (CIs).

5.3 ITHACA study cohort

5.3.1 Pre-transplant patient cohort

Key clinical and demographic information of ITHACA study participants is summarised in Table 5.1.

5.3.1.1 Patient demographics

The sex distribution of patients was equitable between males and females (51.9% vs 48.1%) and did not differ significantly according to thymectomy status (p = 0.94). Study participants were predominantly Caucasian (72.2%) while the rest of the cohort was comprised of other ethnic minorities (Table 5.1). This is representative of the ethnic diversity seen in paediatric patients who are registered onto the national heart transplant waiting list and is also similar to observations from previous UK-based paediatric heart transplant studies. [4, 398]

The overall median age of participants at the point of study inclusion was 8.7 years (IQR = 3.3 – 13.7 years). Although patients in the early thymectomy group tended to be younger compared to study participants in the late thymectomy and non-thymectomy groups (7.8 years vs 13.8 years vs 8.1 years respectively), this difference did not approach statistical significance (p = 0.30).

Variables	N of 54 (%)	Early Thymectomy (n = 21)	Late Thymectomy (n = 9)	No Thymectomy (n = 24)	*p*
Sex at birth					
Male	28 (51.9)	10	5	13	
Female	26 (48.1)	11	4	11	0.94
Ethnicity					
Caucasian	39 (72.2)	17	6	16	
Black	3 (5.6)	2	-	1	
Asian	7 (13.0)	-	3	4	
Other	5 (9.2)	2	-	3	0.17
Age at pre-transplant PBMC sample (years)					
<1	3 (5.6)	2	-	1	0.19
1 – 10	25 (46.3)	12	3	10	
>10	20 (37.0)	4	6	10	
No sample	6 (11.1)	3	-	3	
Median (IQR)		7.8 [2.4 – 9.4]	13.8 [5.6 – 15.0]	8.1 [3.3 – 13.9]	0.30
Cardiac Diagnosis‡					
Congenital Heart Disease	19 (35.2)	16	1	-	
• Single Ventricle Defect	10 (52.6)	11	-	-	
• Biventricular (Acyanotic)	3 (15.8)	2	-	1	
• Biventricular (Cyanotic)	6 (31.6)	3	1	-	
Acquired Cardiomyopathy	32 (59.3)	5	7	20	<0.001
EBV Serostatus (Pre-transplant)					
Positive	24 (44.4)	8	2	14	
Negative	30 (55.6)	13	7	10	0.08
CMV Serostatus (Pre-transplant)					
Positive	19 (35.2)	6	4	9	
Negative	35 (64.8)	15	5	15	0.75
Procedure at Thymectomy†					
Staged cardiac surgery	19 (61.3)	16	3	-	
VAD insertion/ECMO	11 (35.5)	5	6	-	0.04
Type of Thymectomy†					
Partial	3 (10.0)	2	1	-	
Total	13 (43.3)	10	3	-	
Unknown	14 (46.7)	9	5	-	0.74
Time from thymectomy to baseline sample collection (years)					
Median (IQR)		7.8 [2.0 – 9.4]	1.0 [0.3 – 4.0]	-	0.07

Table 5.1: Baseline clinical and demographic details of patients recruited to the ITHACA study. **ECMO** – Extracorporeal Mechanical Oxygenation; **IQR**: Interquartile Range; **PBMC**; Peripheral Mononuclear Cell; **VAD** – Ventricular Assist Device; ‡excluding 3 non-thymectomy patients with a primary renal pathology; †Pre-transplant thymectomy patients only (n = 30).

5.3.1.2 Indications for transplantation

As expected, ACM was the primary indication for transplantation in a majority of children (59.3%) while the remaining 35.2% of patients had a pre-existing diagnosis of a univentricular, biventricular cyanotic and biventricular acyanotic CHD in decreasing frequency (Table 5.1). This was consistent with current trends in the paediatric indications for urgent cardiac transplantation. [417] The median age at early thymectomy was 0.02 years (IQR: 0.01 – 0.34) and 6.6 years (IQR: 1.9 – 12.3) at late thymectomy ($p < 0.001$). Patients with an underlying CHD were also more likely to have undergone an early thymectomy compared to those with ACM and a similarly significant association was found between late thymectomy and ACM ($p < 0.001$). This was indicative of the increased use of staged cardiac surgery for CHD patients, which was the most common procedure at which an early thymectomy was performed (16/21 patients) while VAD/ECMO insertion which is often required as bridging therapy for ACM patients was the principal intervention that resulted in a late thymectomy (6/9 patients). Of the 6 patients who didn't have baseline PBMC samples available for immune profiling, 2 participants still had an intact thymus at the point of study inclusion, 3 had undergone early thymectomy for CHD (2/3) and ACM (1/3), while the final participant had undergone a late thymectomy for ACM.

5.3.1.3 HLA class I and II coverage

Table 5.2 shows the coverage for HLA class I-restricted tetramers in the ITHACA cohort. The total patient coverage was 82%. While all patients in the late thymectomy group had tetramers available for their corresponding HLA type, 5/21 patients (23.8%) in the early thymectomy group and 5/24 (20.8%) in the no thymectomy group had HLA types that could not be matched to the available class I-restricted tetramers. This difference in tetramer coverage was not statistically significant ($p = 0.30$).

Patient Group	N	HLA CLASS I					
		A2	A11	A24	B7	B8	B35
Early Thymectomy	21	7	0	2	4	5	5
Late Thymectomy	9	5	1	4	2	0	1
No Thymectomy	24	8	4	4	7	4	3
Total	**54**	**20**	**5**	**10**	**13**	**9**	**9**

Table 5.2: **Coverage of HLA class I-restricted tetramers among ITHACA participants**. 44/54 patients were successfully matched to at least one class I-restricted tetramer that corresponded to their HLA type.

Similar patient coverage was observed for the HLA class II-restricted tetramers, with a total coverage of 76%. No HLA class II-restricted tetramers were available for 6/21 (28.6%) early thymectomy, 1/9 (11%) late thymectomy and 6/24 (25%) of patients with no thymectomy (p = 0.41).

Patient Group	N	HLA CLASS II			
		DR4	DR7	DR17	DR52b
Early Thymectomy	21	2	10	3	5
Late Thymectomy	9	3	3	1	4
No Thymectomy	24	5	3	4	9
Total	**54**	**10**	**16**	**8**	**18**

Table 5.3: Coverage of HLA class II-restricted tetramers among ITHACA participants. 41/54 patients were successfully matched to at least one class II-restricted tetramer that corresponded to their HLA type.

5.3.2 Pre-transplant viral serology

5.3.2.1 Pre-transplant EBV and CMV exposure are co-dependent factors

The overall seroprevalence rates for EBV and CMV at baseline sampling were 44.4% and 35.5% respectively. A higher proportion of CMV seronegative patients were also EBV seronegative (25/54 patients) while 15/54 patients were noted to have serological evidence that was consistent with prior exposure to both EBV and CMV. Baseline CMV serostatus was found to be predictive of prior EBV exposure with an almost 3-fold increase in the likelihood of a concordant EBV serostatus that was independent of patient age (Adjusted Odds Ratio = 2.6; 95% CI = 1.4 – 4.7). Pre-transplant EBV and CMV serostatus were not associated with either cardiac diagnosis, sex at birth, ethnicity, age group at pre-transplant PBMC sampling or thymectomy status ($p > 0.05$).

5.3.2.2 Early thymectomy is associated with higher pre-transplant EBV DNAemia

EBV DNA was detectable in whole blood samples obtained from 8/24 (33.3%) of the EBV seropositive patients. This subgroup consisted of 5 patients with an early thymectomy and a further 3 patients with an intact thymus. Although there was no association between thymectomy status and pre-transplant EBV DNAemia ($p = 0.15$), patients with an early thymectomy had significantly higher circulating EBV DNA compared to the non-thymectomy subgroup (9440 [8120 – 2200] vs 1000 [1000 – 5240] copies/ml; $p = 0.03$]). One of the early thymectomy patients (NUTH019) exhibited an aberrant EBV antibody profile that was indicative of an acute EBV infection (strong detection of VCA IgM and IgG) despite serological evidence in their clinical records of previous EBV exposure at least 4 years prior.

5.3.3 Demographic and clinical data for non-ITHACA healthy controls

Limited demographic and clinical information was available for the 18 non-ITHACA healthy controls whose samples were included in this study. This is summarised in Table 5.4. The CMV serostatus could not be evaluated for this cohort due to the limited availability of stored serum. Similarly, relevant data on ethnicity and HLA type were unavailable for these patients.

Variables	N of 18 (%)
Sex at birth	
Male	10 (55.6)
Female	8 (44.4)
Age at PBMC sample (years)	
Median (IQR)	6.9 (3.9 – 12.0)
EBV Serostatus	
Positive	5 (27.8)
Negative	13 (72.2)

Table 5.4: Summary of demographic and clinical information for non-ITHACA healthy controls.

5.3.4 Post-transplant patient cohort

Figure 5.1 outlines the thymectomy status of ITHACA participants between the pre-transplant and post-transplant study timepoints while Table 5.5 summarises the relevant peri-transplant demographic and clinical information for this cohort of patients. Of the 44 transplanted patients, 4 were lost to follow up, either due to death from transplant-related complications (1/44), transition to adult services (1/44) or transfer to a shared care centre overseas (2/44). Clinical information was available for 41 patients at the 3-month study timepoint and for the 40 patients who had reached the 6-month study timepoint at the time of data analysis.

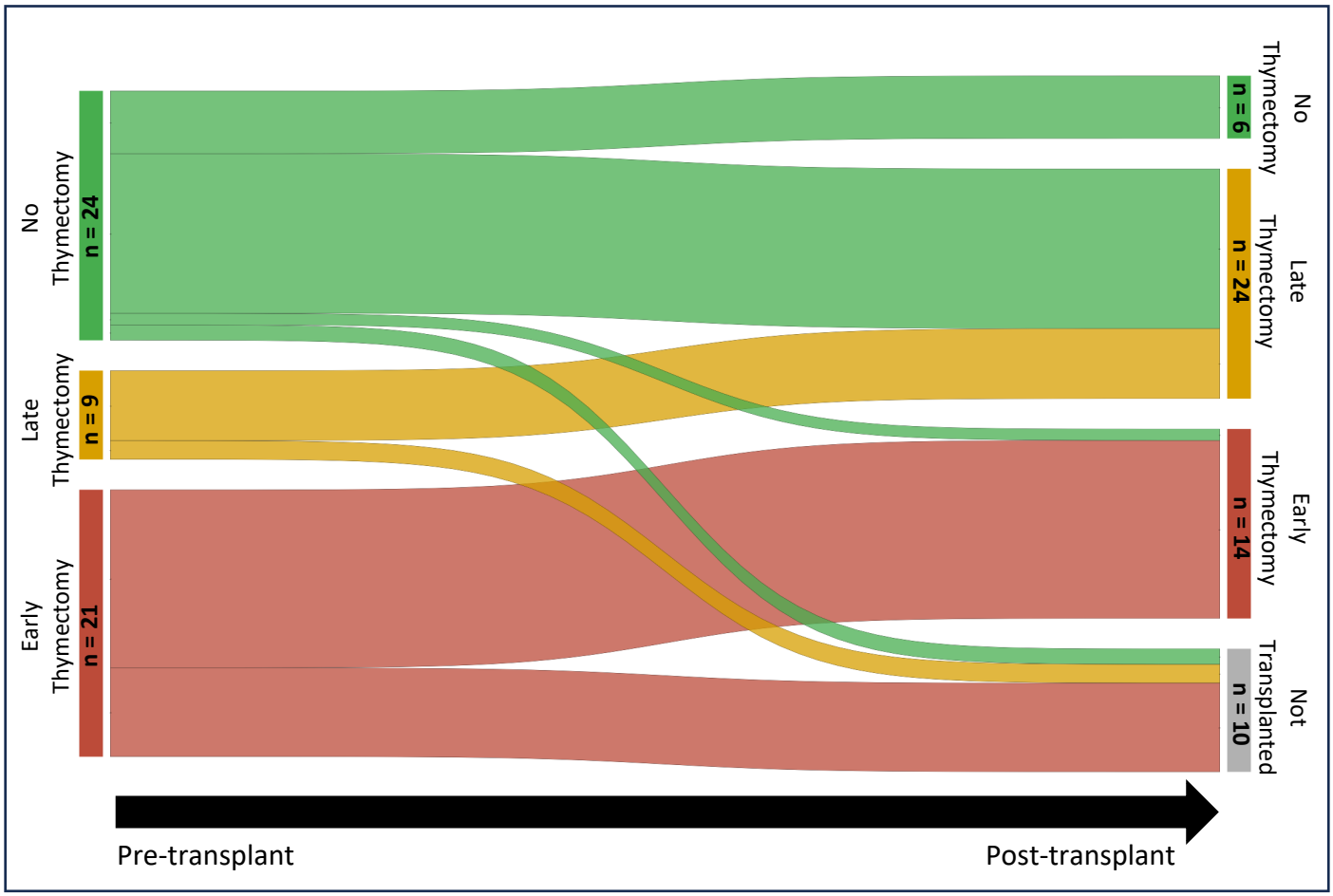


Figure 5.1: Sankey diagram illustrating the post-transplant reallocation of ITHACA recruits (n = 54) according to their thymectomy status. Of the 23 non-thymectomy patients who proceeded to transplant, 1 (4.2%) underwent an early thymectomy and 16 (66.7%) had a late thymectomy at the time of transplant. The 10 participants who did not receive a heart transplant during the study period consisted of 1 non-thymectomy, 1 late thymectomy and 8 early thymectomy patients.

5.3.4.1 Post-transplant patient demographics

The post-transplant patient cohort had similar sex, age and ethnic distributions to the pre-transplant group. However, there was a significant association between sex and thymectomy status, with the no thymectomy group being composed of only males (p = 0.03). Although patients with an early thymectomy were transplanted at a younger age compared to the late and non-thymectomy groups, this was not found to be significant (p = 0.08).

Unlike findings from the overarching ITHACA cohort, transplantation rather than staged cardiac surgery represented the main procedure at which a thymectomy was performed (44.4% vs 30% respectively). However, this was due to the fact that more patients with ACM (70.5%) had proceeded to organ transplant compared to children with CHD (29.5%), with a large proportion of the former undergoing a late thymectomy at the point of transplant (94%). The median age at early thymectomy was 0.11 years (IQR: 0.01 – 0.45) and 10.7 years (IQR: 3.7– 13.7) at late thymectomy (p < 0.001)

A partial thymectomy was performed in 18.4% of patients while total thymectomy was undertaken in 34.2% of transplanted children. The extent of thymectomy could not be verified with certainty from the surgical notes of 47.4% of patients. Importantly, there was no significant association between the extent of thymectomy and thymectomy status (p = 0.63) or a significant difference in the median age at transplant between the patients who underwent a partial versus total thymectomy (11.3 years [1.0 – 14.2] vs 5.7 years [2.7 – 8.9], p = 0.94).

Variables	N of 44 (%)	Early Thymectomy (n = 14)	Late Thymectomy (n = 24)	No Thymectomy (n = 6)	*p*
Sex at birth					
Male	22 (50.0)	5	11	6	
Female	22 (50.0)	9	13	-	0.03
Ethnicity					
Caucasian	34 (77.3)	14	14	6	
Black	1 (2.3)	-	1	-	
Asian	6 (13.6)	-	6	-	
Other	3 (6.8)	-	3	-	0.08
Age at Transplant (years)					
<1	2 (4.5)	2	-	-	0.14
1 – 10	20 (45.5)	8	9	3	
>10	22 (50.0)	4	15	3	
Median (IQR)		7.5 [1.1 – 12.1]	12.4 [5.0 – 14.2]	11.3 [6.1 – 15.2]	0.08
Diagnosis					
Congenital Heart Disease	11 (25.0)	9	2	-	
• Single Ventricle Defect	5 (45.4)	5	-	-	
• Biventricular (Acyanotic)	3 (27.3)	2	1	-	
• Biventricular (Cyanotic)	3 (27.3)	2	1	-	
Acquired Cardiomyopathy	31 (70.5)	5	22	4	
Primary Renal Disease	2 (4.5)	-	-	2	<0.001
EBV Serostatus (Pre-transplant)					
Positive	19 (43.2)	3	11	5	
Negative	25 (56.8)	11	13	1	0.04
CMV Serostatus (Pre-transplant)					
Positive	16 (36.4)	4	10	2	
Negative	28 (63.6)	10	14	4	0.83
EBV Mismatch (D+/R-)					
Yes	15 (34.1)	4	11	-	
No	29 (65.9)	10	13	6	0.10
CMV Mismatch (D+/R-)					
Yes	7 (15.9)	2	4	1	
No	37 (84.1)	12	20	5	0.99
Induction Immunotherapy					
ATG	11 (25.0)	7	4	-	
Basiliximab	33 (75.0)	7	20	6	0.03
Procedure at Thymectomy†					
Staged cardiac surgery	11 (30.0)	8	3	-	
VAD insertion/ECMO	10 (26.3)	5	5	-	
Transplant	17 (44.7)	1	16	-	< 0.001
Type of Thymectomy†					
Partial	7 (18.4)	3	4	-	
Total	13 (34.2)	6	7	-	
Unknown	18 (47.4)	5	13	-	0.68
Time from thymectomy to transplant (years)					
Median (IQR)		7.4 [0.8 – 12.1]	0.002 [0 – 0.8]	-	<0.001

Table 5.5: **Clinical and demographic details of all transplanted patients on the ITHACA study**. **ATG** – Anti-Thymocyte Globulin; **D+/R- –** Donor seropositive/Recipient seronegative; **IQR**: Interquartile Range. †Post-transplant thymectomy patients only (n = 38).

5.3.4.2 HLA Class I and II coverage

Tables 5.6 and 5.7 highlight the respective coverage for HLA class I- and class II-restricted tetramers in all transplanted patients. The total class I tetramer coverage was 84% while that for class II tetramers was 80%. There was no significant difference in the coverage for patient samples assessed with HLA class I-restricted tetramers across early, late and non-thymectomy patients (14% vs 18% vs 13%; p = 0.82). A similarly non-significant result was obtained for coverage HLA class II-restricted tetramers (Early (14% vs 23% vs 25%; p = 0.14). Of note, 2/44 transplanted patients had neither class I nor II tetramers (both from the late thymectomy subgroup).

Patient Group	N	HLA CLASS I					
		A2	A11	A24	B7	B8	B35
Early Thymectomy	14	5	0	2	2	4	6
Late Thymectomy	24	9	4	7	8	1	1
No Thymectomy	6	2	1	1	1	2	1
Total	**44**	**16**	**5**	**10**	**11**	**7**	**8**

Table 5.6: Coverage of HLA class I-restricted tetramers among transplanted ITHACA participants. 37/44 patients were successfully matched to at least one class I-restricted tetramer that corresponded to their HLA type.

Patient Group	N	HLA CLASS II			
		DR4	DR7	DR17	DR52b
Early Thymectomy	14	1	6	3	5
Late Thymectomy	24	6	4	4	10
No Thymectomy	6	2	1	0	1
Total	**44**	**9**	**11**	**7**	**16**

Table 5.7: Coverage of HLA class II-restricted tetramers among ITHACA participants. 35/44 patients were successfully matched to at least one class II-restricted tetramer that corresponded to their HLA type.

5.3.5 Immunosuppression therapy

5.3.5.1 Induction immunosuppression

Three-quarters of all transplant patients received basiliximab as part of their induction immunosuppression. Patients without an early thymectomy were more likely to receive basiliximab compared to those with an early thymectomy (Odds ratio = 6.5 [95% CI: 1.2 – 38.2]; $p = 0.03$). This wasn't associated with the underlying indication for transplantation, EBV/CMV mismatch or recipient pre-transplant EBV serostatus ($p > 0.05$). The use of ATG was dependent on the transplant centre, with its use being employed solely at NuTH. This was reflective of some of the variance in the induction immunosuppression protocol used at the two recruitment sites (appendix F). All transplanted patients who were recruited from NuTH (n = 20) received ciclosporin, azathioprine and methylprednisolone in addition to either ATG or basiliximab as part of their induction immunosuppression while patients recruited from GOSH (n = 24) received methylprednisolone, mycophenolate mofetil (MMF) and basiliximab.

5.3.5.2 Maintenance immunosuppression

There was no association between thymectomy status and the use of calcineurin inhibitors (CNI) or adjunct immunosuppression therapy at either 3 months or 6 months post-transplantation (Tables 5.8 and 5.9 respectively). The median doses of the CNIs and their respective trough levels as measured in patients serum at the 3- and 6-month study timepoints were not found to differ significantly according to thymectomy status ($p > 0.05$).

Variables	N of 41 (%)	Early Thymectomy (n = 14)	Late Thymectomy (n = 21)	No Thymectomy (n = 6)	*p*
CNI (at 3 months)					
Ciclosporin	7 (17.1)	4	3	0	
Tacrolimus	34 (82.9)	10	18	6	0.39
Adjunct therapy					
None	4 (5.6)	2	2	0	
MMF/Aza only	21 (46.3)	5	12	4	
MMF/Aza + Prednisolone	11 (37.0)	5	4	2	
Prednisolone only	5 (11.1)	2	3	0	0.79
CNI dose [mg/day] (IQR)					
Ciclosporin	210 (140 – 310)	185 (150 – 215)	310 (140 – 325)	-	0.57
Tacrolimus	4.9 (3.6 – 7.0)	4.0 (4.0 – 5.6)	5.0 (3.0 -7.0)	5.5 (4.5 – 8.4)	0.37
CNI trough level [ng/dl] (IQR)					
Ciclosporin	208 (190 - 244)	212 (194 – 235)	208 (180 – 275)	-	0.99
Tacrolimus	9.0 (7.0 – 10.3)	10.0 (9.3 – 11.1)	8.4 (6.8 – 9.5)	8.2 (7.1 – 9.1)	0.17

Table 5.8: **Maintenance immunosuppression at 3-months post-transplantation**: **Aza**: Azathioprine; **CNI**: Calcineurin Inhibitor; **MMF**: Mycophenolate Mofetil

Variables	N of 40 (%)	Early Thymectomy (n = 14)	Late Thymectomy (n = 20)	No Thymectomy (n = 6)	*p*
CNI (at 6 months)					
Ciclosporin	2 (5.0)	1	1	0	
Tacrolimus	38 (95.0)	13	19	6	0.99
Adjunct therapy					
None	3 (7.5)	2	1	0	
MMF/Aza only	22 (55.0)	7	12	5	
MMF/Aza + Prednisolone	8 (20.0)	3	4	1	
Prednisolone only	5 (12.5)	2	3	0	0.92
CNI dose [mg/day] (IQR)					
Ciclosporin	173 (105 – 240)	105 (–)	240 (–)	-	-
Tacrolimus	4.8 (3.0 – 7.0)	3.9 (2.8 – 5.2)	6.0 (3.0 – 8.0)	6.5 (4.5 – 8.8)	0.14
CNI trough level [ng/dl] (IQR)					
Ciclosporin	105 (9 - 200)	9 (–)	200 (–)	-	-
Tacrolimus	8.6 (6.3 – 9.9)	6.8 (5.5 – 8.7)	9.1 (7.8 – 10.5)	9.1 (6.7 – 9.9)	0.07

Table 5.9: **Maintenance immunosuppression at 6-months post-transplantation**: **Aza**: Azathioprine; **CNI**: Calcineurin Inhibitor; **MMF**: Mycophenolate Mofetil. Due to the small number of patients on Ciclosporin (n = 2), the *p*-values for the difference in dose and trough level according to thymectomy status could not be estimated with accuracy.

5.3.6 EBV and CMV mismatched transplantation

Twenty-five patients (56.8%) were EBV seronegative at the time of transplantation while 28/44 patients (63.6%) were CMV seronegative. Although omnibus testing showed a significant association between baseline EBV serostatus of transplanted patients and thymectomy status (p = 0.04), post hoc pairwise comparison with adjustment of the significance level of α (adjusted $p < 0.017$) did not reveal a significant association between the non- and early thymectomy (p = 0.02) or between the early and late thymectomy groups (p = 0.12).

An EBV mismatched organ transplant (i.e. donor seropositive and recipient seronegative) was performed in just over a third of transplanted patients (34.1%) while a smaller proportion were CMV mismatched (15.9%). Patients who received an organ transplant that was both EBV and CMV mismatched made up 13.6% (6/44) of the study cohort. There was no association between EBV or CMV mismatch and thymectomy status (Table 5.5). There was no significant difference in the median age at transplantation between patients who received an EBV mismatched heart and those who didn't (12.6 years [IQR = 5.1 – 14.0] vs 8.7 years [IQR: 2.9 – 13.9], p = 0.59). This was similar for CMV mismatched vs non-mismatched patients (13.1 years [IQR: 6.8 – 13.9] vs 8.9 [3.3 – 14.0], p = 0.78).

5.4 Post-transplant clinical outcomes

5.4.1 Transplant-related morbidity and mortality

One late thymectomy patient (NUTH003) died from multiorgan failure 55 days after receiving a heart transplant. Twelve patients (27.3%) showed evidence of acute graft rejection in the early post-transplant period. Four patients (NUTH004, NUTH006, NUTH007 and GOSH023) developed moderate-severe acute cellular rejection within 3 months of transplantation. This required treatment with a short course of methylprednisolone (NUTH006, NUTH007 and GOSH023) and immunoadsorption therapy using pulsed methylprednisolone and 2 doses of rituximab (NUTH004). Histological evidence of (mild) grade 1 graft rejection was noted in 7 other patients within 3 months of transplantation that did not require active treatment. However, it is possible that the incidence of mild 'asymptomatic' graft rejection is underrepresented in this cohort as routine post-transplant endomyocardial biopsy was performed as a standard of care only for patients who were followed up at the GOSH study site. No incidence of graft loss was documented for any patient during the study period.

The most common non-EBV-related infection seen in the early post-transplant period was mild respiratory illness. This was predominantly viral in origin and consisted of upper respiratory tract infections (URTIs) due to respiratory syncytial virus (1 case), Rhinovirus (2 cases), Adenovirus (1 case), SARS-Cov-2 (2 cases) and non-specific URTIs (3 cases). One patient developed a community acquired pneumonia that was managed with oral antibiotics as an outpatient.

CMV viraemia was detected in 6/44 patients within 3 months of transplantation. Of these, 2 were cases of viral reactivation while the remaining 4 patients developed a primary infection following a CMV mismatched transplant. Only the patients with CMV reactivation were treated with IV ganciclovir while those with primary CMV infection showed evidence of viral clearance by 6 months post-transplant without any antiviral intervention. Of note, the two patients with CMV reactivation were started on antiviral treatment by their local centre prior to the 3-month study time point and so did not have any detectable CMV DNA in blood samples collected as part of this study.

5.4.2 EBV mismatch increases the risk of early post-transplant primary EBV infection

Post-transplant viral serology was available for 14/15 patients who received an EBV mismatched transplant. Of these patients, 8/14 (57.1%) developed a primary EBV infection within the first 3 months of transplantation while 2/14 (14.3%) patients acquired a primary infection 6-12 months after transplant. The median EBV DNA copy level at primary infection was 15,010 copies/ml (IQR = 3,990 – 79,150 copies/ml). EBV mismatched transplantation was associated with a significant increase in the risk of acquiring a primary EBV infection in the early post-transplant period (HR = 9.3 [95% CI: 1.1 – 76.5]; $p = 0.04$). This increased risk was independent of thymectomy status ($p = 0.45$), the choice of induction immunosuppression ($p = 0.11$) and maintenance CNI ($p = 0.92$).

Primary EBV infection was mostly asymptomatic in this cohort. However, 1 patient with an early thymectomy (NUTH022) who received a heart transplant at the age of 12 years developed adverse symptoms consistent with severe acute infectious mononucleosis 4 weeks after acquiring an EBV infection. This consisted of pyrexia, pneumonitis with bilateral pleural effusions that required non-invasive respiratory support and profound EBV DNAemia (peak viral load of 7×10^6 copies/ml) in the absence of EBV antibodies. Cross-sectional imaging with positron emission tomography and computed tomography (PET-CT) did not reveal any significant lymphadenopathy that was suggestive of EBV-PTLD. This patient was managed symptomatically alongside a reduction in their immunosuppression therapy with subsequent resolution of their symptoms. The first serological detection of VCA IgM and VCA IgG was observed during the convalescence phase, 7 weeks after the acute clinical episode.

5.4.3 EBV reactivation

For the purpose of this study, EBV reactivation was defined as the post-transplant detection of EBV DNA in the blood sample of any patient who was known to have been EBV seropositive at the time of SOT. Viral reactivation was observed in 8/19 (42.1%) EBV seropositive children. Early reactivation occurred in 7 children within the first 3 months of transplantation while the remaining 1 seropositive patient first demonstrated evidence of EBV reactivation at 12 months post-transplant. The median EBV DNA copy level at reactivation was 2,640 copies/ml (IQR = 1,270 – 4,880 copies/ml). Of note, 7/8 patients with viral reactivation received an EBV concordant transplant (i.e., EBV seropositive status in both donor and recipient). Neither thymectomy status, age at transplant, induction/maintenance immunosuppression or CMV serostatus were associated with an increased risk of EBV reactivation ($p > 0.05$).

5.4.4 Early detection of circulating EBV DNA is associated with persistent EBV DNAemia after transplant and is predictive of chronic viraemia

Measurable EBV DNAemia within the first 3 months of SOT from either primary infection or viral reactivation had a significant association with the persistence of circulating viral DNA at up to 12 months post-transplant ($p = 0.002$). Figure 5.2 shows the median EBV DNA titres (viral copies/ml of whole blood) across the first 12-months of transplantation for patients with primary infection and EBV reactivation. Patients with primary EBV infection had significantly higher levels of EBV DNAemia compared to those with viral reactivation at 3-months post-transplantation ($p = 0.04$). However, this difference was lost at 6 months with the convergence of rising EBV titres in both primary infection (47,200 copies/ml [IQR: 26,500 – 86,300 copies/m]) and viral reactivation (44,600 copies/ml [IQR:

10,000 – 71,500 copies/ml]) [p = 0.80]. A notable decline in EBV DNAemia was observed at 12-months post-transplantation in patients with viral reactivation (18,000 copies/ml [IQR: 1,790 – 50,600 copies/ml], while children with a primary infection demonstrated a progressive rise in circulating EBV DNA (81,600 copies/ml [IQR: 9,860 – 207,000 copies/ml]). There was no significant difference in EBV DNAemia according to thymectomy status for the 3-, 6-, and 12-month study timepoints (p > 0.05).

Early detection of EBV DNAemia within the first 3 months of transplantation proved to be beneficial for the prognostication of persistent EBV viraemia at 12 months post-transplantation. The overall positive predictive value was 80.0% (95% CI: 64.6 – 95.4%) with a negative predictive value of 81.3% (95% CI: 66.3 – 96.3%).

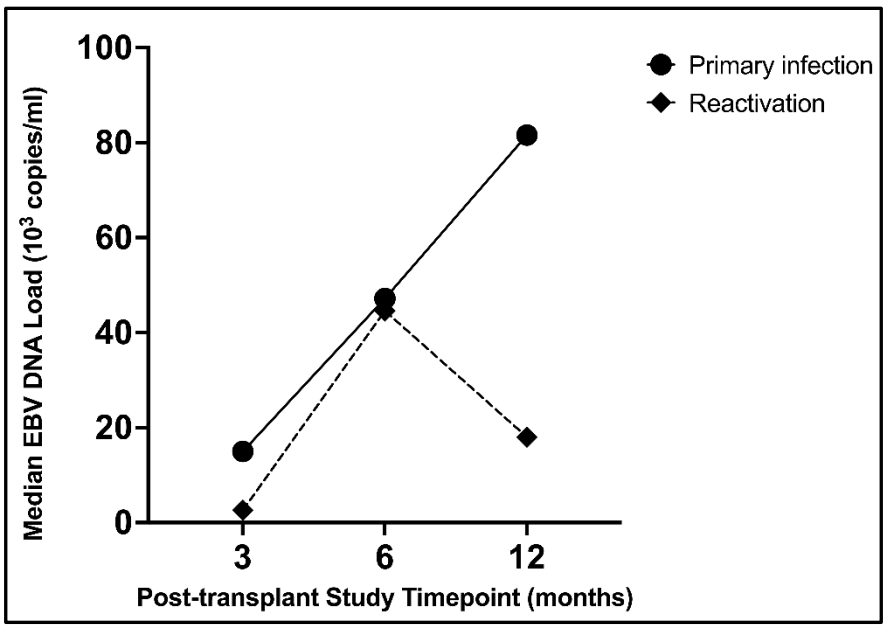


Figure 5.2: Median EBV DNA load (viral copies/ml of whole blood) of patients with either primary EBV infection or viral reactivation during the first 12-months post-transplantation (n = 18).

5.4.5 Early-onset EBV-PTLD is preceded by clinical evidence of poor viral control

EBV-related PTLD had occurred in 2/44 patients at the time of data analysis. Both episodes were within the first year of transplantation. Both patients were recipients of an EBV mismatched organ and had acquired a primary EBV infection within 3 months of transplantation.

Patient 1 (GOSH001) had undergone a late total thymectomy during transplantation for a dilated cardiomyopathy at the age of 1.5 years. The patient had received basiliximab, methylprednisolone and MMF as induction therapy and was maintained on standard immunosuppression with tacrolimus and MMF at the point of primary EBV infection. EBV DNA load at 3 months post-transplant was 324,000 copies/ml and there was no serological evidence of a humoral response to the acute infection. At 4.5 months post-transplant, the patient presented to their local transplant centre with significant right-sided cervical lymphadenopathy and was diagnosed with localised PTLD. Histology revealed a polymorphic lesion (EBV and CD20 positive) with Hodgkin-like features (variable expression of CD30 and CD15). Quantitative EBV DNA load at EBV-PTLD diagnosis was 714,000 copies/ml with no evidence of VCA or EBNA antibody response on serological assessment. The patient was successfully managed with reduction of immunosuppression (MMF discontinued and tacrolimus target trough level reduced from 8-12 μg/l to 5-8 μg/l) and 4 doses of weekly rituximab (375mg/m^2/dose). EBV DNA load had diminished to 4,360 copies/ml by 6-months post-transplant with a corresponding emergence of EBV VCA IgG. A reduced level of immunosuppression was still in

place at 12-months post-transplantation. The patient remained clinically stable with no evidence of EBV-PTLD recurrence or graft rejection. However, EBV DNAemia persisted (40,200 copies/ml) with re-emergence of detectable EBV VCA IgM at 12-months post-transplantation. EBV monitoring is currently ongoing for this patient.

Patient 2 (NUTH025) had undergone an early total thymectomy during staged cardiac surgery at 2 months old for congenitally corrected transposition of the great arteries (ccTGA). The patient was transplanted at 13 years old and received induction therapy with ATG, ciclosporin, azathioprine and methylprednisolone. The patient was maintained on standard immunosuppression. This consisted of ciclosporin, azathioprine (both switched to Tacrolimus and MMF respectively at ~4 months post-transplant as per local transplant centre protocol) and prednisolone. EBV DNAemia was noted within 3 months of transplantation, rising from 5,090 copies/ml at 3-months post-transplantation to 65,800 copies/ml at 6 months alongside a primary CMV infection (peak DNA level of 66,300 copies/ml). Similar to patient GOSH001, NUTH025 exhibited a delayed humoral response to primary EBV infection with no detectable EBV antibodies at the point of EBV-PTLD diagnosis. The patient presented to their local hospital with an acute abdomen due to a perforation of the small bowel that required surgical resection. Cross-sectional imaging demonstrated multiple bilateral lung nodules consistent with PTLD. This was confirmed on histological assessment of the resected bowel tissue as EBV positive DLBCL. EBV DNA load at diagnosis was 14,000 copies/ml. The patient was managed with reduction of immunosuppression (MMF and oral prednisolone discontinued with a reduction in the target trough level of tacrolimus from 6-8 μg/l to 4-5 μg/l) and 4 doses of weekly rituximab. Active treatment of their EBV-PTLD is ongoing.

5.4.6 Discussion

This chapter provides a detailed analysis of the pre- and post-transplant clinical characteristics of a cohort of paediatric patients at high risk of EBV-PTLD. The seroprevalence of EBV and CMV in the ITHACA cohort and among healthy controls was lower than previous reports for other similarly aged UK residents and paediatric transplant populations. [418 – 420] Both EBV and CMV exposure are known to correlate strongly with age and have historically exhibited a progressive rise in seroprevalence across childhood and adolescence. [419, 421] However, this study did not identify a significant relationship between age and viral seroprevalence. Other factors such as ethnicity and blood product requirement are recognised as independent predictors of EBV and CMV exposure. [422, 423] The fact that our ITHACA cohort was mostly Caucasian, an ethnicity known to have lower EBV/CMV seroprevalence compared to other ethnic minorities, [422] might be a contributor to the lower proportion of pre-transplant EBV and CMV seropositivity observed among study recruits. In addition, the reporting of EBV/CMV seroprevalence in transplant patients has primarily involved recipients of haemopoietic stem cells (HSCT) and renal transplants, [424 – 426] both of which are associated with an increased exposure to blood products prior to transplantation. It is important to note that even though 30/54 patients had undergone at least 1 pre-transplant intervention that would have involved exposure to blood products and the risk of EBV/CMV infection, the majority of these study participants remained seronegative at the time of transplantation. This is in contrast to findings from the early 2000s when a similar sized study of paediatric cardiothoracic transplant patients in the UK observed an overall pre-transplant EBV seroprevalence of 73%. [427] This is similar to previous

estimates for children in England. [428] However, a significant decrease in the EBV seroprevalence of young children (< 10 years) over the past two decades to a level that is similar to the ITHACA cohort has recently been described. [429] This has been observed alongside an increase in the age at primary EBV infection in high income countries during the same time period. [430] It is possible that this changing trend in the age at first exposure to EBV is also reflected in the study cohort. This is of major clinical relevance to recipients of SOTs as pre-transplant EBV seronegative status is a well-established risk factor for the development of EBV-PTLD.

Another key study finding was the significant increase in the risk of early primary EBV infection observed in patients who received an EBV mismatched transplant. EBV mismatch is considered to be the most important predictor for EBV-PTLD and is associated with an increased risk of early onset disease (within 12-months of transplant). [312, 431, 432] Studies in both children and adults who received a non-cardiac transplant have shown a comparatively longer time of 9-14 months between EBV mismatched transplantation and primary EBV infection. [433 – 435] To date, no study has reported on the time interval between EBV mismatched paediatric heart transplantation and the development of a primary EBV infection. One prospective study of 44 paediatric heart and lung transplant recipients noted a median interval of 30.5 days between transplantation and the detection of EBV DNAemia in 19 (43%) patients. [427] However, their analysis didn't look specifically at EBV mismatch but at the time from transplant to EBV DNAemia as a consequence of either primary infection or viral reactivation. It is possible that paediatric heart transplant recipients are more susceptible to an earlier emergence of primary EBV infection following EBV mismatched transplantation compared to other paediatric SOT cohorts. Indeed, findings from the ITHACA study also suggests that EBV reactivation after heart transplantation occurs at a significantly higher rate and much earlier than has been reported for other SOT cohorts. [434] Collectively, these results suggest a predisposition in paediatric heart transplant recipients towards aberrant EBV control.

The occurrence of EBV-PTLD in 2/15 (13.3%) of the EBV mismatched ITHACA participants who developed a primary infection within 3 months of their transplant lends further validity to this assertion. The fact that both patients demonstrated clinical evidence of dysregulated viral control weeks prior to the onset of EBV-PTLD (rising EBV DNA titres and absent anti-EBV humoral response) indicates that some underlying immune disruptive mechanism(s) must be at play. Similar albeit less profound immune dysregulation might also explain the chronicity of EBV viraemia in patients who had detectable EBV DNA within the first 3 months of transplantation either from primary infection or reactivation but had not developed EBV-PTLD at the time of data analysis. It is worth mentioning that chronic EBV high viral load (HVL) (defined as EBV DNA of >16,000 copies/ml in ≥ 50% of blood samples for 6 months or more) after primary infection is a recognised risk factor for late-onset PTLD (> 1-year post-transplant) in paediatric heart transplant patients but not in other childhood SOT recipients. [436-439] This has been attributed to changes within their T-lymphocyte compartment which displays upregulated markers of terminal exhaustion and altered memory differentiation that is distinct from the phenotype observed in paediatric kidney and liver transplant recipients with HVL. [440]

Although the incidence of EBV-PTLD in this study cohort was too low at the time of data analysis to investigate its association with other relevant covariates such as thymectomy status and induction/maintenance immunosuppression, this might become possible if there is a further increase

in the incidence of EBV-PTLD with ongoing post-transplant follow up of study participants. A further limitation of this study is that its constrained sample size could have underpowered the ability to detect clinical differences in EBV characteristics that exist according to thymectomy status. Nevertheless, the study findings explored in this chapter provide compelling clinical evidence of dysregulated EBV control in a subset of patients at high risk of PTLD. The next two chapters of this thesis will explore the innate and adaptive immune cell populations of the ITHACA study cohort, specifically detailing the impact of thymectomy on the phenotypic and functional states of immune cell subsets that are integral to the host's ability to mount an effective response to an EBV infection and its potential relationship to the risk of EBV-PTLD.

Chapter 6. Broad immune landscape of the ITHACA study cohort

6.1 Introduction

Robust anti-viral and anti-tumour host responses are mediated through essential mechanistic crosstalk between innate and adaptive immune cells. [441, 442] Exploiting the systemic pathways through which these immune cells interact with the tumour microenvironment has gained increasing relevance in recent years for disease risk stratification, prognostication and the development of novel immune therapies for EBV-PTLD and other virus-driven malignancies. Paediatric recipients of a liver transplant who go on to develop EBV-PTLD have been found to have a diminished frequency of CD56dim NKG2A+ KIR− NK cells in the setting of CMV co-infection and persistent EBV DNAemia, indicating its potential use as a predictive biomarker. [443] Similar examination hasn't been conducted for other circulating innate immune cell populations that are engaged in anti-EBV response(s).

To date, our understanding of the immunological landscape of patients at risk of EBV-PTLD has focussed primarily on the T-lymphocyte compartment with minimal emphasis on the contribution(s) of innate immune cells to a patient's risk profile. Similarly, the temporal effects of thymectomy on the innate immune signatures of children who acquire a primary EBV infection and/or chronic EBV DNAemia after SOT haven't been explored. It therefore remains unclear if aberrations in innate cell subsets such as NK cells, NKT-like cells, DCs, Monocytes or γδ T-lymphocytes play a role in the aetiology of EBV-PTLD, particularly in heart transplant recipients.

This chapter provides a detailed analysis of the pre- and post-transplant innate immune cell compartment of patients recruited to the ITHACA study and their relationship to thymectomy status, EBV infection and immunosuppressive therapy.

6.2 Aims of the chapter

1. To determine if there is a difference in the pre-transplant immune signature of the innate immune cell compartment according to age at thymectomy.
2. To analyse the association between innate cell populations and age at thymectomy in the setting of post-transplant immunosuppression.
3. To examine if there is a difference in the post-transplant immune signature of the innate cell compartment according to age at thymectomy, EBV infection and immunosuppression therapy.

6.3 Flow cytometry data analysis

High dimensional spectral flow cytometry data were obtained from pre- and post-transplant patient samples using the 24-colour broad innate/adaptive immune flow panel described in chapter 3 (section 3.1.2). After all quality control measures relating to spectral unmixing had been performed, FCS files were imported into the OMIQ platform for downstream immune phenotyping of innate cell subsets using the manual gating strategy outlined in section 3.4.5.

In addition, exploratory analysis using dimensional reduction with automated uniform manifold approximation and projection (UMAP) and semi-supervised clustering with FlowSOM was conducted to determine if this computational technique was capable of visually identifying any differences in the frequencies of innate cell subsets and/or the intensity of their surface marker expression based on the EBV status of study participants and other relevant co-variables. UMAP is a well-recognised computational technique for visualising high dimensional spectral flow data with superior resolution compared to other modalities, [444] while FlowSOM is considered to be one the fastest and best clustering algorithms for large cytometric datasets. [445] The following workflow was used in OMIQ for this purpose: First, Archsinh scaling with a co-factor of 6,000 was used for each fluorescent channel to ensure that > 99% of cell events were on scale and that there was a unimodal distribution of the negative cell population around 0. After dead cells and aggregates were manually gated out, automated cleaning of FCS files was performed with flowAI to check for (and remove) any anomalous regions of the files that could be attributable to outlier events and variances in flow rate during sample acquisition. [446] FlowAI settings were as follows: all FCS files used, all fluorescent channels and time feature selected, Anomaly detection method = All methods, FR: Seconds fraction = 0.1, FR: Alpha (significance) = 0.01, FR : decompose flow rate selected, FS: Change point detection penalty = 500, FS: Max changepoints = 3, FM: Dynamic range check side = both limits, FM: Negative value remover = 1. Automated cleaning didn't flag up any anomalous data that required removal from the FCS files that were included in this study.

In order to facilitate meaningful data exploration in such a large dataset, each patient-related FCS file was sub-sampled to ~150,000 live CD45+ CD3- singlets (i.e. all live PBMCs excluding T-lymphocytes) per study timepoint. For the specific sub-analysis of NK cells, NKT-like cells, DCs and monocytes, each patient-related FCS file was sub-sampled to ~25,000 live singlets of the cell subset under investigation on the basis of their lineage marker as identified by conventional gating methods (e.g. total NK cells identified as CD3- CD56+). Next, normalisation of the data to adjust for batch effects due to different

time points of data acquisition was performed for all fluorescent channel with fdaNorm. The fdaNorm algorithm estimates the density for each fluorescent channel and each patient sample, locates the major peaks for the sample and subsequently matches/aligns these peaks across all samples using techniques from functional data analysis. [447] After data normalisation, UMAP analysis was performed for visualisation of the different cell populations of interest. UMAP settings were as follows: all FCS files used, all fluorescent parameters related to the cell subsets of interest (except CD45, CD3, CD4, CD8, γδT and Live/Dead), neighbours = 80, minimum distance = 0.7, components = 2, metric = euclidean, learning rate = 1, epochs = 250, random seed = 8125. Data were then clustered using FlowSOM with the following settings: all files used, clustering features CD56, CD16, CD14, CD11c, CD123, IgD, CD38, CD19 and CD20, UMAP_1, UMAP_2, with xdim = 25 and ydim = 25, rlen = 10, Distance Metric = euclidean, consensus metaclustering with k = 10 – 30, Random Seed = 6823. These settings were based on previously validated workflows for similar sized spectral flow panels, [339, 377] complemented by expert recommendations from the OMIQ technical application specialist team.

After the FlowSOM analysis, metaclusters were annotated into commonly recognised biological cell populations based on their surface marker expression. This was achieved by generating a hierarchical heatmap of the surface markers in the panel as expressed by each metacluster. This workflow permitted verification and translation of FlowSOM clusters into identifiable immune cell populations via the heatmap. The resulting cell populations were then analysed by similar clustered heatmapping according to covariate groups (e.g. EBV status) with a euclidean distance metric to indicate the similarity of the cell populations. These populations could then be visualised for comparison between samples using the UMAP parameters.

6.4 Statistical analysis

Pre-transplant flow cytometry data were analysed independent to the post-transplant study timepoints to account for ITHACA recruits who hadn't yet proceeded to transplantation including those whose post-transplant samples hadn't been processed at the time of data analysis for this thesis. Furthermore, the analysis of post-transplant data was primarily focussed on the 3- and 6-month study timepoints (i.e. < 12 months post-transplant) for reasons that have been elucidated in chapter 5 (section 5.1).

The relative abundances of NK cells and NKT-like cells for each patient sample taken at the pre-transplant, 3- and 6-month study timepoints were calculated as percentages of the total gated live lymphocyte population while that of monocytes and DCs were calculated as percentages of live CD45+ cells. The abundances of immune cell subsets of interest within these major populations were expressed as the percentage of their cell lineage of origin. A dual platform method was used to estimate the absolute counts (cells/µl) for lymphocyte subsets in patients who had a corresponding full blood count (FBC) sample taken at the time of study-related blood sampling. Dual platform cell counting involved the multiplication of the flow cytometry-derived percentage of the lymphocyte subset by the absolute lymphocyte count derived from an automated haemanalyser. This technique has been shown to be a reliable and reproducible way of estimating absolute cell counts in clinical trials. [448]

Comparisons of pre-/post-transplant cell frequencies were performed according to thymectomy group, induction/maintenance immunosuppression agents, EBV status and other relevant study covariates using either Wilcoxon rank sum for the comparison of medians between 2 continuous variables or the Kruskal-Wallis test when >2 continuous variables were being compared. Similar analyses were performed to compare absolute cell counts in patients who had a contemporaneous FBC taken during blood sampling for the study. Correlation analysis was performed by Spearman rank correlation coefficient *r*. Wilcoxon signed rank test was used to compare median cell counts and proportions of paired samples (where available) at 3 months and 6 months post-transplantation. The *p*-value level of significance was set at <0.05 with post hoc adjustment when appropriate for multiple pairwise comparison by thymectomy status using the Benjamini-Hochberg method. All analyses were conducted with the statistical software STATA (version 18.0), while graphs and figures were generated with GraphPad Prism version 10.0 software (GraphPad Software Inc, San Diego, CA, USA).

6.5 Findings

6.5.1 Identification of major non-T lymphocyte immune cell lineages with high dimensional reduction and cluster analysis

An assessment of live non-T lymphocyte PBMCs (i.e. CD45+ TCRγδ- CD4- CD8-) with high dimensional reduction and cluster analysis was used to visually map the major innate immune cell lineages of interest (Figure 6.1).

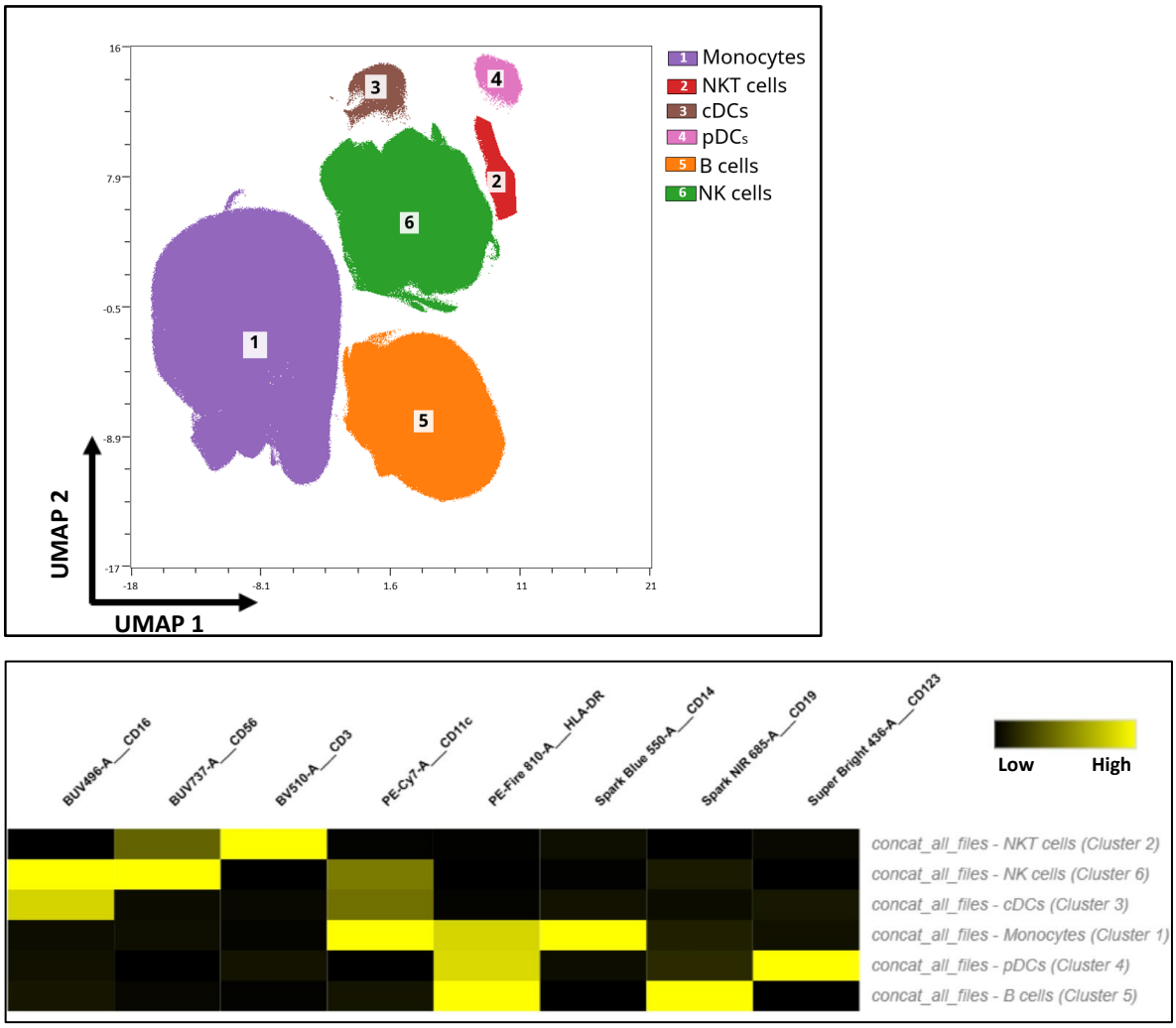


Figure 6.1: High-dimensional cytometry analysis of PBMCs identifying major non-T lymphocytes cell lineages. The concatenated UMAP plot shows the major non-T lymphocyte cell lineages that were identified using FlowSOM. The heatmap highlights the median expression of the lineage-defining surface markers (CD3, CD11c, CD14, CD16, CD19, CD56, CD123 and HLA-DR) that were used to identify each FlowSOM cluster as a distinct immune cell population.

This automated, non-biased resolution of the innate immune cell lineages of interest served as confirmation of the panel's high quality and provided a further platform for profiling the immune landscape of study samples.

6.5.2 Non-T lymphocyte and myeloid lineages are not altered by early thymectomy

Manual gating analysis of PBMC samples across the first 6 months of transplantation showed no significant effect of early thymectomy on absolute counts of the major non-T lymphocyte and myeloid cell lineages (Figure 6.2).

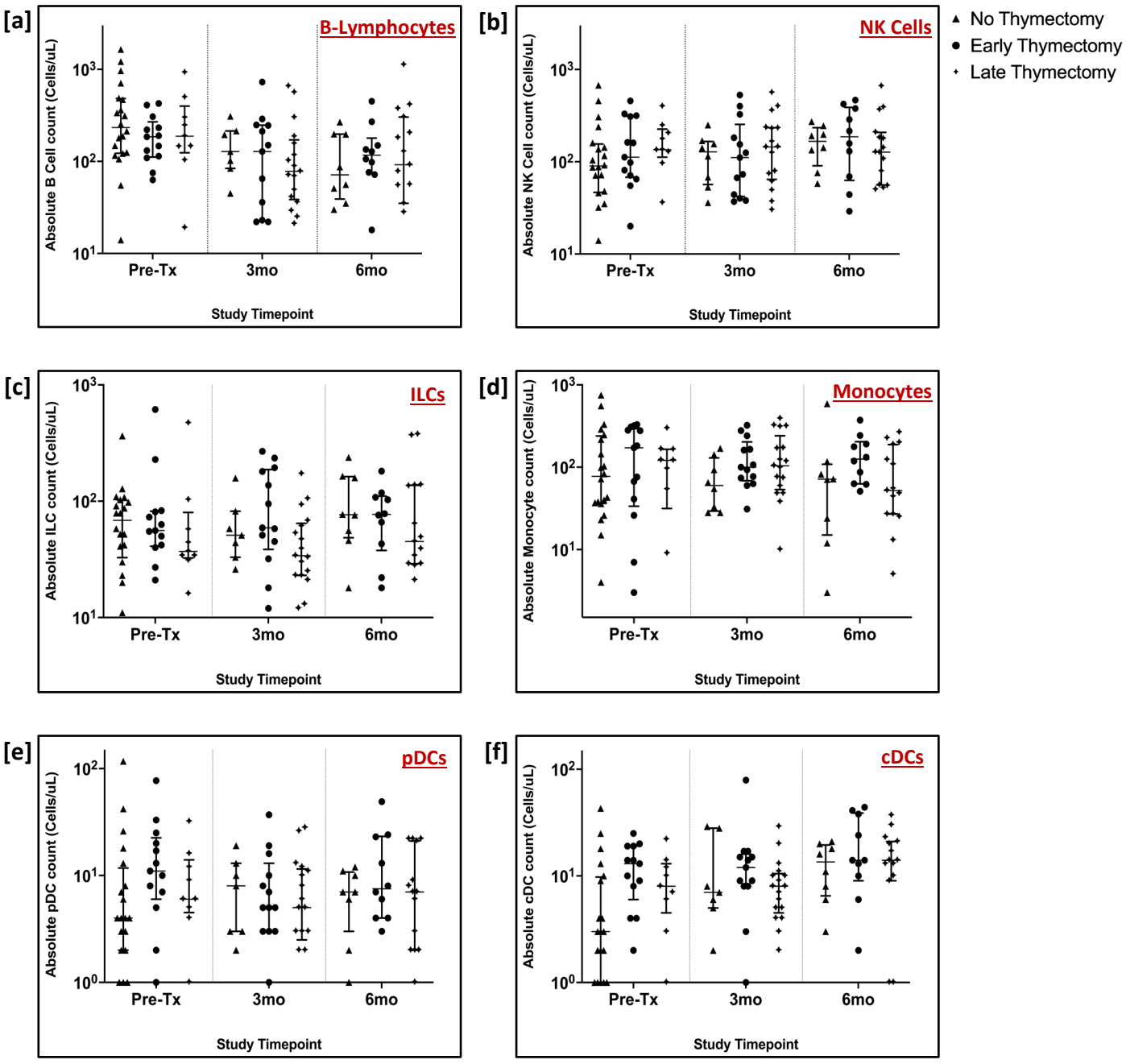


Figure 6.2: Absolute cell counts (cells/µl) for the non-T lymphocyte immune compartment. Comparison was performed by thymectomy status for **[a]** B-lymphocytes; **[b]** NK cells; **[c]** Innate Lymphoid cells (ILCs); **[d]** Monocytes; **[e]** Plasmacytoid dendritic cells (pDCs); **[f]** Conventional dendritic cells (cDCs). Cell counts were log-transformed (log 10). *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. All *p* values were > 0.05.

6.5.3 NKT cells are significantly depleted after transplantation in early thymectomy patients

Prior to transplantation, the absolute count of NKT cells in both early and late thymectomy patients was observed to be lower than in the non-thymectomy cohort, although this difference did not reach statistical significance [p = 0.62 and 0.69 respectively] (Figure 6.3).

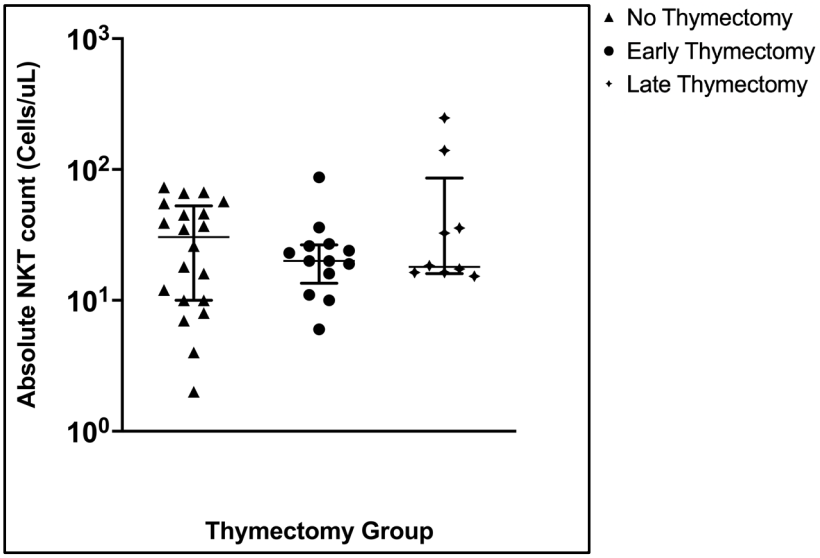


Figure 6.3: Baseline absolute NKT cell count (cells/µl) in ITHACA patients prior to transplantation. Comparison of absolute cell counts (cells/µl) by thymectomy status. *P* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. All *p* values were > 0.05. Cell counts have been log-transformed (log 10).

Unlike the other lymphocyte populations (excluding conventional T-lymphocytes), circulating NKT cells were substantially depleted after transplantation, specifically within the early thymectomy patient group. NKT cells in early thymectomy patients were significantly lower compared to non-thymectomy (p = 0.03) and late thymectomy patients (p = 0.02) at 3 months post-transplantation. This difference in the NKT immune compartment was more prominent at 6 months post-transplantation, with early thymectomy patients having a median absolute count of 16 cells/µl [IQR = 11 – 22] compared to non-thymectomy (97 cells/µl [IQR = 41 – 113; p: 0.0002]) and late thymectomy patients (38 cells/µl [IQR = 26 – 77; p: 0.002]) [Figure 6.4].

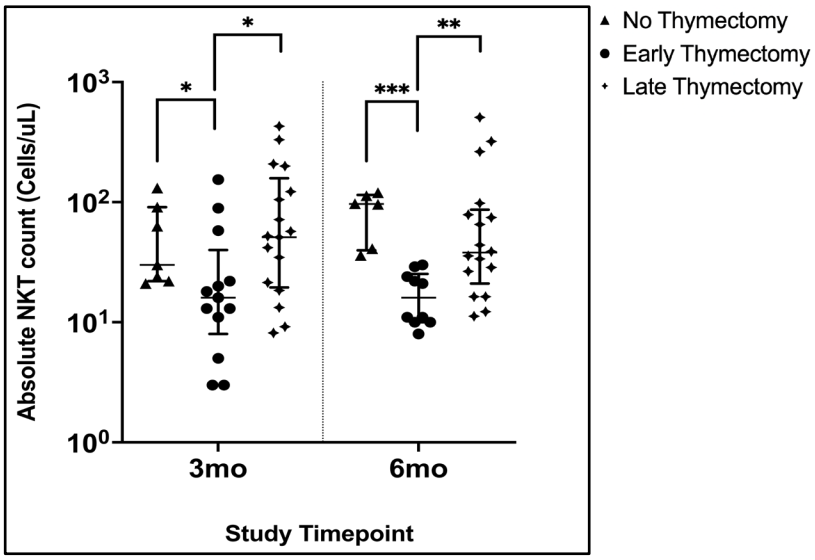


Figure 6.4: Absolute NKT cell counts (cells/µl) in ITHACA participants stratified by thymectomy status at 3- and 6-months post-transplantation. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In order to assess NKT cell reconstitution after transplant, analysis was performed on the subset of patients who had undergone a thymectomy prior to transplantation and had paired study samples available (and analysed) at baseline, 3 months and 6 months post-transplantation (n = 16). This revealed a significant rise in the NKT cell count of non-thymectomy patients at 6 months post-transplantation compared to baseline, while NKT cell counts remained relatively static in both early and late thymectomy patients during the early post-transplant period (Figure 6.5).

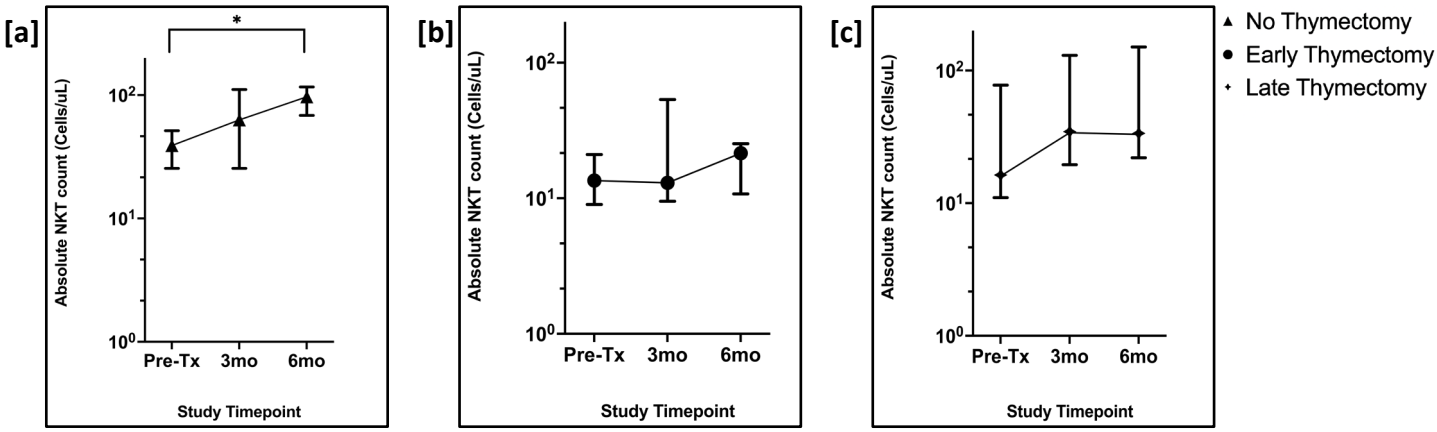


Figure 6.5: NKT cell reconstitution according to thymectomy status. Wilcoxon signed rank test was used to compare pre- and post-transplant NKT cell counts of **[a]** Non-thymectomy (n = 5); **[b]** Early thymectomy (n = 6); and **[c]** Late thymectomy (n = 5) patients who had paired samples available across all 3 study periods. Analysis of early and late thymectomy patients was restricted to study participants who had undergone thymectomy prior to the day of cardiac transplant. Median cell counts are shown by dots with their corresponding interquartile range indicated by error bars. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

There was no significant difference in NKT cell count according to induction immunosuppression ($p = 0.51$), maintenance immunosuppression with either ciclosporin or tacrolimus ($p = 0.67$), and the use of mycophenolate mofetil ($p = 0.22$). However, there was a significant negative correlation between EBV DNA load and NKT cell count in patients with EBV DNAemia at 3 months post-transplantation ($r = -0.61$, $p = 0.02$).

6.5.4 Altered composition of monocytes subsets is associated with induction immunosuppression and a disruption in EBV control

In view of the functional diversity of the 3 main monocyte subsets in immunological patrol (classical), antigen presentation (intermediate) and antiviral recognition (non-classical), [449] percentages of these cell subsets were compared across the first 6 months of transplantation for patients with available paired samples (n = 31). Focus was paid on determining the effects of immunosuppressive therapy on the peri-transplant composition of monocytes in children at high risk of EBV-PTLD due to the lack of effect of thymectomy on the overall monocyte population (see section 6.5.1).

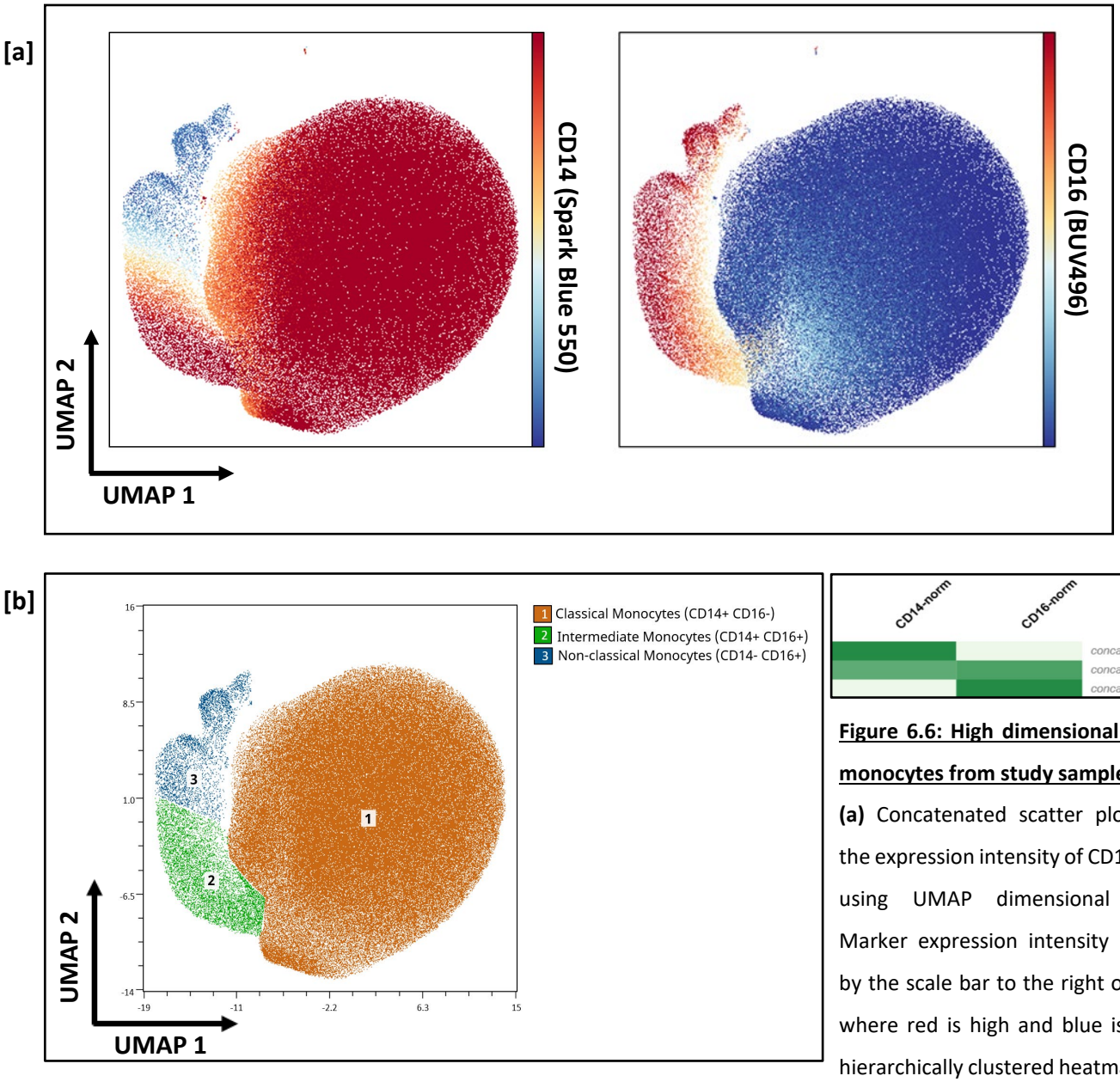


Figure 6.6: High dimensional analysis of monocytes from study samples:

(a) Concatenated scatter plots showing the expression intensity of CD14 and CD16 using UMAP dimensional reduction. Marker expression intensity is indicated by the scale bar to the right of each plot, where red is high and blue is low; **(b)** A hierarchically clustered heatmap was then used to identify FlowSOM clusters as monocyte subsets based on the intensity of CD14 and CD16 surface marker expression.

The 3 main monocyte subsets were first identified using semi-supervised dimensional reduction and cluster analysis as described in section 6.3 (Figure 6.6). The pre-transplant distribution of the 3 monocyte subsets consisted of classical monocytes representing 92.7% (IQR: 90.1 – 96.8), intermediate monocytes making up 4.3% (IQR: 2.1 – 6.3) and non-classical monocytes at 2.1% (IQR: 1.0 – 4.1). These proportions were consistent with the expected distribution of subsets within the monocyte compartment as described in healthy populations. [449] These subsets can broadly be delineated into CD16- versus CD16+ monocytes, with the latter acting as an important driver of early post-transplant cell-mediated immunity through pro- and anti-inflammatory cytokine production, induction of T-lymphocyte activation and proliferation through antigen presentation and direct anti-viral activity. [450, 451]

While much focus has been given to understanding the effects of maintenance immunosuppression on the monocyte compartment, [452] less is known about how induction immunosuppression might contribute to the altered phenotypic profile seen in monocyte subsets after SOT. To address this gap in the literature, percentages of the monocyte subsets were compared between all patients who received ATG and those who underwent induction immunosuppression with Basiliximab to determine if the composition of the monocyte compartment was influenced by the choice of induction immunosuppression. This comparison is illustrated in Figure 6.7.

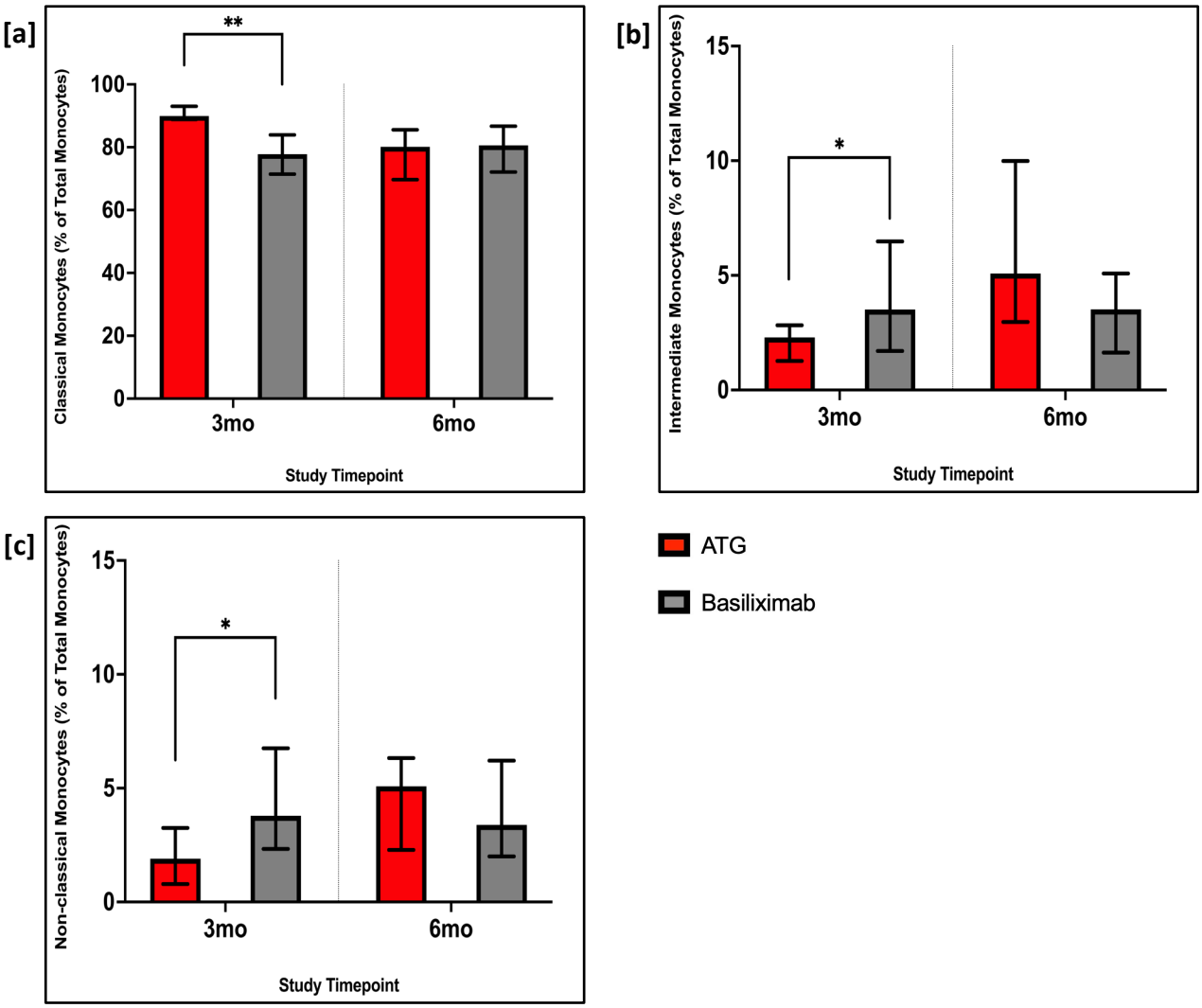


Figure 6.7: Distribution of monocyte subsets in ITHACA patients during the first 6 months of transplantation. Significant differences in the percentage distribution of all three monocyte subsets were observed at 3 months post-transplantation in the ITHACA cohort (n = 44). **[a]** Classical Monocytes, **[b]** Intermediate Monocytes, **[c]** Non-classical Monocytes. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

There was no significant difference in the pre-transplant percentages of classical, intermediate and non-classical monocytes between patients who underwent induction immunosuppression with ATG versus Basiliximab ($p > 0.05$). Likewise, the baseline percentages of the monocyte subsets in both patient groups were comparable to those of healthy non-transplanted children ($p > 0.05$). The frequency of total CD16+ monocytes (i.e. intermediate and non-classical monocytes) at 3 months post-transplantation was 4.5% (IQR: 2.4 – 7.0) in children who received induction immunosuppression with ATG. This was significantly lower than the total CD16+ monocytes in healthy non-transplanted patients (7.3% [IQR: 6.5 – 10.1; $p = 0.01$]). In contrast, total CD16+ monocytes was higher at 3 months post-transplantation in patients who received Basiliximab (9.3% [IQR: 6.7 – 15.1]) and this difference trended towards statistical significance when compared to non-transplanted patients ($p = 0.059$).

Furthermore, at 3 months post-transplantation, patients who received ATG had a significantly higher percentage of classical monocytes compared to those who had received induction with Basiliximab ($p = 0.007$). Induction immunosuppression with ATG was also associated with significantly lower percentages of intermediate and non-classical monocytes at the same study time point (Figure 6.7b-c). By 6 months post-transplantation, higher percentages of intermediate and non-classical monocytes were observed in patients who received ATG than in patients treated with Basiliximab, although these were no longer significantly different ($p = 0.19$ and $p = 0.51$ respectively).

In view of these findings and the known importance of CD16+ monocyte expansion in antiviral control, it was hypothesised that lower proportions of circulating intermediate and non-classical monocytes in the early post-transplant period would contribute to poor EBV immune surveillance in the ITHACA cohort. To explore this hypothesis, the total CD16+ monocyte population was compared between EBV naïve healthy patients and transplant patients who had acquired a primary/reactivated EBV infection at 3 months post-transplantation. Instead of the expected expansion of CD16+ monocytes in the latter group, this cohort had significantly lower CD16+ monocytes compared to EBV naïve healthy children (6.2% [IQR: 3.3 – 8.3] vs 7.3% [IQR: 2.3 - 13.2]; $p = 0.046$). In addition, at 3 months post-transplantation, a significant inverse relationship was observed between EBV DNA load and the two CD16+ monocyte subsets (Figure 6.8).

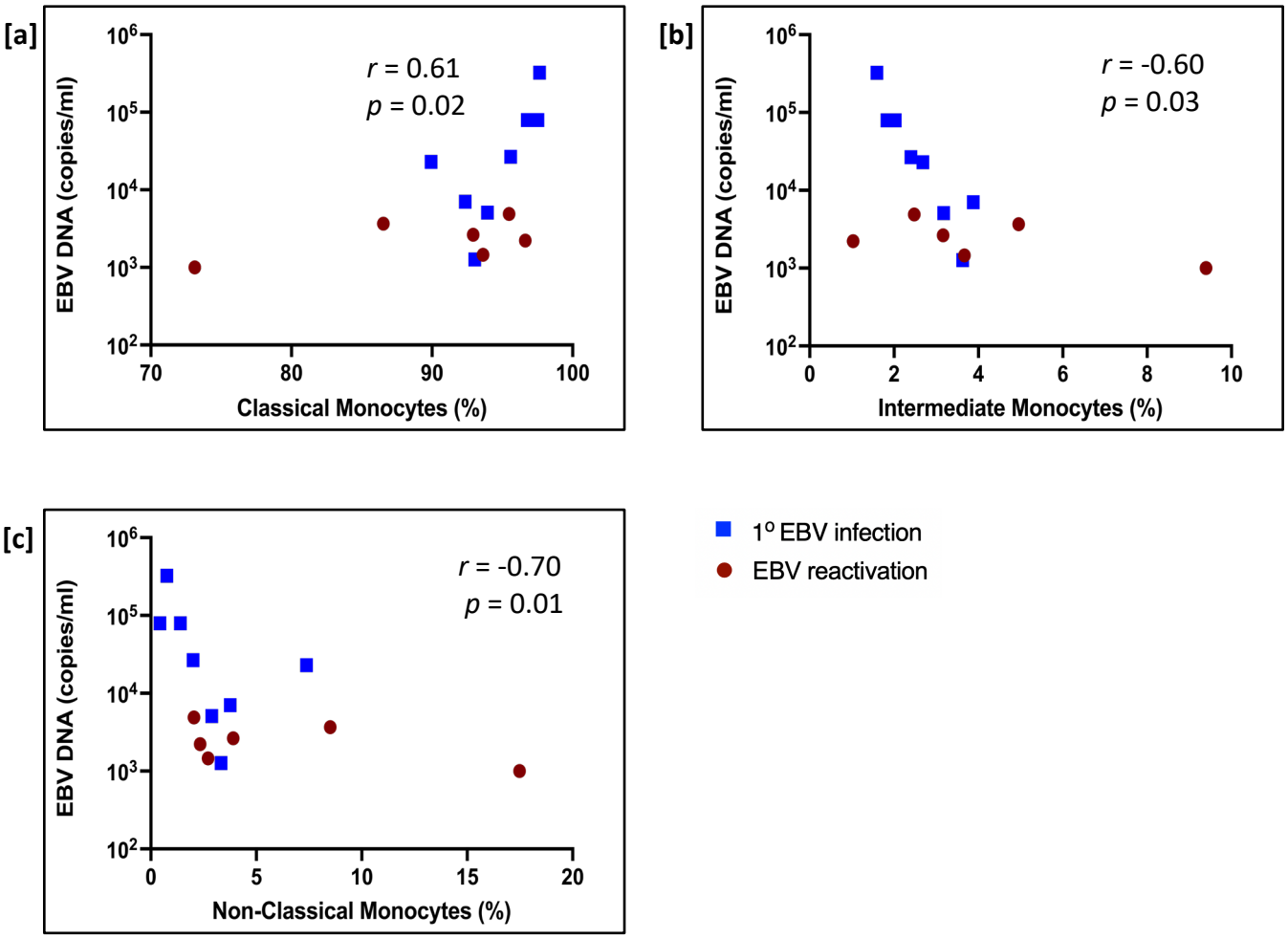


Figure 6.8: Correlation of circulating EBV DNA load (viral copies/ml) at 3-months post-transplantation and percentages of the three main monocyte subsets. [a] A significant positive correlation was observed between EBV DNA load and the percentage of CD14+ CD16- classical monocytes. A significant negative correlation existed with **[b]** CD14+ CD16+intermediate and **[c]** CD14- CD16+ non-classical subsets. r = Spearman's rho. The level of significance for *p*-values was set as < 0.05.

Finally, the expression of representative markers of activation and T-lymphocyte co-stimulation was used as a proxy measure of the functional status of monocytes in patients who had either acquired primary EBV infection or undergone viral reactivation within 3 months of transplantation. For this purpose, HLA-DR expression was evaluated on the total monocyte population, while CD11c – an integrin adhesion molecule known to be specifically upregulated on the surface of CD16+ monocytes during inflammatory stimuli [453, 454] – was assessed on the intermediate and non-classical subsets (Figure 6.9). Here, a significant negative correlation was observed between EBV DNA load and the expression of HLA-DR on monocytes ($p = 0.005$). A similar finding was noted between EBV DNA load and the expression of CD11c on intermediate ($p = 0.049$) and non-classical monocytes ($p = 0.027$).

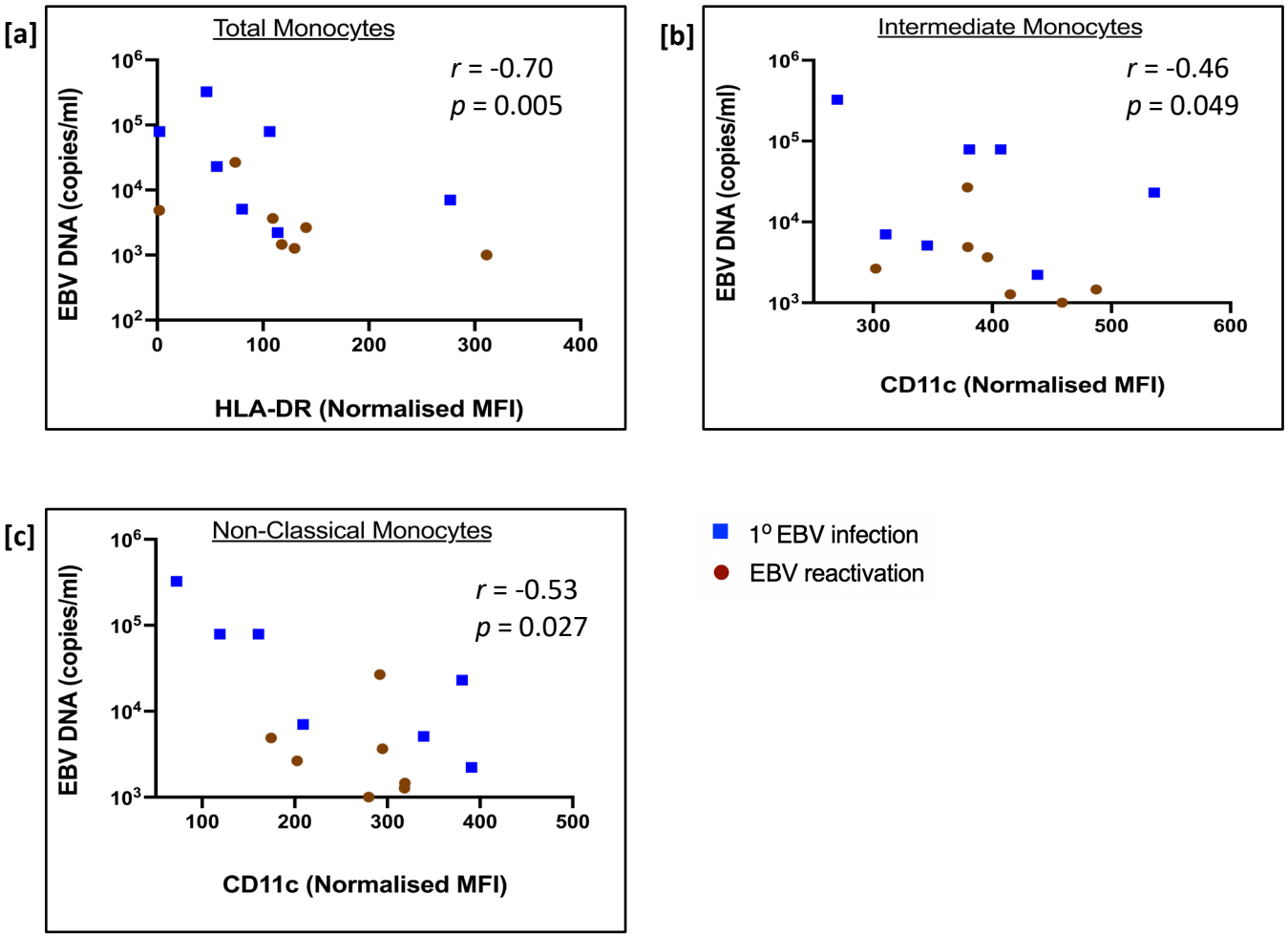


Figure 6.9: Correlation of circulating EBV DNA load (viral copies/ml) at 3-months post-transplantation with the normalised mean fluorescence intensity (MFI) of activation markers expressed by monocytes. The expression of relevant surface markers included**: [a]** HLA-DR on total monocytes; **[b]** CD11c on intermediate monocytes and **[c]** CD11c on non-classical monocytes. r = Spearman's rho. The level of significance for *p*-values was set as < 0.05.

6.5.5 The overall relative frequency of NK cells is influenced by induction and maintenance immunosuppression

Both induction and maintenance immunosuppression were noted to have a significant impact on the relative frequency of NK cells within the lymphocyte compartment. At 3 months post-transplantation, patients who received ATG as part of their induction immunosuppression were found to have a significantly higher proportion of NK cells compared to those who were treated with Basiliximab (Figure 6.10). However, this difference was no longer significant at 6 months post-transplantation (p = 0.46). This relative increase in the proportion of NK cells following induction with ATG was suspected to be as a result of the shift in composition of the lymphocyte compartment due to a selective depletion of T-lymphocytes by ATG. This premise was supported by the presence of a strong negative correlation between the post-transplant frequency of CD3+ T-lymphocytes and the total percentage of NK cells at 3 months (r = -0.76; $p < 0.001$) and the absence of a significant difference in the absolute count of NK cells at the same study timepoint (p = 0.97).

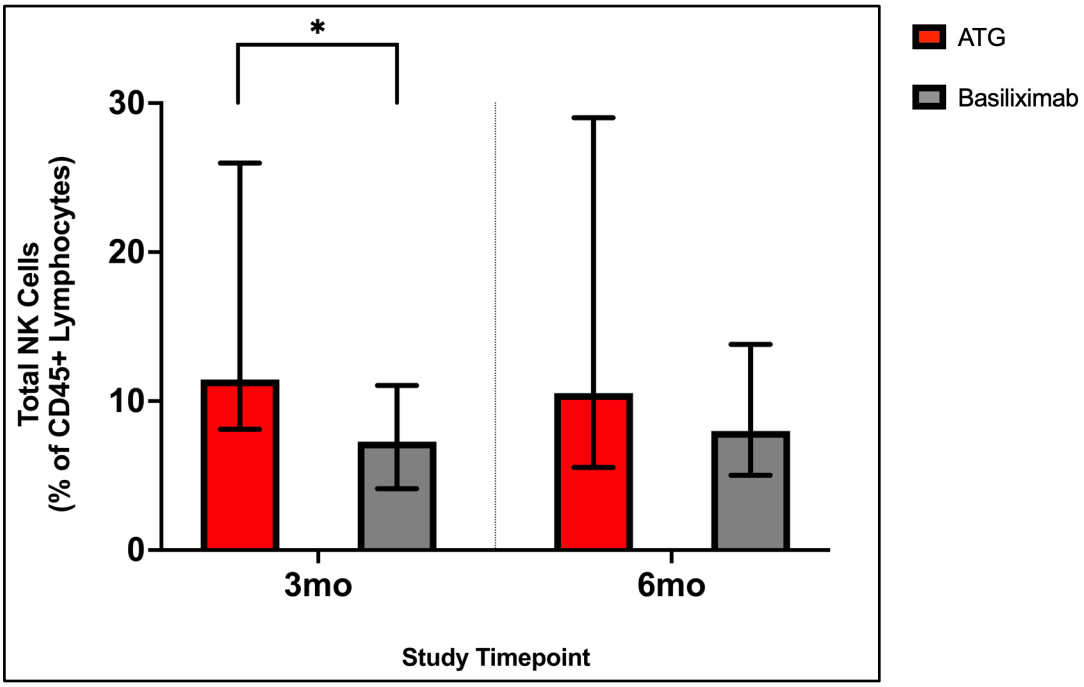


Figure 6.10: Percentage of total NK cell population within the lymphocyte compartment at 3 and 6-months post-transplantation according to choice of induction immunosuppression. **ATG** = Anti-Thymocyte Globulin. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

The choice of CNI backbone as part of maintenance immunosuppression (tacrolimus versus ciclosporin) had no impact on the total frequency of NK cells at either 3- or 6-months post transplantation ($p > 0.05$). However, patients whose maintenance immunosuppression included mycophenolate mofetil (MMF), irrespective of CNI choice or prednisolone, had a significantly lower frequency of NK cells at 3 months post-transplantation compared to those who received a CNI +/- prednisolone but without MMF (Figure 6.11). MMF has been shown to inhibit NK cell proliferation and cytotoxicity, possibly in a dose-dependent manner. [455-457] Interestingly, patients in the ITHACA study were noted to be on a significantly higher total daily dose of MMF at 3 months post-transplantation compared to 6 months after transplant (800mg [IQR: 500 – 1200] vs 500mg [IQR: 250 – 880], $p = 0.025$).

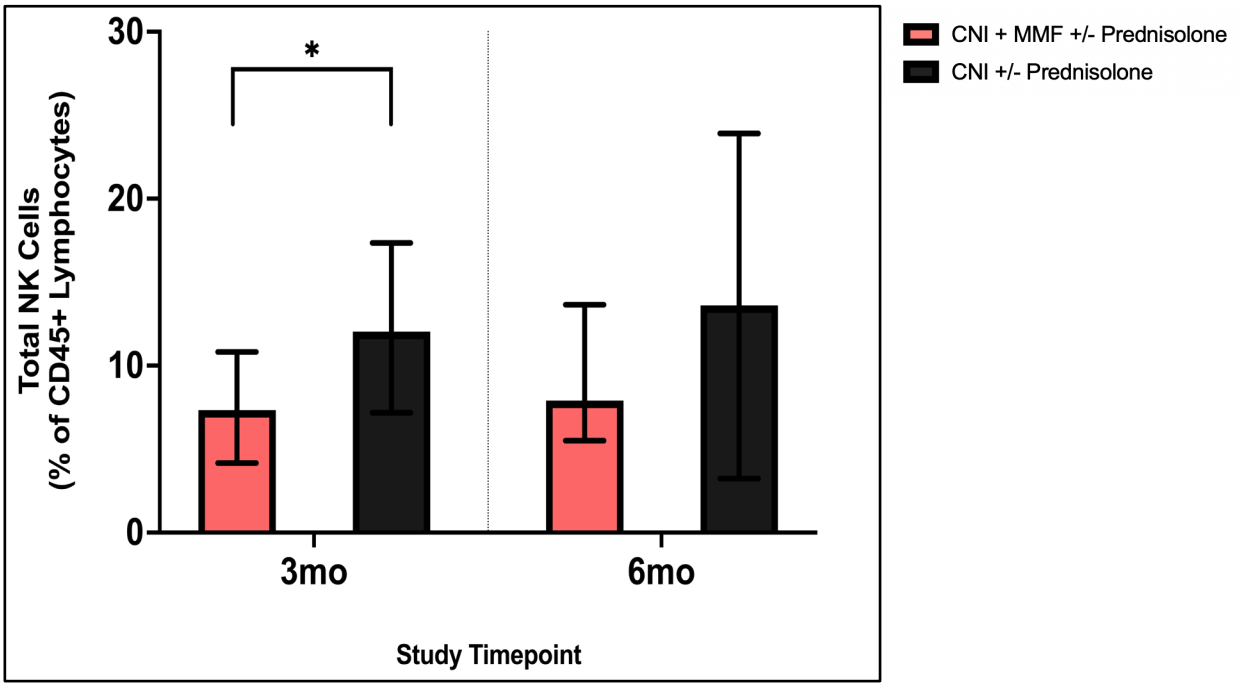


Figure 6.11: Percentage of total NK cell population within the lymphocyte compartment at 3 and 6-months post-transplantation according to choice of adjuvant maintenance immunosuppression. All patients received a Calcineurin inhibitor (CNI) as part of their regimen. MMF = Mycophenolate Mofetil. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

6.5.6 High dimensional reduction and cluster analysis identifies changes in the landscape of NK cell subsets related to maintenance immunosuppression

Since the use of MMF with(out) prednisolone was found to significantly affect the overall frequency of NK cells, further analysis was performed to identify any differences within the NK cell compartment that could help to explain this phenomenon. The diversity of the NK cell population was mapped out using dimensional reduction and subsequently probed for differential clustering of subsets based on the expression of relevant surface markers (see section 3.4.5). Thirteen cell clusters were identified (Figure 6.12), all of which corresponded to the established maturation pathway of NK cells from the CD56^{bright} subsets through to the CD57+ terminally differentiated and senescent phenotypes. [458, 459]

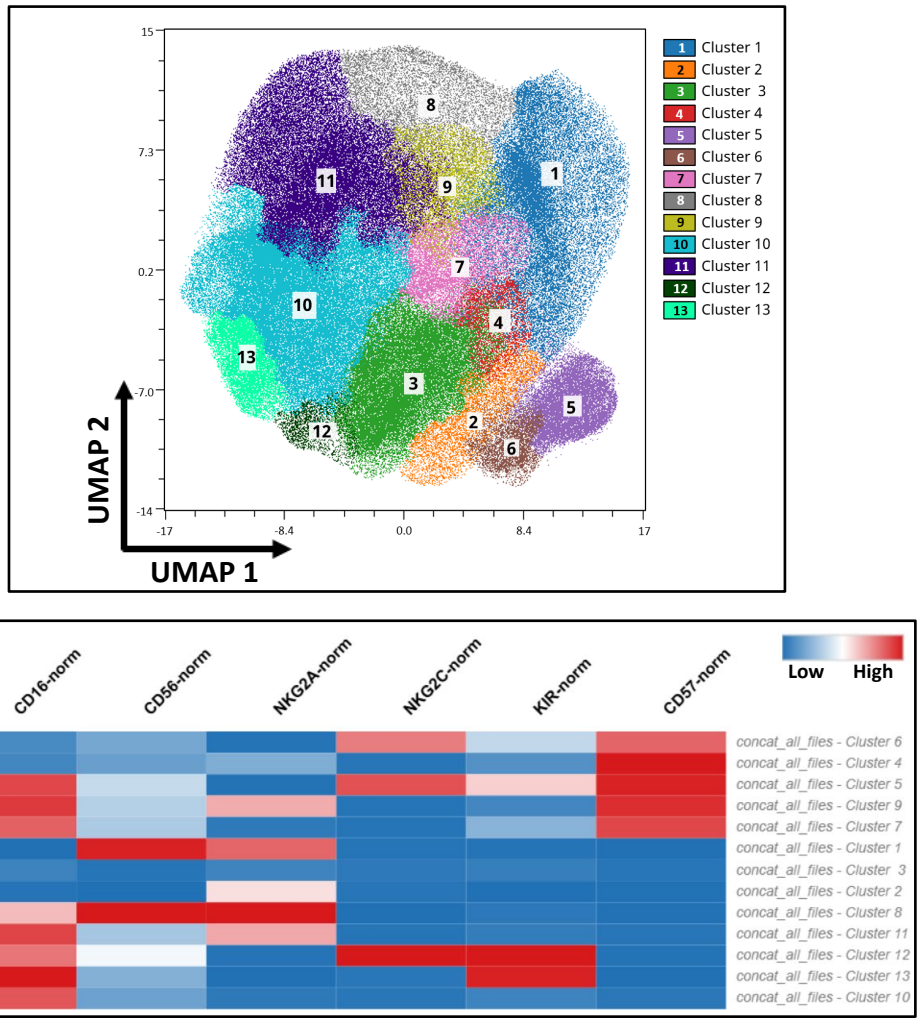


Figure 6.12: High dimensional analysis of NK cells from study samples: The concatenated UMAP plot shows the 13 NK cell clusters that were identified using FlowSOM. The heatmap highlights the expression of relevant NK cell surface markers. Clusters aligned closely with the maturation pathway of NK cells as they differentiate from CD16- CD56bright early NK cells (cluster 1) and CD16+ CD56bright intermediate NK cells (cluster 8), to diverse mature CD56dim NKG2A+/- NKG2C+/- KIR+/- subsets (clusters 2, 3 and 10 – 13) and terminally differentiated CD56dim NKG2C+/- CD57+ subsets (clusters 4 – 7 and 9).

Patients who received MMF with(out) prednisolone had significantly higher proportions of NK cell subsets with a senescent phenotype (clusters 4, 7, 9) and a lower proportion of terminally differentiated (cluster 12) phenotype at 3 months post-transplantation (Figure 6.13a). In addition, they had a significantly lower proportion of CD56bright CD16+ intermediate NK cells (cluster 8) compared to patients on a CNI with or without prednisolone (p = 0.026). These changes were independent of EBV and/or CMV infection.

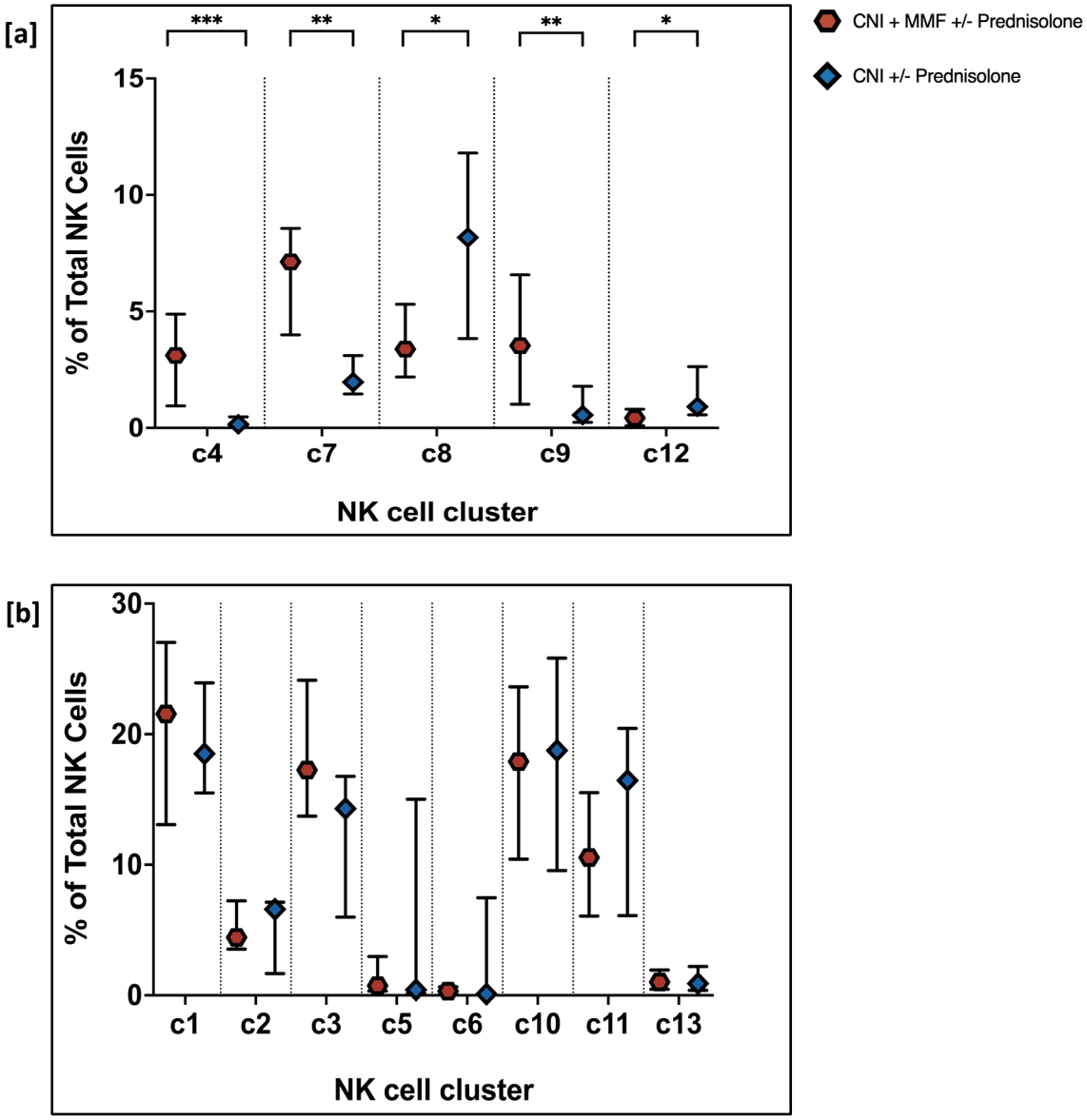


Figure 6.13: Cluster frequencies of NK cell subsets at 3-months post transplantation, compared between patients on maintenance immunosuppression with CNI + MMF +/- prednisolone versus CNI +/- prednisolone. Clusters c1 – c13 were annotated based on their expression of subset-defining NK surface markers. **[a]** Clusters with significant differences between the two groups, **[b]** Clusters without any significant differences. **CNI**: Calcineurin inhibitor; **MMF**: Mycophenolate Mofetil. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

Of interest, patients receiving MMF had a significantly lower proportion of the cluster 12 (i.e., NKG2C^{hi} KIR+) subset compared to their non-MMF counterparts (p = 0.03). Furthermore, a two-fold decline in the NKG2C^{hi} KIR+ subset was observed in patients on MMF between the pre-transplant and 3-months post-transplant time point (0.80% vs 0.43%; p = 0.02). The NKG2C^{hi} KIR+ subset is well recognised for its antiviral function, specifically undergoing expansion in response to CMV infection. [196] A previous study has linked the concurrent use of CNIs and MMF with both early and late reactivation of CMV in transplanted patients. [460] However, in this study, only 1 of the 11 patients on MMF known to be CMV seropositive prior to transplantation experienced viral reactivation within 6-months of their transplant. This low incidence of CMV reactivation in the ITHACA cohort could be due to the high coverage of patients with antiviral prophylaxis during this high-risk period. Changes within the NK cell compartment were not found to be related to thymectomy status or the choice of induction immunosuppression (p > 0.05).

6.5.7 EBV DNAemia in the early post-transplant period is associated with a depletion of early NK cells and a marked expansion of the CD16dim CD56dim population

The relative frequency of circulating CD56bright NK cells was significantly lower in patients with detectable EBV DNA in their blood at 3 months post-transplantation compared to patients without EBV DNAemia (p = 0.024). This was specifically driven by a lower number of circulating early NK cells i.e. CD16- CD56bright subset in this group of patients (Figure 6.14b).

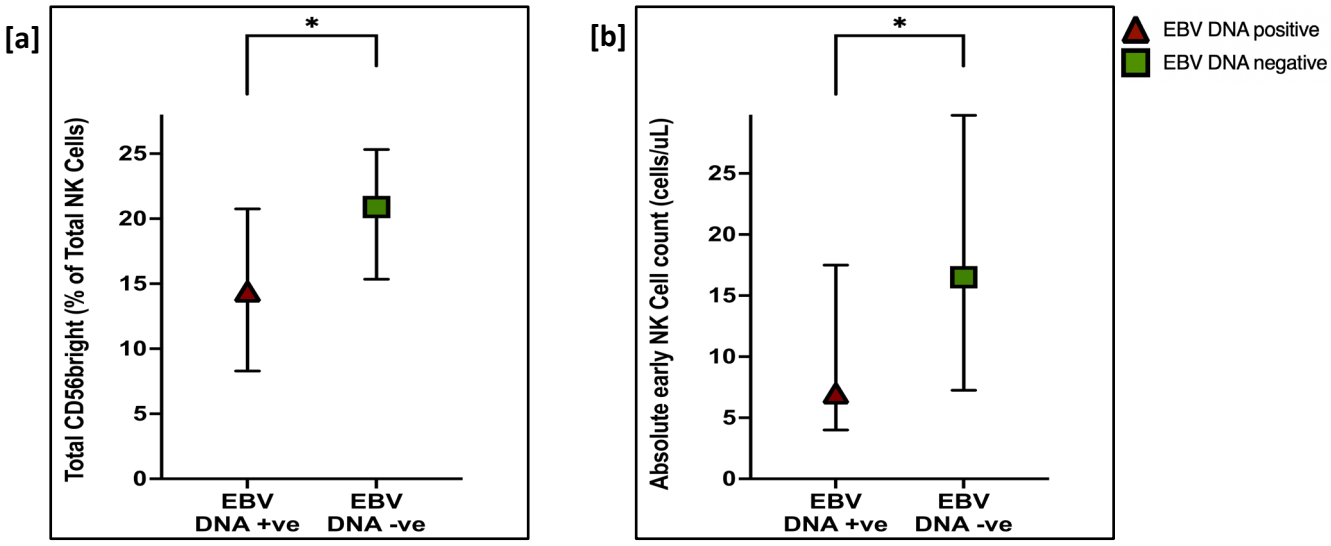


Figure 6.14: Comparison of [a] Relative frequency (%) of total CD56bright NK cells and [b] Absolute early NK cell count (cells/ul) according to EBV DNA status at 3 months post-transplantation. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

A prominent expansion of the total CD16dim CD56dim NK cell population (clusters 2, 3, 4 and 6) was also observed in patients who had acquired EBV DNAemia within 3 months of their transplant, rising from 22.2% (IQR: 14.4 – 30.5) before transplant to 40.4% (IQR: 28.4 – 44.6) at 3 months post-transplant (p = 0.047). A similar increase in this cell subset was observed in transplanted patients without EBV DNAemia, albeit to a much lesser degree (21.0% [IQR: 11.5 – 24.3] vs 25.0% [IQR: 21.8 – 29.8]; p = 0.03). As illustrated in Figure 6.15, the proportion of CD16dim CD56dim NK cells therefore differed significantly between patients with EBV DNAemia at 3 months post-transplant and those without detectable viraemia (p = 0.012).

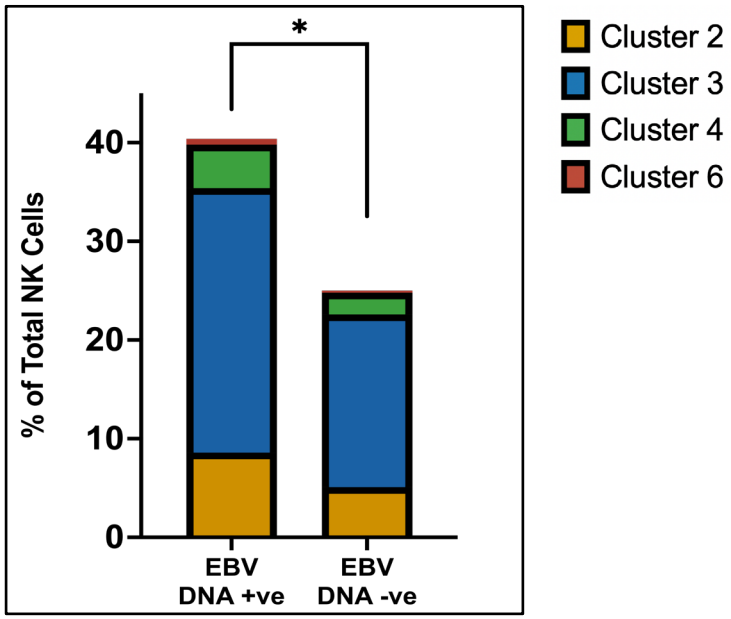


Figure 6.15: Stacked bar chart illustrating the cumulative frequency of the CD16dim CD56dim NK cell subsets identified using high dimensional reduction and cluster analysis. The cumulative frequency was compared according to EBV DNA status at 3 months post-transplantation. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

6.5.8 Paediatric heart transplant patients fail to expand the CD56dim NKG2A+ KIR- NK cell population in response to EBV DNAemia

Contrary to published observations from immunocompetent children with acute/reactivated EBV, there was no evidence of a preferential proliferation of the CD56dim NKG2A+ KIR- subset in ITHACA patients with a primary EBV infection or viral reactivation (Figure 6.16).

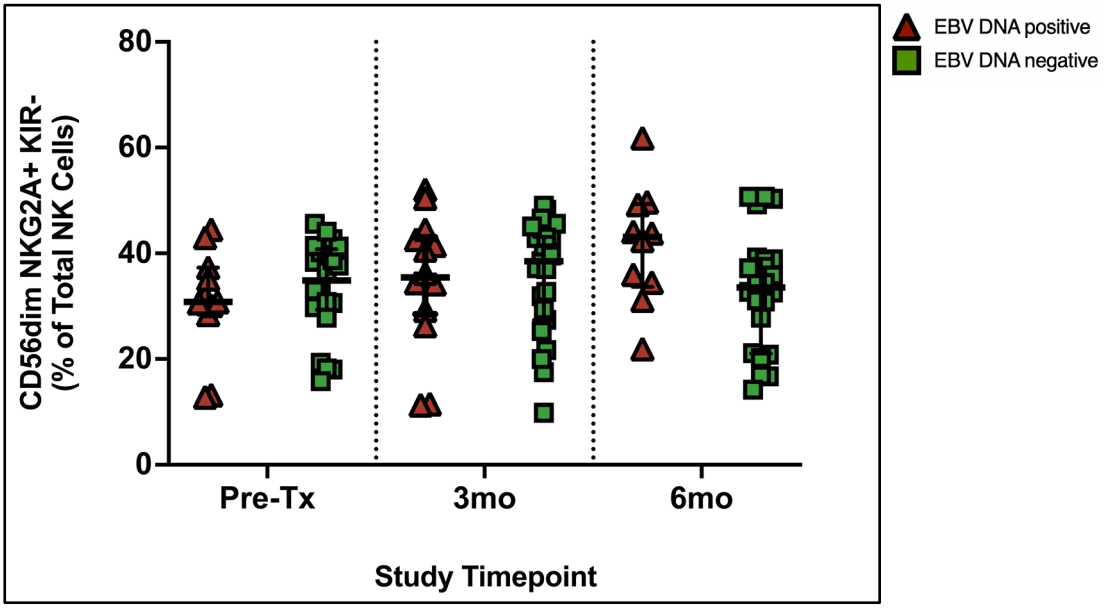


Figure 6.16: Frequency of CD56dim NKG2A+ KIR- NK cell subset in patients who acquired a primary EBV infection or viral reactivation during the early post-transplant period. There was no significant difference in the baseline frequency of the NK cell subset between the two patient groups prior to transplantation and similarly at 3 months and 6 months post-transplantation (all *p*-values > 0.05).

Patients with primary/reactivated EBV infection displayed a similar proportion of CD56dim NK2A+ KIR- NK cells as patients without detectable viraemia. Temporal changes in the frequency of the CD56dim NKG2+ KIR- population during EBV DNAemia were also examined in patients who had paired samples taken prior to and shortly after experiencing an acute EBV infection or viral reactivation. There was no noticeable increase in the frequency of this NK cell subset from baseline (31.3% [IQR: 28.4 – 37.3) compared to the point of detectable circulating EBV DNA at 3 months post-transplantation (34.5% [IQR: 26.3 – 42.6]; p = 0.38). All patients who had acquired a primary EBV infection/viral reactivation within 3 months of their transplant continued to experience EBV DNAemia at 6 months post-transplantation and still showed no evidence of a significant increase in the frequency of CD56dim NK2A+ KIR- NK cells compared to 3 months post-transplant (43.1% [IQR: 34.5 – 49.2; p = 0.29]).

6.6 Discussion

This chapter provides a comprehensive analysis of the innate immune landscape in thymectomised children prior to and after cardiac transplantation. It offers evidence that demonstrates a significant post-transplant perturbation to various immune cell populations involved in anti-EBV host responses. As expected, none of the innate immune cell populations were directly impacted by thymectomy prior to transplantation. Although limited evidence has illustrated an increased frequency of CD56bright NK cells and the acquisition of HLA-DR$^{lo/-}$ monocytes in non-transplanted early thymectomy patients, [152] neither of these immunological changes were evident in this study. It is important to note that such changes to the innate immune composition have only been described in early thymectomised patients presenting with heart failure in adulthood, [152] and could therefore be mediated by other factors unrelated to age at thymectomy.

The most notable period of pervasive disruption to the innate immune landscape was at 3 months post-transplantation. While thymectomy status did not play a key role in the post-transplant alteration of most non-T lymphocyte cell lineages, a key finding was the significant depletion of circulating NKT cells observed in early thymectomy patients after transplantation. This is the first study to describe this phenomenon in children considered to be at high risk of EBV-PTLD. It is unclear why the impact of thymectomy on this subset of unconventional T-lymphocytes was only significant during the post-transplant period. It is possible that extrathymic homeostatic proliferation of NKT cells helps to support their repertoire following thymectomy but is unable to provide adequate compensation in the setting of chronic post-transplant immune stimulation. This potentially explains the observed thymectomy-related variation in NKT cell reconstitution seen in ITHACA participants with paired samples across the early post-transplant period. The importance of this finding is underscored by the strong negative correlation that was noted between circulating EBV DNA load and NKT cell count around the time of primary infection and viral reactivation. NKT cells are a key component of the host's early anti-EBV response, acting to restrict viral replication either directly through antiviral IFNγ and IL-2 activity or by activating critical NK cell, DC and EBV-specific T-lymphocyte responses. [461] The important contribution of NKT cells to EBV immunity is further emphasised by the fact that patients with X-linked lymphoproliferative disease (XLP), an inborn error of immunity associated with a lack of NKT cells, are uniquely predisposed to fulminant EBV infections and EBV-related B cell lymphomas. [462]. Although the size of the study sample and the limited number of patients with primary EBV/viral reactivation after transplant did not permit a more detailed

exploration of the intersection between age at thymectomy, EBV infection and NKT cells, it is hypothesised that the immunological milieu of early thymectomy patients during the immediate post-transplant period (when patients are at their nadir of immunity) might be akin to an XLP-like immune microenvironment. This offers one possible facet of aberrant EBV-immunity in early thymectomy patients that could contribute to their increased risk of EBV-PTLD.

The changes to other innate immune cell populations involved in early anti-EBV responses were primarily mediated by immunosuppressant medications during the early post-transplant period. The restricted expansion of CD16+ monocytes in ITHACA participants who received ATG induction is supported by evidence from *in vitro* studies that show a significant reduction of CD16 on human monocytes following stimulation with ATG. [463] These ATG-stimulated monocytes also exhibit immunosuppressive activity through dampening of T-lymphocyte proliferation by various mechanisms such as the induction of monocyte tryptophan catabolism via the enzymatic activity of Indoleamine 2,3-dioxygenase (IDO). [463, 464] A recent case report identified an association between persistent EBV infection of varying severity and CD16 deficiency on monocytes and NK cells. [465] The fact that ITHACA participants with EBV DNAemia had a significantly lower frequency of total CD16+ monocytes around the time of primary EBV acquisition/reactivation compared to EBV naïve healthy patients is suggestive that the reduction of CD16+ monocytes during the early post-transplant period contributes to the disruption of EBV control after transplantation. It is important to highlight that while persistent EBV infection (n = 5), EBV-PTLD (n = 1) and severe IM-type illness (n = 1) were observed in ITHACA patients who received ATG, both persistent EBV DNAemia (n = 8) and EBV-PTLD (n = 1) were also seen in patients who received Basiliximab. Therefore, while the quantitative loss of anti-EBV CD16+ monocytes could be considered as an important contributor to poor EBV control, it is possible that this is compounded by an impairment of its functional state by immunosuppressive therapy. Impaired secretion of TNF-α by intermediate monocytes stimulated with EBV peptides has been used to identify an "over-immunosuppressed" microenvironment in kidney transplant patients who received induction therapy with either ATG or Basiliximab. [466, 467] Similar to the ITHACA study, other researchers have observed a shift towards CD16+ monocytes after Basiliximab induction therapy in adult kidney transplant patients. [468] However, contrary to observations in other studies, these cells were shown to still retain capacity to produce pro-inflammatory cytokines such as TNF-α and IFN-γ upon stimulation. [468]. While the ITHACA study did not include the functional analysis of monocyte subsets, an impairment of their anti-EBV activity might be inferred from the significant

negative correlation that was observed between EBV DNA load and the expression of monocyte activation markers (HLA-DR and CD11c) irrespective of the induction agent that was used. Further work is required to determine if the Basiliximab-related impairment of CD16+ monocyte anti-EBV activity is either age and/or dose-dependent or specific to cardiac transplant patients.

The adverse impact of antimetabolite immunosuppression on the NK cell population has been reported extensively and aligns with findings from this study of a much lower NK cell frequency in patients on MMF during the early post-transplant period. However, this is the first study to identify a phenotypic shift towards senescent/terminally differentiated NK cell subsets in these patients. Although the cytotoxic function of senescent NK cells is preserved, their proliferative capacity in response to antigenic stimulation (e.g., by EBV peptides) is drastically impaired. [469, 470] These cells also have shorter telomeres and are predisposed to apoptosis. [471] The accumulation of such cells at the expense of more immunologically robust CD16+ CD56dim NK cells has consequences for long-term immunity by contributing to premature immunological ageing and defective anti-viral immunity. [469] The significantly lower proportion of CD16+ CD56bright NK cells observed in patients receiving MMF also has implications for EBV-PTLD pathogenesis. This NK cell subset has been shown to exhibit important antitumour characteristics as evidenced by its potent cytotoxicity when stimulated by the Raji EBV+ lymphoid cell line. [472]

Another notable change within the NK cell population was the depletion of early NK cells and converse expansion of the CD16dim CD56dim subset in patients with EBV DNAemia. The observed contraction of early NK cells during EBV DNAemia is consistent with similar findings in the setting of acute IM. [195, 196] This most likely represents a migration of this cell subset from peripheral circulation to tonsillar tissue, which is known to be enriched with CD56bright NK cells during active EBV infection, as opposed to an increased differentiation into more mature subsets. Unlike early NK cells, the CD16dim CD56dim subset has received little scientific scrutiny to date. There is compelling evidence to suggest that CD16dim CD56dim NK cells are an intermediate subset between the CD16- CD56bright early NK cells and CD16+ CD56dim mature subset. [456] Expansion of this NK cell subset has been shown to be associated with impaired IFN-γ production during HIV infection and poor immune response in HIV patients on antiretroviral therapy. [473, 474] CD16dim CD56dim NK cells have also been shown to be pervasive in patients with IEIs such as transporter associated with antigen processing (TAP) deficiency and in some cases of familial hemophagocytic lymphohistiocytosis (FHL) and common variable immunodeficiency (CVID), all of which can be associated with chronic EBV

viraemia and EBV-associated lymphoproliferative disorders. [456, 475, 476] As this NK subset has not previously been reported in healthy non-transplanted patients with active EBV infection, it is unclear if their expansion is unique to EBV infection in the setting of SOT or whether this is a strategy typically employed by EBV to circumvent host responses. Interestingly, EBV lytic proteins are known to interfere with antigen presentation to EBV-specific T-lymphocytes through the downregulation of TAPs. [477, 478] More detailed investigation of this NK cell subset during EBV DNAemia in healthy children and transplant patients is warranted.

Finally, the failure of ITHACA patients to adequately expand CD56dim NKG2A+ KIR- NK cells in response to EBV DNAemia is indicative of one of the mechanisms through which EBV potentially evades early detection by the host's innate immune system after SOT. The preferential proliferation of this NK cell subset during EBV infection is a well-known phenomenon and the suboptimal control of EBV as a result of a diminished frequency of CD56dim NKG2+ KIR- NK cells has been demonstrated in paediatric liver transplant patients with EBV-PTLD. [443] The reason behind this impaired expansion is uncertain. It is possible that this process is mediated by the use of long-term immunosuppressive medications, some of which have been implicated in anti-NK cell activity. While tacrolimus has been implicated in the selective downregulation of activation receptors such as NKG2D on NK cells, antimetabolites like MMF/azathioprine have been shown to directly impair the expansion of NKG2A+ KIR- NK cells. [322, 479] All ITHACA patients who developed EBV-PTLD and severe IM-like illness were on a combination of tacrolimus and MMF/azathioprine at diagnosis. Similarly, all patients in this study who had persistent EBV DNAemia were on tacrolimus. Further targeted research is needed to examine the direct effect(s) of tacrolimus on NKG2A+ KIR- NK cells as well as any potential synergistic effect it might have when administered in combination with MMF or azathioprine.

In conclusion, the evidence presented in this chapter demonstrates a pervasive disruption to the innate immune landscape during the early post-transplant period which is heavily modulated by induction and maintenance immunosuppression therapy whilst being mostly independent of thymectomy status. These alterations to the innate immune microenvironment carry profound consequences for anti-EBV host responses at the point of primary infection or viral reactivation, along with associated implications for the risk of developing EBV-PTLD. As these cellular changes only provide a partial explanation for the increased risk of EBV-PTLD in paediatric cardiac transplant patients, the next chapter will focus on examining the distinctive effects of thymectomy-induced T-lymphopaenia on the risk of EBV-PTLD.

Chapter 7. T-lymphocyte immunity of the ITHACA study cohort

7.1 Introduction

The role of impaired T-lymphocyte surveillance in the pathogenesis of EBV-PTLD has largely focussed on adults and paediatric recipients of either HSCT or non-cardiac SOTs. Recent retrospective studies have provided convincing evidence to implicate early thymectomy in the pathogenesis of EBV-PTLD. However, there have been no prospective studies to date that explore the evolution of systemic and anti-EBV specific T-lymphocyte immunity in the context of age at thymectomy and subsequent SOT or how these factors may contribute to an increased risk of EBV-PTLD.

This chapter provides a detailed analysis of the pre- and post-transplant T-lymphocyte compartment of patients recruited to the ITHACA study and their relationship to thymectomy status, EBV infection and immunosuppression therapy.

7.2 Aims of the chapter

1. To determine if there is a difference in the pre-transplant immune profile of the T-lymphocyte compartment based on age at thymectomy.
2. To analyse the relationship between T-lymphocyte subsets and age at thymectomy in the setting of post-transplant immunosuppression.
3. To examine if there are differences in the post-transplant immune signature of T-lymphocytes according to age at thymectomy, EBV infection and immunosuppression therapy.
4. To evaluate the phenotypic and functional profile of EBV-specific T-lymphocytes in thymectomised patients after EBV exposure (primary infection and reactivation).

7.3 Flow cytometry analysis

High dimensional spectral flow cytometry data were obtained from pre- and post-transplant patient samples using the 30-colour T-lymphocyte immune panel described in chapter 3 (section 3.1.3). After all quality control measures relating to spectral unmixing had been performed, FCS files were imported into the OMIQ platform for downstream immune phenotyping of innate cell subsets using the manual gating strategy outlined in section 3.4.5.

Development and implementation of the workflow and parameter settings used in the high dimensional analysis of tetramer-labelled EBV-specific T-lymphocytes has been described in detail in chapter 6 (section 6.3). In brief, after FCS files had undergone scaling and automated cleaning, they were sub-sampled to ~22,000 live CD45+ CD3+ singlets per sample before undergoing normalisation with the fdaNorm algorithm to correct for potential batch effects. Once data had been normalised, UMAP analysis was performed for global visualisation of the TCRγδ, CD4+ T_H, CD8+ CTL populations using the sub-sampled live CD45+ CD3+ singlets from all 155 study and healthy control samples that were available across the first six months of transplantation (~3.4 x10^6 cells). To facilitate more detailed sub-clustering of the different T-lymphocyte populations of interest, up to 3.0 x10^6 cells each of TCRγδ, CD4+ T_H and CD8+ CTLs were manually gated before processing using dimensional reduction and cluster analysis pipeline. UMAP settings were as follows: all FCS files used, all fluorescent parameters related to the cell subsets of interest (except CD45 and Live/Dead), neighbours = 65, minimum distance = 0.6, components = 2, metric = euclidean, learning rate = 1, epochs = 250, random seed = 8125. Data were then clustered using FlowSOM with the following settings: all files used, clustering features CD27, CD28, CD57, CCR7, CD45RA, CD8, CD4, CD31, CD25, CD127, γδT, UMAP_1, UMAP_2, with xdim = 25 and ydim = 25, rlen = 10, Distance Metric = euclidean, consensus metaclustering with k = 10 – 30, Random Seed = 5460. Next, a hierarchically-clustered heatmap was used to annotate metaclusters into biologically recognisable cell populations based on their surface marker expression. Once this had been achieved, the resulting cell populations were analysed according to covariate groups (e.g. thymectomy status) by clustered heatmapping and visualised for comparison between samples using the UMAP parameters.

7.4 Statistical analysis

Pre-transplant T-lymphocyte flow cytometry data were analysed independent to data from the post-transplant study timepoints to account for ITHACA recruits who were still awaiting transplantation as well as those whose post-transplant samples hadn't been processed at the time of data analysis for this thesis. Similar to statistical analyses in chapters 5 and 6, the evaluation of post-transplant data was primarily focussed on the 3- and 6-month study timepoints (i.e. < 12-months post-transplant) for reasons that have been outlined in chapter 5 (section 5.1). The relative abundance of T-lymphocytes for each patient sample taken at the pre-transplant, 3- and 6-month study timepoints was calculated as the percentage of CD45+ live events in the total gated live lymphocyte population. The abundances of subsets of interest within the CD4+ and CD8+ lineages were expressed as the percentage of their cell lineage of origin (i.e. CD3+ CD56- lymphocytes) unless stated otherwise. A dual platform method was used to estimate the absolute counts (cells/µl) for CD4+ and CD8+ T-lymphocytes and their subsets in patients who had a corresponding full blood count (FBC) sample taken at the time of study-related blood sampling (see section 6.4). Comparisons of pre-/post-transplant cell frequencies were performed according to thymectomy group, induction/maintenance immunosuppression agents, EBV status and other relevant study covariates using either Wilcoxon rank sum for the comparison of medians between 2 continuous variables or the Kruskal-Wallis test when >2 continuous variables were being compared. Similar analyses were performed to compare absolute cell counts in patients with a contemporaneous FBC. Wilcoxon signed rank test was used to compare median cell counts and proportions of paired samples at 3 months and 6 months post-transplantation. Correlation analysis was performed by Spearman rank correlation coefficient *r*. The *p*-value significance was set at <0.05 with post hoc adjustment when appropriate for multiple pairwise comparison by thymectomy status using the Benjamini-Hochberg method. All analyses were conducted with the STATA (version 18.0), while graphs and figures were generated with GraphPad Prism version 10.0.

7.5 Findings

7.5.1 Early thymectomy exacerbates age-related changes in the T-lymphocyte compartment

Analysis of pre-transplant patient samples demonstrated an age-related contraction in the absolute counts of CD3+ T-lymphocytes, γδT, CD4+ T_H and CD8+ CTL lineages across both thymectomy and non-thymectomy groups (Figure 7.1).

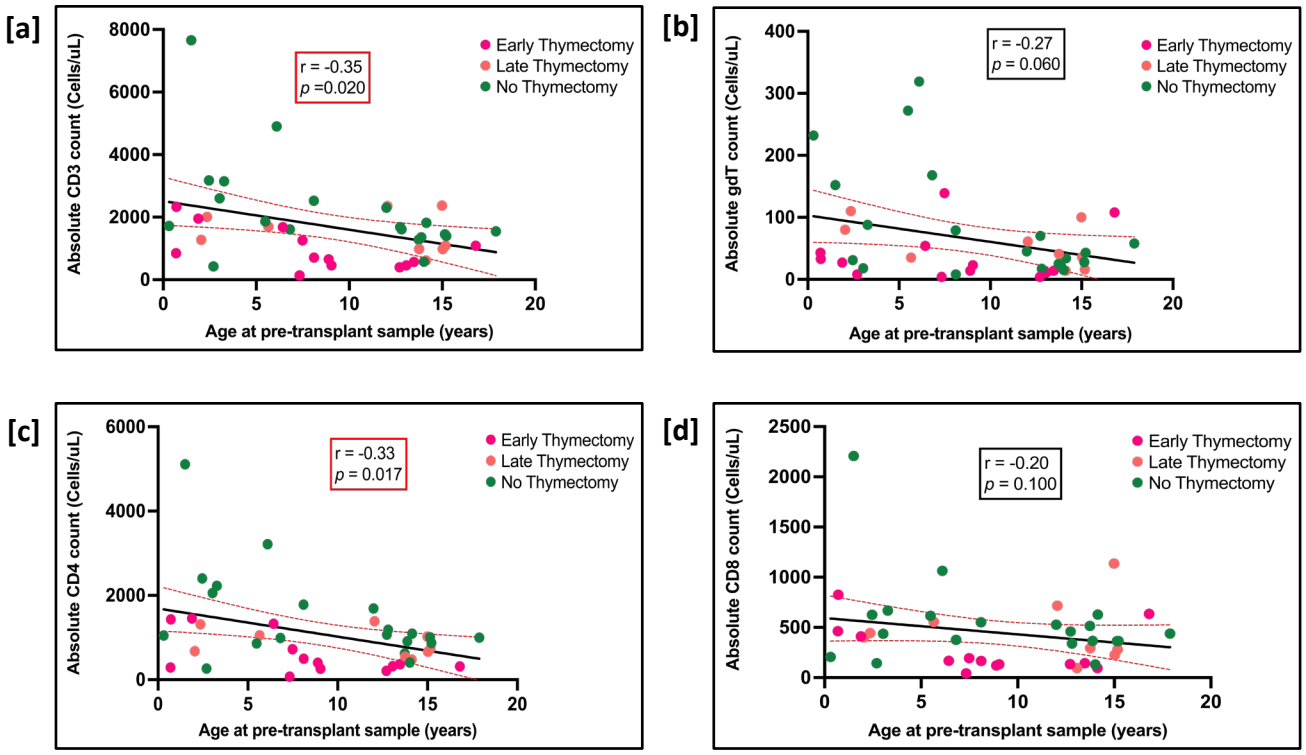


Figure 7.1: Correlation between pre-transplant T-lymphocyte subsets (cells/μL) and patient age (years) by thymectomy status. **[a]** Absolute CD3 T-lymphocyte count, **[b]** Absolute γδT count, **[c]** Absolute CD4 T_H count, **[d]** Absolute CD8 CTL count. Spearman's rank correlation coefficient (r) and its associated *p*-value have been highlighted in red when statistically significant (i.e. $p < 0.05$). The 95% CI for the line of best fit is represented by the two red dashed lines.

Similar age-dependent changes in the T-lymphocyte compartment have been described in healthy paediatric populations. A significant decline in the absolute CD3+ T-lymphocyte and CD4+ T_H counts typically occurs during early childhood to reach adult levels by adolescence in contrast to γδT and CD8+ CTLs which exhibit a more gradual contraction during this period. [100] In this study, the trend was noted to be more pronounced in patients who had undergone an early thymectomy ≥5 years prior. This subgroup of patients had a median CD3+ T-lymphocyte count that was significantly lower than that of the non-thymectomy group (610 cells/μl [456 – 1088] vs 1612 cells/μl [1398 – 1864]; $p < 0.001$). Likewise, their absolute CD4+ T_H count (345 cells/μl [260 – 500]) was significantly lower compared to similarly-aged children with late thymectomy (745 cells/μl [548 – 1052]; $p = 0.014$) and

no thymectomy (1004 cells/µl [873 – 1185]; $p < 0.001$), as was the median CD8+ CTL count in early thymectomy patients (140 cells/µl [120 – 167]) when compared with their late thymectomy (295 cells/µl [225 – 717]; $p = 0.03$) and non-thymectomy (449 cells/µl [364 – 552]; $p = 0.002$) counterparts. Analysis of the naïve T-lymphocyte subsets was performed to determine if the age-related changes within this compartment were similarly more profound in early thymectomy patients. A significant negative correlation was observed between patient age and the absolute RTE, naïve CD4+ T_H and naïve CD8+ CTL counts (Figure 7.2).

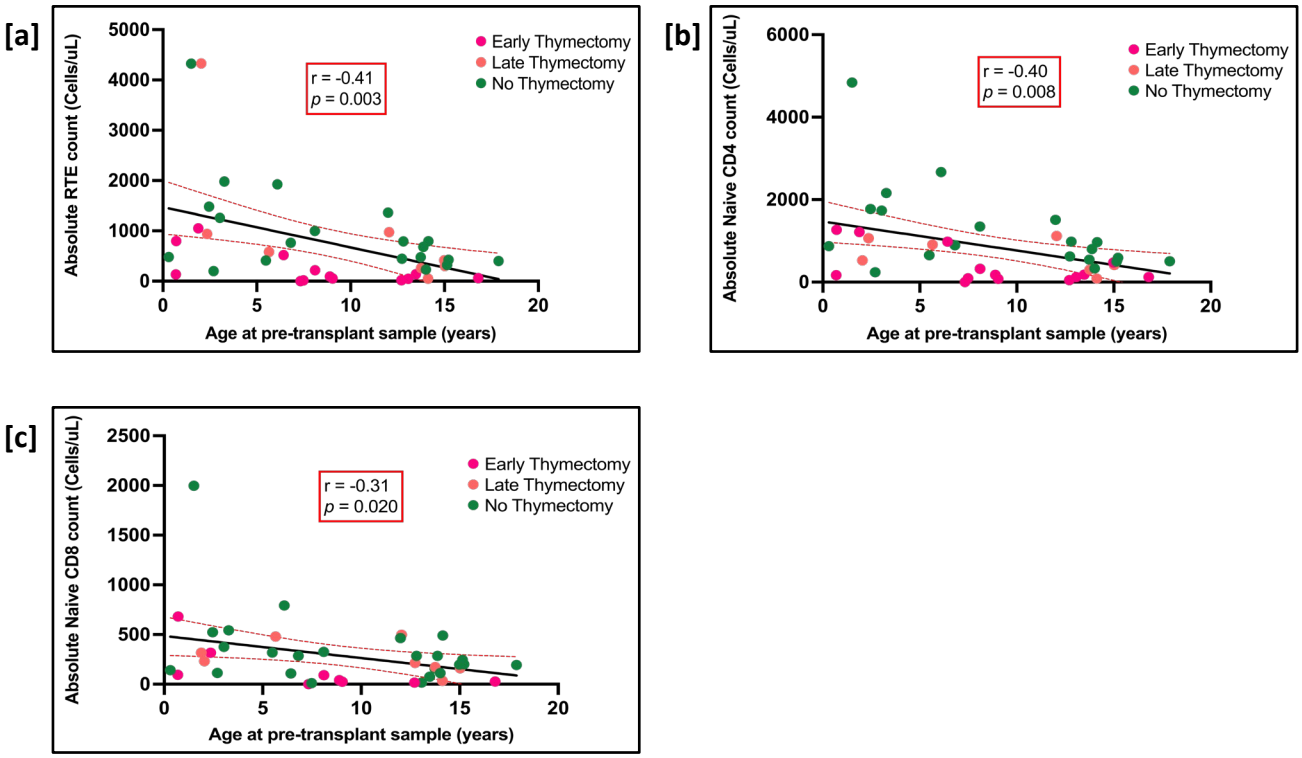


Figure 7.2: Correlation between pre-transplant recent thymic emigrant (RTE), naïve T-lymphocyte subsets (cells/µL) and patient age (years) by thymectomy status. [a] Absolute RTE count**, [b]** Absolute Naïve CD4 T_H count, **[c]** Absolute Naïve CD8+ CTL count**.** Spearman's rank correlation coefficients (r) and associated *p*-values have been highlighted in red when statistically significant (i.e. $p < 0.05$). The 95% CI for the line of best fit is represented by the two red dashed lines.

While a progressive age-dependent decline in recent thymic emigrants and naïve T-lymphocyte counts was expected in the non-thymectomy group as a result of thymic involution, the age-related change in the absolute counts of these subsets was notably more pronounced in patients who had undergone an early thymectomy ≥5 years prior. Their median RTE count was 58 cells/µl (20 – 136) compared to 396 cells/µl (260 – 582) in the late thymectomy group [$p = 0.014$] and 579 cells/µl [(411 – 792) in the non-thymectomy group [$p < 0.001$]. Likewise, their median naïve CD4+ T_H count was noted to be 125 cells/µl (79 – 183) compared to 476 cells/µl (300 – 910) in the late thymectomy group

[p = 0.03] and 726 (542 – 981) in the non-thymectomy group [p < 0.001]. A significant difference was also observed between the median naïve CD8+ CTL count of early thymectomy patients aged ≥ 5 years (27 cells/µl [17 – 76]) compared to similarly-aged children with a late thymectomy (197 cells/µl [160 – 480]; p = 0.001) and no thymectomy (285 cells/µl [200 – 326]; p < 0.001). These findings were in contrast to previous studies that observed a replenishment of the naïve T-lymphocyte pool ≥ 5 years after early thymectomy in non-transplanted children. [480]

7.5.2 Extensive alteration to the pre-transplant αβ T-lymphocyte profile is a distinct feature of early thymectomy

Evidence of a significant alteration to the relative frequencies (Figure 7.3) and absolute counts (Figure 7.4) within the T-lymphocyte compartment prior to transplantation was observed in children with an early thymectomy compared to age-matched children with late and no thymectomy.

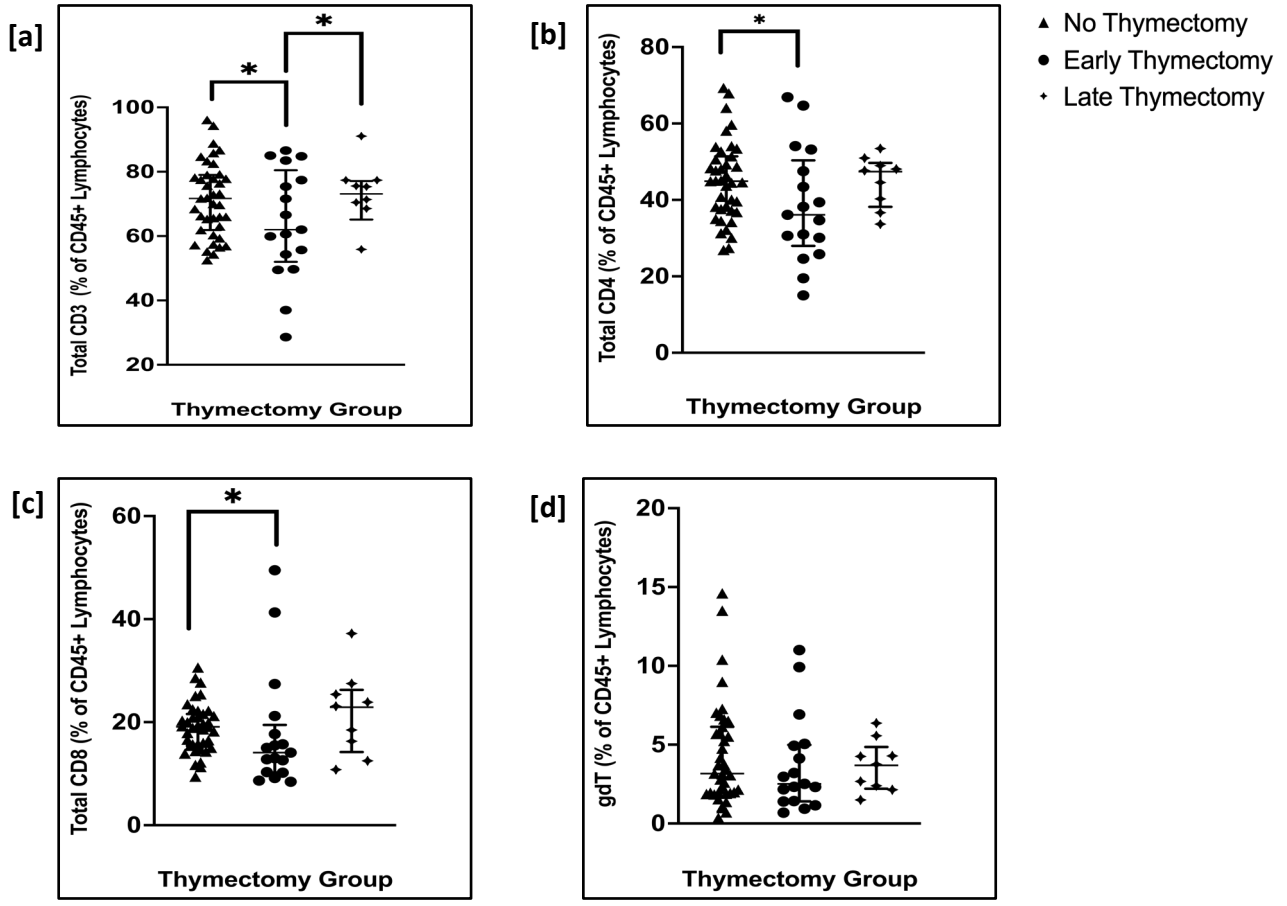


Figure 7.3: Percentage distribution of the main T-lymphocyte subsets according to thymectomy group (pre-transplant).

[a] Proportions of total CD3+ T-lymphocytes **[b]** Proportions of total CD4+ T_H, **[c]** Proportions of CD8+ CTLs; **[d]** Proportions of γδ T-lymphocytes. All p values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

Early thymectomy resulted in a significantly lower proportion of total CD3+ T-lymphocyte counts compared to late and no thymectomy (Figure 7.3a). A similar adverse effect on the proportion of circulating CD4+ T_H and CD8+ CTLs was observed in comparison to age-matched non-thymectomy patients while frequency of γδ T-lymphocytes was similar across the three thymectomy groups (Figure 7.3b-d). An analysis of absolute cell counts revealed an identical trend of significantly lower αβ T-lymphocyte subsets in the early thymectomy group compared to non-thymectomy patients (Figure 7.4a-c) with preservation of the γδ T-lymphocyte repertoire (Figure 7.4d).

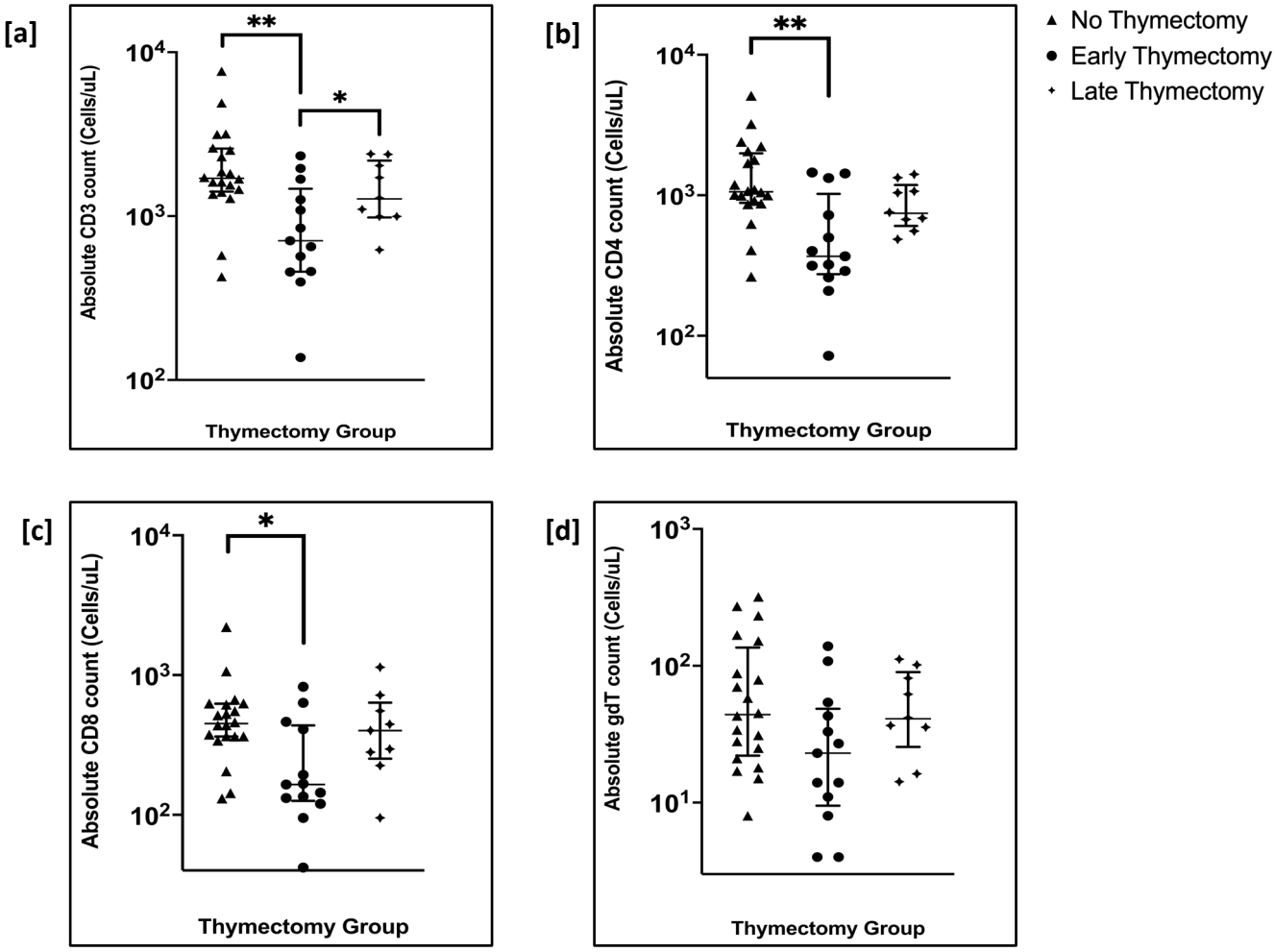


Figure 7.4: Absolute cell counts for the pre-transplant T-lymphocyte compartment. Comparison of absolute cell counts (cells/µl) by thymectomy status for **[a]** Total CD3+ T-lymphocytes **[b]** Total CD4+ T_H, **[c]** Total CD8+ CTLs; **[d]** Total γδ T-lymphocytes. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

As highlighted in section 7.5.1, ≥ 5 years post-thymectomy was identified as an important effect modifier for differences in the T-lymphocyte subsets between the early and late thymectomy groups, with patients who were at least 5 years post an early thymectomy having significant αβ T -

lymphopaenia prior to transplantation compared to age-matched children with a late thymectomy. The number of circulating regulatory T-lymphocytes (Tregs) were also adversely affected by pre-transplant thymectomy. Both early and late thymectomy patients exhibiting significantly lower absolute counts of Tregs compared to the non-thymectomy group, while the difference between the early and late thymectomy groups approached statistical significance (Figure 7.5).

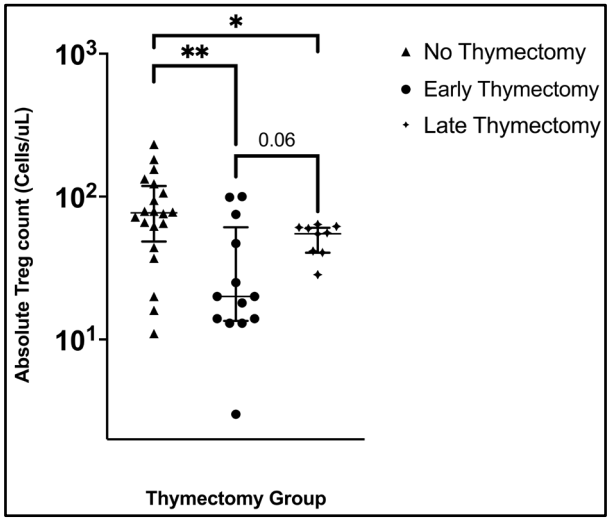


Figure 7.5: **Impact of thymectomy on circulating regulatory T-lymphocyte (Treg) counts (cells/µl).** All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.3 Early thymectomy establishes a significant perturbation of the naïve αβ T-lymphocyte milieu prior to transplantation

The pre-transplant immune landscape of the naïve αβ T-lymphocyte repertoire was interrogated to determine if the changes observed in the main T-lymphocyte lineages were instigated by a depletion of the naïve T-lymphocyte compartment due to early thymectomy. This revealed extensive changes to the naïve αβ T-lymphocyte repertoire as a consequence of early thymectomy (Figure 7.5).

Early thymectomy induced a profound disruption in thymic activity, resulting in a significantly lower number of circulating RTEs compared to the late thymectomy and non-thymectomy groups (Figure 7.5a). An identical effacement of the naïve CD4+ T_H, CD8+ CTLs and Treg populations was observed, all of which were found to be significantly lower in patients with an early thymectomy compared to the late thymectomy and non-thymectomy cohorts (Figure 7.5b-d).

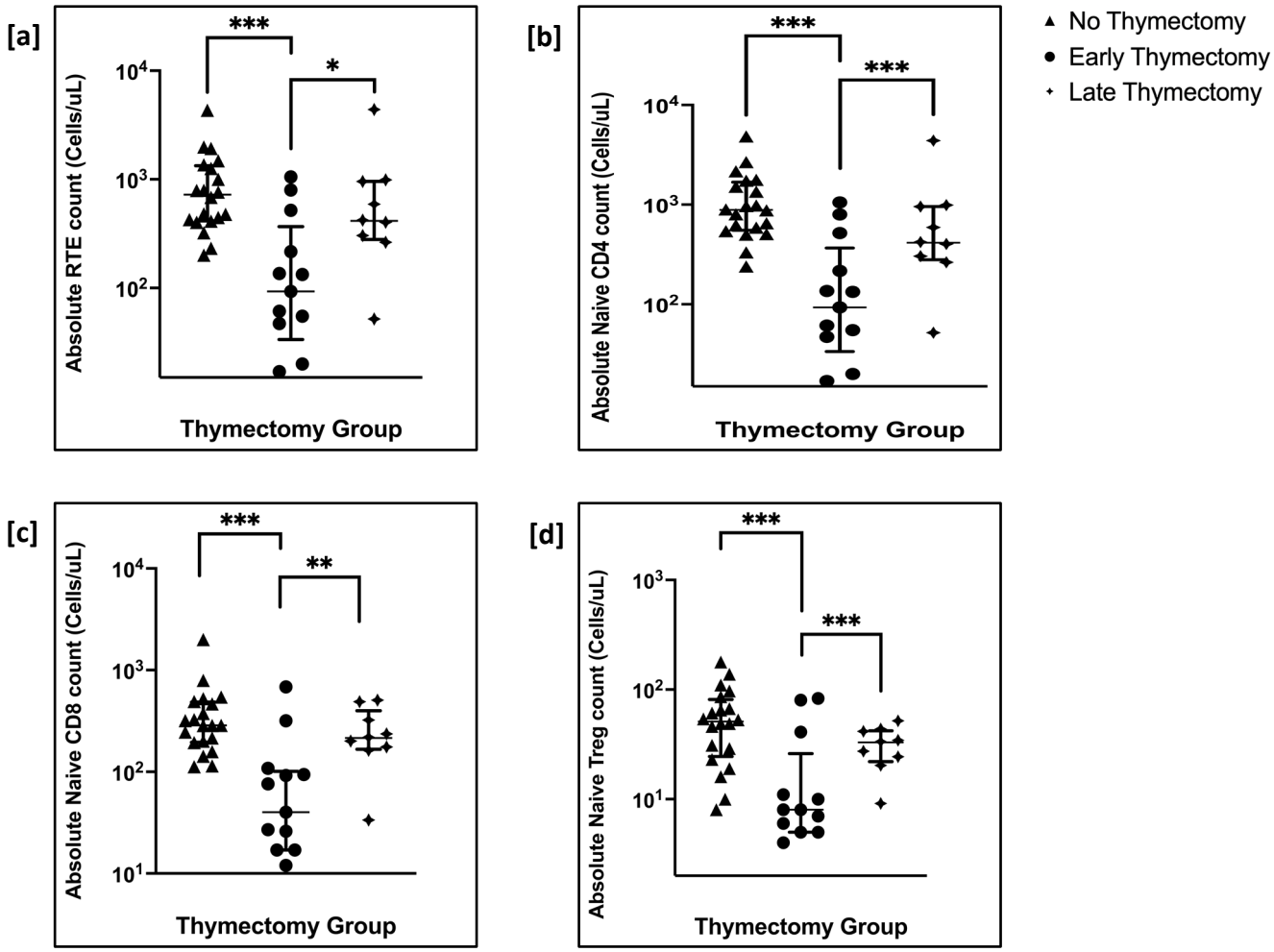


Figure 7.5: Impact of early thymectomy on the pre-transplant naïve αβ T-lymphocyte repertoire. Comparison of absolute cell counts (cells/µl) by thymectomy status for **[a]** Recent thymic emigrants (RTE), **[b]** Naïve CD4+ T_H, **[c]** Naïve CD8+ CTLs; **[d]** Naïve regulatory T-lymphocytes (Tregs). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.4 Early thymectomy prior to transplantation skews the T-lymphocyte profile towards a terminal memory phenotype characterised by the emergence of TEMRAs

The loss of naïve CD4+ T_H and CD8+ CTLs in early thymectomy patients was accompanied by a marked expansion in the proportion of circulating memory/effector cells within these immune populations (Figure 7.6). The pre-transplant memory compartment of CD4 T_H cells in early thymectomy patients was 49.3% (IQR: 25.2 – 69.6) compared to 20.1% (IQR: 13.1 – 30.8) in non-thymectomy patients (p = 0.0003) and 27.9% (IQR: 18.9 – 45.4) in late thymectomy patients (p = 0.21). The frequency of memory/effector CD8+ CTLs was also higher in the early thymectomy group (65.4% [IQR: 34.2 – 80.2] compared to the non-thymectomy (35.8% [IQR: 22.1 – 48.6]; p = 0.008) and late thymectomy patients (29.6% [27.7 – 42.1]; p = 0.07).

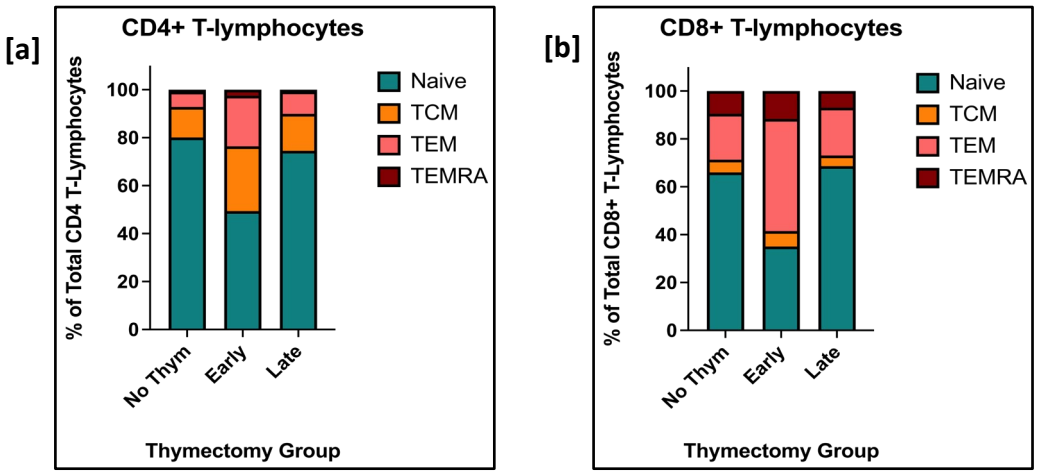


Figure 7.6: Stacked bar charts showing the proportional distribution of the naïve and memory/effector subsets within the **[a]** CD4+ TH and **[b]** CD8+ CTL populations according to thymectomy status. **TCM**: Central memory T-lymphocyte; **TEM**: Effector T-lymphocyte; **TEMRA**: CD45RA re-expressing terminal effector T-lymphocytes.

Early thymectomy patients exhibited an immune phenotype that was consistent with a significant progression towards the terminal differentiation of composite cells within the memory/effector compartment compared to non-thymectomy patients. This was characterised by significantly higher frequencies of central memory (T_{CM}), effector (T_{EM}) and CD45RA re-expressing terminal effector cells (TEMRA) within the CD4+ T_H population ($p < 0.01$). No significant differences were noted in the proportions of T_{CM} and T_{EM} between early and late thymectomy patients. However, late thymectomy patients were found to have significantly fewer CD4+ TEMRAs compared to their early thymectomy counterparts ($p = 0.02$). A similar marked progression towards terminal differentiation was observed in the memory/effector compartment of CD8+ CTLs in the early thymectomy group (Figure 7.6b). These patients had a significantly higher proportion of CD8+ T_{EM} compared to non-thymectomy patients ($p = 0.007$), while the difference in frequency of CD8+ T_{CM} was noted to trend towards statistical significance ($p = 0.058$). Early thymectomy patients had the highest proportion of CD8+ TEMRAs (11% [IQR: 5.0 – 18.6]), but this didn't differ significantly with the late (7.1% [IQR: 6.0 - 10.6]) and non-thymectomy patients (9.1% [IQR: 4.0 – 16.6]); $p = 0.61$.

7.5.5 Early thymectomy is associated with a persistent effacement of αβ T-lymphocytes during the early post-transplant period

The disruption to the αβ T-lymphocyte repertoire was sustained during the early post-transplant period, with early thymectomy patients maintaining significantly lower total T-lymphocytes, CD4+ T_H, CD8+ CTLs and Tregs at 3-months post-transplantation compared to the late and non-thymectomy cohorts (Figure 7.7). Whereas the main T-lymphocyte subsets at 6 months post-transplantation remained significantly lower in the early thymectomy group compared to the late thymectomy group, the previously observed differences between the early and non-thymectomy patients at baseline and 3 months post-transplantation were less pronounced.

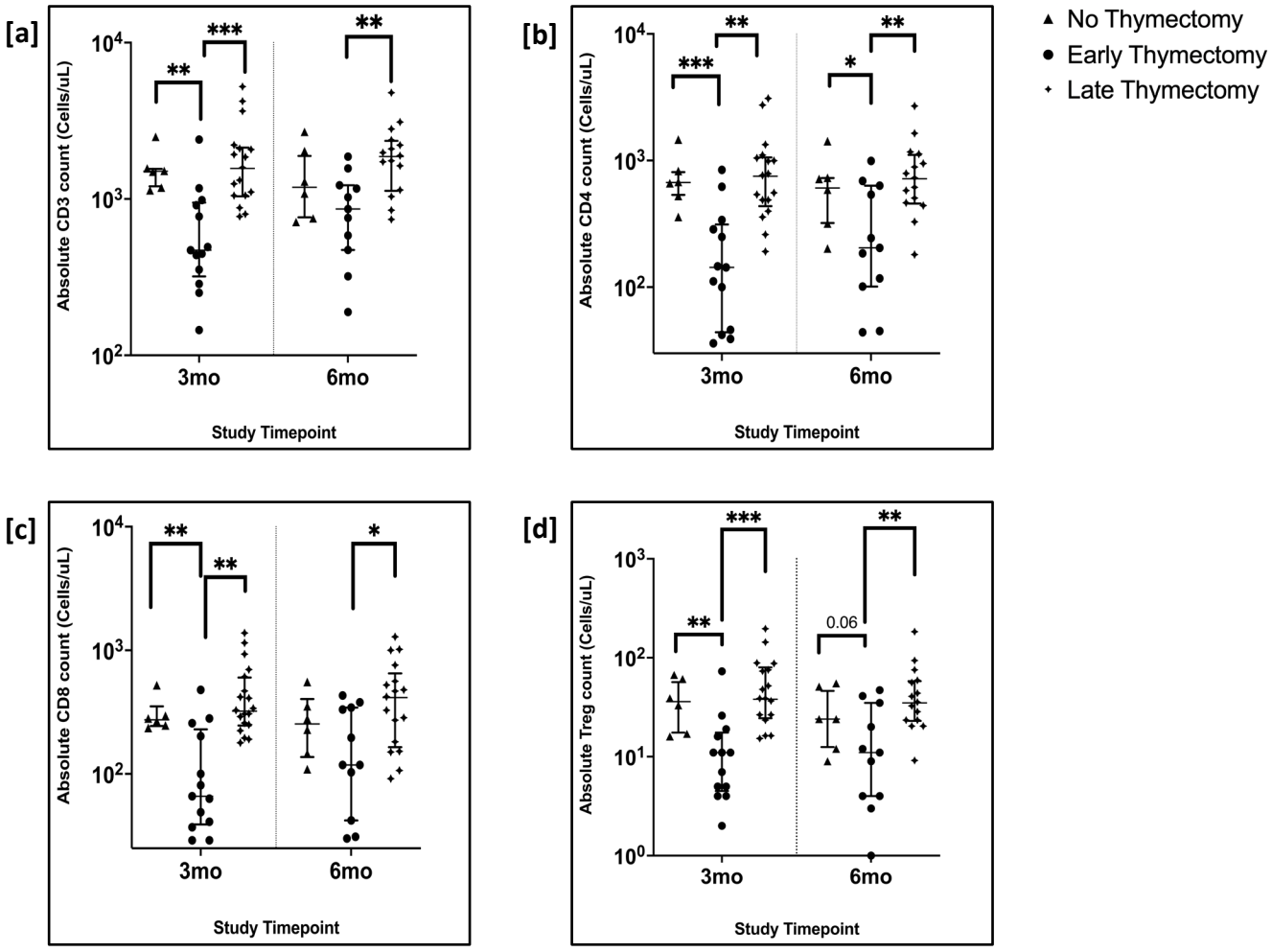


Figure 7.7: Impact of early thymectomy on the post-transplant αβ T-lymphocyte repertoire. Comparison of absolute cell counts (cells/µl) by thymectomy status for **[a]** Total CD3 T-lymphocytes, **[b]** Total CD4+ T_H, **[c]** Total CD8+ CTLs; **[d]** Total regulatory T-lymphocytes (Tregs). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Next, the naïve and effector memory compartments of the αβ T-lymphocyte lineages were assessed to determine if the attenuated differences in CD8+ CTL and Treg counts between non-thymectomy and early thymectomy patients at 6 months post-transplantation were as a result of either (residual) thymic recovery or homeostatic proliferation of differentiated subsets. Figure 7.8 illustrates the comparison of post-transplant absolute counts for RTE, naïve CD8 T-lymphocytes naïve Tregs between the thymectomy groups, while Figure 7.9 shows a similar comparison of post-transplant absolute counts for the CD8 CTL and Treg total memory compartments.

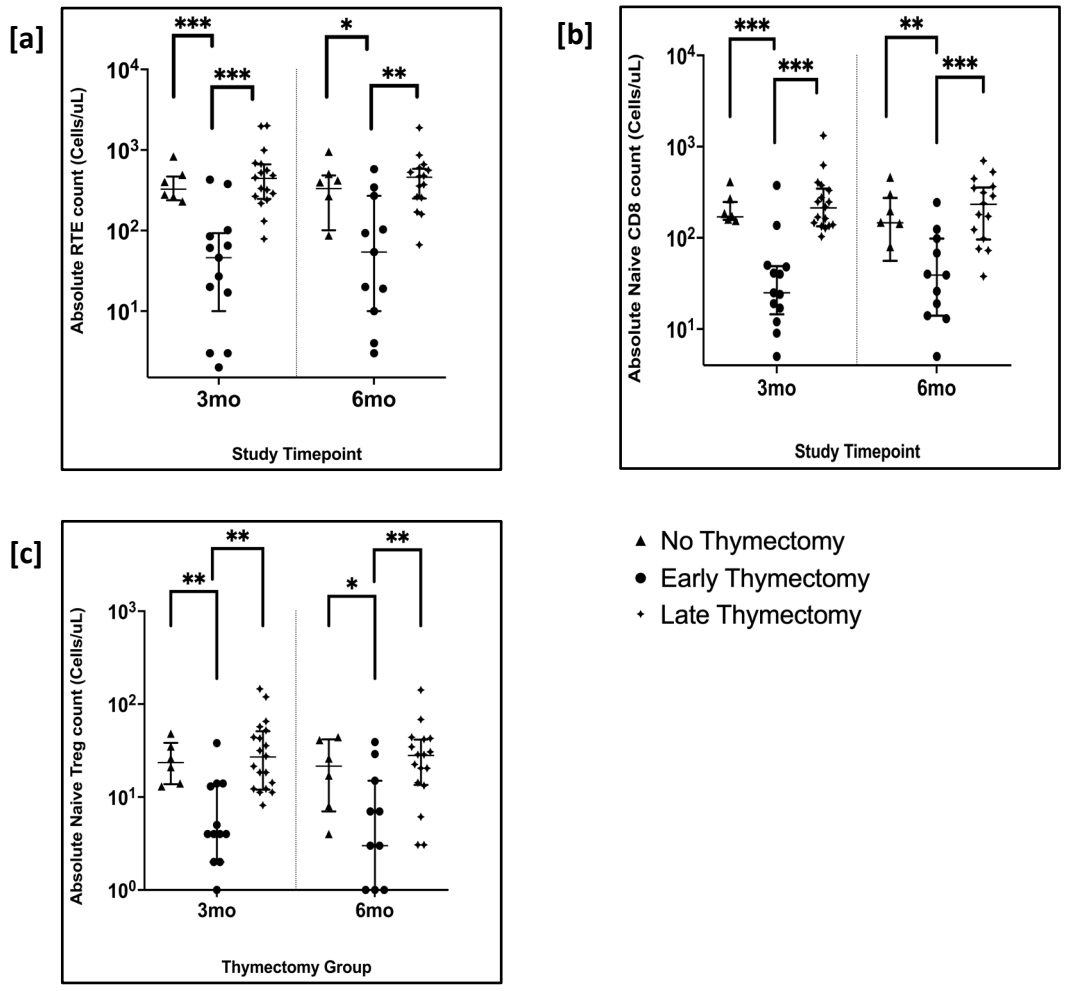


Figure 7.8: Impact of early thymectomy on post-transplant thymic output and naïve αβ T-lymphocyte repertoire. Comparison of absolute cell counts (cells/µl) by thymectomy status for **[a]** Total Recent Thymic Emigrants (RTE), **[b]** Naïve CD8+ CTLs, **[c]** Naïve regulatory T-lymphocytes (Tregs). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

This showed that early thymectomy patients maintained a significantly impaired thymic output and naïve T-lymphocyte compartment at 6 months post-transplantation compared to non-thymectomy patients. However, within their total memory T-lymphocyte compartment, early thymectomy patients had significantly higher total memory CD8+ CTL counts compared to non-thymectomy and late thymectomy patients at 3 months post-transplantation, but no significant difference at the 6 months study timepoint. Similarly, they had significantly lower memory Tregs at 3 months post-transplantation but similar counts at 6 months (Figure 7.9).

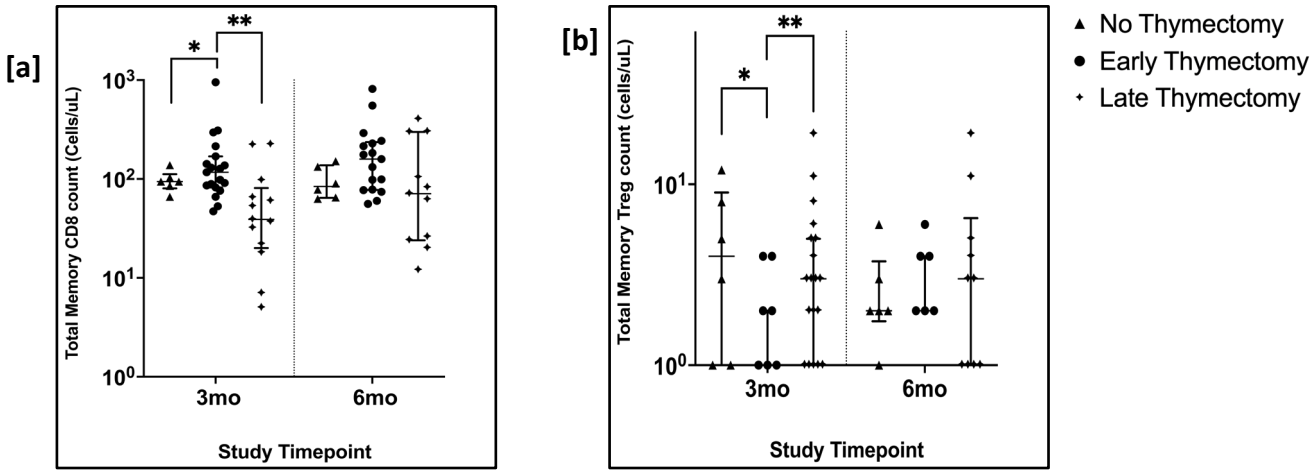


Figure 7.9: Impact of early thymectomy on post-transplant total memory compartments of CTLs and Tregs. Comparison of absolute cell counts (cells/µl) by thymectomy status for **[a]** Total memory CD8+ CTLs, **[c]** Total memory regulatory T-lymphocytes (Tregs). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Cell counts have been log-transformed (log 10). Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

These findings suggest that homeostatic proliferation within the CD8+ CTLs and Treg memory compartments are likely contributors to the observed changes in the overall counts of the αβ T-lymphocyte lineages at 6 months post-transplantation. Indeed, studies in patients undergoing allogeneic stem cell transplantation and lymphodepleting therapies demonstrate that memory CD8+ CTLs undergo much quicker homeostatic proliferation than their CD4+ T_H counterpart – albeit in the setting of preserved thymic function. [481] Animal studies in thymectomised and partially lymphodepleted mice have also identified a similar pattern. [482, 483] Such rapid expansion of memory T-lymphocytes during this critical period tends to be oligoclonal in nature with a restricted diversity of TCRs against new antigens e.g., viruses. [484]

While the timeline for post-transplant homeostatic expansion of Tregs is still debated, there is reasonable evidence to suggest that the early post-transplant period in SOT patients could be characterised by an initial fall in Tregs that is then followed by a more rapid expansion shortly after.[485, 486] It is important to note that the temporal reconstitution of T-lymphocytes after thymectomy and cardiac transplantation has not previously been studied in either a paediatric or adult cohort.

7.5.6 CD57 defines distinct phenotypic populations within the αβ and γδ T-lymphocyte lineages

Following dimensional reduction, semi-supervised cluster analysis of concatenated FCS files identified the three main T-lymphocyte lineages of interest (Figure 7.7). In addition, CD57+ populations were observed to cluster as distinct T-lymphocyte populations. The CD57+ subsets of the αβ and γδ T-lymphocyte populations were noted to have a close spatial relationship in their clustering that was independent of their lineage and differentiation surface markers.

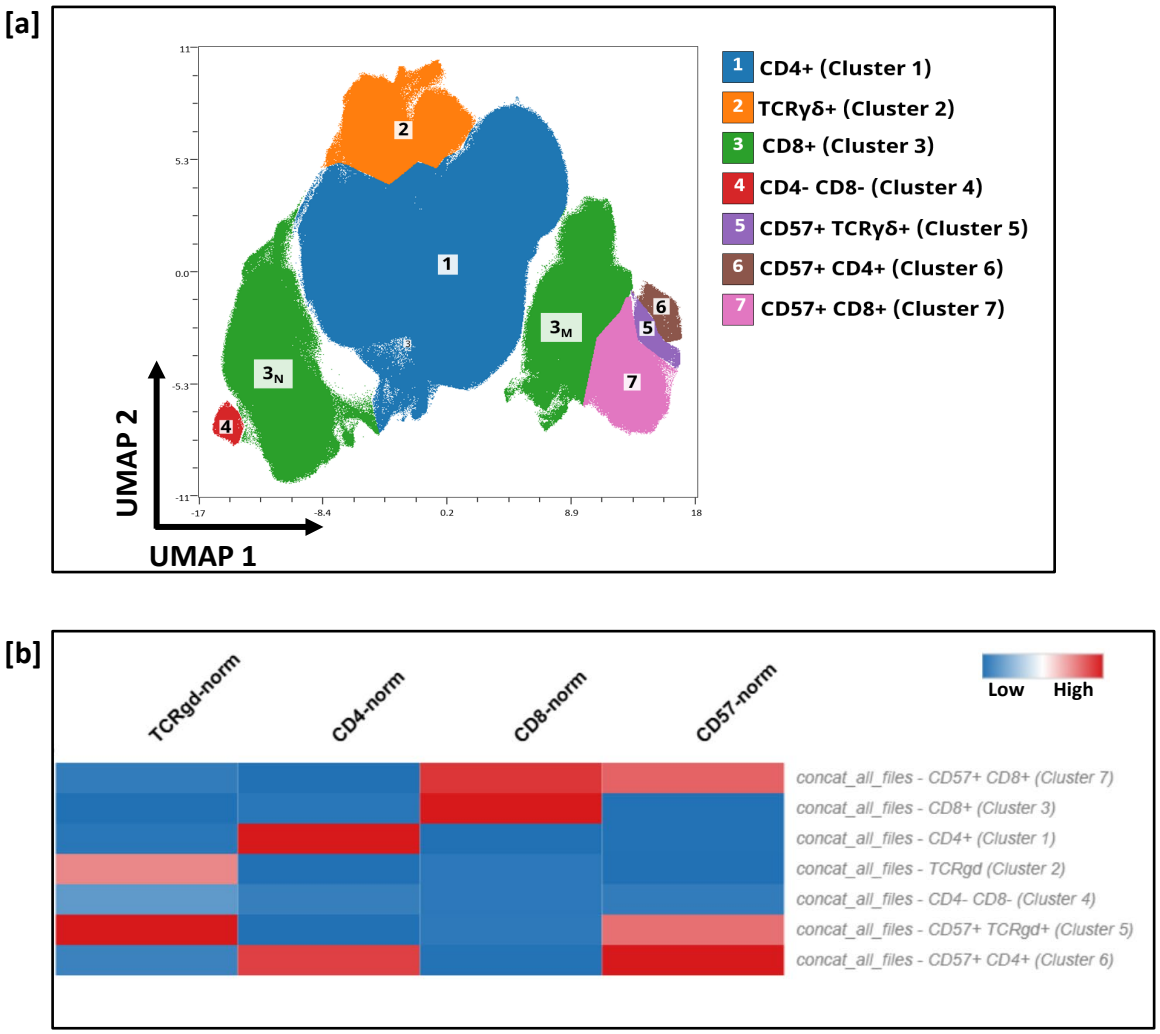


Figure 7.10: Global visualisation of the concatenated T-lymphocyte compartment in ITHACA participants and age-matched non-thymectomy controls. **[a]** semi-supervised Uniform Manifold Parameters (UMAP) in combination with FlowSOM analysis identified 7 cell clusters that corresponded to the 3 main T-lymphocyte lineages (γδT, CD4+ T_H, CD8+ CTLs) and CD4-CD8- population. Naïve and effector memory CD8+ CTLs clustered separately as clusters 3_N and 3_M respectively. The CD57+ subsets for the αβ T-lymphocyte lineages were annotated as discrete subsets because they clustered separately from their main populations **[b]** A cluster heatmap showing the expression levels of γδT, CD4, CD8 and CD57 in each cell cluster.

7.5.7 Early thymectomy is associated with an increase in CD57+ T-lymphocytes in the absence of cytomegalovirus exposure

Very few studies have explored T-lymphocyte senescence in the context of early childhood thymectomy. One such study that examined premature immunological ageing after total thymectomy in the first 2 weeks of life found no significant difference in CD57 expression on CD4+ T_H and CD8+ CTLs between young adults thymectomised in early childhood (YATEC) and their age-matched controls. [138] Of relevance however is the fact that they identified a subset of YATECs with frequencies of CD57+ T-lymphocyte that were similar to elderly patients aged > 75 years. [138] The authors also identified that CMV seropositive YATECs exhibited a more immunosenescent T-lymphocyte profile with significant loss of TCR repertoire diversity compared to CMV seropositive age-matched controls. However, they did not go on to explore if the CMV seropositive status of these non-thymectomised age-matched controls had obfuscated any differences in CD57 expression that could be attributable to early thymectomy. The only other study to investigate thymectomy-induced immunosenescence found that children aged 5 – 12 years who had undergone either a partial or total thymectomy within the first 6 weeks of life had significantly higher CD57+ T-lymphocytes in both the CD4 and CD8 compartments. [150] However, this study did not consider CMV serostatus in their analysis.

CMV is a well-recognised modulator of CD57 expression on T-lymphocytes, specifically CD8+ CTLs, with CD57+ cells being linked to a restricted proliferative capacity, shorter telomeres and an increased predisposition to apoptosis. [487, 488] CMV status has been shown to be just as important as advanced age in predicting T-lymphocyte immunosenescence in overtly healthy individuals and those awaiting SOT. [488, 489] In view of its significant role in cellular senescence and the conflicting reports from previous studies, the analysis of CD57 expression according to thymectomy status was stratified by CMV status. Compared to CMV seronegative patients, CMV seropositive patients had significantly higher CD57+ CD4+ T_H (1.8% [IQR: 0.8 – 3.6] vs 0.7% [IQR: 0.4 – 1.8]; $p = 0.02$) and CD57+ CD8+ CTLs (10.1% [IQR: 2.9 – 41.5] vs 5.3% [IQR: 2.4 – 12]; $p = 0.04$) prior to transplant. CMV seropositive early thymectomy patients in particular had the highest proportion of CD57+ T-lymphocytes prior to transplantation although this wasn't significantly different from CMV seropositive late- and non-thymectomy patients (Figure 7.11).

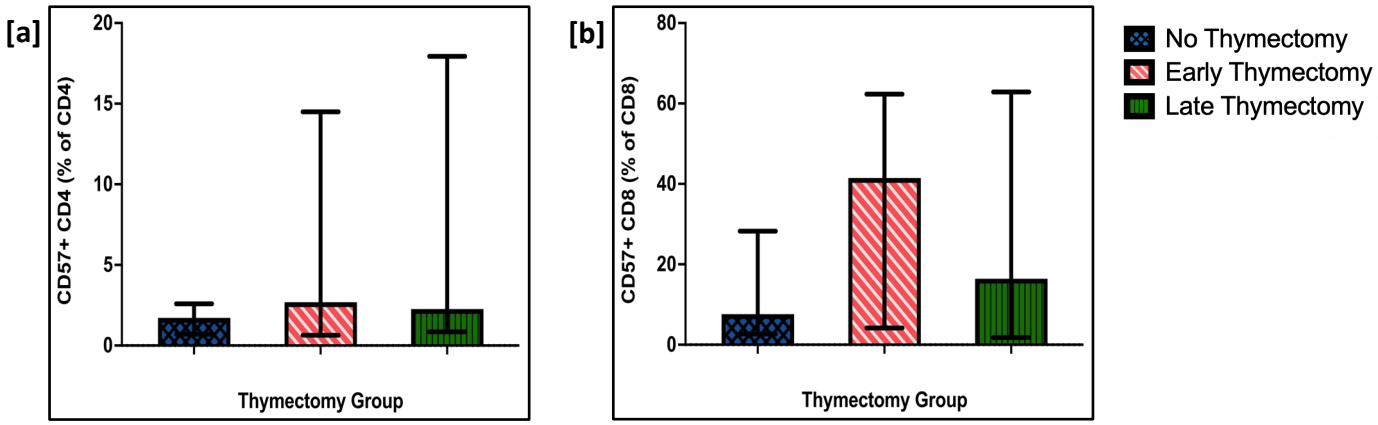


Figure 7.11: Pre-transplant CD57+ cells (%) within the αβ T-lymphocyte populations of CMV seropositive patients stratified according to thymectomy status. **[a]** CD57+ CD4+ T_H and **[b]** CD57+ CD8+ CTLs represented as percentages of the total CD4+ and CD8+ compartments respectively. Error bars represent interquartile ranges. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

However, in CMV seronegative patients, early thymectomy was associated with significantly higher proportions of CD57+ CD4+ T_Hs and CD57+ CD8+ CTLs prior to transplantation (Figure 7.12). There was a notable depletion of CD57+ T-lymphocytes in all patients after transplantation. This resulted in a loss of significance in the thymectomy-related differences seen in CMV seronegative patients. This phenomenon has been described in kidney transplant recipients, who demonstrated a transient but significant loss of CD57+ CD4 T_H within the first 3 months of transplantation followed by a gradual rebound to pre-transplant levels. [489] This process was ascribed as being a sequela of induction immunosuppression therapy. No study has examined if CD57+ CD8+ CTLs demonstrate the same peri-transplant trajectory or whether thymectomy would have an impact on the extent and speed of its reconstitution. The analysis of ITHACA samples that have been collected at 12 months and 24 months post-transplantation might offer better insight on this topic.

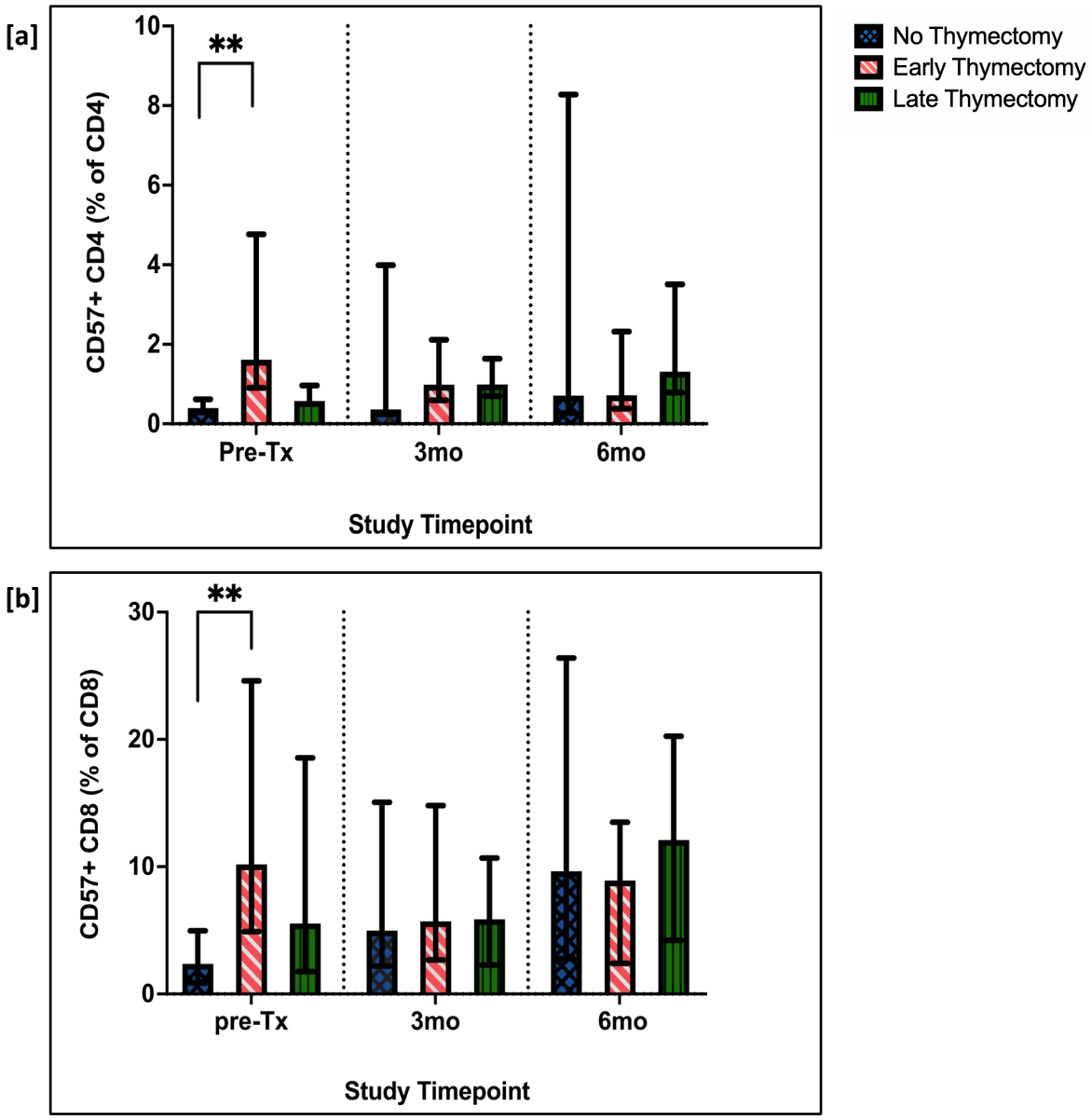


Figure 7.12: Peri-transplant proportions of CD57+ cells (%) within the αβ T-lymphocyte populations of CMV seronegative patients stratified according to thymectomy status. **[a]** CD57+ CD4+ T_H and **[b]** CD57+ CD8+ CTLs represented as percentages of the total CD4+ and CD8+ compartments respectively. Error bars indicate interquartile ranges. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.7 Early thymectomy is associated with an increased expression of immune checkpoint proteins CD39 and PD-1 within the T-lymphocyte memory compartment

In order to investigate if the expression of exhaustion markers varied by thymectomy status before and after transplant, the median fluorescence intensity (MFI) of PD-1, TIM-3, LAG-3, CTLA-4 and CD39 were examined within the CD4+ T_H and C8+ CTL memory compartment (i.e. excluding naïve CD45RA+ CCR7+ T-lymphocytes). Particular focus was placed on the examination of the αβ lineages because these cell populations had exhibited clear differences according to thymectomy status on earlier analysis in this chapter. Out of the aforementioned exhaustion markers in the T-lymphocyte panel, only CD39 on both CD4+ T_H and CD8+ CTLs, and PD-1 on CD4+ T_H alone were found to be significantly upregulated in early thymectomy patients.

A significantly higher expression of CD39 – an ectoenzyme with potent anti-T-lymphocyte immunosuppressive activity – [490] was present in the memory compartment of both CD4+ T_H and CD8+ CTLs in early thymectomy patients prior to transplant and at 3 months post-transplantation (Figure 7.12). At 6 months, the expression of CD39 in both T-lymphocyte populations was still observed to be higher than that of the non-thymectomy group, albeit no longer statistically significant (p = 0.22 and p = 0.15 respectively).

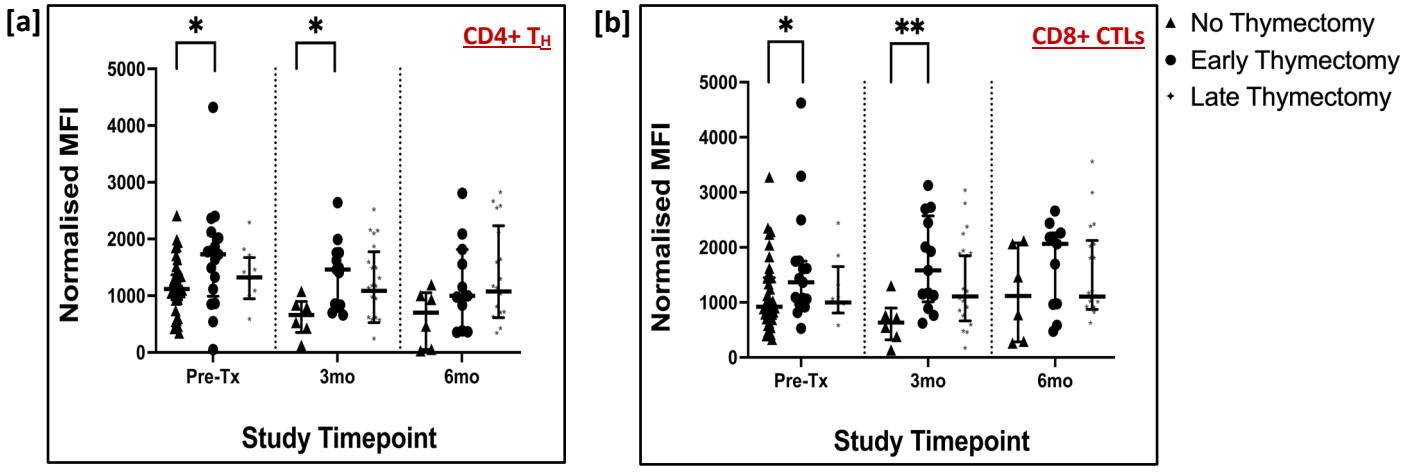


Figure 7.12: Stratified comparison of median fluorescent intensity (MFI) of CD39 within the memory compartment of αβ T-lymphocyte populations according to thymectomy status. Early thymectomy patients demonstrated significantly higher expressions of CD39 than non-thymectomy patients at baseline and 3 months post-transplantation on **[a]** CD4+ T_H and **[b]** CD8+ CTLs memory cells. All p values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

The expression of PD-1 was similarly observed to be significantly higher in early thymectomy patients at baseline and 3 months post-transplantation (Figure 7.13). Analogous to CD39, its expression was still higher than the non-thymectomy and late thymectomy groups at 6 months post-transplantation but did not reach statistical significance (p = 0.15 and p = 0.13 respectively).

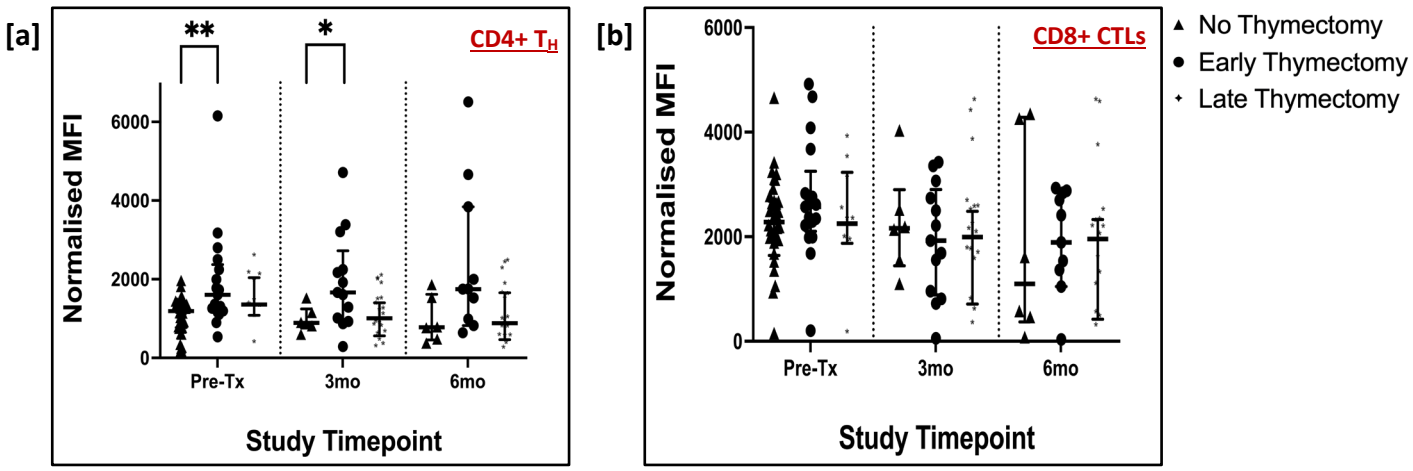


Figure 7.13: Stratified comparison of median fluorescent intensity (MFI) of PD-1 within the memory compartment of αβ T-lymphocyte populations according to thymectomy status. Early thymectomy patients demonstrated significantly higher expressions of PD-1 than non-thymectomy patients at baseline and 3 months post-transplantation on **[a]** CD4+ T_H but not on **[b]** CD8+ CTLs memory cells. All p values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.8 Induction immunosuppression with anti-thymocyte globulin (ATG) is an important contributor to the altered αβ T-lymphocyte landscape seen after cardiac transplantation

The lymphodepletive effect of ATG was noted to further compound the pervasive lymphopaenia that had been established by thymectomy prior to transplant. All 11 patients who received induction immunosuppression with ATG had undergone either early (n = 7) or late (n = 4) thymectomy prior to transplant, resulting in significantly lower baseline T-lymphocyte counts (except for the γδT population) compared to patients treated with non-depleting basiliximab (Figure 7.14). The profound adverse effect of ATG on circulating αβ T-lymphocytes was still evident at 6 months post-transplantation.

All major T-lymphocyte populations remained significantly lower after ATG compared to basiliximab. The only exception was CD8+ CTLs. While patients treated with basliximab had comparable CD8+ CTL populations prior to and after transplant, those who received ATG showed some evidence of reconstitution after an initial decline in circulating CD8+ CTLs. This partial recovery of CD8+ CTLs was considered as most likely being due to homeostatic proliferation within the CD8 memory compartment, a phenomenon that has already been discussed in this chapter (see section 7.5.5).

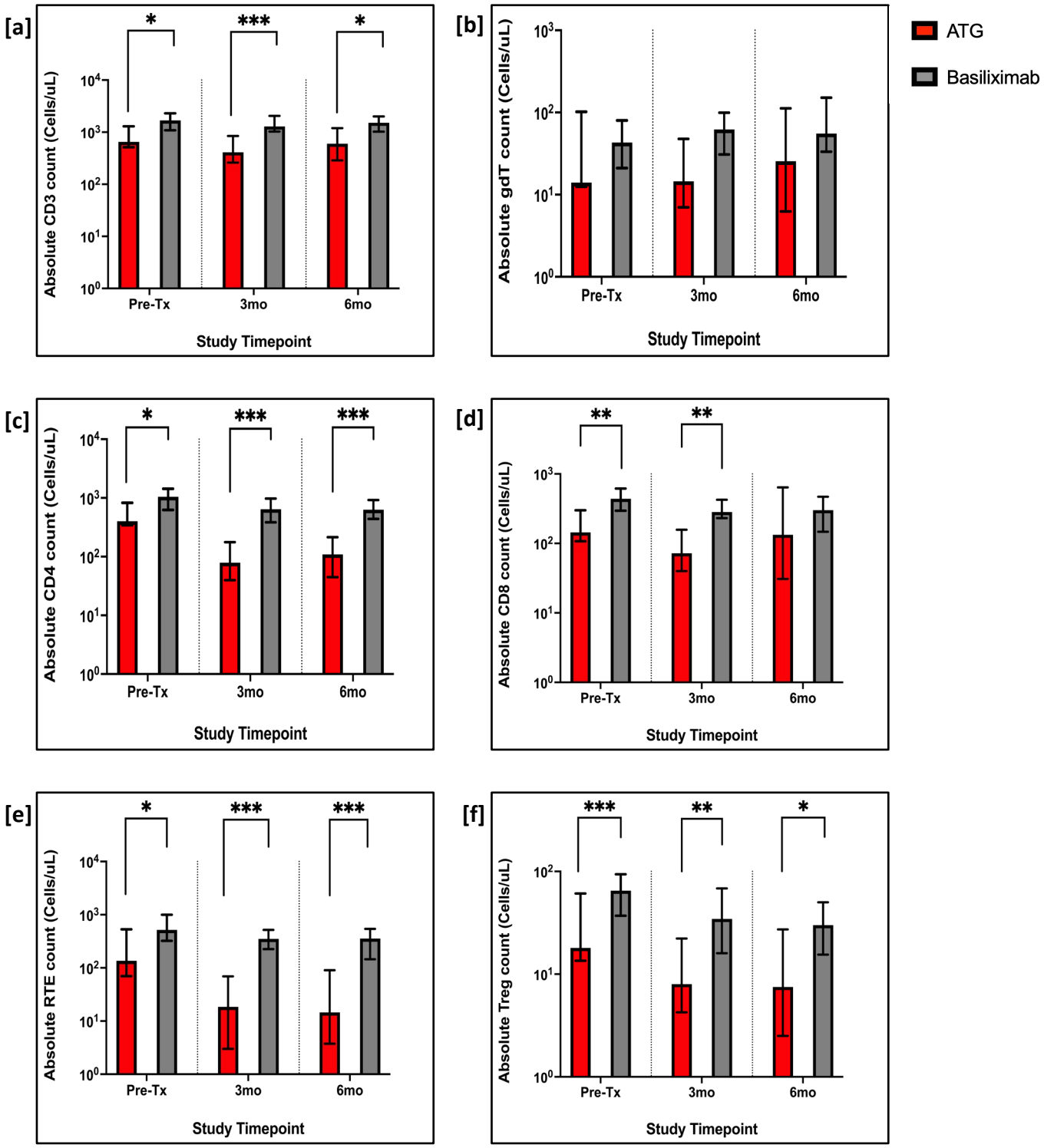


Figure 7.14: Absolute counts of T-lymphocyte populations in ITHACA patients during the first 6 months of transplantation and stratified by choice of induction immunosuppression. Significant differences in the absolute counts of all αβ T-lymphocyte populations were observed at baseline and after transplant. **[a]** Total CD3+ T-lymphocytes; **[b]** γδT; **[c]** CD4+ T_H; **[d]** CD8+ CTLs; **[e]** RTEs; **[f]** Treg. Cell counts have been log-transformed (log 10). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * *p* <0.05; ** *p* <0.01; *** p <0.001. **CTL** = cytotoxic T-lymphocyte; **RTE** = Recent Thymic Emigrant; **Treg** = Regulatory T-lymphocytes.

Within the CD4+ T_H compartment, ATG was associated with a shift in phenotype towards an effector/terminal memory signature after transplant (Figure 7.15). This was characterised by significantly lower proportion of naïve CD4+ T_H compared to patients treated with basilixmab in tandem with significantly higher proportions of CD4+ TCM, TEM and TEMRAs.

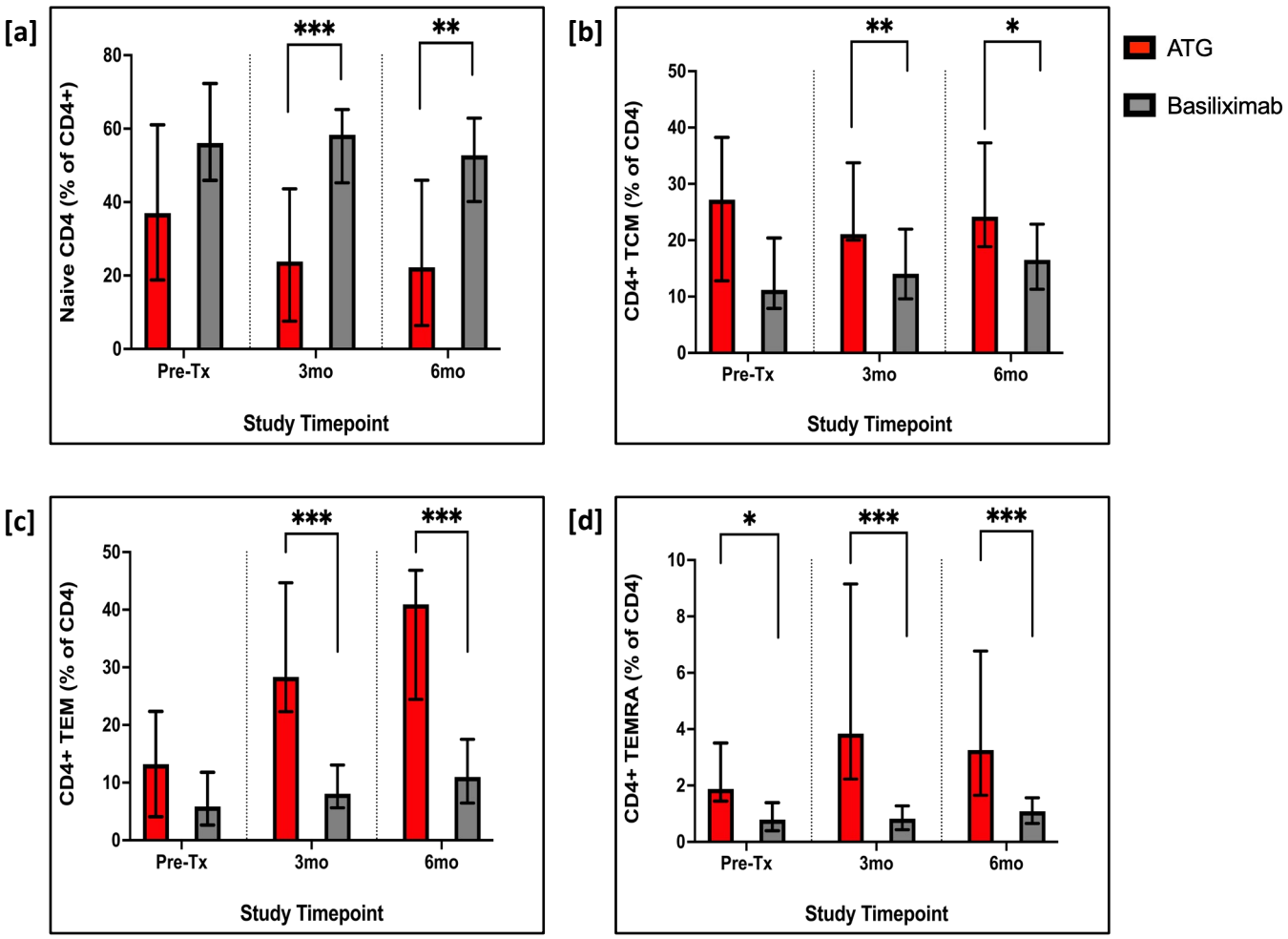


Figure 7.15: Distribution of key immune cell subsets within the CD4+ compartment of ITHACA patients across the first 6 months of transplantation and stratified by choice of induction immunosuppression. Significant differences in the percentage distribution of all four subsets were observed at 3 months and 6 months post-transplantation in the ITHACA cohort (n = 44). **[a]** Naïve (CD45RA+ CCR7+), **[b]** Central memory (CD45RA- CCR7+), **[c]** Effector memory (CD45RA- CCR7-), **[d]** Terminal memory (CD45RA+ CCR7-). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

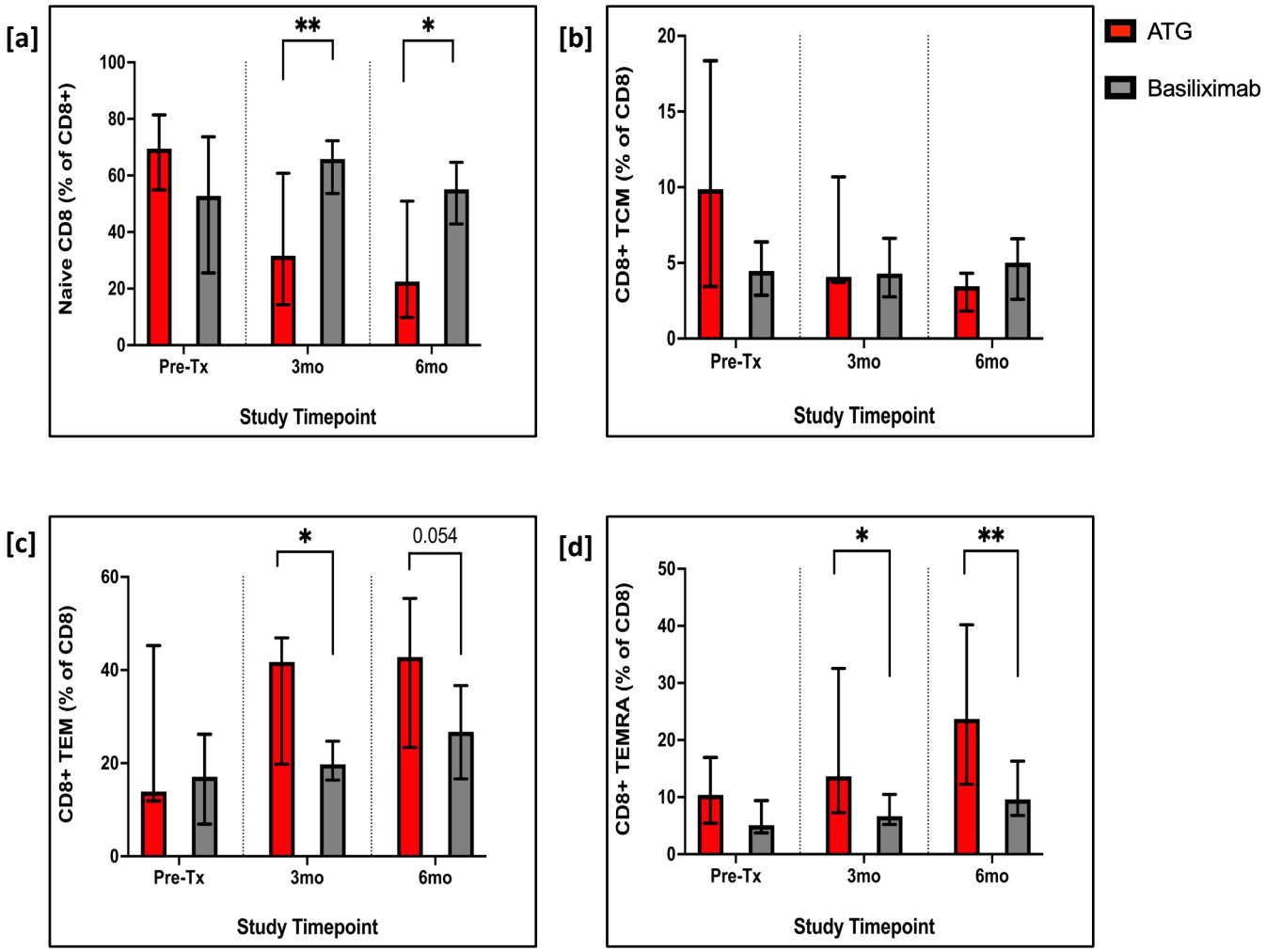


Figure 7.16: Distribution of key immune cell subsets within the CD8+ compartment of ITHACA patients across the first 6 months of transplantation and stratified by choice of induction immunosuppression. Differences of varying significance in the percentage distribution were observed at 3 months and 6 months post-transplantation in the ITHACA cohort (n = 44). **[a]** Naïve (CD45RA+ CCR7+), **[b]** Central memory (CD45RA- CCR7+), **[c]** Effector memory (CD45RA- CCR7-), **[d]** Terminal effector memory (CD45RA+ CCR7-). All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The effect of ATG on the CD8+ compartment was more variable, with significant differences observed in the naïve, effector memory (TEM) and terminal effector memory (TEMRA) subsets after transplant, but not in the central memory (TCM) population.

7.5.9 Impaired EBV control is associated with CD8+ lymphocytosis and immunological features suggestive of T-lymphocyte immunosenescence

Even after thymectomy and lymphodepletive immunosuppression, acute EBV/viral reactivation exhibited a capacity to elicit CD8+ proliferation. There was evidence of a progressively rising frequency of CD8+ CTLs following early post-transplant primary infection/viral reactivation, which was still sustained at 6 months post-transplantation in the setting of persistent EBV DNAemia. EBV DNA positive patients had a significantly higher proportion of circulating CD8+ CTLs compared to EBV DNA negative patients at each of the early study time points (Figure 7.17). Patients who acquired a primary infection/viral reactivation during the early post-transplant period were noted to already have significantly higher CD8+ CTLs at baseline compared to EBV DNA negative patients (i.e. before acquiring a primary infection/viral reactivation). It is possible that this finding was driven by a subclinical asymptomatic (latent) infection in patients who would later go on to become early EBV reactivators. However, this doesn't fully explain the pre-transplant difference as both early EBV reactivators and those with post-transplant primary EBV infection had similar CD8+ percentages prior to transplant (21.6% [IQR: 19.4 – 30.6] vs 22.5% [IQR: 18.2 – 27.7]; p = 0.54).

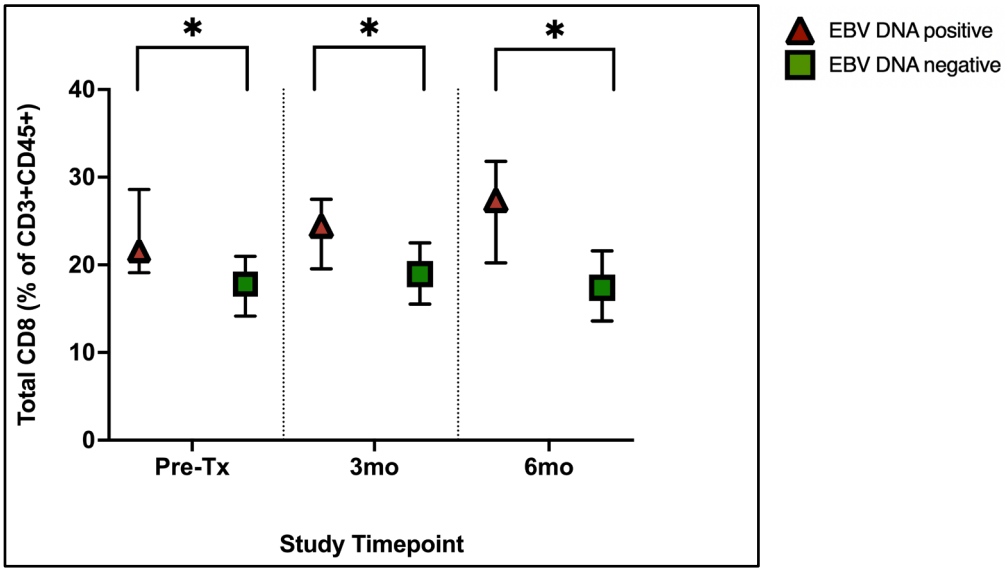


Figure 7.17: Total frequency of CD8+ CTLs according to circulating EBV DNA status at baseline and during the early post-transplant period. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

Patients with persistent EBV DNAemia had an approximately 2.5 times higher frequency of CD57+ CD4+ T_H at 6 months post-transplantation compared to EBV DNA negative patients (2.8% [IQR: 0.9 – 4.3] vs 1.2% [IQR: 0.7 – 2.0]; p = 0.025). There were notably strong correlations between the circulating EBV DNA load and the proportions of naïve and effector memory CD8+ CTLs subsets

(Figure 7.18). There was a significant negative correlation between EBV DNA load and the proportion of circulating naïve CD8+ CTLs while a strong positive correlation between viral load and CD8+ TEMs was also observed (Figure 7.18a and 7.18b respectively). There was evident clustering of patients according to early versus late thymectomy, with early thymectomy patients having noticeably lower naïve T-lymphocytes than their late thymectomy counterparts but higher effector memory cells. Significant positive correlations were also evident between EBV DNA load and circulating proportions of both CD57+ CD8+ CTLs and CD57-expressing CD8+ TEMRAs.

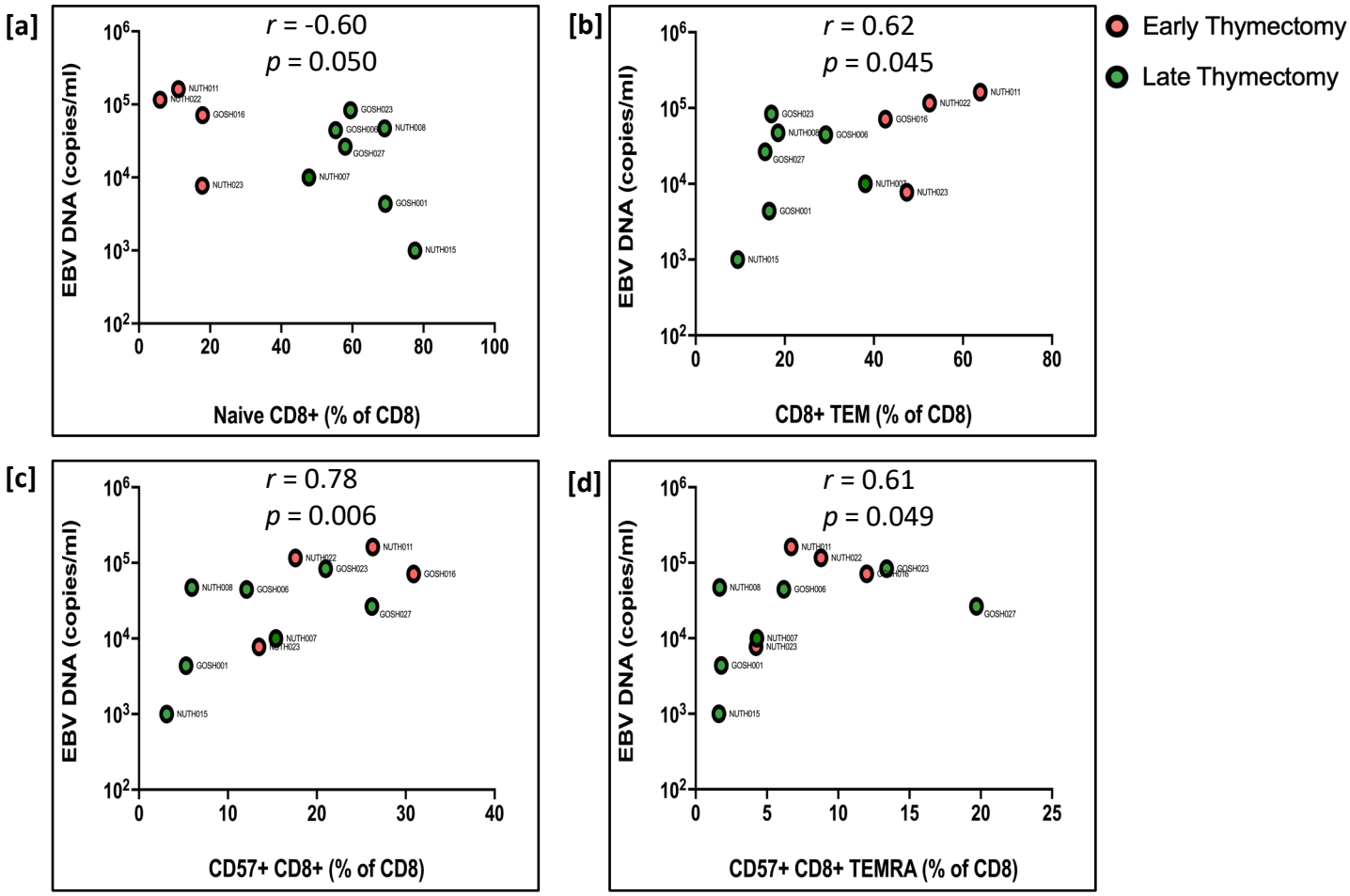


Figure 7.18: Correlation of circulating EBV DNA load (viral copies/ml) at 6-months post-transplantation and percentages of CD8+ CTL subsets. [a] A significant negative correlation was observed between EBV DNA load and the percentage of naïve CD8+ CTLs (CD45RA+ CCR7+). A significant positive correlation existed with **[b]** CD8+ TEM (CD45RA- CCR7-), **[c]** CD57+ CD8+ CTLs and **[d]** CD57+ CD8 TEMRAs (CD45RA+ CCR7-). Each data point has been labelled with its corresponding patient ID number. r = Spearman's rho. The level of significance for *p*-values was set as < 0.05.

7.5.10 Tetramer analysis of EBV-specific T-lymphocytes

HLA class I/II restricted tetramer staining was possible for 30/44 transplanted patients. These patients were suitably matched to at least one tetramer in the T-lymphocyte flow panel and had been confirmed to be EBV positive on viral serology and/or DNA PCR. Patients were therefore sub-grouped as either seropositive only (i.e. EBV antibody positive with undetectable circulating EBV DNA) or EBV DNA positive (irrespective of serostatus). CD4+ EBV-specific T-lymphocytes (CD4+ EBVTLs) were detectable in 22/30 patients, while CD8+ EBV-specific T-lymphocytes (CD8+ EBVTLs) were observed in 19/30 patients. CD4+ EBVTLs made up 0.01% (IQR: 0.003 – 0.03) of the cumulative CD4+ T_H lymphocytes in these patients, and CD8+ EBVTLs made up 0.2% (IQR: 0.03 – 0.90) of the total analysed CD8+ CTLs. CD4+ EBVTL responses are known to be 10-fold lower than CD8+ responses to the same viral epitopes, and the proportional responses observed in this study were concordant with previously observed frequencies of CD4+ and CD8+ EBVTLs in healthy (adult) EBV carriers and healthy seropositive children. [491-495] Tables 7.1 and 7.2 outline the EBV and thymectomy statuses of the ITHACA patients who met the criteria for CD8+ and CD4+ EBV-specific tetramer staining respectively.

EBV Status	**N**	**Patient Group**		
		No thymectomy	**Early thymectomy**	**Late thymectomy**
Seropositive only	**9**	3	2	4
EBV DNA detected	**10**	1	4	5
Total	**19**	**4**	**6**	**9**

Table 7.1: EBV profile and thymectomy status of ITHACA patients with detectable CD8+ HLA class I-restricted EBV-specific CTLs.

EBV Status	**N**	**Patient Group**		
		No thymectomy	**Early thymectomy**	**Late thymectomy**
Seropositive only	13	5	3	5
EBV DNA detected	9	0	4	5
Total	**22**	**5**	**7**	**10**

Table 7.2: EBV profile and thymectomy status of ITHACA patients with detectable CD4+ HLA class II-restricted EBV-specific T_H lymphocytes.

Dimensional reduction with UMAP and clustering with FlowSOM were used for deep immunophenotyping of EBV-specific T-lymphocytes according to thymectomy status (early vs non-thymectomy) and EBV profile (persistent viraemia vs seropositive only.

7.5.10.1 EBV-specific cytotoxic T-lymphocytes exhibit a predominantly effector/terminal effector memory phenotype

Eight clusters of CD8+ EBVTLs were identified based on their expression of key differentiation and functional surface markers (Figure 7.19). PE-labelled tetramers confirmed a restriction of CD8+ latent EBVTLs to cluster 3, while all other clusters were identified by APC-labelled tetramers as being CD8+ lytic EBVTLs. Effector memory (CD45RA- CCR7-) and terminal effector memory (CD45RA+ CCR7-) subsets made up 50% (IQR: 26.5 – 64.4) and 34.2% (IQR: 23.8 – 52.1) of CD8+ EBVTLs respectively. These proportions did not differ significantly according to thymectomy status ($p > 0.05$).

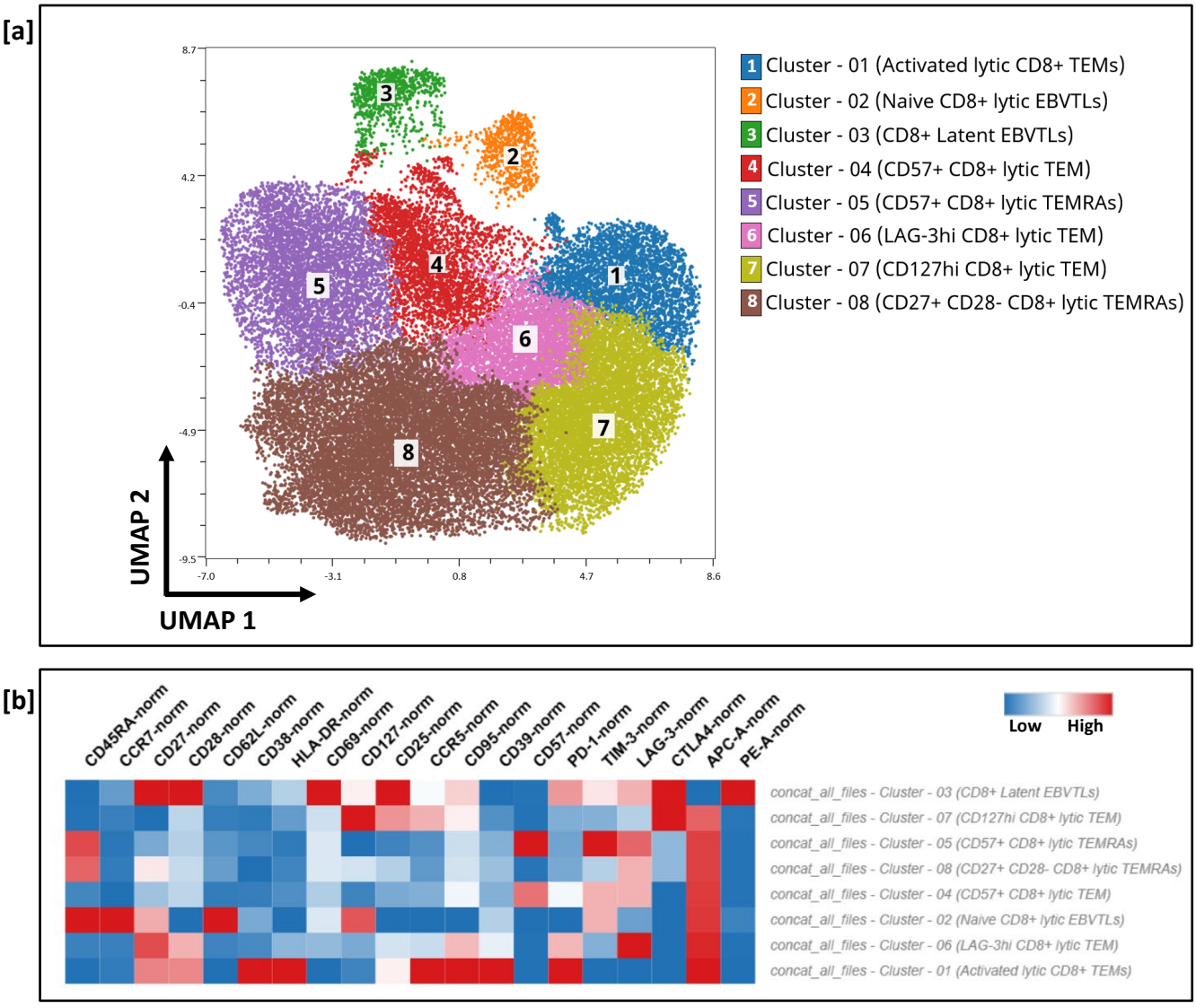


Figure 7.19: High dimensional analysis of CD8+ EBV-specific T-lymphocytes from ITHACA study participants. **[a]** Eight clusters were identified. These consisted of HLA class I-restricted latent EBVTLs (cluster 3) and HLA class I-restricted lytic EBVTLs (cluster 1-2 and 4-8). Lytic CD8+ EBVTLs encompassed various stages of T-lymphocyte differentiation from naïve (cluster 2) to effector (clusters 1, 4, 6, 7) and CD45RA re-expressing terminal effector (clusters 5 and 8) subsets. **[b]** Cluster phenotypes were determined by their median expression of relevant differentiation and functional surface markers.

7.5.10.2 Disruption of CCR5+ CD8+ EBV-specific cytotoxic T-lymphocytes is linked to thymectomy and persistent EBV DNAemia

CC chemokine receptor 5 (CCR5) is well recognised as an important chemokine receptor required for the migration of T-lymphocytes to sites of infection and inflammation. [495] Its expression on T-lymphocytes reflects a memory/effector phenotype and is indicative of priming for cytotoxic responses to viral epitopes. [496] In the context of EBV infection, not only does it serve as a marker of activated and memory virus-specific CD8+ CTLs, but there is also suggestion that an increased circulation of CCR5+ CD8+ EBVTLs contributes to sustained plasma clearance of EBV DNA. [497, 498]

To determine if this relevant CD8+ subset was impacted by age at thymectomy and/or persistent EBV DNAemia, its frequency and expression profile were examined across the 8 clusters of CD8+ EBVTLs identified high dimensional analysis. The cumulative frequency of CCR5+ CD8+ EBVTLs was found to be significantly lower in both early and late thymectomy patients compared to the non-thymectomy group (Figure 7.20).

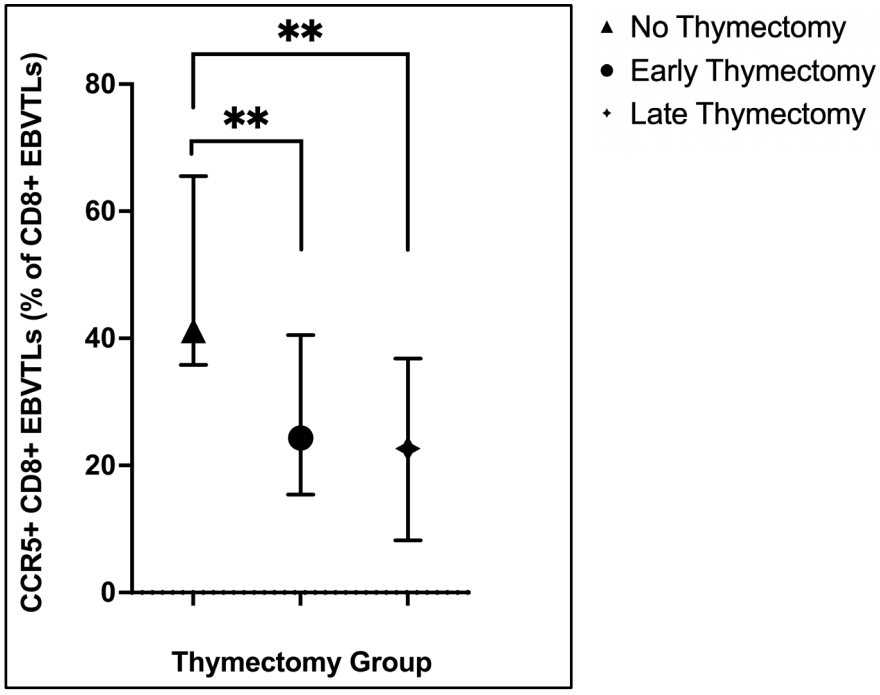


Figure 7.20: Frequency of circulating CCR5+ CD8+ EBV-specific T-lymphocytes according to thymectomy status. All *p* values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Next, the signal intensity of CCR5 was examined in clusters that showed the highest level of expression (clusters 1, 3 and 7).

A notable variation in the MFI of CCR5 was seen across these 3 clusters (Figure 7.19b). While low expression was observed on CD8+ latent EBVTLs (cluster 3), an intermediate expression was noted on CD127hi lytic EBV TEMs (cluster 7) in contrast to activated lytic CD8+ TEMs (cluster 1), which exhibited a high expression of CCR5. Although the low expression of CCR5 on CD8+ latent EBVTLs cannot be readily explained, the expression profiles seen on the other CD8+ EBVTL clusters are biologically plausible. CD127hi lytic EBV TEMs represent a distinct subset of CD8+ antigen-specific CTLs known as memory precursor effector cells (MPECs). These cells are fated to eventually provide long-lived immunological memory after encountering antigenic stimulation. [499] Studies have shown that MPECs are less cytotoxic and are capable of shutting down their effector function more rapidly than short-lived effector cells (SLECs). [500] Such manoeuvring allows them to escape rapid and recurring degranulation *in vivo* during an infection, thus conserving resources for long-term survival. [501] It therefore stands to reason that this cell subset would express lower levels of CCR5 in comparison to activated EBVTLs as a mechanism to facilitate its survival through the avoidance of antigen-induced terminal differentiation and apoptosis. The high expression of CCR5 on activated EBVTLs was in keeping with its expected upregulation in the setting of viral antigen stimulation. [498] The MFI of CCR5 on activated CD8+ EBVTLs was found to be significantly lower in patients with persistent EBV DNAemia compared to those who were EBV seropositive but DNA negative ($p = 0.001$) [Figure 7.21a], while the difference in its expression on CD8+ latent EBVTLs between the two groups approached statistical significance (Figure 7.21b).

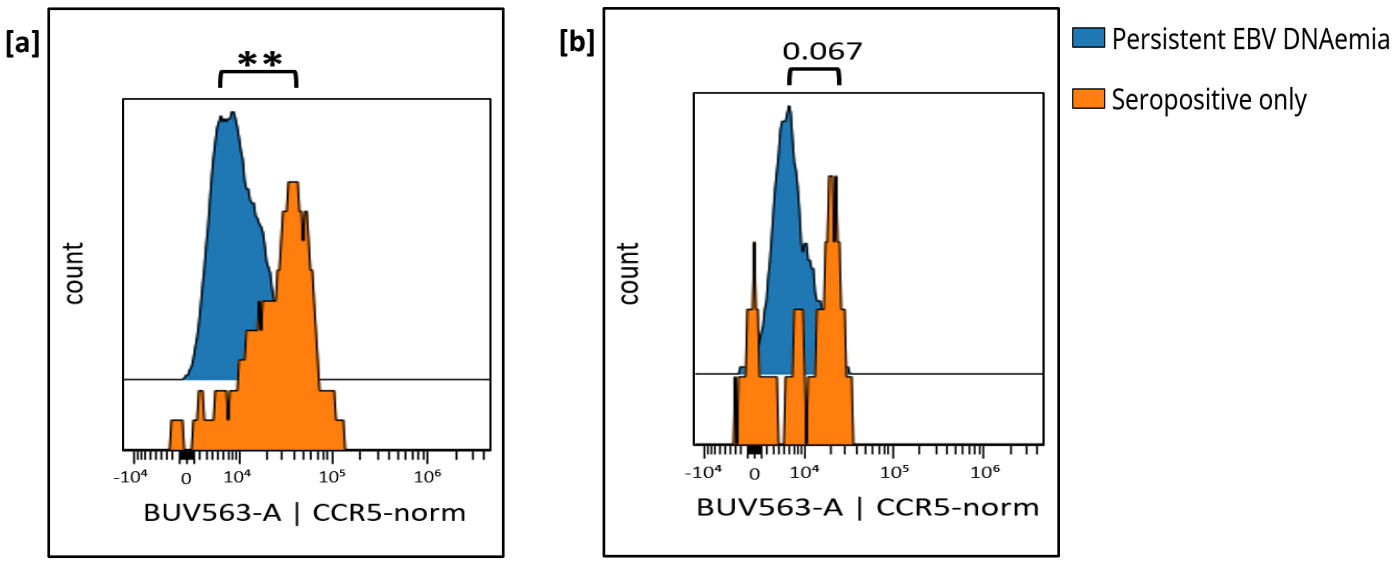


Figure 7.21: Histogram overlay plots comparing the expression of CCR5 in activated CD8+ lytic EBVTLs according to persistent EBV DNAemia and seropositive only patients. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.10.3 Increased immunosenescence and cellular exhaustion of EBV-specific cytotoxic T-lymphocytes is seen after early thymectomy

Clusters 4 and 5 were identified as immunosenescent subset of HLA class I-restricted lytic EBVTLs based on their expression of CD57 (Figure 7.19b). Figure 7.22a illustrates the percentage distribution of CD57+ EBVTLs according to thymectomy status. The frequency of CD57+ CD8+ TEM EBVTLs did not differ according to age at thymectomy (p = 0.81). However, early thymectomy patients had a significantly higher proportion of CD57+ CD8+ TEMRA EBVTLs compared to non-thymectomy patients (6.3% [IQR: 3.2 – 8.0] vs 0.3% [IQR: 0 - 8.4]; p = 0.032). In addition, the expression of PD-1 in both the CD8+ TEM and TEMRA EBVTL compartments was markedly higher in early thymectomy patients compared to the non-thymectomy group (Figure 7.22b).

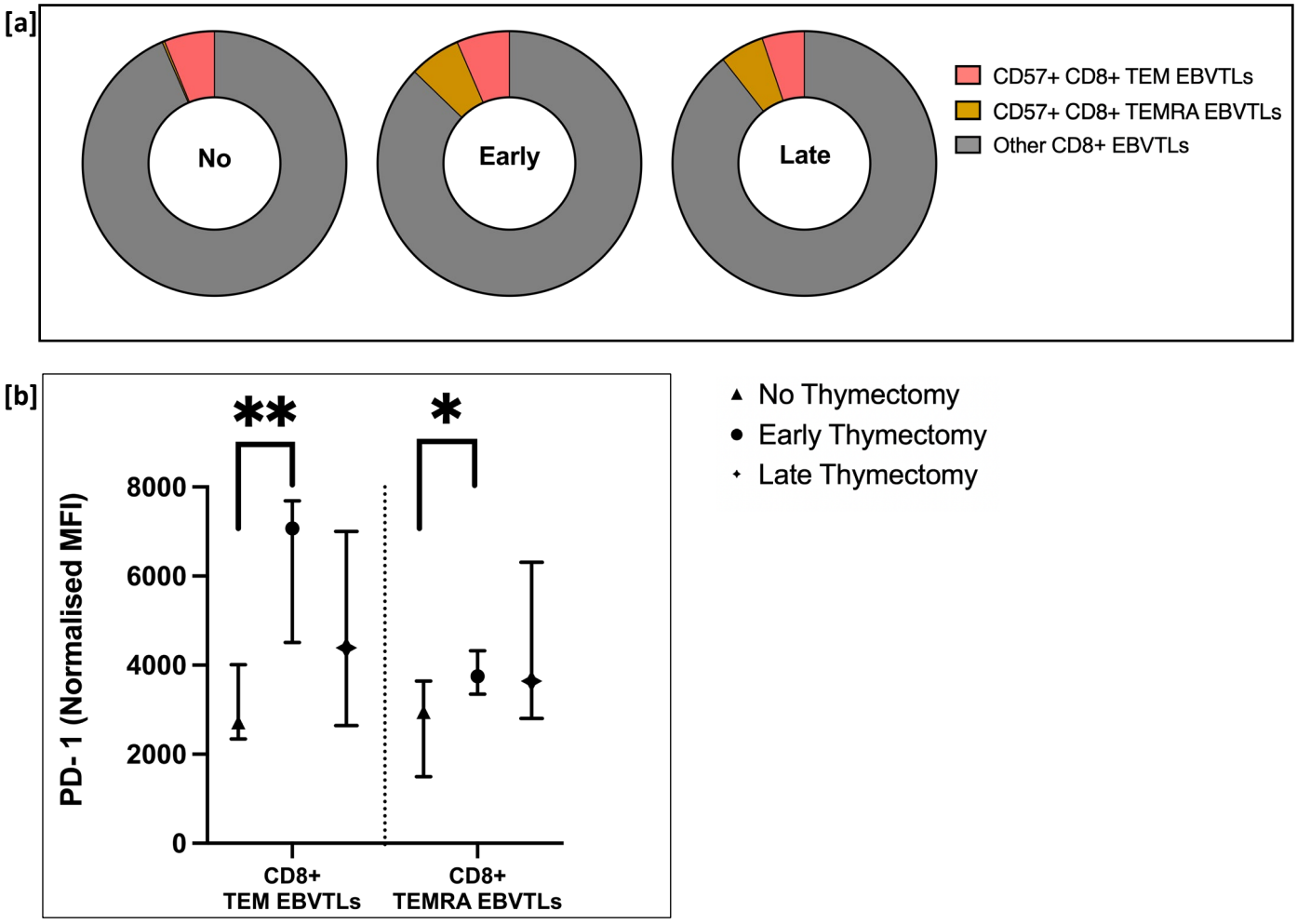


Figure 7.22: Features of immunosenescence and cellular exhaustion in CD8+ lytic EBV-specific T-lymphocytes. [a] Percentage distribution of CD57+ CD8+ EBVTLs according to thymectomy status. **[b]** Expression of PD-1 within the TEM and TEMRA EBVTL compartments. All p values were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001. **TEM:** Effector T-lymphocytes; **TEMRA**: CD45RA re-expressing terminal effector T-lymphocytes.

7.5.10.4 High expression of PD-1 and CD39 on activated effector EBV-specific cytotoxic T-lymphocytes is associated with early thymectomy and persistent EBV DNAemia

Of the total CD8+ EBVTLs, activated CD8+ effector EBVTLs (cluster 1) constituted 6.3% (IQR: 3.2 – 14.2) in early thymectomy patients, 5.9% (IQR: 3.0 – 11.2) in the late thymectomy group and 4.6% (IQR: 1.6 – 14.6) in the non-thymectomy cohort (p = 0.76). Early thymectomy patients expressed significantly higher levels of both CD38 and HLA-DR compared to non-thymectomy patients (Figure 7.23a). Similarly, the expression of these activation markers was markedly higher in patients with persistent EBV DNAemia compared to those who were seropositive but with undetectable circulating viral DNA (p = 0.009 and p = 0.043 respectively).

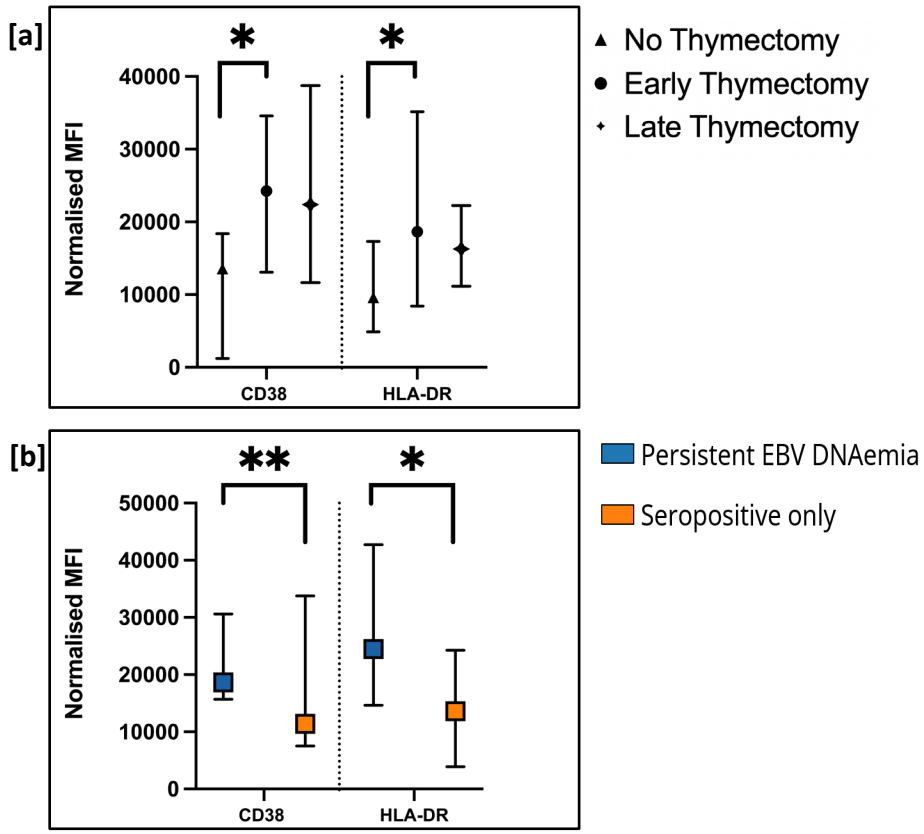


Figure 7.23: Expression profile of CD38 and HLA-DR on activated CD8+ lytic EBVTLs. Comparative analysis was performed based on **[a]** Thymectomy status and **[b]** EBV profile. All p values derived from analysis according to thymectomy status were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

Further immune profiling revealed a substantially higher expression of PD-1 and CD39 related to early thymectomy (Figure 7.24a). A nearly identical profile was seen with persistent EBV DNAemia alongside a significantly reduced expression of CCR5 compared to seropositive patients (Figures 7.24b and 7.24c respectively).

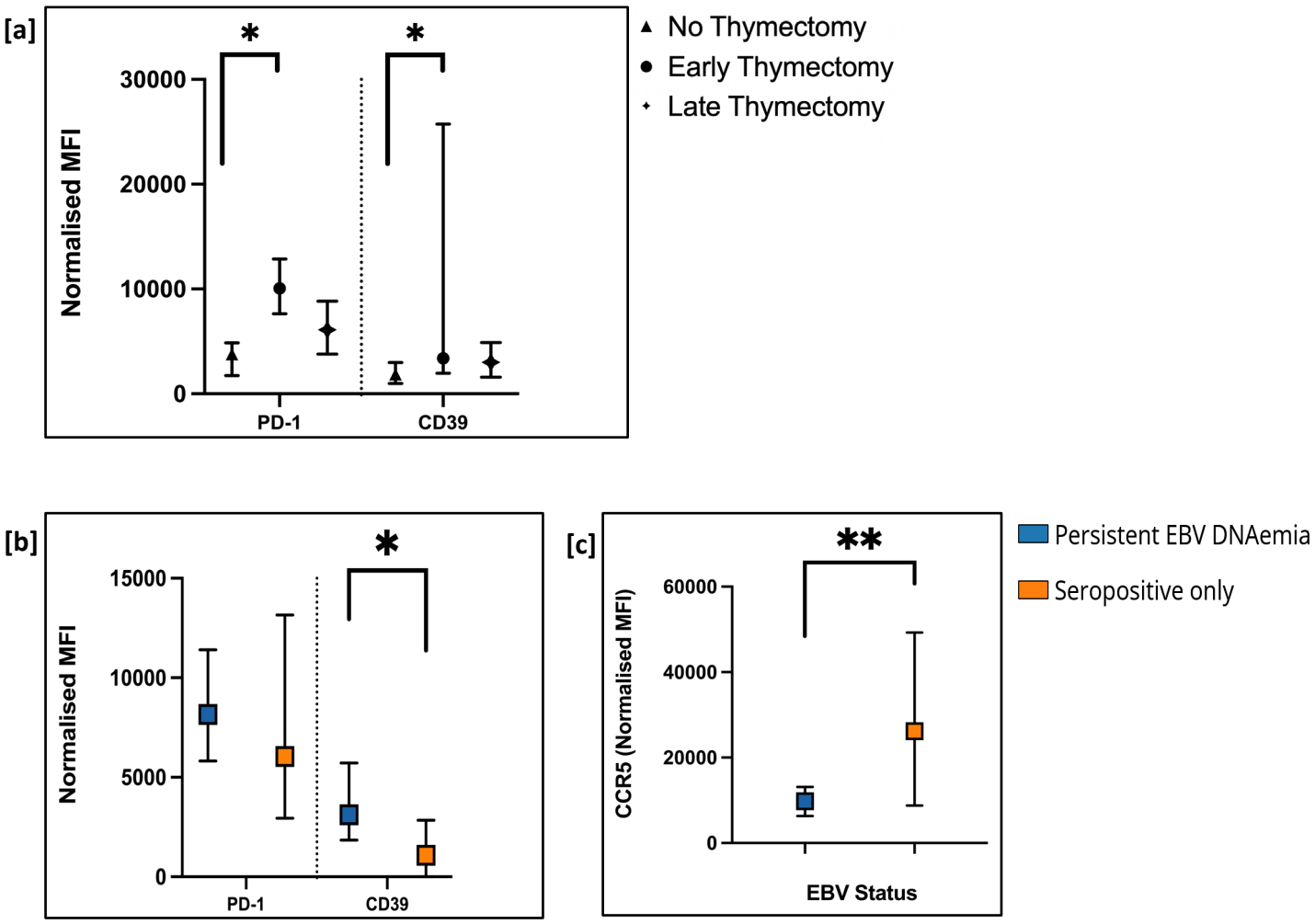


Figure 7.24: Expression profile of immune checkpoint proteins and CCR5 on activated CDB+ lytic EBVTLs. **[a]** PD-1 and CD39 expression according to thymectomy status; **[b]** PD-1 and CD39 expression according to EBV profile; **[c]** CCR5 expression according to EBV profile. All *p* values derived from analysis according to thymectomy status were adjusted for multiple pairwise comparison using the Benjamini-Hochberg method. Significant results are indicated: * p <0.05; ** p <0.01; *** p <0.001.

7.5.10.5 CD4+ EBV-specific T-lymphocytes

Interrogation of the HLA class II-restricted CD4+ EBVTLs was limited to a descriptive analysis. This was due to the low cumulative count (n = 3,786 cells) which did not permit robust stratification of subsets by thymectomy status and EBV profile. Seven clusters of CD4+ EBVTLs were identified based on their expression of relevant surface markers (Figure 7.25).

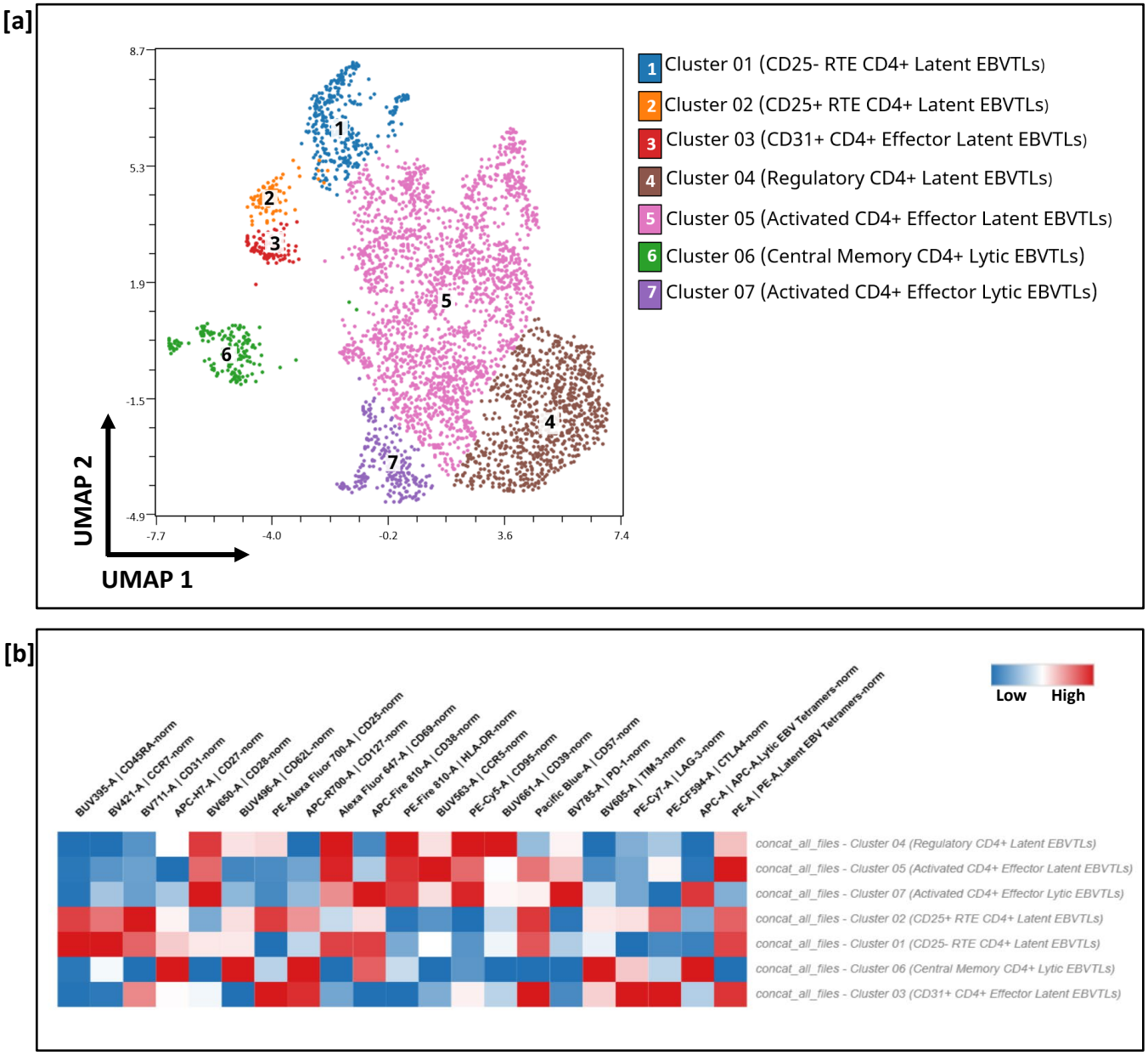


Figure 7.25: High dimensional analysis of CD4+ EBV-specific T-lymphocytes from ITHACA study participants. **[a]** Seven clusters were identified. These consisted of HLA class II-restricted lytic EBVTLs (clusters 6 and 7) and HLA class II-restricted latent EBVTLs (clusters 1 – 5). Latent CD4+ EBVTLs included RTEs (clusters 1 – 2), regulatory (cluster 4) and effector (clusters 3 and 5) subsets while lytic CD4+ EBVTLs consisted of a central memory (cluster 6) and activated effector subset (cluster 7). **[b]** Cluster phenotypes were determined by their median expression of relevant differentiation and functional surface markers. **RTE**: Recent thymic emigrants.

Detectable HLA class II-restricted EBVTLs were predominantly against latent EBV peptides (95.6% [IQR: 73.7 – 100]) while lytic CD4+ EBVTLs made up the remaining 4.4% (IQR: 0 – 26.3). The phenotype of clusters 1 and 2 was consistent with recent thymic emigrants (i.e. CD31+ CD45RA+ CCR7+) and made up 8.1% (IQR: 0 – 33.2) of the CD4+ EBVTL population. CD25+ RTE EBVTLs (cluster 2) were distinguished their CD25- counterpart (cluster 1) due to the recognised upregulation of CD25 on RTEs that have undergone homeostatic peripheral expansion in the absence of antigenic stimulation. [502] CD25- RTE EBVTLs were more prominent, representing 5.4% (IQR: 0 – 25) of the total CD4+ EBVTL population, while CD25+ RTEs were infrequent [0% (IQR: 0 – 2.8)]. An effector phenotype (CD45RA- CCR7-) was seen in 55.5% (IQR: 29.3 – 81.8) of CD4+ latent EBVTLs (clusters 3 and 5). Cluster 5 represented the most abundant subset of CD4+ EBVTLs (52.6% [IQR:17.6 – 80.0]). This cluster had a high expression of CCR5, CD69 and HLA-DR, indicating an early activation status. CD69+ CD4+ latent EBVTLs have been shown to exhibit cytotoxic function through INF-γ production and are an important feature of acute anti-EBV host responses. [235] Furthermore, a low frequency of CD69+ CD4+ EBVTLs has been linked to the defective control of latent EBV infection. [503] The co-expression of CD57, CD39, PD-1 and CTLA4 within this cluster of cells (Figure 7.25b) is indicative that their functional capacity is also likely hampered by multi-pathway immune checkpoint activity and cellular senescence.

The next most prominent cluster (cluster 4) was phenotypically aligned with CD4+ EBTVLs that exhibit an immune regulatory/suppressive function (CD45RA- CCR7- CD25+ 127lo/-). This cluster of cells made up 2.1% (IQR: 0 – 9.1) of CD4+ EBVTLs. Unlike thymic-derived natural Tregs, these antigen-specific Tregs are generated in the periphery from naïve T-lymphocytes that encounter a cognate antigen under the auspices of DCs. [504] There is scant published evidence on the functional role of EBV-specific Tregs in the setting of transplantation or iatrogenic immunosuppression. However, the induction of LMP-1-specific Tregs has been shown to inhibit pro-inflammatory Th1 responses to other EBV proteins, which may in turn facilitate viral persistence and promote EBV-associated malignancies. [505] As expected, the frequency of CD4+ T_H response to lytic EBV epitopes was much less prominent compared to latent EBV epitopes (see Figure 7.25a). Lytic CD4+ EBVTLs consisted of central memory (cluster 6) and activated effector phenotypes (cluster 7) respectively. Activated effector CD4+ lytic EBVTLs represented a maximum of 4.4% of CD4+ EBVTLs, while the central memory subset made up 0.7% (IQR: 0 – 16.7). The co-expression of CD57 and PD-1 was a feature of this cluster of cells while those with a central memory phenotype were characterised by the co-expression of the immune checkpoint proteins TIM-3 and LAG-3.

7.5.11 EBV-specific T-lymphocyte responses are impaired in paediatric heart transplant recipients

EBV-specific T-lymphocyte responses against BZLF-1 (lytic viral epitope), LMP-2 and EBNA-1 (latent viral epitopes) were assessed by IFN-γ ELISPOT assay in a subset of 10 ITHACA patients with persistent EBV DNAemia and 8 healthy EBV seropositive controls. Both the magnitude and distribution of T-lymphocyte responses to these immunodominant viral proteins were observed to differ between the two study groups (Figure 7.26).

The net production of IFN-γ in response to EBV epitopes was nearly two-fold higher in seropositive healthy controls compared to transplanted patients with persistent EBV DNAemia (Figure 7.26a). The T-lymphocyte response in the latter group was primarily driven by ex vivo stimulation with BZLF-1. This accounted for approximately 75% of the overall T-lymphocyte response in this cohort. In contrast, IFN-γ production against BZLF-1 made up less than 1% of the total EBV-specific T-lymphocyte response in seropositive healthy controls. While immune responses to LMP-2 and EBNA-1 dominated the cytotoxic anti-viral landscape of seropositive healthy controls, they were found to be less prominent in transplanted patients. Of note, IFN-γ production against LMP-2 made up just 6.6% of the T-lymphocyte response in transplanted patients with persistent EBV DNAemia compared to 50% in seropositive healthy controls (Figure 7.26b).

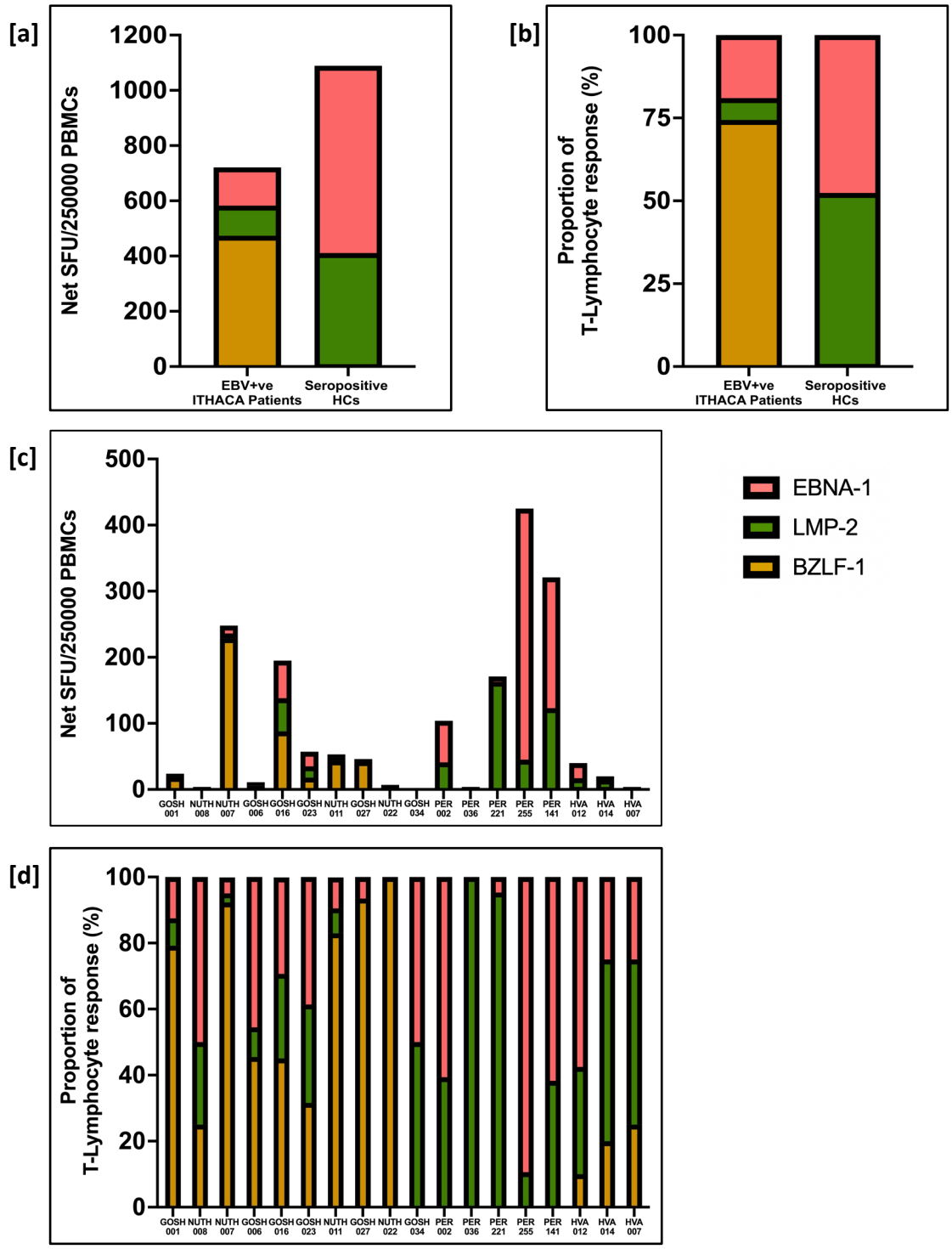


Figure 7.26: EBV-specific T-lymphocyte responses in a subset of ITHACA participants with persistent DNAemia (n = 10) and EBV seropositive healthy controls (n = 11). [a] Magnitude of IFN-γ production represented as the cumulative (net) spot forming units (SFU) of PBMCs stimulated with EBNA-1, LMP-2 and BZLF-1; [b] Percentage distribution of T-lymphocyte responses per 250,000 PBMCs after incubation with viral epitopes (EBNA-1, LMP-2 and BZLF-1) for at least 18 hours; [c] Magnitude of ELISPOT responses in PBMC for each of the 10 ITHACA participants with persistent EBV DNAemia (prefix 'GOSH' and 'NUTH') and 8 seropositive healthy controls (prefix 'PER' and 'HVA'); [d] Individual percentage distribution of T-lymphocyte responses per 250,000 PBMCs for each of the 10 ITHACA participants and 8 healthy controls.

These findings are in keeping with previous observations in healthy adults with chronic EBV carriage, who exhibit a higher frequency and magnitude of T-lymphocyte responses to lytic viral antigens compared to latent EBV epitopes. [356, 506] LMP-2 comprises the isoforms LMP-2a and LMP-2b and is an integral viral epitope expressed during the type III latency program observed in EBV-PTLD. [289] One of the key roles of LMP-2 in lymphomagenesis is its ability to mimic B-cell receptor (BCR) signalling and activate pro-survival pathways (e.g., PI3K/AKT). [507] These mechanisms effectively support the survival and proliferation of EBV-infected cells. In addition, it facilitates EBV latency by inhibiting the viral lytic cycle, thus contributing to the evasion of EBV infected cells from immune detection. [508, 509] The similarly inferior antigenic response to EBNA-1 in patients with persistent EBV DNAemia compared to seropositive healthy controls represents another viable pathway for establishing cell transformation and lymphomagenesis. Not only does EBNA-1 maintain a latent reservoir of EBV in infected B-lymphocytes through episomal persistence, but it also plays a crucial role in the evolution of high-grade lymphomas by direct transcriptional re-programming that increases the activity of c-MYC. [510] This reinforces cell transformation through enhanced proliferation in conjunction with an increased resistance to apoptosis.

The exact mechanisms by which the above study findings might interact with age at thymectomy remain unclear. It is hypothesised that early thymectomy patients will have a smaller magnitude and a lytic-skewed distribution in antigen-specific T-lymphocyte responses as a result of reduced immunological diversity and premature immunological ageing. The subset of ITHACA participants on which the ELISPOT assay was performed consisted of 3 early thymectomy (NUTH011, NUTH022, GOSH016) and 7 late thymectomy participants (NUTH007, NUTH008, GOSH001, GOSH006, GOSH023, GOSH027, GOSH034), thus posing challenges with drawing any firm conclusions when stratified by age at thymectomy. Neither the magnitude nor distribution of T-lymphocyte responses to the viral epitopes deployed in this study appeared to follow a set pattern in relation to thymectomy status. However, since the ELISPOT data in this section represents findings from less than 50% of EBV positive transplanted study participants, it is possible that further analysis of cryopreserved study samples will elucidate key differences in IFN-γ production that are related to age at thymectomy.

7.6 Discussion

The findings presented in this chapter offer compelling evidence in support of the hypothesis that early thymectomy in children undergoing cardiac transplantation establishes long-term and profound alterations to the T-lymphocyte compartment. Whilst the analysis of remaining samples will be important, this appears to result in the dysregulated immune surveillance of EBV. The data robustly demonstrate that early thymectomy leads to a sustained depletion of naïve CD4+ and CD8+ T-lymphocytes, as well as Tregs, at a critical period in the development of cell-mediated immunity. In contrast, non-thymectomised and late-thymectomy groups showed a relatively preserved or partially reconstituted T-lymphocyte compartment, indicating that later thymectomy supports better immunological outcomes. Importantly, the profound lymphopaenia observed in early thymectomy patients persisted beyond transplantation, suggesting that peripheral expansion and homeostatic proliferation cannot fully compensate for absent thymopoiesis in the setting of lifelong immunosuppression.

These findings have significant clinical implications. Thymic output is necessary not just for numerical T-lymphocyte recovery but also for the diversity and quality of the TCR repertoire. The skewed memory phenotype and increased expression of senescence markers (CD57, PD-1, CD39) in early thymectomy patients suggest a prematurely aged immune state, potentially limiting responsiveness to oncogenic viral antigens. The integration of EBV-specific tetramer staining into the high-dimensional T-lymphocyte flow panel provided novel insights into EBV-specific immunity after thymectomy and transplantation. Early thymectomy patients had fewer detectable EBVTLs and those that were present displayed increased PD-1 and CD39 expression, along with reduced CCR5. These are classical features of T-cell exhaustion and impaired cellular trafficking, both of which could compromise effective EBV control. Furthermore, persistent EBV DNAemia correlated with diminished naïve CD8+ CTLs and elevated CD57+ memory T-lymphocytes. This association strongly supports a mechanistic link between early thymectomy, immune senescence, and failure to clear latent viral infections. The observation is particularly pertinent for transplant recipients, where chronic EBV is a known risk factor for PTLD development. [327] These findings echo previous studies showing that a compromised thymic environment or senescent T-lymphocyte profile increases susceptibility to opportunistic viral reactivation in immunosuppressed populations. [511, 512]

Due consideration has been given to the limitations of this study. The small cohort size, particularly for tetramer-positive patients, limited statistical power for certain analyses. This is especially true for CD4+ EBV-specific T-lymphocytes, where event counts were low, precluding in-depth statistical analyses. ATG was used exclusively in thymectomised patients, potentially amplifying the observed lymphopaenia compared to non-thymectomy patients. While attempts have been made to separate these effects, residual confounding still remains a possibility. Future analysis using multivariate modelling could help address this. In addition, follow up was limited to the 6-month post-transplant window, which may not fully capture late reconstitution dynamics or clinical events such as EBV-PTLD. Future analysis of study data and samples collected at 12-months and 24-months post-transplant would be valuable to assess whether the observed immunological impairments observed in the early post-transplant period are sustained and translate to higher rates of persistent EBV DNAemia or malignancy. Finally, given the emphasis on thymopoiesis, the study would have been strengthened by TCR sequencing to assess clonality and diversity, which are critical correlates of functional T-lymphocyte immunity. Incorporating future studies on this aspect of T-lymphocyte immunity into the ITHACA study would provide broader insight into the complexity of EBV-PTLD pathogenesis.

Despite its limitations, this study had several significant strengths to support the scientific robustness of the findings in this chapter. First, the use of a 30-colour spectral flow cytometry panel coupled with high dimensional analysis provided a comprehensive view of the T-lymphocyte landscape. This approach overcomes some of the limitations of conventional gating strategies and aligns with emerging standards for in-depth bioinformatic analysis in translational immuno-oncology. Furthermore, the use of HLA-restricted tetramer analysis of EBV-specific CD8+ and CD4+ T-lymphocytes allowed direct interrogation of antiviral immunity, thus moving beyond low resolution immunophenotyping. Finally, few studies on the immunological risk of EBV-PTLD have stratified patients by the timing of thymectomy. This allowed for nuanced comparisons and reinforced the emerging theory that early thymectomy poses a more substantial risk for dysregulated T-lymphocyte function and impaired EBV control.

Chapter 8. Final conclusions and future works

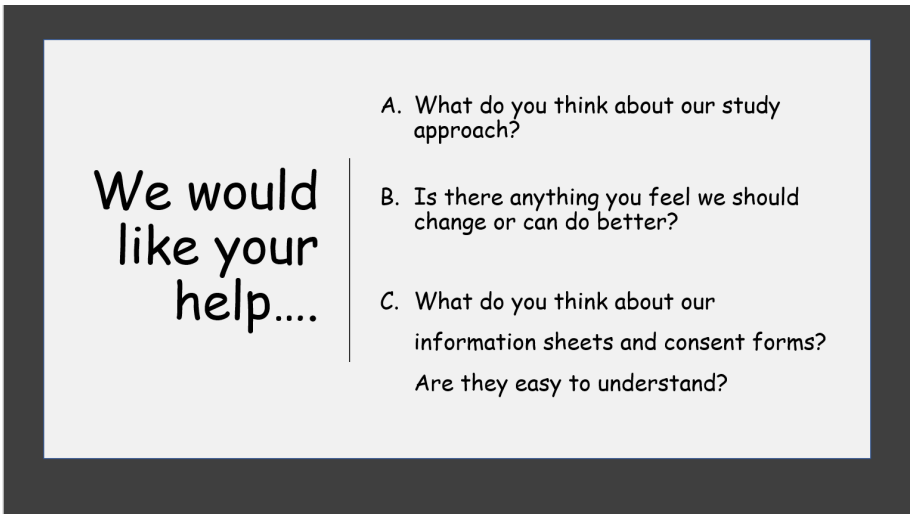
Advancements in our understanding of the cellular landscape in both healthy and neoplastic states have been propagated in recent years by the implementation of full spectrum flow cytometry in translational research. [513, 514] Not only has this cutting-edge single-cell technology deepened our insight of the complex immunological interface between the host and oncogenic drivers, but it has also been leveraged to identify relevant prognostic biomarkers and unveil previously unknown cellular targets for novel therapies in paediatric oncology. [515] The identification of immunological prognosticators and therapeutic targets are of particular relevance to EBV-PTLD, an insidious group of lymphoid malignancies that pose substantial morbidity and mortality for children after SOT. This is especially important for paediatric heart transplant recipients, who are affected disproportionately by EBV-PTLD compared to other childhood SOT cohorts and for whom the role of treatment-related thymectomy in infancy has emerged as a potential candidate for risk stratification. [4, 324] As the incidence of heart transplantation continues to increase in children who have already undergone staged cardiac surgery and thymectomy in infancy, [516 – 519] so does the need for unravelling the immunological consequences of early thymectomy and lifelong immunosuppressive therapy on EBV-specific T-lymphocyte activity, the broader immune landscape and the associated risk of EBV-PTLD.

This study demonstrated an extensive adverse impact of early thymectomy on the T-lymphocyte milieu of children undergoing heart transplantation. Its association with a significant depletion of RTEs, naïve CD4+ T_H and CD8+ CTLs, the emergence of TEMRAs, expansion of senescent CD57+ T-lymphocytes and the increased expression of immune checkpoint proteins within the T-lymphocyte memory subset, showcases a prematurely ageing adaptive immune system that could be considered as primed for dysregulated EBV control and potential capitulation to EBV-induced aberrant lymphoproliferation. Indeed, this could be inferred from the significant positive correlation observed between EBV DNAemia and the relative frequencies of senescent CD57+ CD8+ CTL subsets as well as the increased proportion of senescent and exhausted CD8+ EBVTLs after early thymectomy. Interestingly, the effects of late thymectomy were less prominent and mostly consisted of immunophenotypic signatures that mirrored the non-thymectomy cohort. That being said, the occurrence of persistent EBV DNAemia and EBV-PTLD within the late thymectomy group probably indicates that a subset of patients exist for whom thymectomy, irrespective of age at procedure, can result in immunological changes that harbour an increased risk of poor EBV control and development of EBV-PTLD. One of the most intriguing findings in this project was the association of both early and

late thymectomy with a reduced circulation of CCR5+ CD8+ EBVTLs. This thymectomy-induced impairment in the trafficking of CD8+ EBVTLs to sites of EBV propagation could partially explain why patients with early and late thymectomy experienced EBV-related complications. The future use of high-throughput RNA sequencing to examine the clonal diversity of the TCR repertoire after early versus late thymectomy would deepen our understanding of how age at thymectomy contributes to pathological EBV immune responses. It is important to highlight that the immunological aberrations mentioned thus far are mostly based on findings from univariate statistical analysis of early post-transplant data (i.e. the first 6 months post-transplant). Further multivariate analysis and predictive risk modelling with the inclusion of the 12- and 24-months post-transplant data will be necessary to elucidate how these variables interact with each other and whether these changes are sustained in the longer term. In addition, the observed disruptions of NK cell and monocyte-mediated anti-EBV activities in relation to the use of MMF and ATG respectively are indicative of the treatment-related pathways through which EBV might be able to exploit the host's immune landscape to establish lymphomagenesis. The lack of functional markers for NK cells and monocytes in the broad immune panel limited the extent to which the effects of age at thymectomy could be examined in these important early responders to EBV infection. Further work is required to develop a spectral flow cytometry panel that focusses on the functional capacity of NK cells and monocytes of ITHACA participants. This will help to elucidate if/how age at thymectomy affects innate anti-EBV responses.

Finally, the results of this project raise the important question of how immunosuppressive therapy and thymectomy-induced disruptions to both systemic and EBV-specific immunity correlate with changes within the tumour immune microenvironment (TIME) of EBV-PTLD to influence treatment outcomes. There is emerging evidence to suggest that the TIME of mature high-grade B-cell lymphomas and PTLD that exhibit a "T-lymphocyte inflamed" signature are associated not only with better survival outcomes but are also more amenable to immunotherapies such as bispecific antibodies. [520, 521] It remains unknown whether the poorer clinical outcomes often seen after EBV-PTLD in paediatric heart transplant patients is related to a more tolerogenic TIME as a consequence of thymectomy-induced immunosenescence and cellular exhaustion. Addressing this knowledge gap will not only provide key insight to the complex mechanisms involved in EBV-driven oncogenesis relevant to both PTLD and other B-cell lymphomas in immune competent patients but also contribute to the identification of new therapeutic targets for EBV-PTLD that improve the long-term survival outcomes of transplant patients.

Appendix A

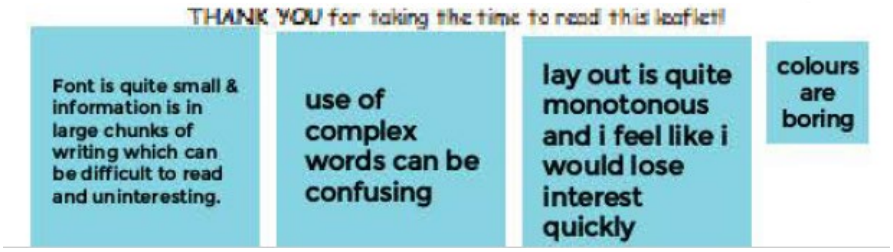


The wording of question 2 could be altered so it says something like "I have had by questions answered" to make sure that they have asked everything the wanted rather than just been given the chance

The Assent Form is very easy to understand. However, I believe that it may be good to put key information at the bottom to give a quick overview of the timeline and/or FAQ's.

I think in terms of things going wrong you could use phrases such as " just in case" etc to reiterate that you don't expect anything to go wrong, it is just to reassure that there is support.

I think that this information sheet is really well suited to the age range! It is able to stay simple while still managing to explain well enough to understand



it's still consent so i think this is ok

Deferred consent - is it OK?

Yes, in some situations asking for consent isn't appropriate at the time, or someone isn't in the right mind frame or physical state to give consent.

Yes, in an emergency it's a good time to get a sample but a bad time to get consent.

Appendix B

Open access Protocol

BMJ Open

Immunology of THymectomy And childhood CArdiac transplant (ITHACA): protocol for a UK-wide prospective observational cohort study to identify immunological risk factors of post-transplant lymphoproliferative disease (PTLD) in thymectomised children

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ABSTRACT

Introduction Paediatric heart transplant patients are disproportionately affected by Epstein-Barr virus (EBV)-related post-transplant lymphoproliferative disease (PTLD) compared with other childhood solid organ recipients. The drivers for this disparity remain poorly understood. A potential risk factor within this cohort is the routine surgical removal of the thymus—a gland critical for the normal development of T-lymphocyte-mediated antiviral immunity—in early life, which does not occur in other solid organ transplant recipients. Our study aims to describe the key immunological differences associated with early thymectomy, its impact on the temporal immune response to EBV infection and subsequent risk of PTLD.

Methods and analysis Prospective and sequential immune monitoring will be performed for 34 heart transplant recipients and 6 renal transplant patients (aged 0–18 years), stratified into early (<1 year), late (>1 year) and non-thymectomy groups. Peripheral blood samples and clinical data will be taken before transplant and at 3, 6, 12 and 24 months post-transplant. Single cell analysis of circulating immune cells and enumeration of EBV-specific T-lymphocytes will be performed using high-dimensional spectral flow cytometry with peptide-Major Histocompatibilty Complex (pMHC) I/II tetramer assay, respectively. The functional status of EBV-specific T-lymphocytes, along with EBV antibodies and viral load will be monitored at each of the predefined study time points.

Ethics and dissemination Ethical approval for this study has been obtained from the North of Scotland Research Ethics Committee. The results will be disseminated through publications in peer-reviewed journals, presentations at scientific conferences and patient-centred forums, including social media.

Trial registration number ISRCTN10096625.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This is the first prospective study to monitor the temporal immune response to Epstein-Barr virus (EBV) infection within a group of patients known to be at a high risk of developing post-transplant lymphoproliferative disease.
⇒ The integration of data from clinical parameters, in-depth immunophenotyping and EBV-specific T-lymphocyte functional assay permits robust analysis of potential predictive immune biomarkers.
⇒ The multicentre study design optimises recruitment of participants.
⇒ The primary study limitation is the low incidence of paediatric heart transplantation, which may affect the rate of study recruitment.

INTRODUCTION

Post-transplant lymphoproliferative disease (PTLD) is the most common childhood cancer in paediatric recipients of a solid organ transplant. This heterogeneous group of life-threatening lymphoid malignancies is typically driven by Epstein-Barr virus (EBV) infection.[1] PTLD comprises a histological spectrum that ranges from indolent non-destructive B-cell lymphoid infiltrates through to more aggressive and destructive polyclonal or monoclonal B-cell lymphomas, for example, Burkitt lymphoma and diffuse large B-cell lymphoma. These destructive PTLD subtypes are indistinguishable from high-grade mature B-cell lymphomas seen

in immunocompetent children.[2] Their incidence is allograft-dependent and ranges from 1%–2% in renal transplant recipients to as high as 10%–15% in heart transplant patients.[1 3]

PTLD has one of the worst clinical outcomes among childhood lymphomas. The estimated 2-year event-free survival (EFS) is 70% compared with 94% in sporadic cases within the general paediatric population.[4–6] In even starker contrast is the survival outcome for paediatric heart transplant patients, who ostensibly have an inferior EFS compared with other non-cardiac organ transplants.[7 8] The reasons for this potential disparity are still poorly understood, although partly ascribed to a higher incidence of therapeutic complications from organ rejection and treatment-related toxicities.[9]

The pathogenesis of PTLD is complex and multifactorial. It involves the interplay between EBV-driven lymphoproliferation, iatrogenic immunosuppression and the suspected functional exhaustion of T-lymphocytes due to graft-initiated chronic antigen stimulation.[10] The role of EBV is clearly established in children, many of whom experience primary infection from an EBV-mismatched organ.[1 3 4] In vitro and in vivo studies focused mainly on adult monomorphic PTLD have demonstrated distinct patterns of viral protein expression in infected B-lymphocytes.[10 11] These expression patterns likely influence both the cellular composition of the tumour immune microenvironment and aberrant immune signalling, which result in immune escape of tumour cells.[10]

In paediatric heart transplant patients, cardiac surgery via median sternotomy in early childhood often requires routine thymectomy in order to access the heart and great vessels. Our earlier study retrospectively examined risk factors for PTLD in the largest UK cohort of paediatric orthotopic heart transplant patients to date.[3] The risk of PTLD was found to be significantly higher in children with congenital heart disease (CHD) (HR=3.2; 95% CI=1.4 to 7.4) and early thymectomy in infancy (HR=2.7; 95% CI=1.3 to 5.2).[3] Furthermore, children with CHD had persistently lower T-lymphocyte counts compared with children transplanted for acquired cardiomyopathy (CD4+: 430 cell/µL vs 963 cells/µL, p<0.01 and CD8+: 367 cells/µL vs 765 cells/µL, p<0.01).[3] Similar studies have identified marked phenotypic and functional disruptions to the T-lymphocyte compartment in paediatric heart transplant patients compared with children receiving a liver or kidney transplant.[12 13]

During the first year of life, the thymus plays a crucial role in the development of cell-mediated immunity, providing a microenvironment for precursor T-lymphocytes to proliferate and differentiate into mature (naïve) T-lymphocytes.[14] While it has been shown that neonatal thymectomy alone is associated with premature immunosenescence and an increased risk of viral infections such as Cytomegalovirus (CMV),[14] little is known about the immunological consequences of early thymectomy in immunosuppressed transplant patients. In particular, there are no data on the synergetic impact of thymectomy and transplant-related immunosuppression on EBV-specific immunity and the subsequent risk of developing PTLD.

Study aims and hypothesis

This study aims to investigate the development of EBV-specific immune responses following childhood heart transplant. Specifically, it aims to identify the impact of early thymectomy—compounded by iatrogenic immunosuppression—on EBV immunology and the risk of PTLD.

We hypothesise that a combination of early thymectomy and lifelong immunosuppression therapy establishes a tolerogenic immune profile consisting of dysregulated, exhausted and senescent immune cell subsets, poorly able to control EBV infection. This dysfunctional immune microenvironment permits the uncontrolled proliferation of EBV-infected B-lymphocytes and the subsequent development of PTLD.

METHODS AND ANALYSIS

Study design and setting

The Immunology of THymectomy And Childhood CArdiac transplant (ITHACA) study is a prospective nationwide cohort study recruiting children (0–18 years) from the two UK centres currently commissioned to provide paediatric heart transplant services: The Freeman Hospital, Newcastle upon Tyne and Great Ormond Street Hospital for Children, London. The study cohort will consist of 34 prospective heart transplant recipients and a non-thymectomy age-matched control group made up of 6 renal transplant recipients. All patients meeting the study's eligibility criteria will be identified and recruited through their local transplant teams at the time of listing for cardiac or renal transplantation. The study opened to patient recruitment in March 2022 and is expected to recruit until June 2024.

Inclusion criteria

- Aged 0–18 years.
- Actively listed on the National Health Service Blood and Transplant (NHSBT) waiting list for a primary organ transplant or awaiting transplant with a living related donor kidney or recently transplanted with pretransplant blood samples available.
- Written informed consent.

Exclusion criteria

- Has a pre-existing diagnosis of an inherited or acquired immunodeficiency.
- Has an underlying thymic disorder.
- Has previously received a bone marrow or organ transplant.
- Has had a previous cancer diagnosis.
- Withheld consent.
- Weight under 2.5 kg.

Informed consent

Informed consent will be obtained from the parent/carer of the eligible child or from the patient themselves if over

the age of 16 years. Assent may be given by children<16 years of age who wish to do so.

A deferred consent approach will be employed for potential study participants who attend a research site's transplant service for transplantation in a critical/life-threatening clinical situation. This will involve the collection of baseline study samples from potential participants at the point of presurgical workup without written informed consent being received. Discussion about the study, the giving of participant information sheet (PIS) and receiving of written informed consent/assent will be offered at a more appropriate time before further follow-up blood tests are taken. Such cases will require that the clinical team considers obtaining informed consent prior to transplant to be inappropriate. This will be documented in the patient's clinical notes. Study samples collected under such circumstances will be processed for storage but not analysed until written informed consent is obtained. Any patient who has study samples collected by deferred consent but subsequently declines enrolment in the study will have their samples destroyed in a timely manner according to local laboratory standard operating procedures. Additional consent will be sought to store specimens for future ethically approved research.

Assessment and procedures

Children will undergo blood sampling for study-specific investigations during routine clinical visits prior to transplant and at 3, 6, 12 and 24 months post-transplant (figure 1). No additional study-related hospital visits are planned. Medical therapy will be applied as clinically indicated and per the local post-transplantation protocols. A total of 5–20 mL of blood will be taken at each study time point for all participants. This will coincide with their usual transplant-related investigations in adherence to WHO guidance for blood sampling in child health research.[15] In the case of a prolonged interval between the pretransplant blood sample and subsequent organ transplant (eg, >6 months) additional blood samples will be specifically requested from the patient/parent and verbal consent recorded in the medical record. Relevant clinical data will also be collected from participants at each study time point (online supplemental table 1).

Study investigations will consist of:

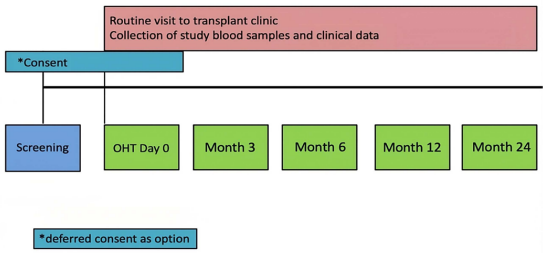

Figure 1 Immunology of THymectomy And Childhood CArdiac transplant study flow chart. OHT, orthotopic heart transplant.

- Immunophenotyping of circulating immune cell subsets.
- EBV serology.
- EBV-specific T-lymphocyte quantification.
- EBV-specific T-lymphocyte functional analysis.

Immunophenotyping of circulating immune cell subsets

Circulating immune cell populations will be analysed in Newcastle University laboratories. Mononuclear cells (MNCs) will be isolated and cryopreserved from blood samples taken at each study time point. In addition, biobanked MNC samples from age-matched healthy children will be included for analysis as non-thymectomy, non-transplant controls. Circulating immune cell subsets will be identified within samples using high-dimensional spectral flow cytometry (Aurora system (Cytek)). The 5-laser Aurora spectral cytometer enables an in-depth analysis of up to 40 cell surface markers at a time.[16] This is highly valuable in phenotypically characterising various immune cell populations and their subsets (including T-lymphocytes, B-lymphocytes, Natural Killer [NK] cells and dendritic cells) in low volume paediatric samples.[17] These samples will be analysed simultaneously in batches to mitigate interassay variation. A comprehensive list of cell surface markers has been selected to comprise the following:

1. Major innate/adaptive cell lineages.
2. Main T-lymphocyte subsets as well as putative T-helper subsets.
3. Recent thymic activity.
4. Cellular exhaustion and senescence.
5. Key innate/adaptive cells involved in immune response to EBV infection.

Two full spectrum flow panels have been validated for this purpose. A 24-colour panel has been designed to probe the wider circulating immune landscape of the study cohort (table 1). This is complemented by a targeted 30-colour T-lymphocyte panel that will elucidate any temporal changes in the T-lymphocyte compartment after transplantation (table 2).

EBV serology

Routine blood samples for evaluation of EBV serology will be tested centrally in the Newcastle upon Tyne NHS Hospitals Foundation Trust (NUTH) virology laboratories. EBV and CMV viral load will be measured from whole blood using PCR assays. Serum and/or plasma will be tested for antibody response to key EBV proteins including IgM/IgG for viral capsid antigen and IgG for EBV Nuclear Antigen 1. The results for viral load assay will be reported as total titres while antibody testing will be reported as a binary detected/not detected based on NUTH laboratories reference cut-off values.

EBV-specific T-lymphocyte quantification

An optimised panel of Human Leucocyte Antigen (HLA)-restricted peptide-Major Histocompatibility Complex (pMHC) I/II tetramers will be used to evaluate

Table 1 Cell surface markers of interest used in the broad immune full spectrum flow for the Immunology of THymectomy And Childhood CArdiac transplant study

Cell surface marker	Fluorophore	Antibody clone	Purpose
CD45	Spark YG 593	HI03	Leucocytes
TCRγd	PerCP-eFluor 710	B1.1	γd T-lymphocyte
CD3	BV 510	SK7	Pan T-cell, NK T-like cells
CD4	PerCP	SK3	CD4 T-helper lymphocytes
CD8	BUV 805	SK1	CD8 cytotoxic T-lymphocytes
CD11c	PE-Cy7	B-ly6	Dendritic cell differentiation
CD14	Spark Blue 550	63D3	Monocyte differentiation
CD16	BUV 496	3G8	Monocyte, NK cell and dendritic cell differentiation
CD19	Spark NIR 685	HIB19	B lymphocytes
CD20	BV 786	2H7	B lymphocytes
CD21	PE-Cy5	B-ly4	B lymphocyte differentiation
CD24	PE-AF 610	SN3	B lymphocyte differentiation
CD27	APC-H7	M-T271	T/B lymphocyte differentiation
CD38	APC-Fire 810	HIT2	Monocyte, dendritic cell, T/B lymphocyte activation and differentiation
CD56	BUV 737	NCAM16.2	NK cells
CD57	Pacific Blue	NK-1	NK and CD8 T lymphocyte immune senescence
CD123	Super Bright 436	6H6	Plasmacytoid dendritic cells
CD127	APC-R700	HIL-7R-M21	Cytokine receptor; T lymphocyte differentiation
KIR (CD158)	BUV 605	DX27	NK cells
NKG2A (CD159a)	APC	REA110	NK cells
NKG2C (CD159c)	PE	REA205	NK cells
IgD	BV 480	IA6-2	B lymphocyte differentiation
IgM	BV 570	MHM-88	B lymphocyte differentiation
HLA-DR	PE-Fire 810	L243	Monocyte activation, dendritic cell lineage, NK cell lineage discrimination,
Viability	Live/Dead Blue	–	Live cells

NK, Natural Killer.

EBV-specific CD8 and CD4 T-lymphocyte immunity at the time points outlined in figure 1.[18] This panel will be tailored to maximise coverage of participants who have the most commonly expressed HLA genotypes within our study cohort. Combinations of differently fluorescently labelled pMHC tetramers presenting purified peptides of dominant lytic and latent EBV epitopes,[19] relevant for the patient HLA genotype, will be included in the T-lymphocyte panel where applicable (table 2). These customised tetramers will be procured from the National Institute of Health Tetramer Core Facility, Atlanta, Georgia, USA.

EBV-specific T-lymphocyte functional analysis

To assess the functional capacity of EBV-specific T-lymphocytes in an HLA-unbiased manner, effector cytokine production of interferon gamma will be determined by ELISpot following stimulation with pools of overlapping peptides (JPT PepMix) representing the full sequences of a panel of EBV latent and lytic cycle proteins.[20 21]

Data collection

Study data will be collated and managed using Research Electronic Data Capture (REDCap) electronic case report forms.[22] REDCap is a secure, web-based platform specifically designed to support data acquisition for research studies. It provides restricted user rights to protect identifiable data, including audit trails for tracking data handling and export and procedures for data integration with external sources.[23]

Outcome measures

Primary outcomes measures:

1. Proportions of circulating innate and adaptive immune cell subsets before and at 3, 6, 12 and 24 months post-transplant.
2. Frequency of detectable EBV-specific T-lymphocyte immunity.
3. Functional capacity of EBV-specific T-lymphocytes.

Secondary outcome measures:

Table 2 Cell surface markers of interest used in the T lymphocyte full spectrum flow panel for the Immunology of THymectomy And Childhood CArdiac transplant study

Cell surface marker	Fluorophore	Antibody clone	Purpose
CD45	Spark YG 593	HI03	Leucocytes
CD45RA	BUV 395	5H9	T lymphocyte differentiation
CD3	BV510	SK7	Pan T-cell, NK T-like cells
CD4	PerCP	SK3	CD4 T-helper lymphocytes
CD8	BUV 805	SK1	CD8 cytotoxic T lymphocytes
TCRγd	PerCP-eFluor 710	B1.1	γd T lymphocyte
CD25	PE-AF700	CD25-3G10	Regulatory T lymphocytes
CD27	APC-H7	M-T271	T lymphocyte differentiation
CD28	BV650	CD28.2	T lymphocyte differentiation
CD31	BV711	WM59	Recent thymic emigrants
CD38	APC-Fire 810	HIT2	T lymphocyte activation/differentiation
CD39	BUV 661	TU66	Regulatory T lymphocytes
CD57	Pacific Blue	NK-1	CD8 T lymphocyte immune senescence
CD62L	BUV 496	SK-11	T lymphocyte differentiation
CD69	AF 647	FN50	T lymphocyte activation
CD95	PE-Cy5	DX2	T lymphocyte activation/differentiation
CD127	APC-R700	HIL-7R-M21	Chemokine receptor; T lymphocyte differentiation
HLA-DR	PE-Fire 810	L243	T lymphocyte activation
CCR4	BB 700	1G1	Chemokine receptor; T lymphocyte differentiation
CCR5	BUV 563	2D7/CCR5	Chemokine receptor; T lymphocyte differentiation
CCR6	BV 480	140 706	Chemokine receptor; T lymphocyte differentiation
CCR7	BV 421	G043H7	T lymphocyte differentiation
CXCR3	AF 488	GH025H7	Chemokine receptor; T lymphocyte differentiation
CXCR5	BV 750	RF8B2	Chemokine receptor; T lymphocyte differentiation
PD-1	BV 785	EH12.2H7	Co-inhibitory receptor; T lymphocyte exhaustion
LAG-3	PE-Cy7	3DS223H	Co-inhibitory receptor; T lymphocyte exhaustion
TIM-3	BV 605	F38-2E2	Co-inhibitory receptor; T lymphocyte exhaustion
CTLA4	PE-CF594	BNI3	Co-inhibitory receptor; T lymphocyte exhaustion
Tetramer (lytic)	APC	–	EBV-specific T lymphocytes
Tetramer (latent)	PE	–	EBV-specific T lymphocytes
Viability	Live/Dead Blue	–	Live cells

EBV, Epstein-Barr virus; NK, Natural Killer.

1. Incidence of EBV infection.
2. Time from transplantation to EBV viraemia.
3. Time from EBV viraemia to seroconversion.

DATA ANALYSIS PLAN

Sample size calculation

As the study objectives are largely descriptive, no sample size calculation is necessary. Instead, we selected our sample size such that the study is feasible and large enough to conduct comprehensive analyses. Therefore, our sample size is based on a national average of 30 childhood cardiac transplants per year,[24] and a 60% recruitment rate. All eligible patients listed on the NHSBT register will be approached for consent, recognising that not all of these patients will receive a transplant during the lifetime of this study. However, any pretransplant blood samples that have been obtained are equally important to achieve the study's primary outcome measure.

Statistical analysis

Data from spectral flow cytometry will be analysed using the OMIQ platform (https://www.omiq.ai/). Automated clustering and dimensionality reduction will be used to identify immune cell populations by FlowSOM and Uniform Manifold Approximation and Projection, respectively. These techniques overcome the practical

challenges associated with manual gating and user bias when analysing datasets from large flow panels.

Statistically different immune signatures between patients with early thymectomy and late or non-thymectomy, EBV+ and EBV− serostatus and between time points, will be identified by linear and generalised linear mixed models (statistical significance defined as $p<0.05$). Continuous variables will be assessed by Pearson correlation using the single linkage method to group patients by expression values, and non-continuous variables by non-parametric Spearman correlation, as appropriate. Viral loads will be serially quantified at each study time point to correlate changes in immune responses with the volume of circulating virus-infected cells. Tetrameric frequencies for population groups (eg, early vs late/non-thymectomy) will be compared using Mann-Whitney U test and between time points for paired patient sample using Wilcoxon signed-rank test.

Patient and public involvement

The Young Person's Advisory Group North England (YPAGne) was involved in the development of the study design, patient facing documents (eg, PIS) and the informed consent process. Ongoing consultation with YPAGne will continue to influence participant recruitment, outcome measure priorities and the acceptability of study methods.

ETHICS

The ITHACA study has both research governance and ethical approvals (IRAS project ID: 298986; REC reference number: 21/NS/0142) and is adopted onto the National Institute for Health Research Clinical Research Network portfolio. Study organisation and sponsorship will be provided by The Newcastle upon Tyne Hospitals NHS Foundation Trust including coverage of insurance and NHS indemnity.

No significant risks are anticipated for enrolled participants. Study samples will be taken at the same time as routine transplant investigations during clinic visits, thereby avoiding any additional discomfort or hospital attendance. The estimated volume of study-related blood samples required at each assessment time point is based on guidance from the WHO for trial-related blood volumes in children.[15] This has been determined to be safe without any risk of causing anaemia.

DISSEMINATION

Study findings will be disseminated widely through publications in high impact peer-reviewed journals, national and international conferences, and stakeholder events. We will engage YPAGne to identify patient-centred forums to facilitate discussion of study progress and any relevant findings with the general public. Published data will be made available via a public data repository, with a digital object identifier included in any published manuscript to aid discovery and outline access conditions. Potentially identifiable data, including patient sex, date of birth and date of transplant, will not be shared. Any unpublished data will only be shared with other parties where a data access agreement has been negotiated by Newcastle University Legal Services team on behalf of the study's chief investigator.

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Contributors UTO and SB conceptualised the study. UTO, SB, CMB, ARG and HML contributed to the study design. SB (chief investigator) and coapplicants UTO, CMB and ARG developed the protocol with contributions from PH, MO, GP, AK, JS and ZR. UTO drafted and prepared the study protocol manuscript and received comments from the coauthors. All authors reviewed and approved the final version of the manuscript for publication.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Data analysis plan section for further details.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

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Appendix C

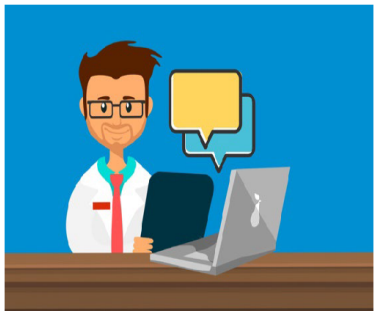
Information Sheet for ages 6 – 10 years
ITHACA Study
Version/Date: 2.6.1 / 02.12.21

REC Reference: 21/NS/0142
IRAS number: 298986

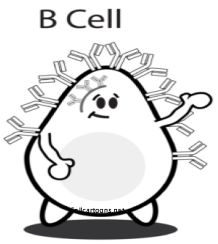
ITHACA
Immunology of THymectomy And Childhood CArdiac Transplant

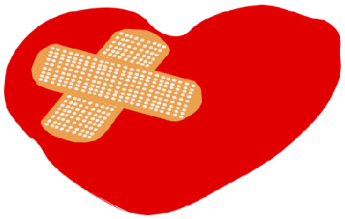
Participant Information Leaflet to be read with children 6 – 10 years

What is a research study?
A research study is the way we try to find out answers to questions.



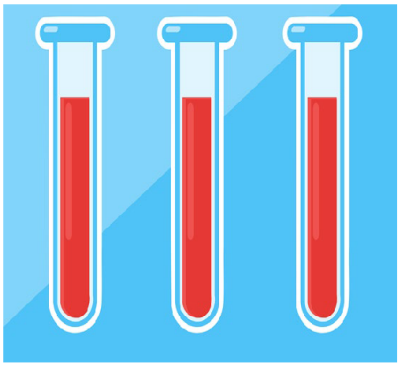
Why is this study being done?
After a transplant, some children can become poorly with an illness called post-transplant lymphoproliferative disease (PTLD). This happens when cells in the blood called "B" cells become infected with a bug called Epstein-Barr Virus (EBV). This only happens in a tiny number of children with transplants but is more likely to happen in those who have a new heart. We want to look at how well the cells in the blood of young children with a transplant are able to fight off EBV infection. We also want to look at how well these cells can either protect children from getting PTLD or make them more likely to get it.



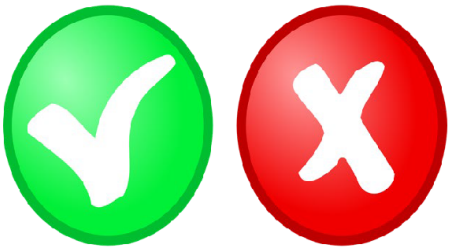


Why me?
We are asking children around the country, who like you, have recently had or are about to have a transplant. We would like to include 40 children with transplants in the study.

What will happen to me if I join the study?
We would like to take a small amount of extra blood samples during your other blood tests. We would like to do this just before your transplant and at some of your visits to see the doctors and nurses in clinic with your mum/dad/carer.
We will also collect some information about you from your doctors and nurses while you are in the study.



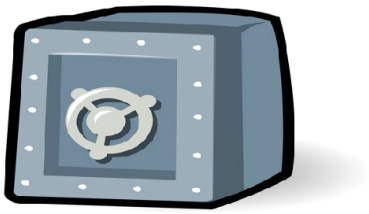
Do I have to say yes?
No, not at all. It is up to you!
Just say no if you don't want to join. Nobody will mind. If you join but then change your mind later, that's ok as well. It won't change the way the doctors and nurses will look after you.



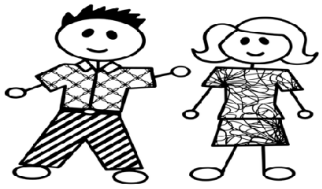
Will taking part in the study help me?
No, it won't help you right now. But it will help us learn more about PTLD and how to prevent other children from getting it in the future.

Will anyone else know that I am taking part in the study?
We will keep anything we find out about you private. We will only tell your parents and the doctors looking after you. The people who are responsible for checking that the study is done properly may also ask to look at information about you. You will be given a special study number so that we don't have to use your name in the study.

What will happen to the blood samples I give?
We will do some extra tests on the blood samples you give us. If you agree, we will store any of your leftover blood in a safe place so that we can use this for other research in the future.



What if something goes wrong?
Just in case there is a problem, we will work with you and your parents to help make it better.



If you have any questions or would like to know more about this study, you can contact us at any time.
Email Dr Simon Bomken and Dr Ugo Offor at **nuth.ithaca@nhs.net** or call **+44 (0) 191 2824068.**

THANK YOU for taking the time to read this information sheet!

REC Reference: 21/NS/0142
IRAS number: 298986

ITHACA
Immunology of THymectomy And Childhood CArdiac Transplant

Participant Information Leaflet for young people 11 – 15 years

We would like to invite you to take part in our research study. Before you decide, it is important that you understand *why* the research is being done and *what* it would involve for you.

This leaflet is divided into two parts. **Part 1** tells you the purpose of this study and what will happen to you if you take part. **Part 2** gives you more details about how the study will run.

Please **take time** to read this information carefully. You can talk about it with your family and/or friends if you want.

Ask us if there is anything you don't understand, or if you have any questions.

Thank you for reading this!

PART 1 – To give you first thoughts about the study

After an organ transplant, children and young people have a weaker immune system. This causes a small risk of developing a condition called posttransplant lymphoproliferative disease (PTLD). PTLD occurs when a group of white blood cells called 'B cells' grow out of control and transform (change) into harmful cells that can make you feel poorly. Most of the time, this is because of an infection of 'B cells' by a very common virus called Epstein-Barr Virus (EBV) which can also cause glandular fever.

Most people with transplants **DO NOT** develop PTLD, but the risk is slightly higher after a heart transplant compared to most other organs. We still do not understand the reason for this. We think there might be some differences in how well the immune system of children and young people with heart transplants works compared to people with other organ transplants.

We want to measure the number and function of different immune cells circulating in the blood just before transplant and at regular periods after transplant. We will also look at how well the immune system is able to produce immune cells to fight an EBV infection. This will help us to better understand how the immune system recovers after transplant and identify immune patterns that either protect people from developing PTLD or increase their chances of getting the disease.

Why have I been asked to take part?

We are asking patients under 18 years old from around the UK, who like you, have been put on the transplant waiting list. We plan to involve 40 children and young people who have had a transplant in this study.

Do I have to take part?

No! It is entirely up to you. If you do decide to take part:

- We will ask you if you'd like to sign a form to say that you agree to take part (an assent form).

- Your parents/carers will also be asked to sign a similar form (a consent form).

- You will be given this information sheet and a copy of your signed assent form to keep. You are free to stop taking part at any time during the study without giving a reason. If you decide to stop, this will not affect the care you receive.

What will happen to me if I take part?

We would like to take no more than an extra 3 teaspoons of blood during your usual blood tests. This will happen during routine appointments with your local transplant team and **will not usually** need additional hospital visits or extra needles. The first blood samples will be taken before your transplant. We would like to have talked to you about the study before taking your first blood samples. This may not be possible if your transplant team thinks there isn't a good time to do so before your operation. If this happens, any blood samples we take will be stored and not used until you have agreed to be a part of the study. If you decide not to take part, then we will destroy your blood samples as soon as

possible. If you do decide to take part, further samples will be taken during your regular follow up clinic visits at 3, 6, 12 and 24 months after your transplant.

At the beginning of the study, we will collect information on your medical care from the transplant team. We will continue to keep in touch with the transplant team about your health and how you are getting on after each of your regular clinic visits for up to 2 years after your transplant.

Is there anything else to be worried about if I take part?

Since the blood samples will be taken during your regular transplant tests, there will be no additional pain or discomfort from taking part in this research study.

Blood tests can be uncomfortable, but we have numbing cream or cold spray, so it doesn't hurt as much.

Will taking part in the study help me?

No, but the information we get might help us understand how PTLD develops. This can help us discover ways to prevent people in the future from getting PTLD and/or develop new and better treatments for it.

Contact for further information

If you would like any further information about this study, or if you are interested in taking part, you could contact:

Name: Dr Simon Bomken **Designation:** Consultant Paediatrician **Department:** The Sir James Spence Institute, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191-282-4068	**Name:** Dr Ugo Offor **Designation:** Paediatric Registrar **Department:** The Sir James Spence Institute Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191-233-6161
Study email: nuth.ithaca@nhs.net	

Thank you for reading so far – if you are still interested, please continue to read the additional information in Part 2 before making any decision.

PART 2 – More detailed information about the study if you still want to take part

What will happen to the samples I give you?

We will run extra tests on your blood samples at NHS laboratories, Newcastle University research laboratories and other partnering laboratories in the country.

With your permission, any of your leftover blood samples can be stored safely within the Newcastle University biobank. This can be used for other approved research in the future. This is entirely your choice. Your involvement in this study will not be affected by your decision whether to allow us store and use your leftover samples in the future. If you ask at any time, your remaining blood samples will be destroyed.

Who will know that I am taking part in the study?

We will keep your information confidential. This means we will only tell people who have a need or right to know. If you and your parents agree, we will write to your family doctor/GP to tell them that you have decided to take part in the study. The letter will explain what the study is about but will not include any information about your blood test results.

What will happen to the information you collect about me?

Any information you give us will be kept completely private. We will only use a research code number to identify you. All personal details that could identify you will be kept safe and secure on password protected NHS computers. We will keep identifiable information about you such as contact details and assent forms for no more than 5 years after the study has finished.

What will happen to the results of the study?

The results of the study will be published in medical papers which can be found on the internet and may also be presented at national/international conferences. We will make sure to remove any personal information that could identify you before the research results are published.

Who is organising and paying for the study?

The study is organised by The Newcastle upon Tyne Hospitals NHS Foundation Trust. It is paid for by Cancer Research UK and The Lymphoma Research Trust. None of the researcher doctors are paid for including you in the study.

Who has checked and approved this study?

Before any research in the NHS goes ahead, it has to be checked by a group of people called a Research Ethics Committee. They make sure that the research study is safe and that you are being treated properly. This study has been checked and approved by North of Scotland (2) Research Ethics Committee.

What if I don't want to do the study anymore?

Just tell your parents and the research doctors that you don't want to take part anymore. You don't have to give any reason why. It is **YOUR CHOICE**.

What should I do if I have a problem with this study?

If you have any concerns about this study, you should ask to speak to the researcher doctors or ask your parent/carer to talk to them and they will do their best to answer your questions. If you are still unhappy after this, you or your parent/carer can speak to the hospital's complaint team:

You can contact the Patient Advise and Liaison Service (PALS). This service is confidential and can be contacted on freephone: 0800 032 0202.

If you would prefer to make a formal complaint you can contact the Patient Relations Department through any of the details below:
Telephone: 0191 233 1382 or 0191 233 1454
Email: patient.relations@nuth.nhs.uk
Address: Patient Relations Department, The Newcastle upon Tyne Hospitals NHS Foundation Trust, The Freeman hospital, Newcastle upon Tyne, NE 7 7DN

THANK YOU for taking the time to read this leaflet!

Immunology of THymectomy And Childhood CArdiac Transplant

Participant Information Sheet – 16 years and over

We would like to invite you to take part in our research study. Before you decide, it is important that you understand why the research is being done and what it would involve for you.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Please take time to read this information carefully and discuss it with others if you wish. If there is anything that is not clear, or if you would like more information, please feel free to ask us.

Part 1 – To give you first thoughts about the study

What is the purpose of the study?

After an organ transplant, patients on lifelong anti-rejection medications (known as immunosuppressive treatment) have a small risk of developing post-transplant lymphoproliferative disease (PTLD). This occurs when a group of white blood cells called 'B cells' grow out of control and transform (change) into cancerous cells. Most cases of PTLD are caused by infection of 'B cells' by a very common virus called Epstein-Barr Virus (EBV) which can also cause glandular fever.

Whilst most people with transplants **DO NOT** develop PTLD, the risk is slightly higher after a heart transplant compared to most other organs (1 in every 10 heart transplant patients under 18 years old will develop PTLD). The reason for this increased risk is still poorly understood. Our previous study showed that very early removal of the thymus (a gland in the neck needed to develop a healthy immune system) during major childhood heart surgery might play an important role in this.

The purpose of this study is to examine the quantity and function of different immune cells circulating in the blood just before transplant and at regular periods after transplant. We will also look at how well the immune system is able to produce antibodies and other specific immune cells to fight an EBV infection. We intend to compare the immune cell patterns of patients undergoing a heart transplant with those

receiving other types of organ transplants. This will help us to better understand how the immune system recovers after transplant and identify immune patterns that either protect patients from developing PTLD or increase their risk of getting the disease.

Why have I been invited to participate in this study?

We are inviting all patients under 18 years old from around the UK, who like you, have been put on the transplant waiting list. We aim to involve 40 participants who received a transplant in this study.

Do I have to take part?

No, this study is entirely **voluntary**. It is up to you to decide to take part in the study. If you agree to take part, we will ask you to sign a study consent form.

You are free to withdraw from the study at any time without giving a reason why. This will not affect the standard of care you receive in any way.

Can I take part?

Children with an organ transplant under the age of 18 years are able to take part. In order to be enrolled in the study;

- You should be listed on the national transplant list or awaiting a living related kidney transplant.

You cannot take part in the study if you:

- Have a pre-existing diagnosis of an inherited or acquired immunodeficiency.
- Have an underlying thymic disorder.
- Have previously received a bone marrow or organ transplant.
- Have had a previous cancer diagnosis.

What will happen if I decide to take part?

We will ask you to sign a study consent form to show that you have agreed to take part. We will give you a copy of this to keep as well. We would like to take up to an extra 3 teaspoons of blood during your usual investigations. This will occur during routine appointments with your local transplant team and **will not usually** require additional hospital visits. We aim to discuss the study with you before taking the first blood samples. The first blood samples will usually be taken once you have agreed to participate in the study. However, if your transplant team thinks that this discussion cannot happen at an appropriate time before your transplant, then any blood samples taken for our study will be stored but not used until you have given full consent to be a part of this study. If you decide not to participate in the study, your blood samples will be destroyed as soon as possible. If you do decide to participate in the study, subsequent samples will be taken during your routine follow up clinic visits at 3, 6, 12 and 24 months after your transplant. In the case of a prolonged period between the first blood sample being taken and transplant, we may ask your permission to take an additional blood sample before transplant.

At the beginning of the study, we will collect information on your medical history from the transplant team. We will continue to follow up with the transplant team about your health and clinical progress after each of your routine clinic visits for up to 2 years after your transplant.

Are there any possible disadvantages or risks from taking part?

Apart from taking a slightly larger blood sample, this research will not affect you. It does **NOT** alter your treatment. Since the blood samples will be taken during your routine transplant tests, there will be no additional pain or discomfort from taking part in this research study.

What are the possible benefits of taking part?

You will not gain any direct benefit from the study, but the information gathered from our research might help us to better understand how PTLD develops. This is an important step for us to develop future tests that tell us which patients are more likely to develop PTLD. With this knowledge, we could possibly identify ways to reduce the chance of transplant patients getting PTLD and/or create new treatments for this condition.

Will my taking part in the study be kept confidential?

Yes. All information about you will be kept private. The details are included in Part 2.

Will my family doctor/General Practitioner (GP) be informed of my participation?

It is optional for your GP to be informed about your participation in the study. If you agree, we will write to your GP to inform them that you have agreed to take part in the study. The letter will explain the aims of the study but will not include any information about your specific blood test results.

What if there is a problem?

Any complaint about the way you have been treated during the study or any possible harm you might experience will be addressed. The detailed information on this is given in Part 2.

Contact for further information

If you would like any further information about this study, or if you are interested in participating, you could contact:

Name: Dr Simon Bomken **Designation:** Consultant Paediatrician **Department:** The Sir James Spence Institute, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191–282–4068	**Name**: Dr Ugo Offor **Designation:** Paediatric Registrar **Department:** The Sir James Spence Institute, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191–233–6161

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2 – More detailed information you need to know if you still want to take part.

What will happen to the samples I give?

Your study samples will be analysed in NHS laboratories, Newcastle University research laboratories and other collaborating laboratories in the UK.

If you consent, any of your leftover blood samples can be stored within the Newcastle University biobank and used for future approved research. This is optional; your involvement in this study will not be affected by your decision whether to allow storage and future use of your leftover samples. If you request at any time, your remaining blood samples will be destroyed.

What will happen if I don't want to carry on with the study?

If at any time you change your mind about being involved with this study, you are free to withdraw without giving a reason. Your decision will not affect the standard of your care in any way. Unless you state otherwise, any blood samples taken while you were involved in the study will continue to be stored and used for research as detailed above. You are free to request that your blood samples are destroyed at any time during or after the study. Any data collected and/or generated up to the time of your withdrawal may still be kept and used for analysis.

Will my taking part in the study be kept confidential?

All information that is collected about you during the course of this study will be coded with a study number and kept private. The information will be available to the study team, authorised collaborators, ethical review committee, Newcastle University and the study sponsor (The Newcastle upon Tyne Hospitals NHS Foundation Trust), who can ask to access the study data to ensure that we are complying with research study regulations. They are all bound by the same confidentiality rules.

Every effort will be taken to maintain your privacy. Information about you may be stored electronically on an NHS secure server, and paper documents will be kept in a key-locked filing cabinet or restricted access office at the Sir James Spence Institute, Royal Victoria Infirmary. Study results will be published in a scientific journal but nothing that could identify you will be mentioned in any report or publication.

How will you use information about me?

As the study sponsor's, The Newcastle upon Tyne Hospitals NHS Foundation Trust is the data controller and is responsible for looking after your information and using in properly. We will need to use information from you and your medical records for this

research project. This will include identifiable information such as your name, NHS number, hospital number, and contact details. People will use this information to do the research or to check your records to make sure that the research is being done properly. We will keep such identifiable information about you for a maximum of 5 years after the study has finished.

People who do not need to know who you are will not be able to see your name or contact details. Your data will have a code number instead. We will keep all information about you safe and secure. We will write our reports in a way that no-one can work out that you took part in the study. De-identified research data will be stored indefinitely at Newcastle University.

What are my choices about how my information is used?

You can stop being part of the study at any time, without giving a reason, but we will keep any information about you that we have already collected.

We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

Where can I find out more about how my information is used?

You can find out more about how we use your information:

- at www.hra.nhs.uk/information-about-patients/
- by asking a member of our research team
- by sending an email to richard.oliver2@nhs.net, or
- by ringing us on 0191-213-8946.

What will happen to the results of the study?

The results of the study will be published in medical journals which are available via the internet and may also be presented at national/international conferences. Your name will not be used when the research results are published. The study is expected to run for at least 2 years.

How have patients and the public been involved in this study?

Potential participants were involved in reviewing the Participant Information Sheet. In designing this study, we have taken into account patient opinions on the frequency of participant visits and the tests that we will carry out.

Who is sponsoring, organising and funding the study?

The study is organised and sponsored by The Newcastle upon Tyne Hospitals NHS Foundation Trust. The study is funded through financial support from Cancer

Research UK and the Lymphoma Research Trust. None of the researchers are paid for recruiting you into the study.

Who has reviewed and approved the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by North of Scotland (2) Research Ethics Committee.

What if something goes wrong?

The study team recognise the important contribution that volunteers make to medical research and make every effort to ensure your safety and well-being. The Newcastle upon Tyne Hospitals NHS Foundation Trust, as the study sponsor, has arrangements in place in the unlikely event that you suffer any harm as a direct consequence of your participation in this study.

In the event of harm being suffered, while the Sponsor will cooperate with any claim, you may wish to seek independent legal advice to ensure that you are properly represented in pursuing any complaint. The study doctor can advise you of further action and refer you to a doctor within the NHS for treatment, if necessary. NHS indemnity operates in respect of the clinical treatment which may be provided if you needed to be admitted to hospital.

Complaint statement

What if I wish to complain about the way in which the study has been conducted?

If you have a concern about any aspect of this study, you should ask to speak to the researcher investigators who will do their best to address your concerns. Alternatively, you may wish to send us an email at **nuth.ithaca@nhs.net**. If you remain unhappy and wish to complain formally, the normal National Health Service complaints mechanism is available to you. You will not be treated any differently if you decide to make a complaint. You can contact the Patient Advise and Liaison Service (PALS). This service is confidential and can be contacted on Freephone: **0800 032 0202.**

Alternatively, if you wish to make a formal complaint you can contact the Patient Relations Department through any of the details below:
Telephone: **0191-223-1382** or **0191-223-1454**
Email: patient.relations@nuth.nhs.uk
Address: Patient Relations Department
The Newcastle upon Tyne Hospitals NHS Foundation Trust
The Freeman Hospital
Newcastle upon Tyne
NE7 7DN

Further information and contact details

We hope this information sheet has answered all of your questions. If you would like further information about participating in research, please visit the following website: www.crn.nihr.ac.uk/can-help/patients-carers-public/how-to-take-part-in-a-study.
or you could contact the hospital Clinical Research Facility:

nuth.paedoncresearch@nhs.net

01912821893 (Clinical Lead)

01919177591 (Research Nurse)

REC Reference: 21/NS/0142
IRAS number: 298986

Immunology of THymectomy And Childhood CArdiac Transplant

Participant Information Sheet – Parents/Guardians

We would like to invite your child to take part in our research study. Before you decide, it is important that you understand why the research is being done and what it would involve.

Part 1 tells you the purpose of this study and what will happen to you and your child if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Please take time to read this information carefully and discuss it with others if you wish. If there is anything that is not clear, or if you would like more information, please feel free to ask us.

Part 1 – To give you initial information about the study

What is the purpose of the study?

After an organ transplant, patients on lifelong anti-rejection medications (known as immunosuppressive treatment) have a small risk of developing post-transplant lymphoproliferative disease (PTLD). This occurs when a group of white blood cells called 'B cells' grow out of control and transform (change) into cancerous cells. Most cases of PTLD are caused by infection of 'B cells' by a very common virus called Epstein-Barr Virus (EBV) which can also cause glandular fever.

Whilst most people with transplants **DO NOT** develop PTLD, the risk is slightly higher after a heart transplant compared to most other organs (1 in every 10 heart transplant patients under 18 years old will develop PTLD). The reason for this increased risk is still poorly understood. Our previous study showed that very early removal of the thymus (a gland in the neck needed to develop a healthy immune system) during major childhood heart surgery might play an important role in this.

The purpose of this study is to examine the quantity and function of different immune cells circulating in the blood just before transplant and at regular periods after transplant. We will also look at how well the immune system is able to produce antibodies and other specific immune cells to fight an EBV infection. We intend to compare the immune cell patterns of patients undergoing a heart transplant with those

receiving other types of organ transplants. This will help us to better understand how the immune system recovers after transplant and identify immune patterns that either protect patients from developing PTLD or increase their risk of getting the disease.

Why have we been invited to participate in this study?

We are inviting all patients under 18 years old from around the UK, who like your child, have been put on the transplant waiting list. We aim to involve 40 participants who received a transplant in this study.

Do we have to take part?

No, this study is entirely **voluntary**. It is up to you and your child (wherever possible) to decide to take part in the study. If you agree to take part, we will ask you to sign a study consent form.

If your child is able to understand the research and is happy to take part and can write their name, they will be asked to sign an assent form with you, if they want to.

You and your child are free to withdraw from the study at any time without giving a reason why. This will not affect the standard of care your child receives in any way.

Can we take part?

Children with an organ transplant under the age of 18 years are able to take part. In order to be enrolled in the study;

- Your child should be listed on the national transplant list or awaiting a living related donor kidney transplant

You cannot take part in the study if your child:

- Has a pre-existing diagnosis of an inherited or acquired immunodeficiency.
- Has an underlying thymic disorder.
- Has previously received a bone marrow or organ transplant.
- Has had a previous cancer diagnosis.
- Weighs less than 2.5kg.

What will happen if we decide to take part?

We would like to take no more than an extra 3 teaspoons of blood during your child's usual investigations. This will occur during routine appointments with your local transplant team and **will not usually** require additional hospital visits. We aim to discuss the study with you and your child before transplant. The first blood sample will usually be taken once you and your child have agreed to participate in the study. However, if your child's transplant team thinks that this discussion cannot happen at an appropriate time before your child's transplant, then any blood samples taken for our study will be stored but not used until you and/or your child have given full consent to be a part of this study. If you and/or your child decide not to participate in the study, your child's blood samples will be destroyed as soon as possible and no further samples will be taken. If you and your child decide to participate, subsequent samples

will be taken during routine follow up clinic visits at 3, 6, 12 and 24 months after your child's transplant. In the case of a prolonged period between the first blood sample being taken and transplant, we may ask your permission to take an additional blood sample before transplant.
At the beginning of the study, we will collect information on your child's medical history from the transplant team. We will continue to follow up with the transplant team about your child's health and clinical progress after each routine clinic visits for up to 2 years after your child's transplant.

Are there any possible disadvantages or risks from taking part?

Apart from taking a slightly larger blood sample, this research will not affect your child. It does **NOT** alter your child's treatment. Since the blood samples will be taken during your child's routine transplant tests, there will be no additional pain or discomfort to them from taking part in this research study.

What are the possible benefits of taking part?

Your child will not gain any direct benefit from the study, but the information gathered from our research might help us to better understand how PTLD develops. This is an important step for us to develop future tests that tell us which patients are more likely to develop PTLD. With this knowledge, we could possibly identify ways to reduce the chance of transplant patients getting PTLD and/or create new treatments for this condition.

Will our taking part in the study be kept confidential?

Yes. All information about your child will be kept private. The details are included in Part 2.

Will our family doctor/General Practitioner (GP) be informed of my child's participation?

It is optional for your GP to be informed about your child's participation in the study. If you agree, we will write to your GP to inform them that you and your child have agreed to take part in the study. The letter will explain the aims of the study but will not include any information about your child's specific blood test results.

What if there is a problem?

Any complaint about the way you or your child have been treated during the study or any possible harm your child might experience will be addressed. The detailed information on this is given in Part 2.

Contact for further information

If you would like any further information about this study, or **if you are interested in participating**, you could contact:

Name: Dr Simon Bomken	**Name**: Dr Ugo Offor

Designation: Consultant Paediatrician **Department:** The Sir James Spence Institute, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191–282–4068	**Designation:** Paediatric Registrar **Department:** The Sir James Spence Institute, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP. **Tel:** +44 (0) 191–233–6161
Study email: nuth.ithaca@nhs.net	

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2 – More detailed information you need to know if you still want to take part.

What will happen to my child's samples?

Your child's study samples will be analysed in NHS laboratories, Newcastle University research laboratories and other collaborating laboratories in the UK.

If you and your child consent, any leftover blood samples can be stored within the Newcastle University biobank and used for future approved research. This is optional; your child's involvement in this study will not be affected by the decision whether to allow storage and future use of leftover samples. If you or your child request at any time, the remaining blood samples will be destroyed.

What will happen if we don't want to carry on with the study?

If at any time you or your child change your minds about being involved with this study, you are free to withdraw without giving a reason. If you withdraw, we would not perform any more research procedures on your child. Your decision will not affect the standard of your child's care in any way. Unless you state otherwise, any blood samples taken while your child was involved in the study will continue to be stored and used for research as detailed above. You and your child are free to request that their blood samples are destroyed at any time during or after the study. Any data collected and/or generated up to the time of your child's withdrawal may be kept and used for study analysis.

Will my child's taking part in the study be kept confidential?

All information that is collected about your child during the course of this study will be coded with a study number and kept private. The information will be available to the study team, authorised collaborators, ethical review committee, Newcastle University and the study sponsor (The Newcastle upon Tyne Hospitals NHS Foundation Trust), who can ask to access the study data to ensure that we are complying with research study regulations. They are all bound by the same confidentiality rules.

Every effort will be taken to maintain your child's privacy. Information about your child may be stored electronically on NHS secure servers, and paper documents will be

kept in a key-locked filing cabinet or restricted access office at the Sir James Spence Institute, Royal Victoria Infirmary. Study results will be published in a scientific journal but nothing that could identify your child will be mentioned in any report or publication.

How will you use information about my child?

As the study sponsor's, The Newcastle upon Tyne Hospitals NHS Foundation Trust is the data controller and is responsible for looking after your child's information and using in properly. We will need to use information from your child's medical records for this research project. This will include identifiable information such as your child's name, NHS number, hospital number, and contact details. People will use this information to do the research or to check your child's records to make sure that the research is being done properly. We will keep such identifiable information about your child for a maximum of 5 years after the study has finished.

People who do not need to know who your child is will not be able to see his/her name or contact details. Your child's data will have a code number instead. We will keep all information about your child safe and secure. We will write our reports in a way that no-one can work out that your child took part in the study. De-identified research data will be stored indefinitely at Newcastle University.

What are my choices about how my child's information is used?

Your child can stop being part of the study at any time, without giving a reason, but we will keep any information about your child that we have already collected.

We need to manage your child's records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about your child.

Where can I find out more about how my child's information is used?

You can find out more about how we use your child's information:

- at www.hra.nhs.uk/information-about-patients/
- by asking a member of our research team
- by sending an email to richard.oliver2@nhs.net, or
- by ringing us on 0191-213-8946.

What will happen to the results of the study?

The results of the study will be published in medical journals which are available via the internet and may also be presented at national/international conferences. This research will also contribute to the fulfilment of educational requirements (e.g., a doctoral thesis). We will protect your child's privacy by ensuring that no information

that could identify them is used when the research results are published. The study is expected to run for at least 2 years.

How have patients and the public been involved in this study?

Potential participants were involved in reviewing the Participant Information Sheet, consent forms and study design. In designing this study we have taken into account patient opinions on the frequency of participant visits and the tests that we will carry out.

Who is sponsoring, organising and funding the study?

The study is organised and sponsored by The Newcastle upon Tyne Hospitals NHS Foundation Trust. The study is funded through financial support from Cancer Research UK and the Lymphoma Research Trust. None of the researchers are paid for recruiting your child into the study.

Who has reviewed and approved the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your child's safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by North of Scotland (2) Research Ethics Committee.

What if something goes wrong?

The study team recognise the important contribution that volunteers make to medical research and make every effort to ensure your child's safety and well-being. The Newcastle upon Tyne Hospitals NHS Foundation Trust, as the study sponsor, has arrangements in place in the unlikely event that your child suffers any harm as a direct consequence of participation in this study.

In the event of harm being suffered, while the Sponsor will cooperate with any claim, you may wish to seek independent legal advice to ensure that you are properly represented in pursuing any complaint. The study doctor can advise you of further action and refer your child to a doctor within the NHS for treatment, if necessary. NHS indemnity operates in respect of the clinical treatment which may be provided if your child needed to be admitted to hospital.

Complaint statement

What if I wish to complain about the way in which the study has been conducted?

If you have a concern about any aspect of this study, you should ask to speak to the researcher investigators who will do their best to address your concerns. Alternatively, you may wish to send us an email at **nuth.ithaca@nhs.net**. If you remain unhappy and wish to complain formally, the normal National Health Service complaints mechanism is available to you. You and your child will not be treated any differently if

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nuth.paedoncresearch@nhs.net

01912821893 (Clinical Lead)

01919177591 (Research Nurse)

Appendix D

Confidential

First visit/Baseline assessment

Study ID	_____ (ITHACA study number)
Local hospital number	_____
NHS number	_____
Date of hospital visit	_____
Sex at birth	◯ Male ◯ Female
Date of birth	_____
Ethnicity	◯ Caucasian ◯ Caucasian and Black Caribbean ◯ Caucasian and Black African ◯ Caucasian and South Asian ◯ Caucasian and Asian ◯ Any other mixed ethnicity ◯ Asian ◯ South Asian ◯ Black Caribbean ◯ Black African ◯ Any other ethnic background
Type of organ transplant	◯ Heart ◯ Kidney
Pre-transplant cardiac diagnosis	◯ Congenital Cyanotic ◯ Congenital Acyanotic ◯ Acquired
Pre-transplant renal diagnosis	◯ Congenital ◯ Acquired
Pre-transplant cardiac diagnosis	_____ (Please specify (e.g. hypoplastic left heart, TGA, etc))
Pre-transplant renal diagnosis	_____ (Please specify (e.g multicystic dysplastic kidneys, Horseshoe Kidneys, Autoimmune glopmerulonephritis,etc))
Date of first median sternotomy	_____

Procedure at first median sternotomy	◯ Transplant ◯ Corrective heart surgery ◯ Staged heart surgery ◯ Ventricular assist device insertion ◯ ECMO ◯ Other (please specify)
Other (please specify)	_____
Was any Thymic tissue removed during this procedure?	◯ Yes ◯ No ◯ Not documented (NB: Information may exist in the patient's surgical notes)
Type of Thymectomy	◯ Total ◯ Partial ◯ Unknown
Date of organ transplant	_____
Weight (Kg)	_____ (Please provide most recent weight before transplant)
Height (cm)	_____ (Please provide most recent height before transplant)
Induction immunosuppression	☐ Anti-Thymocyte Globulin (ATG) ☐ Basiliximab ☐ Methylprednisolone ☐ None ☐ Other (Please select all that apply)
Other (please specify)	_____
Recipient's Blood Group	◯ O+ve ◯ O-ve ◯ A+ve ◯ A-ve ◯ B+ve ◯ B-ve ◯ AB+ve ◯ AB-ve

Organ donor's Blood Group	◯ O+ve ◯ O-ve ◯ A+ve ◯ A-ve ◯ B+ve ◯ B-ve ◯ AB+ve ◯ AB-ve ◯ Not documented
Recipient EBV VCA IgG status	◯ Detected ◯ Not detected
Recipient EBV VCA IgM status	◯ Detected ◯ Not detected
Recipient EBNA-1 IgG status	◯ Detected ◯ Not detected
Recipient EBV DNA (copies/ul)	_____
Donor EBV VCA IgG status	◯ Detected ◯ Not detected ◯ Unknown
Donor EBNA-1 IgG status	◯ Detected ◯ Not detected ◯ Unknown
Recipient CMV IgM status	◯ Detected ◯ Not detected ◯ Not done
Recipient CMV IgG status	◯ Detected ◯ Not detected ◯ Not done
Recipient CMV viral load (copies/ul)	_____ (Please enter "N/A" if not available)
Donor CMV IgG status	◯ Detected ◯ Not detected ◯ Unknown
Co-morbidities prior to transplant	_____ (Please list any relevant conditions)
Other relevant comments	_____

Follow up visit

Study ID	_____
Local hospital number	_____
NHS number	_____
Date of clinic visit	_____
Study time point (post-transplant)	◯ 3 months ◯ 6 months ◯ 1 year ◯ 2 years
Sex at birth	◯ Male ◯ Female
Date of birth	_____
Current Weight (Kg)	_____
Current Height (cm)	_____
Organ transplant	◯ Heart ◯ Kidney
Maintenance immunosuppression	☐ Ciclosporin ☐ Tacrolimus ☐ Sirolimus ☐ Mycophenolate Mofetil (MMF) ☐ Azathioprine ☐ Prednisolone ☐ Other (please specify)
Other (please specify)	_____
Current dose of Calcineurin inhibitor (mg)	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Trough level of Calcineurin Inhibitor (ug/L) at this clinic visit	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Target trough level of Calcineurin inhibitor (ng/ml)	_____

Dose(s) of non-CNI immunosuppressant(s)	_____ (CNI = Calcineurin Inhibitor)
Any recent change to the dose(s) of immunosuppression therapy?	◯ Yes ◯ No
Please specify which medication(s)	_____ (Please include date of change)
Indication for change in dosage	_____
Recipient EBV VCA IgG status	◯ Detected ◯ Not detected
Recipient EBV VCA IgM status	◯ Detected ◯ Not detected
Recipient EBNA-1 IgG status	◯ Detected ◯ Not detected
Recipient EBV DNA (copies/ul)	_____
Recipient CMV IgM status	◯ Detected ◯ Not detected ◯ Unknown
Recipient CMV IgG status	◯ Detected ◯ Not detected ◯ Unknown
Any opportunistic infections since the last study visit?	◯ Yes ◯ No
Please specify	_____
Any transplant-related co-morbidities since last the study visit?	◯ Yes ◯ No
Please specify	_____
Other relevant comments	_____

PTLD episode

Study ID	_____
Local hospital number	_____
NHS number	_____
Sex at birth	◯ Male ◯ Female
Date of birth	_____
Weight (Kg)	_____
Height (cm)	_____
Maintenance immunosuppression	☐ Ciclosporin ☐ Tacrolimus ☐ Sirolimus ☐ Mycophenolate Mofetil (MMF) ☐ Azathioprine ☐ Prednisolone ☐ Other (please specify)
Other (please specify)	_____
Current dose of Calcineurin inhibitor (mg/day)	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Trough level of Calcineurin Inhibitor (ng/ml) measured at PTLD diagnosis	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Target trough level of Calcineurin inhibitor (ng/ml)	_____
Dose(s) of other non-CNI immunosuppressant(s)	_____ (CNI = Calcineurin Inhibitor)
Recipient EBV VCA IgG status	◯ Detected ◯ Not detected (Please only enter the result from this clinic visit)

Recipient VCA IgM status	◯ Detected ◯ Not detected (Please only enter the result from this clinic visit)
Recipient EBNA-1 IgG status	◯ Detected ◯ Not detected (Please only enter the result from this clinic visit)
Recipient EBV DNA (copies/ul)	_____ (i.e. Peripheral blood titre (Please only enter the result from this clinic visit))
Recipient CMV IgM status	◯ Detected ◯ Not detected ◯ Unknown
Recipient CMV IgG status	◯ Detected ◯ Not detected ◯ Unknown (Please only enter the result from this clinic visit)
Date of PTLD diagnosis	_____
PTLD Histology (WHO 2016 criteria)	◯ IM-like ◯ Florid follicular hyperplasia ◯ Plasmacytic hyperplasia ◯ Polymorphic ◯ DLBCL ◯ Burkitt/Burkitt-like lymphoma ◯ Hodgkin lymphoma ◯ Other
Please specify	_____
EBV status of PTLD tumour	◯ EBER +ve ◯ EBER -ve
Tumour site(s) involved	_____
Stage of disease (St Jude staging)	◯ Stage I ◯ Stage II ◯ Stage III ◯ Stage IV
PTLD first line treatment	☐ Reduction of Immunosuppression ☐ Rituximab ☐ Chemotherapy ☐ No treatment (Please select all that apply)

Reason for no treatment	◯ Non-destructive PTLD diagnosis ◯ Palliative care ◯ Other
Please specify	_____
How many doses of Rituximab given?	_____
Chemotherapy regime	◯ COP ◯ GRAB ◯ Protocol 901 ◯ Other
Please specify	_____
Response to first line treatment	◯ Complete response ◯ Partial response ◯ No response ◯ Progressive disease
Any further lines of therapy given?	◯ Yes ◯ No
Please provide details	_____
Other relevant comments	_____

Death episode

Study ID	_____
Local hospital number	_____
NHS number	_____
Sex at birth	◯ Male ◯ Female
Date of birth	_____
Date of death	_____
Primary cause of death	_____ (Please provide all relevant details)
Was a post-mortem performed?	◯ Yes ◯ No
Post-mortem findings (if available)	_____
Other relevant comments	_____

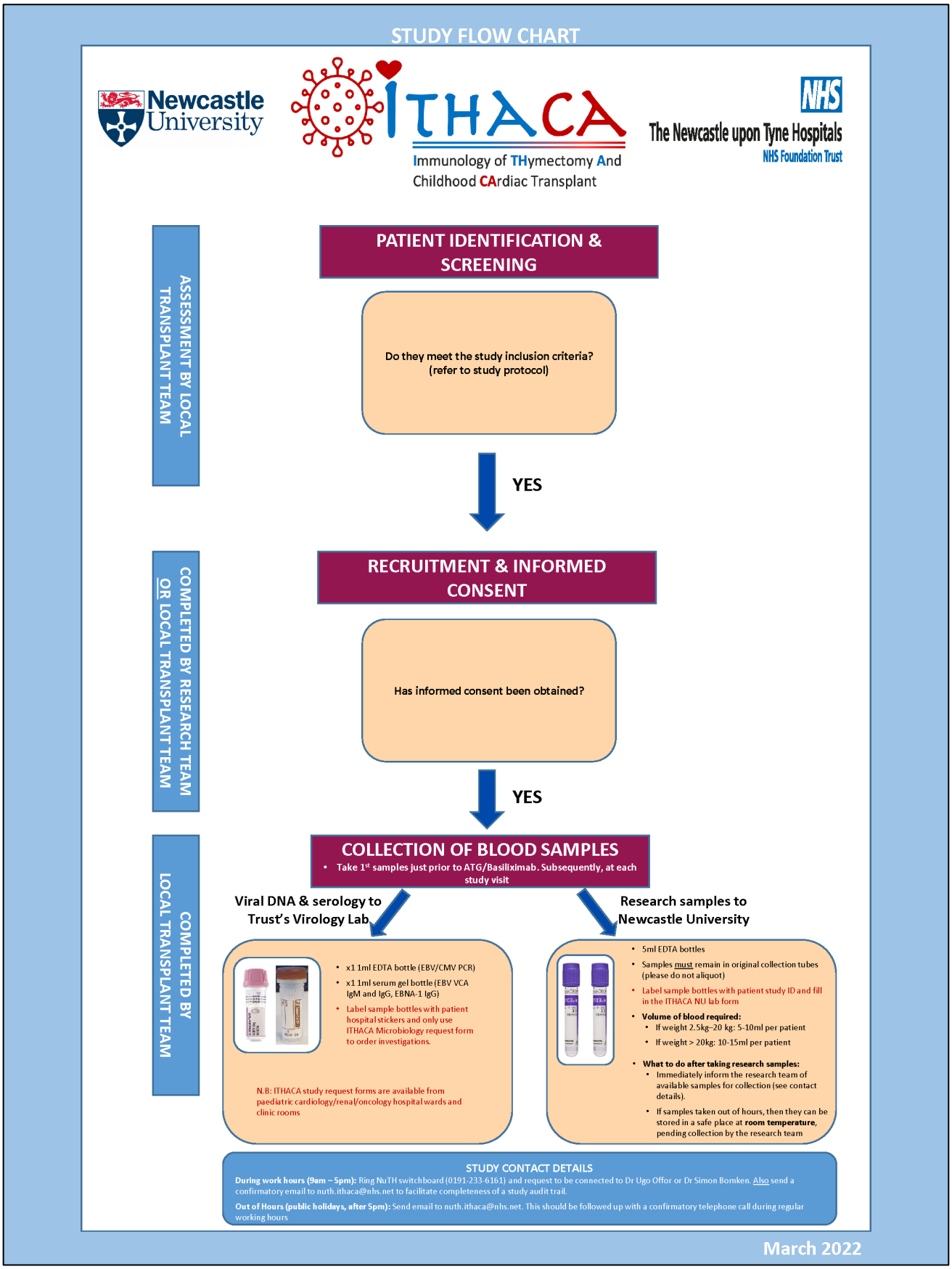
Follow up visit V2

Study ID	_____
Local hospital number	_____
Date of clinic visit	_____
Study time point (post-transplant)	◯ 3 months ◯ 6 months ◯ 1 year ◯ 2 years
Sex at birth	◯ Male ◯ Female
Date of birth	_____
Weight (Kg)	_____
Height (cm)	_____
Organ transplant	◯ Heart ◯ Kidney
Maintenance immunosuppression	☐ Ciclosporin ☐ Tacrolimus ☐ Sirolimus ☐ Mycophenolate Mofetil (MMF) ☐ Azathioprine ☐ Prednisolone ☐ Other (please specify)
Other (please specify)	_____
Dose of Calcineurin inhibitor (ng/ml)	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Trough level of Calcineurin Inhibitor (ng/ml)	_____ (Calcineurin Inhibitor = Ciclosporin/Tacrolimus)
Target trough level of Calcineurin inhibitor (ng/ml)	_____

Dose(s) of other immunosuppressant(s)	_____
Any recent change to the dose(s) of immunosuppression therapy?	◯ Yes ◯ No
Please specify which medication(s)	_____ (Please include date of change)
Indication for change in dosage	_____
Recipient EBV VCA IgG status	◯ Detected ◯ Not detected
Recipient EBV VCA IgM status	◯ Detected ◯ Not detected
Recipient EBNA-1 IgG status	◯ Detected ◯ Not detected
EBV DNA (copies/ul)	_____
Recipient CMV IgM status	◯ Detected ◯ Not detected ◯ Unknown
Recipient CMV IgG status	◯ Detected ◯ Not detected ◯ Unknown
Any episode(s) of acute graft rejection since last study visit?	◯ Yes ◯ No (Please consider clinical symptoms and/or histological evidence of rejection)
How many?	_____
Type of acute graft rejection (ISHLT 2004 criteria)	☐ ACR - 1R ☐ ACR - 2R ☐ ACR - 3R ☐ AMR 1 ☐ AMR 2 ☐ AMR 3 (NB: Select all that apply if episode was a combined ACR/AMR)
Any treatment required?	◯ Yes ◯ No

Please specify	_____
Type of graft rejection (Banff criteria)	_____
Any treatment required?	◯ Yes ◯ No
Please specify	_____
Any opportunistic infections since last study visit?	◯ Yes ◯ No
Please specify	_____
Any transplant-related co-morbidities since last study visit?	◯ Yes ◯ No
Please specify	_____
Other relevant comments	_____

Appendix E


STUDY FLOW CHART
Newcastle University
ITHACA
Immunology of THymectomy And Childhood CArdiac Transplant
NHS
The Newcastle upon Tyne Hospitals
NHS Foundation Trust
ASSESSMENT BY LOCAL TRANSPLANT TEAM
PATIENT IDENTIFICATION & SCREENING
Do they meet the study inclusion criteria? (refer to study protocol)
YES
COMPLETED BY RESEARCH TEAM OR LOCAL TRANSPLANT TEAM
RECRUITMENT & INFORMED CONSENT
Has informed consent been obtained?
YES
COMPLETED BY LOCAL TRANSPLANT TEAM
COLLECTION OF BLOOD SAMPLES
Take 1st samples just prior to ATG/Basiliximab. Subsequently, at each study visit
Viral DNA & serology to Trust's Virology Lab
x1 1ml EDTA bottle (EBV/CMV PCR)
x1 1ml serum gel bottle (EBV VCA IgM and IgG, EBNA-1 IgG)
Label sample bottles with patient hospital stickers and only use ITHACA Microbiology request form to order investigations.
N.B: ITHACA study request forms are available from paediatric cardiology/renal/oncology hospital wards and clinic rooms
Research samples to Newcastle University
5ml EDTA bottles
Samples must remain in original collection tubes (please do not aliquot)
Label sample bottles with patient study ID and fill in the ITHACA NU lab form
Volume of blood required:
If weight 2.5kg–20 kg: 5-10ml per patient
If weight > 20kg: 10-15ml per patient
What to do after taking research samples:
Immediately inform the research team of available samples for collection (see contact details).
If samples taken out of hours, then they can be stored in a safe place at room temperature, pending collection by the research team
STUDY CONTACT DETAILS
During work hours (9am – 5pm): Ring NuTH switchboard (0191-233-6161) and request to be connected to Dr Ugo Offor or Dr Simon Bomken. Also send a confirmatory email to nuth.ithaca@nhs.net to facilitate completeness of a study audit trail.
Out of Hours (public holidays, after 5pm): Send email to nuth.ithaca@nhs.net. This should be followed up with a confirmatory telephone call during regular working hours
March 2022

Supplementary Table 1: Peri-operative and long term immunosuppression regimen for children < 18 years undergoing an orthotopic heart transplant at the Freeman Hospital

Pre-operative	Intra-operative	Post-operative
Ciclosporin • 4mg/kg orally if eGFR >90 ml/min/1.73 m2 • 2mg/kg orally if eGFR 60-90 ml/min/1.73 m2 • Omit if eGFR <60ml/min/1.73 m2 **Azathioprine** • 4 mg/kg orally (no max dose) • Titrate dose depending on severity of induced thrombocytopaenia and/or leucopaenia **Basiliximab (Simulect)** • IV 10mg if body weight < 35kg • IV 20mg if body weight > 35kg • Administer within 2 hours pre-transplant	**Methyl prednisolone** • IV 15mg/kg (max dose 500mg) when the cross-clamp is released	**Anti-thymocyte globulin (rATG)** • IV 2.5mg/kg • Repeat daily doses for up to 7 days post-op if CD3 count > $0.05x10^9$/l **OR** **Basiliximab (Simulect)*** • IV 10mg if body weight < 35kg • IV 20mg if body weight > 35kg • Administer on day 4 post-op **Methylprednisolone** • 3 mg/kg (max dose 125 mg). THREE doses only are given **Calcineurin inhibitors** • **Ciclosporin** IV 1mg/kg twice daily initiated day 1 post-op. Switched to enteral route when absorption is reliable (3x IV dose). Peritransplant trough level of 250mcg/l is gradually reduced to 100-150mcg/l over the first year. • **Tacrolimus** Used if significant side effects with Ciclosporin (typically switched at 3 months post-transplant). Initial trough levels target of 10-15 mcg/l are reduced to 5-10 mcg/l over time. **Metabolic inhibitors** • **Azathioprine** 2 mg/kg daily (no max dose). Initiated a few days after transplant when the platelets and LFTs have normalised. Stopped if the WCC < 4.0 - 6.0 x 109/l. • **Mycophenolate mofetil** Substituted for Azathioprine if needed. Target dose is 600 mg/m2/dose bd. **Prednisolone** • Given to older patients • > 5 years 0.2mg/kg/day from day 2 – 6 weeks. Gradually wean off if no rejection • > 10 years 1mg/kg/day from day 2 and then tailor to a maintenance dose of 0.2mg/kg/day. Gradually wean to 0.1mg/kg/day by 1 year of transplant if no rejection then may be discontinued or maintained for life depending on rejection history.

* Used as **an alternative to ATG** in patients at high risk of sepsis or in those with severe renal dysfunction as it selectively blocks the CD25 T cell receptor preventing their activation. It is used in patients bridged with ECMO but not necessarily a VAD.

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RENAL TRANSPLANT PROTOCOL

DRAFT LATEST AS OF 1 Jan 2021

Patient label		
Name		
Pre transplant dry weight (kg)		
Pre transplant height (cm)		
Surface area (m^2)		
Recipient	**CMV**	**EBV**
Donor	**CMV**	**EBV**

File in patients nursing notes

1. **PRE-TRANSPLANT drugs**
Calculate SA from Ht and Wt (√ (htXWt) :3600
☐ Check meds with Consultant
☐ Check with anaesthetist the drugs they need to give in theatre

Prescribe ☑	Given ☑	
Take on arrival on ward:		
		Tinzaparin prophylaxis (LMW heparin) 50 units/kg subcutaneous Post pubertal patients should wear TED stockings.
		If **recipient is female and Rh –ve**, and **donor Rh +ve** • **Anti-D (Rh0) Immunoglobulin** 1250 units intramuscular give as soon as the transplant is given the go ahead • If drug not available, give within 72 hours of transplant.
Antibiotics 0-2 hours before going to theatre: Repeat dose after 4-6 hours (while still in theatre) (If donor died of infection e.g. meningococcemia, need 10 days appropriate iv antibiotic)		
		Amoxicillin 30 mg/kg iv- Check Penicillin allergy status
		Flucloxacillin 30 mg/kg iv
		Ceftazidime 15 mg/kg iv
1 hour before theatre:		
		Basiliximab intravenous infusion < 35kg : 10 mg ≥ 35kg : 20 mg
To take to theatre:		
		Methylprednisolone 600 mg/m^2 (max 500 mg) intravenously **Prescribe for giving at the time of the arterial anastomosis** by anaesthetist. Send drugs to theatre with patient.

NB: Use most recent virology result to guide antiviral prophylaxis. Send repeat samples to the lab for next office day processing. Change treatment accordingly.

Additional medication plan – check on Tx review meeting

POST - TRANSPLANT

1. **POST-TRANSPLANT DRUGS**
   - ☐ All previous medications and drug charts are discontinued post-transplant
   - ☐ Check medication with **both** PICU and Renal Consultant
   - ☐ In PICU, only intensive care doctors **CAN** prescribe fluids or drugs.

<table>
<tr><th>Prescribe ☑</th><th>Given ☑</th><th colspan="2">All patients</th></tr>
<tr><td colspan="4">Regularly:</td></tr>
<tr><td></td><td></td><td colspan="2">Tacrolimus (Adoport)
• Prescribe Adoport (tablet form) for older children and tacrolimus suspension (1mg/ml) for younger children
• 0.15 mg/kg oral (max 5 mg) twice daily 08:00 & 20:00 Start post- transplant.
• Tacrolimus trough levels are done from day one (8am) and daily thereafter.
• Please DO NOT wait for trough level results to come back before administering Tacrolimus - go ahead and give it.

<table><tr><td>Post-transplant</td><td>Tacrolimus level ug/l</td></tr><tr><td>0 to 8 weeks</td><td>8-12</td></tr><tr><td>8 weeks to 1 year</td><td>5-8</td></tr><tr><td>From 1 year</td><td>3-5</td></tr></table>
Taken with food or on an empty stomach but it is important that the patient is consistent with how the drug is take in relation to food.

IV dosing if oral contraindicated: Consultant to make decision to change the route of administration.
• Give 20% of total daily oral dose as IV infusion over 24 hours.
• Do not infuse through central line, causes permanent contamination.
• Use peripheral cannula. Take levels every 24 hours through central line access.</td></tr>
<tr><td></td><td></td><td colspan="2">Basiliximab intravenous infusion once on day 4 post-operative period.
< 35kg : 10 mg
≥ 35kg : 20 mg</td></tr>
<tr><td></td><td></td><td colspan="2">Patients will have individualised plans pre-transplants:Please see under the folder “Transplant” on shared drive</td></tr>
<tr><td></td><td></td><td colspan="2"></td></tr>
<tr><td></td><td></td><td>TWIST</td><td>PAT-B</td></tr>
</table>

<table>
<tr><td></td><td></td><td>Prednisolone (oral) Start morning after transplant. Dose max as per 1m^2

Day 1: 60 mg/m^2 once daily
Day 2: 40 mg/m^2 once daily
Day 3: 30 mg/m^2 once daily
Day 4: 20 mg/m^2 once daily
Day 5: STOP</td><td>Prednisolone (oral) Start morning after transplant. Dose max as per 1 m^2

<table><tr><th>Time</th><th>Dose (mg/m^2)</th></tr><tr><td>Day 0-2</td><td>60 once daily</td></tr><tr><td>Day 3-7</td><td>40 once daily</td></tr><tr><td>Day 8-14</td><td>30 once daily</td></tr><tr><td>Day 15-21</td><td>20 once daily</td></tr><tr><td>Day 22-28</td><td>10 once daily</td></tr><tr><td>Day 29-90</td><td>10 alternate days</td></tr><tr><td>Month 3 onwards</td><td>5 alternate days</td></tr></table></td></tr>
<tr><td></td><td></td><td>TWIST</td><td>PAT-B</td></tr>
<tr><td></td><td></td><td>Mycophenolate mofetil round to nearest tablet or as liquid
Days 1 -14: 600 mg/m^2 bd
Day 15+ : 300 mg/m^2 bd</td><td>Azathioprine 2 mg/kg daily from day 1 onwards</td></tr>
<tr><td colspan="4">Others</td></tr>
<tr><td></td><td></td><td colspan="2">Tinzaparin prophylaxis (LMW heparin):
50 units/kg subcutaneously
Give once daily for 7 days if >30kg and 10 days if <30kg (or longer if clinical situation indicates – annotate prescription accordingly)
If unavailable, can use Enoxaparin twice daily dose as per BNFC.</td></tr>
<tr><td></td><td></td><td colspan="2">Co-trimoxazole 12 mg/kg once every evening oral – 6 months
Round up to nearest tablet or ml (max 480 mg)</td></tr>
<tr><td></td><td></td><td colspan="2">Analgesia
Patients will have pain control assessed by the anaesthetist, with advice from the renal team.
Typically patients return from theatre with opiate PCA or NCA
Regular Paracetamol to be given
Oromorph is an alternative once PCA discontinued</td></tr>
<tr><td></td><td></td><td colspan="2">Antibiotics:
Cefuroxime should be commenced and continued for 10 days if the donor died of meningitis (30mg/kg/dose – frequency adjusted according to GFR, see BNFC)</td></tr>
<tr><td colspan="4">Viral prophylaxis if required Start day after transplant and adjusting to eGFR</td></tr>
</table>

		If ***recipient is CMV*** negative and ***donor is CMV positive*** ***OR*** if **antibody depletion induction** used **Valganciclovir** – 6 months GFR < 25 or on dialysis: 150 mg/m^2 every 48 hours GFR 25-50: 310 mg/m^2 every 48 hours GFR >50: 520mg /m^2 once daily	If ***recipient is EBV*** negative and ***donor is EBV positive*** **Acyclovir 10mg/kg po** (max 400 mg) –3 months On dialysis: Twice a week dose GFR <10 ml/min/1.73 m^2: Once daily GFR 10-25: Three times daily GFR > 25: Four times daily

Additional medication plan:

Heart Transplant Protocol
Great Ormond Street Hospital NHS Trust

Indications	Drug dose and route	Pg
Peri-op immunosuppression (Day 0)	• **basiliximab** Do not give unless the transplant is definitely going ahead. Should only be given in anaesthetic room or theatres. If a dose is given and the transplant is cancelled, there is a high risk of sensitization and anaphylactic response when a dose is given at the subsequent transplant. <35kg: 10mg iv slow bolus over 3-5 minutes >35kg: 20mg iv slow bolus over 3-5 minutes Two doses only First dose within two hours prior to organ implantation and before going on to by-pass. Second dose on day 4 For ABO mismatch transplants, using the column, basiliximab can be given any time in anaesthetic room. For ABO mismatch using immunoadsorption, give basiliximab **after** immunoadsorption. • **methylprednisolone** 15mg/kg iv **15 minutes prior to cross clamp release**	8 8
Antibiotics – Surgical prophylaxis	• **teicoplanin** ○ < 2 months old (give via IV infusion): 16mg/kg for ONE dose, then 24 hours later, give 8mg/kg every 24 hours. ○ ≥ 2 months and < 12 years old: 10mg/kg every 12 hours for 3 doses then 24 hours later 10mg/kg every 24 hours. ○ ≥ 12 years old: 12mg/kg every 12 hours for 3 doses then 24 hours later 12mg/kg every 24 hours • **ceftazidime** 50mg/kg iv (max 2g) q8h until chest drains are out. • On EPIC: for antibiotic indication select Prophylaxis Heart Transplantation • **liposomal amphotericin (Ambisone)** 3mg/kg iv daily if chest is open (test dose required)	9
Post- op immunosuppression	• **mycophenolate mofetil** iv 600mg/m² twice a day. First dose immediately post op. • **methylprednisolone iv** day 1: 10mg/kg iv daily in the morning as linked order to	

	day 2: 2mg/kg iv daily in the morning as linked order to day 3: 2mg/kg iv daily in the morning as linked order to day 4: 1 mg/kg iv daily in the morning and continue day 5: 1mg/kg iv daily or as per transplant team… • **tacrolimus** orally / ng No tacrolimus for the first 24 hours post-op if ○ post-op ECMO ○ renal dysfunction ○ high dose inotropes (ie adrenaline any dose) or ○ poor urine output (<1mL/kg/hour) Discuss starting with the on call Transplant Consultant. Usual start dose 0.05mg/kg (max 2mg initially) orally twice a day. Take trough level in the morning before second or third dose. Target range is 10-15 ng/mL, **Modigraf sachets** can go down the ng tube. GOSH no longer keep silk NGT. Flush the NGT as standard practice. Round dose to multiple of 0.2mg. Modigraf sachets must be dispersed in a glass. If you don't have a glass, please ask the transplant team. **Switch to Prograf capsules when able to swallow**. Dose conversion is 1:1 but sachets and capsules are not bioequivalent, and not interchangeable. Re-check tacrolimus level the following morning.	
Inotrope support	• **adrenaline** iv infusion as per ICU guideline • **milrinone** iv infusion as per ICU guideline	
Pain	• **morphine** infusion as per icu protocol Use NCA / PCA for appropriate children • **paracetamol** 15mg/kg ng/pr 6 hourly regularly, not prn NB: no NSAIDs for transplant patients	
Fluid balance	• **furosemide** iv infusion as per cicu initially then 0.5 - 1mg/kg iv/orally up to four times a day	
Pacing	**If no pacing wires, pacing failure** • **isoprenaline** iv 0.01 – 0.05mcg/kg/min Via central line 1000 mcg/50mL as per ICU guidelines	
Hypertension	• **sodium nitroprusside** iv continuous infusion as per icu guidelines • **nifedipine oral drops** 0.05 - 0.25mg/kg orally/ng (max 5mg initially) tds when necessary for hypertension Caution: nifedipine drops can cause profound rapid hypotension (Can use sustained release tablets crushed and dispersed in water if oral drops not	

	available.) When able to take oral medication • **amlodipine** 0.1-0.2mg/kg (max 0.4mg/kg or 10mg) orally daily If no renal impairment and persistent high blood pressure • **captopril** initially 0.1mg/kg orally tds increasing to 1mg/kg tds as tolerated. (Adult hypertension dose 12.5 – 25mg tds) For older children • **enalapril** initially 0.05 mg/kg bd	
Prevention oral Candidiasis	• **nystatin** drops 1mL orally 6 hourly	
Prevention of aspiration	• **esomeprazole** iv 0.5mg/kg daily >12mths &<20kg: 10mg iv daily >12mths &>20kg: 10-20mg iv daily >12yrs: 20mg iv daily Switch to oral lansoprazole when tolerating oral medicines. See proton pump inhibitor guideline on intranet.	
When tolerating oral medication		
Oral immunosuppression	• **tacrolimus (Prograf or Modigraf) as before per blood levels** 0-6 months: 10 – 14ng/mL. If post op renal dysfunction, first week levels may be 8 to 10ng/mL 6-12 months: 8 – 12ng/mL From 1 year: 5 - 8ng/mL • **For children < 2 years old mycophenolate mofetil** reduce to 300mg/ m^2 orally twice a day when tacrolimus stable in range. (Round doses up/down to nearest whole / half tablet). Oral liquid 1gm/5mL available. • Steroids: **methylprednisolone**: Continue weaning dose after 1mg/kg iv dose, once tacrolimus in range. When able to take oral medicines convert to oral prednisolone (1:1 on mg basis) once daily in the morning. (One to one conversion on mg basis is a 20% steroid wean) If no signs of rejection, aim to wean to 0.1 to 0.15mg/kg daily by discharge or 4 weeks. Wean off by 3 month biopsy if no previous rejection.	

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