The Introduction and Evaluation of Ki-67 as a read-out method of in vitro lymphocyte proliferation and its application in the investigation of immunodeficiency.

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Contents

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Abbreviations

ALPS – Autoimmune Lymphoproliferative Syndrome

BD – Becton Dickinson

BrdU – bromodeoxyuridine

CF – Cystic fibrosis

CFSE - Carboxyfluorescein diacetate succinimidyl ester

CHH – Cartilage-hair hypoplasia

CMI – Cell mediated immunity

CMV – Cytomegalovirus

CPI – Consumer Price Index

CPM – Counts per minute

CV – Coefficient of Variation

CVID – Common Variable Immune Deficiency

DC – Dendritic cells

DMSO – Dimethyl Sulfoxide

DRESS - Drug reaction with Eosinophilia and systemic symptoms

EdU - 5-ethynyl-2-deoxyuridine

ELISPOT – Enzyme Linked Immunospot

EQA – External Quality Assurance

FCS – Foetal Calf Serum

GOSH – Great Ormond Street Hospital

HSCT – Haemopoietic Stem cell transplant

HSV – Herpes simplex virus

HZ – Herpes Zoster

IE63 – Immediate early protein 63

IFN – Interferon

IRF – Interferon regulatory factor

IT – Information technology

JAK – Janus kinase

LRT - Lower Respiratory Tract

LTT – Lymphocyte Transformation Test

MFI – Mean Fluorescence Intensity

MG – Myasthenia Gravis

MHC – Major Histocompatibility Complex

MM – Multiple myeloma

MS – Multiple sclerosis

MSMD – Mendelian Susceptibility to Mycobacterial disease

NFκB – Nuclear factor kappa-light-chain enhancer of activated B cells

NICE – National Institute for Health and Care Excellence

NK – Natural killer cells

NKG – Natural killer group

NTM – Non-tuberculous mycobacterium

ORF – Open reading frame

PBMC – Peripheral Blood Mononuclear Cells

PHA – Phytohaemoglutanin

PID – Primary Immunodeficiency

PPD – Purified protein derivative

PSA – Prostate specific antigen

RBC – Red Blood Cells

RLH – Royal London Hospital

RT – Room Temperature

SCID – Severe Combined Immune Deficiency

SD – Standard Deviation

SI – Stimulation index

SOP – Standard Operating Procedure

STAT – Signal transducer and activator of transcription proteins

^{3}[H] thymidine incorporation – Tritiated thymidine incorporation

TT – Tetanus toxoid

ULBP – UL16 Binding protein

VZV – Varicella Zoster Virus

Abstract

In the United Kingdom, a significant minority of people, have a primary or secondary immune deficiency, leaving them vulnerable to severe, recurrent, persistent or unusual bacterial, viral and fungal infections. As part of investigating these patients, an in vitro lymphocyte proliferation assay is used to measure cell meditated immunity, where patient lymphocytes are stimulated with mitogens and/or antigens in order to measure the functional response of a patients' T cells. Lymphocyte proliferation assays are also used to monitor immune suppressive therapy and assess immune function after bone marrow transplantation. The method commonly used to measure lymphocyte proliferation is tritiated thymidine incorporation (^{3}[H] thymidine incorporation). However, this assay has issues such as a high Coefficient of Variation (CV), the inability to distinguish different lymphocyte populations and the use of radioactivity. Alternative assays for the measurement of lymphocyte proliferation are welcome. The intra-cellular protein, Ki-67, is widely used as a proliferation marker in vitro, mainly within research. It is expressed in proliferating cells but not in G0 resting cells.

A method was developed for utilising Ki-67 to measure lymphocyte proliferation in vitro. It was optimised as a whole blood assay, for use in healthy controls, by designing a suitable antibody panel, demonstrating the ability to detect Ki-67 in proliferating cells but not unstimulated cells and determining the optimal conditions for red blood cell (RBC) lysing. Eighteen healthy controls and 25 patients being investigated for immune deficiency were tested by Ki-67. Inter- and intra-assay variation were found to be less than 20%. Costings and the practicalities of introducing the Ki-67 assay for routine use were also considered. Normal ranges were devised on the healthy controls and an approach using the mean and 3SDs was adopted. Twenty five patients and 8 Healthy Controls (HC) were tested by Ki-67 assay and ^{3}[H] thymidine incorporation and results compared**.** Concordance was good although there were several discrepancies which were further investigated. The use of the Ki-67 assay in the measurement of antigen-specific T cell responses to VZV antigen was also examined. Different methods were tried to identify Ki-67 positive T cells responding to VZV antigen, however, no significant increase in Ki-67 positive T cells could be identified. Stimulation with Covid peptides and Tetanus toxoid did produce a significant increase in Ki-67 positive T cells, demonstrating the Ki-67 method is suitable for identifying antigen-specific T cells. The Ki-67 assay for lymphocyte proliferation is suitable for use as a routine test for the investigation of immune deficiency and the use of the assay could be extended to other areas of the clinical immunology laboratory.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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The Author

I completed my first degree, a BSc (Hons) in Human Genetics with a 2(i) from the University of Nottingham in 1998. After discovering my interest in Immunology during this time, I went on to study an MSc in Immunology and Allergy at the University of Nottingham, completing this in 1999 with a Distinction. During the MSc I completed a research project investigating how T cell subsets regulate the proliferation of other subsets in vitro, using CFSE dye.

After completing the MSc, I worked as a Research Assistant at the University of Cambridge for 5 years, furthering my research and laboratory management skills. During this time, I worked on murine B cells and discovered that Interleukin-4 decreases the expression at protein and RNA level and abolishes the function of inhibitory receptors CD32 and CD22 on activated B cells. This led to me publishing a first author paper, along with my colleagues.

In 2004, I successfully applied to the Clinical Scientist training programme and started my training at Barts and the London NHS Trust, in Immunology. As part of this training, I studied a part-time MSc in Medical Immunology at King's College, completing this with Distinction in 2006. As part of this MSc, I completed a research project introducing a method of measuring T cell proliferation in patients for the investigation of immune deficiency using CFSE. This was subsequently used as a routine method in the laboratory until 2021.

I completed my Clinical Scientist training in 2007, by undertaking a portfolio of work-based competencies and a *viva voce* examination, giving me a certificate of completion from the Association of Clinical Scientists (ACS). This allowed me to become a Health and Care Professions Council (HCPC) registered Clinical Scientist. As part of my role, I work to validate new assays and collaborate with Clinical colleagues to undertake research and audit to improve patient care.

In October 2017 I started the Higher Specialist Scientific Training (HSST) programme for Senior Scientists. This is a 5-year bespoke work-based learning programme, underpinned by the DClinSci. The HSST comprises a PGDip in Leadership and

Management in the Healthcare Sciences, from University of Manchester Business School, completion of FRCPath Examinations Part I and Part II in Immunology, a work-based portfolio of evidence and a research and innovation component. This thesis is submitted as part of this final component. The Innovation proposal has already been assessed (Appendix 10). Further details and evidence of completion of these components can be found in Appendix 6, 7, 8, 9 and 10.

1. Introduction and Literature review

1.1 Introduction

In the United Kingdom, a significant minority of people, have a primary or secondary immune deficiency, leaving them vulnerable to severe, recurrent, persistent or unusual bacterial, viral and fungal infections. The number of people affected by secondary immune deficiency, has increased due to the success of treatments for autoimmune disease and haematological malignancy. As part of investigating these patients, an in vitro lymphocyte proliferation assay is used as a measure of cell meditated immunity, where patient lymphocytes are stimulated with mitogens and antigens in order to measure the functional response of the lymphocytes. One such antigen is varicella zoster virus (VZV). While in healthy individuals, the virus usually remains latent with no clinical symptoms, patients with primary or secondary immune deficiency can have re-activation of latent VZV leading to the development of Herpes Zoster (HZ) (Weinberg, A. and Levin, 2010). This is particularly true of older adults and those with defects in their cellular immunity (Arvin et al., 1980; Groot et al., 2017; Kumagai et al., 1980; Laing et al., 2015; Sadaoka et al., 2008; Weinberg, A. and Levin, 2010). There is currently little knowledge about the normal cellular immune response to VZV and a need for greater understanding. Also, the current readout for immunity to VZV is by measuring the patients' humoral immune response, in the form of anti-VZV IgG antibodies. It has been shown that these antibody levels do not always correlate with protection against viral re-activation, severity of disease and for determining which patients should be treated with anti-viral prophylaxis or who may require vaccination prior to transplantation (Gershon and Steinberg, 1981; Sadaoka et al., 2008; Webster et al., 1989). Therefore, measuring lymphocyte proliferation to VZV antigens may be a better way of assessing the extent of a patient's immunity to VZV. Examining lymphocyte proliferation in patients with severe or recurrent VZV infection, who have apparent normal VZV-specific humoral immunity, may give

further insights into the cellular immune responses in these patients, which are thought to be protective.

Methods for measuring in vitro lymphocyte proliferation vary between clinical immunology laboratories and include tritiated thymidine (^{3}[H] thymidine incorporation), CFSE (5-(and 6-) carboxyfluorescein diacetate succinimidyl ester), bromodeoxyuridine (BrdU) incorporation and 5-ethynyl-2-deoxyuridine (EdU) measurement. These methods have various advantages and disadvantages which will be discussed. This project will investigate the use of Ki-67 as a measurement of in vitro lymphocyte proliferation. Ki-67 is a human nuclear antigen expressed in proliferating cells, but not in G0 resting cells (Endl, Hollmann and Gerdes, 2001; Gerdes et al., 1984). The use of antibodies against this protein have been used, immunohistochemically, to identify proliferation rates in a variety of tumours and as a prognostic marker (Drach et al., 1992; Hammarsten et al., 2019; Qiao, Shiff and Rigas, 1997). It has also been widely used as proliferation marker in vitro, mainly within research, using flow cytometry to measure proliferation responses to both mitogens and antigens (Gerdes et al., 1984; Shedlock et al., 2010; Simonetti et al., 2019; Soares et al., 2010).

1.2 Aims of this Research Project

The aims of this project are:

- to evaluate and introduce Ki-67 as a measurement of lymphocyte function in a clinical immunology laboratory
- to use this method to examine responses to mitogens and antigens, namely VZV
- T cell proliferation to VZV antigen will be examined:
    - in patients with primary and secondary immune deficiency,
    - patients with recurrent or severe VZV
    - before and after HZ vaccination

This would allow greater investigation of cellular responses to VZV in patients who have recurrent or severe VZV infection and identify those patients at risk of viral re-

activation, who may have normal VZV humoral immunity, as measured by current methods. Once established the method could be extended to other antigens.

In this introduction, I will examine the literature related to the methods employed for investigation of lymphocyte proliferation, including Ki-67 and the clinical relevance of the measurement of cellular immunity to VZV.

1.3 Techniques to investigate in vitro lymphocyte proliferation

The assessment of the proliferative potential of T cells can help determine the immune status of an individual patient (Lastovicka, Rataj and Bartunkova, 2016; Whiteside, 2005). In a clinical immunology laboratory, in vitro lymphocyte proliferation testing is used not only when investigating a patient for a potential immune deficiency but also to monitor immune suppressive therapy and assess immune function after bone marrow transplantation (Poujol et al., 2014). Lymphocyte proliferation can be used as a measure of patients' cell mediated immunity (CMI) for instance, when assessing the safety of live vaccination in a patient with immunodeficiency. However, lymphocyte proliferation is a crude measure of CMI and can be normal in some CMI defects.

1.3.1 Tritiated thymidine incorporation

Historically, the main method used to measure the response of lymphocytes to an antigen or mitogen in a clinical setting, is ^{3}[H] thymidine incorporation. This method exploits the incorporation of thymidine into DNA during its synthesis (Lyons and Parish, 1994). Peripheral blood mononuclear cells are cultured, with the ^{3}[H] thymidine added during the final 4-7 hours. Incorporated radiolabel is measured by a liquid scintillation counter and the results reported as counts per minute (CPM) (Shahid, N. Internal SOP, GOSH Immunology). This assay has many limitations. Firstly, it requires the use of radioisotopes, ideally be kept to a minimum within the laboratory environment. Interpretation of the results can be problematic as a low level of ^{3}[H] thymidine uptake could be due to impaired T cell function, low T cell numbers, a poor batch of ^{3}[H] thymidine or technical difficulties. This can in part be

overcome by the use of an appropriate healthy control sample each time the assay is run. As thymidine is incorporated into DNA during the S phase of cellular proliferation, the information gained in this assay, only relates to the S phase during the time in which the ^{3}[H] thymidine was added (Lastovicka, Rataj and Bartunkova, 2016). Therefore, if the majority of the proliferation occurred in the early part of the culture, the overall degree of proliferation may be underestimated. This method is unable to give a proliferation history of individual cell populations, whereas flow cytometric methods of measuring lymphocyte proliferation can differentiate which population of T cells is responding, giving greater detail on the patients' immune response (Lyons and Parish, 1994; Whiteside, 2005). Most clinical immunology laboratories already have a flow cytometer and expertise in running it. By using a flow cytometric method of lymphocyte proliferation, assays could potentially be expanded further based on demand to examine the phenotype of the proliferating cells in more detail. For example, this could include not just the differentiation of CD4 and CD8 T cells but also the measurement of naïve, effector and memory T cells, in response to mitogen and antigen, alongside activation markers or intracellular cytokine production. Examples of this could include the use of CD45RA for naïve T cells, CD45RO for memory T cells and activation markers such as CD69.

1.3.2 Other methods measuring cellular proliferation

Although ^{3}[H] thymidine is the main current method of measuring cell proliferation in the diagnostic setting, flow cytometric methods are becoming increasingly popular. These assays have been widely used in the research setting and include CFSE, BrdU incorporation and EdU.

The BrdU method is based on a similar principle to ^{3}[H] thymidine incorporation, with BrdU competing with thymidine to incorporate into replicating DNA (Böhmer, 1990). The incorporated BrdU is detected by monoclonal antibodies, and the cells visualised by flow cytometry (Gratzner, 1982). However, a major disadvantage of this method, is that the complementary base pairing in double-stranded DNA blocks the access of the anti-BrdU antibody, requiring the cells to be permeabilised and

the DNA denatured, before staining (Salic and Mitchison, 2008). This can create difficulties in excluding dead cells from the analysis (Lyons and Parish, 1994). It is also time consuming and expensive (Lastovicka, Rataj and Bartunkova, 2016). There is a commercial kit available using another thymidine analogue, EdU (Salic and Mitchison, 2008). EdU has a terminal alkyne group in the place of a methyl group in the 5 position and is incorporated into cellular DNA upon replication (Salic and Mitchison, 2008). The reagent used to detect the EdU once incorporated, is not antibody-based and smaller than the antibodies used to detect BrdU, allowing easier access to the DNA without the use of harsh denaturation (Lastovicka, Rataj and Bartunkova, 2016; Salic and Mitchison, 2008). The EdU method has been compared to ^{3}[H] thymidine measurement in a clinical laboratory setting (Paulo, Patel and McDermott, 2015). The authors used phytohaemagglutinin (PHA) as a mitogen and *Candida albicans* as an antigen, to stimulate mononuclear cells from healthy controls. Significant positive correlation between both methods for PHA stimulation were seen. However, two assays out of twelve using the EdU method, when stimulated with *Candida* resulted in technical failure, leading the authors to question the reproducibility of the assay (Paulo, Patel and McDermott, 2015). This evaluation was only carried out on healthy individuals, and further evaluation would be required to ensure that EdU has the sensitivity to identify abnormal lymphocyte proliferation. One major advantage of the EdU method over ^{3}[H] thymidine, is the ability to include staining of cell populations alongside the proliferation marker (Paulo, Patel and McDermott, 2015). As previously discussed here, this could be useful in being able to distinguish which population of cells is proliferating and identifying those in which proliferation may be impaired. One other such method is CFSE.

1.3.3 CFSE

CFSE is a fluorescein with two acetate groups, allowing it to cross the cell membrane. The subsequent removal of these acetate groups by intracellular esterases, decreases the dye permeability ensuring it concentrates within the cell (Quah and Parish, 2012). Its subsequent coupling to intracellular proteins, via its

succinimidyl ester group, changes the structure of the molecule, causing it to fluoresce (Weston and Parish, 1990). When the labelled cell undergoes division, the fluorescein is halved between the two daughter cells arising from the divided cell (Lyons and Parish, 1994). CFSE is excited by the 488nm argon laser, so the labelled cells can be visualised by flow cytometry, allowing the progeny of a proliferating cell population to be followed up to eight rounds of division (Quah and Parish, 2012). In the first reported use of CFSE labelling, a stepwise halving of the CFSE was seen in the stimulated but not in unstimulated cells. Furthermore, the mean fluorescence intensity of the peaks showed an almost exact halving between the daughter cells of successive generations (Lyons and Parish, 1994). To confirm that the halving of the CFSE in the stimulated cells was due to cell division, the authors analysed the same cells using the BrdU/Hoechst fluorescence quenching method, which allows two rounds of replication of a cell to be quantified, and found that the results correlated (Lyons and Parish, 1994). It has been reported that CFSE is toxic to cells at concentrations of greater than 1mM (Last'Ovicka et al., 2009; Lyons and Parish, 1994; Parish et al., 2009; Quah, Warren and Parish, 2007; Whiteside, 2005). From our own experience, we found that CFSE at concentrations of greater than 5mM caused cell death and routinely used a concentration of 0.6mM CFSE, which does not cause cell toxicity (Walker, E. Internal SOP, RLH). However, Quah et al., found that even when using CFSE at a concentration of 40mM, as long as the cells were labelled in culture medium containing 10% foetal calf serum, no significant toxic effect on the cells was observed (Quah and Parish, 2012).

CFSE labelling has been compared with ^{3}[H] thymidine incorporation on several occasions (Angulo and Fulcher, 1998; Faivre et al., 2015; Fulcher and Wong, 1999; Lastovicka, Rataj and Bartunkova, 2016; R. and D.A., 1998). When comparing lymphocyte responses to PHA in healthy controls and patients with T cell defects, similar results were observed in both CFSE and ^{3}[H] thymidine incorporation assays, with T cell deficient patients showing markedly reduced blast formation and proliferation in both assays (D.A. and J., 1999; Fulcher and Wong, 1999). More recently, Faivre et al., demonstrated that when examining the inhibition of

lymphocyte proliferation by ultraviolet radiation, there was one hundred percent concordance between the CFSE and ^{3}[H] thymidine incorporation methods with high sensitivity and specificity (Faivre et al., 2015). A comparison of 18 patients from our own department who had both CFSE and ^{3}[H] thymidine incorporation assays done, showed good concordance between the two assays with 6 patients having different results. When investigated further, the differences could be accounted for by the assays being carried out at different times, sometimes years apart, differences in doses of immunosuppressant drugs and variation in reporting.

CFSE labelling and ^{3}[H] thymidine incorporation assays have also been compared in their ability to measure *Candida*-specific T cell responses in exposed and non-exposed individuals in a clinical laboratory setting (Angulo and Fulcher, 1998). Patients had lymphocyte proliferation measured by ^{3}[H] thymidine and were grouped into responders, all of which had recurrent vaginal candidiasis and a CPM of greater than 20,000 and non-responders, with a CPM of less than 20,000 and no history of *Candida* infections. The authors found that these two sets of patients could also be differentiated by the CFSE method leading them to conclude that T cell responses to antigen can be reliably measured using the CFSE method and were equivalent to results generated from ^{3}[H] thymidine incorporation (Angulo and Fulcher, 1998).

CFSE labelling has been used in various other applications in a research setting, including in vivo animal studies, to track the migration of lymphocytes (Weston and Parish, 1990) and there are many examples in vitro, including the investigation of delayed type hypersensitivity responses (Tsuge et al., 2007; Verhoef et al., 2005). The method has also been used to determine T and B cell division, in combination with intra-cellular staining, as a marker of cell proliferation in primary immunodeficiency (PID) patients (Ma et al., 2005).

In our immunology laboratory at the Royal London Hospital, the CFSE method was introduced as a routine method for measuring in vitro lymphocyte proliferation in 2006 in order to examine the proliferation of T lymphocytes in greater detail than our previous method, ^{3}[H] thymidine.

This changed during the Covid-19 pandemic, where there were fewer patients coming into hospital for face-to-face appointments. This was particularly marked in the cohort of patients for whom this test was indicated, namely those with primary and secondary immunodeficiency, who were advised to shield during the pandemic. In September 2021, the decision was made to stop using CFSE as a routine measure of lymphocyte proliferation, due to low numbers of requests for testing and staffing pressures. The small number of requests which did come in for lymphocyte proliferation testing, were sent to a reference Immunology laboratory for testing using ^{3}[H] thymidine incorporation.

The CFSE assay was carried out on Thursdays and Fridays each week on patients and a healthy control. After three days of stimulation with PHA and anti-CD3/anti-CD28, the cells were harvested and stained for CD3 and CD4 to differentiate the T cell populations, and after flow cytometry, the number of divisions analysed using FlowJo software. Excerpts of the SOP are shown in Appendix 1. However, this method was time consuming and could only be undertaken on the stated days of the week due to its labour-intensive nature and the complexity of the data analysis. Prior to the Covid-19 pandemic, there was an increasing demand for a greater number of proliferation assays to be carried out, including on other days during the week. There has been evidence that this demand is increasing again now that face-to-face appointments are being re-instated. Sending patient samples to the referral laboratory for testing is associated with costly courier fees and senior laboratory staff time in processing these requests and ensuring that they reach the testing laboratory within the required timeframe. This is not always a smooth process and there is also a risk of loss of samples in transit. One benefit of this research project, is that there is the potential to bring a simpler lymphocyte proliferation assay back in house, avoiding the cost and risks of sending these samples away. The repertoire of antigens and mitogens used to assess immune function in the laboratory could potentially expand, thereby creating an opportunity to introduce an assay which is simpler to undertake than the previous CFSE method, but which would still give detailed information on the proliferation of different T cell populations, leading to a widening of our knowledge of cellular responses to mitogens and antigens,

including VZV. The use of Ki-67 for measuring in vitro lymphocyte proliferation could be a way of addressing these needs.

1.4. Ki-67

1.4.1 Introduction to Ki-67

Ki-67 was discovered during the production of monoclonal antibodies to Hodgkin and Reed-Sternberg cells in 1983, in well 67 of a 96 well plate, giving it the name Ki-67 (Gerdes et al., 1983). Further investigation of this clone revealed that it recognised a human nuclear antigen expressed in proliferating cells, namely G1, S, G2 and mitosis but not in G0 resting cells (Endl, Hollmann and Gerdes, 2001; Gerdes et al., 1984). This led to the monoclonal antibody being used in immunohistochemistry to measure tumour proliferation and in vitro as a measure of cell proliferation (Endl, Hollmann and Gerdes, 2001).

For many years very little was known about the exact function of the Ki-67 protein due to a lack of homology with other known proteins (Scholzen and Gerdes, 2000). Schluter et al, sequenced the cDNA of the human protein and found 16 tandemly repeated sequences containing a highly conserved “Ki-67 motif”, found to be the epitope detected by the monoclonal antibody (Schlüter et al., 1993). The same repeating sequences were found in the murine protein (Starborg et al., 1996). When anti-sense oligonucleotides were used, the inhibition of proliferation in the cell line IM-9 was seen, leading to the authors to conclude that Ki-67 was an absolute requirement for the maintenance of cellular proliferation (Schlüter et al., 1993). Ki-67 expression is mainly located in the nucleoli during interphase (Gerdes et al., 1984; Kill, 1996). It is also thought to be required for nucleolar reorganisation during nucleus formation after mitosis (Bridger, Kill and Lichter, 1998). Whilst it is widely accepted that Ki-67 is absent in resting cells, there has been some discussion over the expression of Ki-67 during the first G1 phase of the cell cycle. Gerdes purported that Ki-67 was expressed late in the G1 phase of cell proliferation while Lopez et al, reported that it was absent in cells during the first G1 phase of proliferation (Gerdes et al., 1984; Lopez et al., 1991). Lopez et al., stimulated

lymphocytes and blocked them at various points in the cell cycle using cell cycle inhibitors. They used DNA and RNA staining to determine which part of the cell cycle the cells were in and then examined Ki-67 expression. They reported that those cells which expressed Ki-67 in G1, were those returning to G1 after mitosis (Lopez et al., 1991). They concluded, therefore that Ki-67 is not expressed in the first G1 phase of the cell. Gerdes et al., support this view stating that 'the nuclear antigen detected by Ki-67 is expressed in GI, S, G2, and M phase of continuously cycling cells, but is absent in G0 cells' (Gerdes et al., 1984). It is unclear whether Ki-67 is expressed in cells which leave the cell cycle and become quiescent. Several authors have concluded that Ki-67 expression increases during S phase, with a further increase during G2, with the highest expression being during metaphase, and decreasing during anaphase and telophase (Braun, Papadopoulos and Müller-Hermelink, 1988; Bruno et al., 1991; Starborg et al., 1996). These findings are summarized in Figure 1.1. These studies were performed on both human and murine cell lines. Due to the nature of its expression, Ki-67 has been widely used as a marker for tumour proliferation and prognosis and in vitro proliferation of cells.

Figure 1.1. Ki-67 expression in the cell cycle

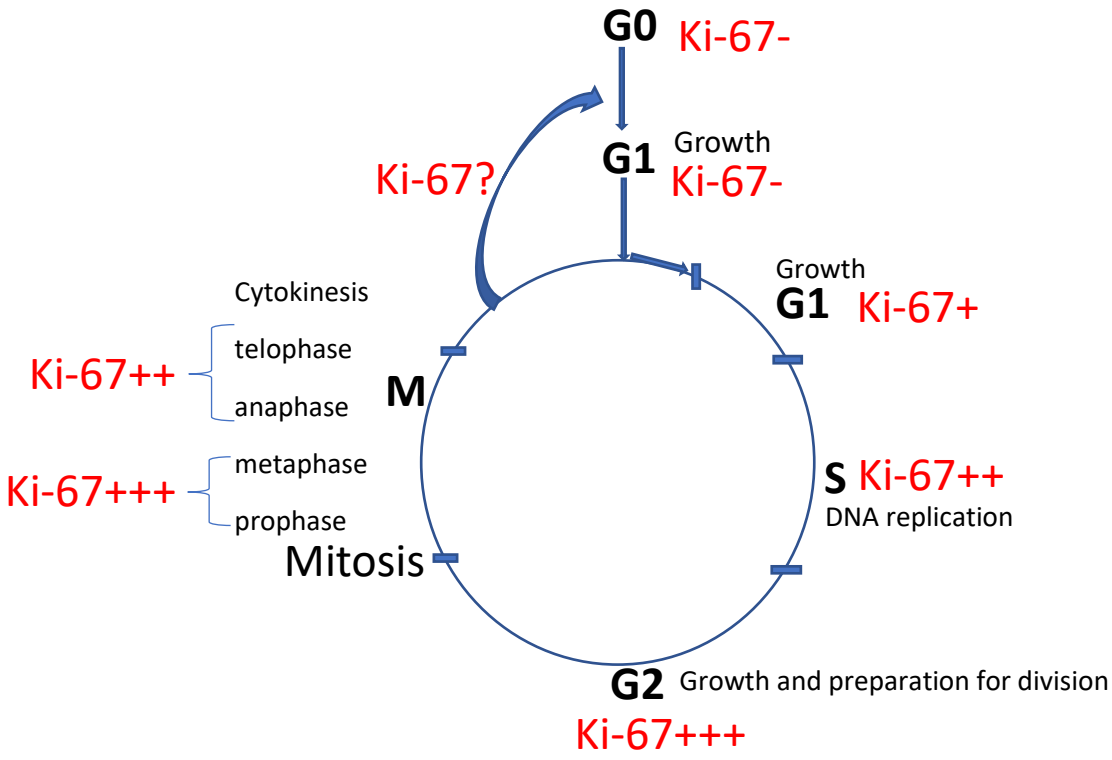


Figure 1.1. Ki-67 expression in the cell cycle. Adapted from Gerdes et al., 1984.

1.4.2 Ki-67 as a proliferation marker in tumours

Clinically, Ki-67 has been widely used during histochemical staining of tumour cells to determine their proliferation rate and rate of death (Qiao, Shiff and Rigas, 1997). A comparison of Ki-67 with BrdU staining of human malignant tumours was carried out by Sasaki et al., showing a linear relationship between the two methods when calculating the percentage of positive cells in patients with a variety of cancers (Sasaki et al., 1988). However, the percentage of Ki-67 positive tumour cells was consistently higher than BrdU positive cells. This led to the proposition that this may be due to the retention of Ki-67 in non-proliferating cells (van Dierendonck et al., 1989). This was supported in a study by Van Oijen et al., in which they arrested tumour cells lines in G/S and G2/M phases using, hydroxyurea, a DNA synthesis inhibitor, and also by transfecting p21 and p53 in these cell lines. They showed that these arrested cells maintained Ki-67 positivity, concluding that this phenomenon

may lead to an over-estimation of the number of proliferating cells when examining tumour proliferation rates (van Oijen et al., 1998).

Ki-67 expression has been used in many tumour types as a marker of disease progression and prognosis, including in soft tissue carcinoma, lymphoma, lung carcinoma and multiple myeloma (MM) ((Martin et al., 2004; Scholzen and Gerdes, 2000; van Oijen et al., 1998). Drach et al., showed that in a study of MM patients, increased Ki-67 staining in CD38 plasma cells in the bone marrow correlated with disease relapse. Also, patients with a high level of Ki-67 expression at diagnosis, had a less favourable disease course with primary resistant disease or early relapse, demonstrating the usefulness of Ki-67 as a prognostic marker in MM (Drach et al., 1992). Additionally in lymphoma, a correlation has been demonstrated between increased Ki-67 expression and increased relapse and inferior two-year event free survival rates in a group of diffuse large B cell lymphoma patients (Yoon et al., 2010). Hammarsten et al., demonstrated that Ki-67 and prostate specific antigen (PSA) levels could be used to subdivide prostate cancer patients. Those with tumours with a low PSA and high Ki-67 level, showed a more advanced tumour stage and a higher risk of prostate cancer death (Hammarsten et al., 2019). The measurement of Ki-67 may be of additional significance in tumours which have treatment protocols relying on the inhibition of proliferating cells. As the expression of Ki-67 is tightly linked to cellular proliferation, the Ki-67 antigen may be a potential target for tumour therapy. Myeloid and epithelial tumour cell lines were treated with antisense oligodeoxyribonucleotides against Ki-67 leading to a specific reduction of the Ki-67 mRNA and protein, inhibition of cellular proliferation and increased apoptosis (Kausch et al., 2003). This was confirmed by the same group in mice. A similar study was undertaken in a human renal carcinoma cell line where the cells treated with antisense mRNA to Ki-67 had decreased Ki-67 protein expression, DNA synthesis and ^{3}[H] thymidine incorporation compared to untreated cells (Zheng et al., 2005).

1.4.3 Ki-67 as a proliferation marker in vitro

Alongside its use clinically in a immunohistochemical capacity, Ki-67 is also widely used as proliferation marker in vitro, mainly within the non-clinical research setting. Gerdes et al., discovered that Ki-67 was upregulated in lymphocytes which had been stimulated with PHA using flow cytometric analysis of the cells (Gerdes et al., 1984).

Ki-67 and CFSE comparison has been performed in peripheral blood mononuclear cells (PBMCs) and Ki-67 shown to give estimates of proliferation comparable to CFSE (Shedlock et al., 2010). Furthermore, cells labelled with CFSE had 19% more cell death than those cells which had not been CFSE labelled on day 5 of culture, consistent with other studies discussed in the previous section of this review. This demonstrates one of the potential advantages of Ki-67 over CFSE. However, this toxicity was mitigated by using the CFSE at a lower concentration and the results remained consistent with Ki-67 proliferation results (Shedlock et al., 2010).

Soares et al., compared Ki-67 in vitro proliferation with BrdU incorporation and Oregon Green, a CFSE derivative (Soares et al., 2010). Whole blood and PBMCs were stimulated with PHA, purified protein derivative (PPD), anti-CD3 and anti-CD28 or left unstimulated and Ki-67 expression measured in CD4+ T cells each day for 6 days of cell culture. Ki-67 expression was found to increase during this time in both whole blood and PBMC culture, compared to cells which had not been stimulated. The frequency of Ki-67 positive CD4 T cells correlated with those from the BrdU and Oregon Green assays. The authors also undertook intra-assay variability studies on the whole blood Ki-67 assay and found the coefficient of variation (CV) to be less than 5% for PHA and PPD stimulated CD4 T cells (Soares et al., 2010). These results indicate that this assay would be a reliable and reproducible assay suitable for introduction into a routine clinical laboratory.

It was demonstrated that Ki-67 could be used in the investigation of lower frequency antigen specific T cell responses, by examining Ki-67 expression in the T cells of infants before and after vaccination with tetanus toxoid (TT). The assay was specific and sensitive enough to identify rare, antigen-specific T cells post-vaccination and it was proposed to be a more sensitive marker for detecting rare T

cell responses than BrdU due to the expression of Ki-67 in all active phases of the cell cycle compared to BrdU only measuring cells in S phase (Soares et al., 2010). Lastovicka et al, when comparing Ki-67 expression with CFSE and ^{3}[H] thymidine incorporation, in human PBMCs, proposed that Ki-67 is the most sensitive assay when examining samples from severely immunocompromised patients, followed by CFSE and then ^{3}[H] thymidine incorporation (Lastovicka, Rataj and Bartunkova, 2016).

Ki-67 has also been used in mice to examine the migration of T cells in mice. Simonetti et al., used Ki-67 alongside DNA staining to track naïve antigen-specific CD8 T cells in mice, in spleen, lymph nodes and peripheral blood, after vaccination with an inactivated adenoviral vector vaccine (Simonetti et al., 2019; Simonetti et al., 2021). They used increased DNA content in an expanding CD8 T cell population, as a marker of proliferating antigen-responsive T cells, concluding that the percentage of specific T cells in S-G_2/M phase in peripheral blood, after priming, were always less than 5%, and three-fold and eleven-fold lower than those in spleen and lymph nodes, respectively (Simonetti et al., 2019).

This literature review has highlighted some issues directly pertinent to my project. Firstly, that Ki-67 as a measure of in vitro proliferation, can be used in whole blood assays, giving CVs which are acceptable for a method in a clinical laboratory setting (Soares et al., 2010). This group, and others, also demonstrate that this method is suitable for detecting lower frequency T cell numbers, such as antigen-specific T cells, post-vaccination. In this project, T cell responses to VZV antigens will be measured. Secondly, it has been demonstrated that Ki-67 is a sensitive assay for determining lymphocyte proliferation in immunocompromised patients, which is relevant to this project as the main patient group will have primary or secondary immunodeficiency (Lastovicka, Rataj and Bartunkova, 2016). These authors also highlight other issues in carrying out proliferation assays which are pertinent to this project, including that the Ki-67 method can be performed on whole blood, which is an advantage over the previous CFSE method, making it a less time-consuming and an easier assay to set up. It also has the advantage over ^{3}[H] thymidine incorporation in that no special extra equipment is required, as a flow cytometer is

a common piece of equipment in a clinical laboratory, and no extra health and safety requirements are needed over radiation and disposal. However, one disadvantage may be that Ki-67 is retained within non-proliferating tumour cells (van Dierendonck et al., 1989; van Oijen et al., 1998). This potential problem would be overcome in this project, as unstimulated cells from the same patient and control samples would always be run alongside stimulated cells, in order to take any background staining into account. Lastly, Lastovicka et al., evaluated the cost of the Ki-67 assay compared to CFSE and BrDU, and concluded that CFSE is the most economical. However, they assumed that only anti-CD3 antibody was used to stain the cells when analysing the results (Lastovicka, Rataj and Bartunkova, 2016). This is not reflected in the CFSE assay which was used in our laboratory, as anti-CD3, anti-CD4 and anti-CD8 antibodies were used, followed by a significant amount of time by the operator in interpreting the results. Therefore, I would conclude that this would not be the case in our laboratory but would investigate this as part of this project, alongside, the cost of sending samples to a reference lab for testing.

1.5 Clinical impact of Research Project

One of the aims of this project is to investigate cellular responses to VZV antigens using the Ki-67 method of in vitro lymphocyte proliferation.

VZV causes chicken pox, a common childhood illness. After primary infection, the virus becomes latent in neurons of cranial nerve and dorsal root ganglia without causing clinical symptoms in immune competent individuals (Weinberg, A. and Levin, 2010). However, the risk of reactivation, leading to Herpes zoster (HZ) or shingles, increases with age, from 0.3% in adults at the age of fifty to 2% in those over 75 (Qi et al., 2016; Weinberg, A. and Levin, 2010). This is thought to be due to the natural reduction in thymic output associated with ageing and the gradual loss of VZV specific memory T cells (M. Levin et al., 2003). Defects in cellular immunity and pregnancy are risk factors for developing severe disease (Duncan and Hambleton, 2015). Furthermore, older adults experience higher morbidity of HZ, notably the occurrence, duration and severity of associated chronic post-herpatic neuralgia (Sadaoka et al., 2008; Weinberg et al., 2017). This can significantly impact

their quality of life. Furthermore, immune deficient patients, can develop overwhelming varicella, leading to hepatitis, pneumonitis and multi-organ failure.

The physiology of VZV re-activation is not well understood, however it is widely recognised that immune surveillance and cell mediated immunity are essential in avoiding viral re-activation (Arvin, 2008; Groot et al., 2017; Hata et al., 2002; Weinberg et al., 2017). The immune response to natural VZV infection begins with the innate immune response by activation of Natural Killer (NK) cells, leading to the lysis of infected cells and the production of anti-viral cytokines, such as IFN-γ, which in turn activates antigen specific T cells (Arvin, 2008). The adaptive arm of the immune system, consisting of humoral and cellular immunity, is responsible for clearing viral infection. Virus specific antibodies capture and neutralise virus particles, however, once the virus has infected its target cell, the cellular arm of the immune response is required. Anti-viral immune responses are thought to be Th1 type immune responses and viral peptides are processed by professional antigen presenting cells and presented as peptide complexes with MHC Class II to CD4 T cells, leading to further T cell activation, cytokine production and amplification of the immune response. Viral peptides are also presented on the surface of an infected cell in MHC Class I-peptide complexes which activate CD8 T cells, releasing toxic granules directly onto the infected cell, leading to apoptosis (Sietske Erosendahl et al., 2014). A small proportion of these T cells go on to become memory T cells, thought to be required to avoid viral reactivation.

VZV, like other viruses, utilises a number of ways to evade detection by the innate and adaptive immune response and to establish latency. These include expressing a limited number of genes during latency, inhibiting programmed cell death pathways of infected cells, interfering with antigen presentation and modulating the immune response to its advantage (Arvin et al., 2022). During primary infection, it is thought that VZV infects T cells within the tonsils, leading to the expression of homing markers allowing their migration to the skin to cause the distinctive lesions identified in chicken pox (Abendroth, Arvin and Moffat, 2010; Arvin et al., 2022). Mucosal dendritic cells (DC) enable the virus to be transported from the respiratory mucosa to the lymph nodes, assisting with viral dissemination. After primary

infection, the incubation time is around 10-21 days before skin lesions appear. This prolonged incubation time leads to the adaptive immune response to VZV being delayed while the virus remains undetected and is able to establish latency in ganglionic neurons (Abendroth, Arvin and Moffat, 2010).

Interferons (IFN) α, β and γ are a vital part of the host innate immune response in preventing the spread of viruses. Ku et al., demonstrated that IFN-α is downregulated in VZV infected epidermal skin cells, enabling VZV to replicate in the skin (Ku et al., 2002). VZV protein Open reading frame (ORF) 66, has also been shown to block the IFN signalling pathway in T cells after exposure to IFN-γ (Schaap et al., 2005). In addition, patients with defects in IFN signalling or production, such as IFN-γ receptor defects, Signal transducer and activator of transcription protein (STAT)-1, TYK2 and NEMO deficiency, have increased susceptibility to VZV, demonstrating the importance of this part of the innate immune response in controlling VZV (Gerada et al., 2020). Also, VZV-infected plasmacytoid DCs have been shown to have decreased ability to produce IFN-α after stimulation through the Toll like receptor (TLR) 9 (Huch et al., 2010).

VZV also interferes with host antigen presentation by downregulating MHC Class I transport from the Golgi to the cell surface in infected cells (Abendroth et al., 2001). This is thought to involve ORF66, demonstrating that this protein may be responsible for several immune evasion strategies by VZV. The downregulation of MHC Class I, enables infected cells to avoid killing by CD8 T cells, but the lack of MHC Class I makes them susceptible to targeting by NK cells. However, VZV has been shown to downregulate UL16 binding protein (ULBP)2 and ULBP3, two of the ligands detected by the NK cell activating receptor, Natural Killer group (NKG) 2D, enabling infected cells to avoid detection by NK cells (Gerada et al., 2020). VZV has also been shown to inhibit IFN-γ induced upregulation of MHC Class II, demonstrating another strategy to avoid immune detection, by CD4 T cells, in this case (Abendroth, Arvin and Moffat, 2010). VZV has been shown to downregulate MHC Class I and II expression on monocyte derived DCs, as well as, co-stimulatory molecules CD86, CD83 and CD80, and reduce the capacity of DCs to stimulate T cell proliferation, thereby interfering with the function of these cells, which are central

to effective immune responses (Morrow et al., 2003). VZV also affects the function of vital cellular transcription factors, involved in the regulation of a host of immune genes, such as STAT-3, Interferon Regulatory factor (IRF)-1 and Nuclear Factor kappa-light-chain enhancer of activated B cells (NFκB), in order to establish latency (Abendroth, Arvin and Moffat, 2010; Arvin et al., 2022). Jones and Arvin demonstrated by microarray analysis, that many NFκB regulated genes were downregulated upon infection with VZV (Jones and Arvin, 2005). This demonstrates interference in a transcription factor which has a wide reaching role in both the innate and adaptive immune response, which would potentially have great advantage for viral survival and the establishment of latency. Finally, VZV gene expression if highly restricted during latency, leading to the successful establishment of latency and avoidance of detection by the immune system (Azarkh, Gilden and Cohrs, 2010). These different mechanisms allow VZV to evade immune detection during primary infection and latency.

1.5.1 Reactivation of VZV

Patients with immunodeficiency have an increased risk of VZV reactivation and increased severity of disease (Groot et al., 2017). Particularly those with cellular immune defects such as HIV, transplant patients and those on immunosuppressive treatments for other conditions (Arvin et al., 1980; Groot et al., 2017; Kumagai et al., 1980; Laing et al., 2015; Sadaoka et al., 2008; Weinberg, A. and Levin, 2010). In the healthy population, it is thought that sub-clinical reactivation of latent VZV may occur, leading to a natural boosting of the immune response and the maintenance of viral latency (Weinberg, A. and Levin, 2010). Some of the first evidence for the importance of the cellular immune response in viral disease came from Good and Zak, who noted that children with agammaglobulinaemia, did not have a second episode of VZV, despite their inability to produce VZV-specific antibodies (Good and Zak, 1956). Ljungman et al., examined VZV re-activation rates in various groups of immunocompromised patients, including bone marrow transplant (BMT) patients and acute leukaemia patients on therapy. Sub-clinical reactivation was also investigated and categorised as those patients with a fivefold increase in titre of

anti-VZV IgG antibody or occurrence of specific IgM antibody without clinical signs of varicella or HZ. In BMT patients, an increase in the lymphocyte proliferation response to VZV antigens, as measured by ^{3}[H] thymidine incorporation, in the absence of clinical symptoms, was taken as an indicator of sub-clinical reactivation. Rates of both clinical and sub-clinical re-activation of HZ were found to be highest in the most severely immunocompromised, namely the BMT patients, consistent with the importance of cellular immunity in avoiding viral re-activation (Ljungman et al., 1986).

Severe and recurrent VZV infection is associated with primary immune deficiencies with defects in cellular immunity, including Combined Immune Deficiency (CID), CID with syndromic features, such as DOCK8 deficiency, Autoimmune Lymphoproliferative Syndrome (ALPS). It is also associated with defects in innate immunity, including Mendelian Susceptibility to Mycobacterial disease (MSMD) and NEMO deficiency (Duncan and Hambleton, 2015). In the PID clinic at the Royal London Hospital a significant minority of patients have recurrent or severe HZ infection. There are also a number of patients who have undergone HSCT for haematological malignancy, at risk of developing HZ or systemic infection. Further investigation of the cellular immune response to VZV would be welcome in these patients.

1.5.2 Measurement of VZV immunity

Currently, the main method of assessing immunity to VZV antigens in a routine clinical setting, is the measurement of anti-VZV IgG and IgM antibodies. The presence of IgG antibodies is associated with protection, in immunocompetent patients and IgM antibodies suggest acute infection. However, as this is a measurement of humoral immunity, this may not be the most effective way of measuring immunity to VZV. Gershon and Steinberg examined anti-VZV antibody levels in patients ranging from 1-85 years old to examine whether anti-VZV antibodies decreased with increasing age and whether this correlated with an increased risk of VZV re-activation (Gershon and Steinberg, 1981). They found no decrease in VZV antibodies with increasing age and also no significant difference

between VZV-specific antibody levels in healthy people verses immunocompromised patients at different ages. Furthermore, there was no significant difference in those patients with disseminated HZ and localised disease, (Gershon and Steinberg, 1981). Although this was looking at total VZV antibodies, these findings suggest that measurement of antibody levels may not be the most effective way of measuring immunity to VZV infection and in assessing the risk of re-activation or severity of disease. Webster et al., examined pre-transplant levels of anti-VZV IgG antibodies in BMT recipients and found no correlation between these antibody levels and the risk of VZV reactivation post-transplant (Webster et al., 1989). Similarly, when examining anti-VZV IgG antibodies in the bone marrow donors, the levels did not correlate with VZV re-activation, leading the authors to conclude that VZV antibody measurement was not useful in determining which patients should be treated with anti-viral prophylaxis, post-transplant (Webster et al., 1989). The role of VZV specific antibodies has been demonstrated to facilitate viral neutralisation and antibody dependant cellular cytotoxicity upon infection, whereas VZV specific T cells are thought to be essential for preventing viral re-activation and disease after infection (Arvin, 2008). When considering the most appropriate measure of immunity to VZV, Sadoaka et al, compared anti-VZV T cells responses by VZV skin test and IFN-γ Enzyme linked Immunospot (ELISPOT)in several different age groups of VZV sero-positive subjects. They also measured anti-VZV IgG antibody levels (Sadaoka et al., 2008). Comparable antibody titres were detected across all the age groups in the study. In contrast, both cellular assays showed a decreased response with increasing age that correlated between the two assays. These findings were in agreement with other reports that cellular immunity to VZV declines with increasing age (Sadaoka et al., 2008).

Several authors have also measured T cell responses to VZV antigens in the context of post-vaccination with the VZV vaccine.

1.5.3 VZV vaccination studies

Prior to 2021, there was a single, live attenuated HZ vaccine available in the UK, given once to those between seventy and seventy-nine (NHS England, 2018).

Individuals who are within this age group but have a contra-indication to receiving live vaccines, are now offered a recombinant vaccine, containing VZV glycoprotein E antigen (UK Health Security Agency, 2021). Due to limited supply, only those patients deemed extremely immunocompromised are eligible to receive this vaccine, such as those with HIV/AIDS with a CD4 T cell count below two hundred, those who have undergone a stem cell transplant in the previous twenty four months and those undergoing prolonged immunosuppression or immunomodulation (UK Health Security Agency, 2021). The papers referenced in this section, relate to the live attenuated vaccine.

Despite the role of cellular immunity in inhibiting viral re-activation, the measurement of anti-VZV IgG antibodies remains the current routine method of measuring whether immunity to the virus has been achieved. Pre- and post-vaccination VZV specific antibody levels were examined in a group of subjects between the ages of 50-59 and a significant increase in post-vaccination VZV-specific antibody titres was found compared to those who were vaccinated with a placebo (Levin et al., 2013). However, they were unable to determine an antibody level which would serve as a surrogate marker of protection from HZ, as VZV antibody titres measured in the placebo recipients who did not develop HZ were lower than those achieved by ZV recipients who did develop HZ. In order to find a surrogate marker for vaccine efficacy, VZV-specific T cell responses in vaccinated older individuals were compared to young adults, who have a much lower risk of developing HZ (Patterson-Bartlett et al., 2007). Elderly subjects had a greater number of VZV-specific CD4 T cells than younger adults, but when examining the frequency of IL-4 and IFN-γ producing cells, these were lower in the older subjects, suggesting that the function, rather than the number of VZV-specific T cells may be defective in older subjects. It was speculated that this increase in number may be an attempt to compensate for a loss of function of these cells. Laing et al., established that vaccination with the live attenuated HZ vaccine increases the breadth of T cell responses to the virus, by examining T cells for reactivity to different VZV proteins, confirming the importance of the cellular response after vaccination (Laing et al., 2014; Laing et al., 2015).

The cellular T cell responses to HZ vaccination in severely immunocompromised individuals, have also been investigated, namely patients receiving a haemopoietic stem cell transplant (HSCT) for the treatment of lymphoma. In vitro lymphocyte proliferation responses to VZV antigen were examined using ^{3}[H] thymidine incorporation at various time-points pre- and post-transplant in patients with or without vaccination with a heat-inactivated live-attenuated HZ vaccine (Hata et al., 2002). Patient were vaccinated pre-transplant and then boosted at several timepoints afterwards. CD4 T cell proliferation to viral antigens appeared earlier in samples taken from patients who had been vaccinated than those who had not. Furthermore, those patients who had been vaccinated had a more robust response seen as higher stimulation indices than those who had not been vaccinated (Figure 1.2).

Figure 1.2. CD4 T cell proliferation to Varicella-Zoster virus antigen among recipients of Haemopoetic stem cell transplants

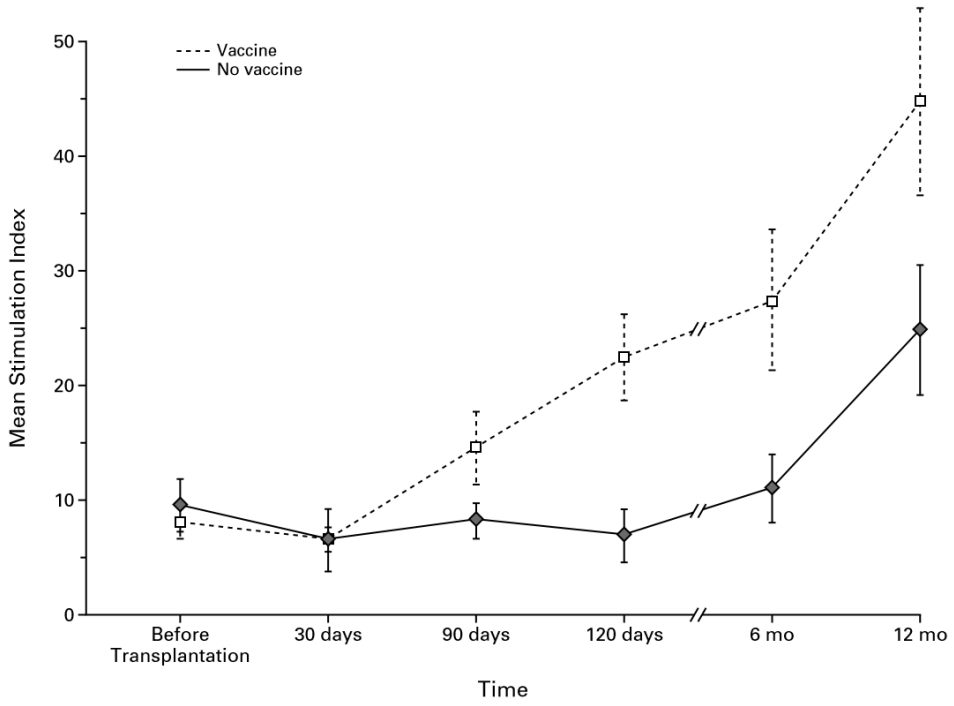


Figure 1.2 CD4 T cell proliferation in response to varicella zoster virus antigen among recipients of hemopoietic stem cells transplants who were randomly assigned to receive inactivated varicella vaccine or no vaccine. In vitro T cell proliferation in response to varicella zoster virus antigen in shown as the mean stimulation index in relation to the time of haemopoietic stem cell transplantation. Taken from Hata et al., 2002.

By examining patients with varying stimulation indices, the authors concluded that having a stimulation index above 5 was highly predictive of protection from HZ and could be used as an immunological correlate with protection (Hata et al., 2002).

Quantification of cellular responses to VZV were investigated by Haredy et al, using a large-volume whole blood method to overcome the difficulties in detecting the low numbers of antigen-specific T cells (Haredy et al., 2019). These cells were identified as $CD3^{+}CD4^{high}$ T cells with CD45RO and granzyme B positivity, that were only found in cell cultures stimulated with VZV antigen but not the mock-stimulated cultures. These cells were detectable in children with a history of varicella infection and VZV IgG antibodies and in young adults (aged 16-45), with a mean of 16% and 28% respectively (Haredy et al., 2019). Children with no history of varicella infection and negative VZV IgG antibodies, showed very low numbers of these cells of less than 1%. A child with acute varicella was found to have a similarly low number of $CD3^{+}CD4^{high}$ T cells, demonstrating the correlation of these cells with the clinical response to varicella. Older subjects (>50 years old) had lower numbers compared to young adults, which is in keeping with other studies of cell mediated immunity in older individuals (Duncan and Hambleton, 2015; M. Levin et al., 2003; M. J. Levin et al., 2003; Sadaoka et al., 2008).

Cutaneous immunity to VZV is thought to be an important factor in the response to HZ and a weak skin response to VZV antigen is associated with post herpatic neuralgia (Imoto et al., 2016). Patel et al., investigated how vaccination against HZ altered the number of skin-resident antigen-specific T cells (Patel et al., 2018). The response to VZV antigen challenge after intradermal VZV vaccination was assessed, in volunteers, older than 70 years of age, by examining the numbers of VZV-specific T cells in peripheral blood and skin biopsies. Peripheral blood antigen-specific CD4 T cells were increased post-vaccination, in addition to an increased T cell accumulation and proliferation at the antigen-challenge site. However, the numbers of skin-resident memory T cells were not affected. The authors concluded that vaccination leads to the recruitment of VZV-specific T cells from the blood which are subsequently activated and proliferate in the skin (Patel et al., 2018). This

led to improved cutaneous VZV-specific immunity in these vaccinated older individuals. This paper is also relevant to my project due the fact the Ki-67 was used to identify proliferating T cells in skin biopsies at the site of VZV antigen challenge.

When considering the significance to this project of the studies discussed above, it is evident that HZ is a considerable health burden, particularly in the elderly and those with impaired immunity, leading to a reduction in patient quality of life and potentially serious complications. The measurement of VZV IgG and IgM antibodies may not be the most effective way of measuring protection against HZ in particular in immunodeficient patients, as several authors have demonstrated that antibody levels do not correlate with a decreased risk of developing HZ, in older adults and in immunocompromised patients (Gershon and Steinberg, 1981; Sadaoka et al., 2008; Webster et al., 1989). Cellular immunity to VZV has been measured by ELISPOT, skin test and ^{3}[H] thymidine incorporation by various groups, particularly when examining the response to vaccination with the live attenuated HZ vaccine (Hata et al., 2002; Laing et al., 2014; Patterson-Bartlett et al., 2007). As the cellular immune responses are widely thought to be vital in preventing VZV re-activation, having a reliable measure of anti-VZV T cell function, which could help stratify patients who are at risk of developing HZ would be a useful tool in managing immunocompromised patients. Developing the Ki-67 in vitro proliferation assay as a routine test would help identify those immunodeficient patients who retain VZV-specific cellular immunological memory, allowing them to potentially avoid post exposure or long-term prophylaxis. It would also allow identification of those patients who have apparent VZV-specific humoral immunity but are at risk of developing clinical HZ as a result of a defect in their cellular immunity. This would enable greater insight into other patient groups which have in increased risk of HZ, including post-stem cell transplant recipients and patients on biological agents to treat autoimmune, rheumatological or neurological diseases. Prior to the advent of the recombinant VZV vaccine, another potential advantage of this method could have been in determining whether older transplant patients should be vaccinated prior to transplant. Those patients who had poor or absent responses to VZV antigen could avoid the live vaccine, due to the danger of systemic disease,

whereas those who demonstrated a response may benefit from a booster, and those with strong responses may not require boosting. The clinical advantages of having this information would have been of benefit to the patient and the wider NHS.

1.6 Conclusion

In conclusion, the measurement of in vitro lymphocyte proliferation is used in clinical immunology laboratories for assessing the cellular immune function of patients being investigated for primary or secondary immune deficiency. From the literature, and experience, recurrent and severe VZV infection can affect patients with primary and secondary immune deficiency and can severely impact their quality of life (Duncan and Hambleton, 2015; Weinberg et al., 2017). Current methods for measuring immunity to VZV, by measuring anti-VZV IgG antibodies, do not always correlate with disease severity or the risk of viral re-activation and measurement of the cellular immune response may be more appropriate (Gershon and Steinberg, 1981; Sadaoka et al., 2008; Webster et al., 1989).

Current methods for measuring in vitro lymphocyte proliferation have some disadvantages, notably a lack of standardisation. Flow cytometric methods of assessing lymphocyte proliferation not only negate the need for radioactivity but also can differentiate which T cell population is responding to a given mitogen or antigen, potentially giving greater detail on the patients' immune response (Lyons and Parish, 1994). However, there is a need to balance complexity of the assay with the demand for testing, time spent on set up and analysis of results, and cost, while maintaining a high quality service. The introduction of the Ki-67 assay may be a way of achieving this. This technique has been used widely in research for the assessment of in vitro lymphocyte proliferation and has shown excellent comparability to other methods of lymphocyte proliferation (Lastovicka, Rataj and Bartunkova, 2016; Shedlock et al., 2010; Soares et al., 2010). These same authors also demonstrate the suitability of the assay for routine use, demonstrating the use of whole blood and examining immunocompromised patients.

This project aims to introduce Ki-67 as a new method for measuring lymphocyte proliferation into a clinical immunology laboratory, and to utilise this to investigate the cellular immune response to VZV antigens in patients with primary and secondary immune deficiency, in healthy controls and in vaccine responses. This would widen current knowledge of cellular responses to VZV both in healthy and patient groups with potential defects in their cellular immunity.

2. Materials and methods

2.1 BD FACSLyric Flow cytometer

At the time of testing, the BD FACSLyric (Becton Dickinson Biosciences, UK) was introduced into the laboratory. Fluorochromes were added to the flow cytometer configuration. BD CompBeads (Becton Dickinson Biosciences, UK) were used to compensate the surface antibody fluorochromes. Whole blood was stimulated with PHA for 3 days at 37°C, 5% CO_2. After this time, the cells were harvested, fixed and permeabilised as per Section 2.8, and some left unstained and the remainder stained with 5μl anti-Ki67 Alexa Fluor 647. These were put into separate tubes and compensations calculated using these positive and negative populations.

All antibodies for surface and intra-cellular staining were from BD Biosciences, UK. See Table 3.1 for a list of fluorochromes and clones.

2.2 Subjects

Healthy controls were recruited from the Immunology department of the Royal London Hospital. Patients with primary and secondary immune deficiency were recruited from the PID clinic at the Royal London Hospital. Spare samples from healthy control and patients were taken from the Immunology laboratory at Great Ormond Street Hospital (GOSH) for method comparison. This work is covered by Ethics REC 11/LO/1689.

2.3 Whole blood proliferation assay

Lithium heparin blood was taken from healthy volunteers and patients and diluted 1 in 10 (100μl blood) in complete media (RPMI + 10% Foetal calf serum (FCS) (Life Science Technologies, UK) in 1ml wells of a sterile 24-well plate (RL Slaughter, UK). Cells were either left unstimulated or stimulated with PHA (Life Technologies, UK)

at 10 μg/ml or anti-CD3, clone OKT3 (Thermofisher Scientific, UK) and anti-CD28, clone CLB-CD28/1, 15E8 (MAST Group Ltd, UK) antibodies at 15 μg/ml and 80 μg/ml respectively. Cells were incubated at 37°C, 5% CO_2 for 4 days. After 4 days, cells were harvested by agitating with a 1ml pipette and then harvesting into a 15ml Falcon tube (RL Slaughter, UK). This was followed by adding 1ml PBS + 10% FCS to each well and agitating again, before harvesting.

2.4 Red blood cell lysis

After cell culture of whole blood, BD Pharmlyse (Becton Dickinson, UK) was used to lyse RBCs before cells culture, after cell culture at harvesting, and after antibody staining. 10ml of 1x BD Pharmlyse was used per 1ml whole blood, as per manufacturer's instructions and incubated for 15 minutes, inverting five times before incubation and once, halfway through. Cells were washed twice in PBS + 10% FCS, by centrifuging at 580g for 7 minutes before antibody staining, see Sections 2.7 and 2.8.

2.5 Peripheral blood mononuclear cell (PBMC) preparation and culture

Lithium heparin blood was taken from healthy volunteers, diluted 1 in 2 in RPMI in 50ml sterile Falcon tubes (RL Slaughter, UK) and layered over Lymphoprep (Stemcell Technologies, UK). Tubes were centrifuged at 800g for 20 minutes, with a brake and acceleration rate of 0 using a Falcon 6/300 Centrifuge. After centrifugation, the mononuclear cell layer was removed and washed twice, in RPMI, by centrifuging at 800g for 10 minutes, brake and acceleration rate 9, before counting the cells and re-suspending them at 1 x 10^6/ml. Cells were re-suspended in culture medium containing either 10% FCS or 10% AB normal human serum (Life Technologies, UK) and incubated at 37°C, 5% CO_2.

2.6 Peptide stimulations

2.6.1 VZV peptide stimulations

Whole blood or PBMCs at 1 x 10^6/ml were left unstimulated or stimulated with PHA, anti-CD3 and anti-CD28, as described in Section 2.3, or VZV peptide (PepMix™ IE63) (Cambridge Biosciences, UK) at a final concentration of 1μg/ml, or DMSO (Life Technologies, UK), diluted to the same volume, according to the manufacturer's instructions. PepMix™ IE63 was made up in 50μl DMSO (Life Technologies, UK) before further dilution to a final concentration of 1μg/ml, as per manufacturer's instructions. PepMix™ IE63 is a mix of 67 overlapping peptides.

Cells were incubated at 37°C, 5% CO_2 for varying timepoints before harvesting and staining. Cells were harvested by agitation and harvesting using a pipette. PBS was added to the well and the plate incubated for 30 minutes to allow any adherent cells to de-adhere, before agitating using a pipette and harvesting into a 15ml Falcon tube. Cells were washed once with PBS, by centrifugation at 580g for 7 minutes before surface staining, Section 2.7, followed by intra-cellular staining, Section 2.8.

Healthy controls which had VZV T cell proliferation performed, were tested for VZV IgG antibodies in the Virology department at RLH, with results of >150 mIU/ml. They also had a history of chicken pox infection in childhood and were below the age of 30.

2.6.2 Whole blood bulk 9 day culture

1ml of whole blood from a HC was diluted with 9ml RPMI + 10% FCS for each stimulation condition and decanted in to a sterile 50ml Falcon tube. PHA was added at a final concentration of 10μg/ml, VZV peptide was added at a final concentration of 1μg/ml or DMSO at the same dilution. One tube was left unstimulated. Lids were loosely fitted, and the cells incubated for 9 days at 37°C, 5% CO_2 at a slight incline. After 9 days, cells were harvested by centrifuging the tubes at 580g for 7 minutes and resuspending cells in 1ml PBC + 10% FCS. Red cell lysis was then carried out, as

in Section 2.4, followed by surface staining, Section 2.7 and intra-cellular staining, Section 2.8.

2.6.3 72 hour PBMC cell culture

PBMCs were prepared from HCs as per Section 2.5. Cells were re-suspended at 1 x 10^6/ml in RMPI + 10% FCS and plated into a sterile 24 well plate. Cells were left unstimulated or stimulated with PHA at a final concentration of 10μg/ml or VZV peptide, at a final concentration of 1μg/ml or DMSO at the same dilution. Cells were incubated for 72 hours at 37°C, 5% CO_2. After 72 hours, cells were harvested by agitation and harvesting using a pipette. PBS was added to the well and the plate incubated for 30 minutes to allow any adherent cells to de-adhere, before agitating again using a pipette and harvesting into a 15ml Falcon tube. Cells were washed once, by centrifugation at 580g for 7 minutes, with PBS + 10% FCS before surface staining, see Section 2.7, followed by intra-cellular staining, Section 2.8.

2.6.4 Covid peptide stimulations

PBMCs were prepared from a HC as per Section 2.5 and resuspended in 3600μl RPMI + 400μl normal human serum. 180μl of cells were plated into a sterile 96 well plate in duplicate. Covid peptides Spike (S), Nucleocapsid (N) and Membrane (M) were used (Miltenyi Biotech, UK) in 1:2, 1:4 and 1:8 serial dilutions at final concentrations of 1, 0.5 and 0.25μg/ml. Samples were incubated for 4 days at 37°C, 5% CO_2. Cells were then harvested by agitation and harvesting using a pipette. PBS was added to the well and the plate incubated for 30 minutes to allow any adherent cells to de-adhere, before agitating using a pipette and harvesting into a 15ml Falcon tube. Cells were washed once with PBS, by centrifugation at 580g for 7 minutes, before surface staining, Section 2.7, followed by intra-cellular staining, Section 2.8. Samples were run in parallel at GOSH for Covid T cell proliferation using ^{3}[H] thymidine incorporation. The subject had Covid infection 10 weeks prior to being tested.

2.6.5 Tetanus antigen stimulation

PBMCs were prepared from a HC as per Section 2.5. Cells were re-suspended at 1 x 10^6/ml in RMPI + 10% FCS and plated into a sterile 96 well plate in duplicate. Cells were left unstimulated, or stimulated with PHA at a final concentration of 10μg/ml, or tetanus antigen (Merck, UK) at a final concentration of 10μg/ml. Samples were incubated for 7 days in culture medium RPMI + 10% AB normal human serum at 37^{o}C, 5% CO_2. Cells were then harvested by agitation and harvesting using a pipette. PBS was added to the well and the plate incubated for 30 minutes to allow any adherent cells to de-adhere, before agitating using a pipette and harvesting into a 15ml Falcon tube. Cells were washed once with PBS, by centrifugation at 580g for 7 minutes, before surface staining, Section 2.7, followed by intra-cellular staining, Section 2.8. The HC which had tetanus cell proliferation done, was also tested for tetanus IgG antibodies in the Immunology department at RLH and had a tetanus IgG level of 2.4 IU/ml, which is consistent with long term protection.

2.7 Surface staining of cells

Cells were harvested from wells and wells washed with PBS + 10% FCS. Whole blood was lysed (BD PharmLyse) and washed once with PBS + 10% FCS, as described in Section 2.4. 50μl of Brilliant Stain buffer (Becton Dickinson, UK) was added to each FACs tube before adding antibodies. 50μl of cells were stained with 5μl anti-CD45 BV510, anti-CD3 BV421, anti-CD14 BB700, anti-CD4 PE-Cy7 and anti-CD8 Alexa 700. See Table 3.1 for a list of fluorochromes and clones. Cells were incubated at RT in the dark for 20 minutes before washing with once with PBS + 10% FCS, by centrifugation at 1000g for 45 seconds, using Hettich E280 microfuge. This was followed by intracellular staining, Section 2.8.

2.8 Intracellular staining

Cells were fixed and permeabilised using Intrastain Kit (Dako, UK).

After centrifugation at 1000g for 45 seconds, using Hettich E280 microfuge, cells were incubated with 100μl Reagent A for 15 minutes at RT in the dark. Cells were

then washed once with PBS + 10% FCS by centrifugation at 1000g for 45 seconds, before adding 100µl Reagent B alongside 5µl anti-Ki67 Alexa Fluor 647. Cells were incubated at RT in the dark for 15 minutes before washing once with PBS + 10% FCS by centrifugation at 1000g for 45 seconds. Cells were re-suspended in 300µl PBS + 10% FCS before analysing on FACSLyric flow cytometer (FACSLyric, BD Biosciences, UK) within 8 hours. Analysis of flow cytometry data was done using BD FACSuite (BD Biosciences, UK).

2.9 Comparison of Ki-67 method with ^{3}[H] thymidine incorporation at referral laboratory

Samples from healthy controls and patients who were having lymphocyte proliferation tests done were run both at GOSH for ^{3}[H] thymidine incorporation and using the whole blood Ki-67 method. Samples were prepared and cultured using the method outlined in Sections 2.3 and 2.4, whole blood proliferation assay, and results analysed and compared. PHA at GOSH was used at a concentration of 2µg/ml, 4µg/ml and 8µg/ml. Anti-CD3 was used at 7.5ug/ml and anti-CD28 was taken from a cell line, therefore no exact concentration was available (Mark Davis, personal communication). Results for lymphocyte and T cells Ki-67 positivity were compared with the results of the ^{3}[H] thymidine incorporation to examine correlation between normal, impaired and absent results, between both assays. The results of both assays were also examined alongside the patient's clinical details.

2.10 Statistical analysis

Statistical analysis was undertaken using GraphPad Prism version 7.9.5 for Windows, GraphPad Software, San Diego, USA). A test of skewness was carried out to determine if the results obtained from healthy controls (HC)s was normally distributed. This allowed appropriate reference ranges to be calculated.

2.11 Figures

Figures were made using GraphPad Prism (version 7.9.5 for Windows, GraphPad Software, San Diego, USA) and created using BioRender.com.

2.12 Cost comparison and practicality analysis

The cost of the Ki-67 assay was calculated using the most recent quotes from the manufacturers for the reagents used. Staff hands-on time was measured by timing each section of the method. The staff hourly rate was calculated using the Barts Health Trust Day Rate for the appropriate Band of staff member (Appendix 3). The New Day Rate was used. The cost of the CFSE assay was calculated using a top-down approach and Consumer Price Index (CPI) added to account for increases in cost since the cost was calculated in 2019. This was done using the Bank of England Inflation calculator (Bank of England Inflation calculator, 2023). Cost of the ^{3}[H] thymidine incorporation was the price quoted by the referral laboratory.

3. Developing an assay to measure T cell proliferation using Ki-67

3.1 Introduction

The aim of this project is to develop and evaluate a method for lymphocyte proliferation using Ki-67 as an in vitro read out. Lymphocyte proliferation testing is used by clinicians when investigating patients with primary and secondary immune deficiency, as a way of measuring T cell function. Samples from patients and a HC are stimulated with mitogen such as PHA and directly through the T cell receptor, by using anti-CD3 with or without anti-CD28. Cells are cultured for several days before being harvested and the proliferation of the patient lymphocytes compared to that of the HC. Methods for doing this differ between different clinical laboratories and include, ^{3}[H] thymidine incorporation, CFSE, BrDU and EdU. Patient samples can also be stimulated with antigen, such as TT and Candida, to measure a T cell response, giving an indication of a protective T cell response.

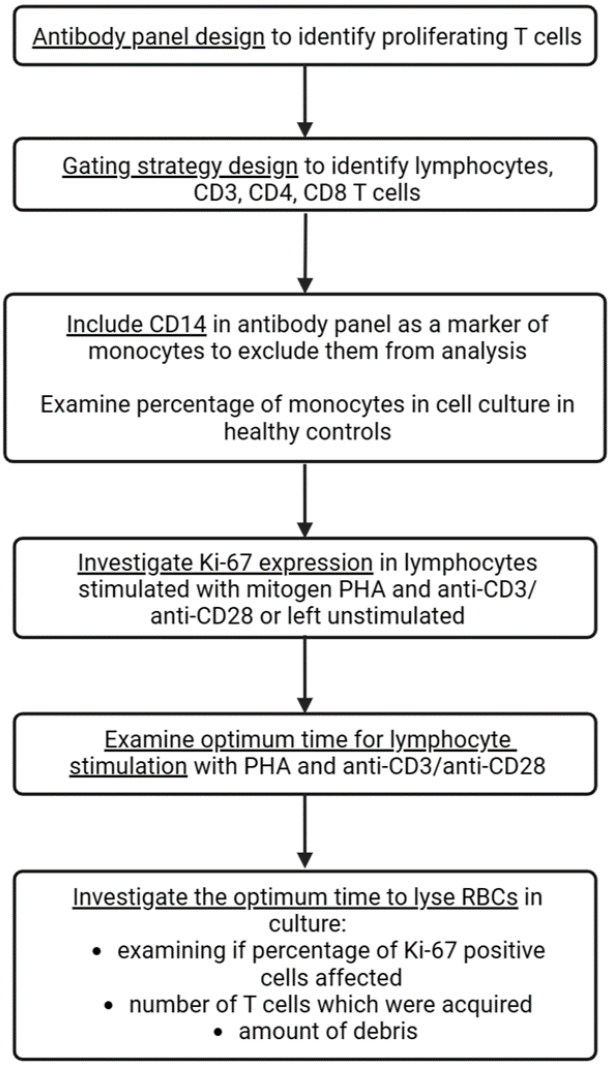
Previously my laboratory had used the CFSE method to examine lymphocyte proliferation. This involved the preparation of PBMCs from whole blood, labelling with CFSE, enumerating the lymphocytes and stimulating them with mitogen or anti-CD3/anti-CD28 for 4 days (Appendix 1). Since the Covid-19 pandemic, CFSE testing has been discontinued and samples sent to a referral laboratory for ^{3}[H] thymidine incorporation. This is described in detail in Section 1.3.3. The introduction of a straightforward in-house method for measuring lymphocyte proliferation would be welcome.

The Ki-67 method had not been used before in the laboratory before and therefore, the method was designed from scratch. This involved designing a panel of antibodies to identify the cell populations of interest. During this time a new flow cytometer was introduced into the laboratory, BD FACSLyric. This is a multi-parameter flow cytometer designed for clinical use and is used routinely in the laboratory. Fourteen parameters can be analysed at one time, including forward,

side scatter and 12 different spectral regions of fluorescence (Hickey, A. Internal SOP, RLH). Some work was required to ensure that the fluorochromes of interest were added to the flow cytometer configuration and that they were correctly compensated. A gating strategy was also designed, based on that used in the CFSE method, and used throughout the project.

One of the aims of creating the Ki-67 method for lymphocyte proliferation was to have a robust but also straightforward method of setting up a proliferation assay, ideally setting up whole blood from patients and HCs rather than preparing PBMCs. This would enable the laboratory to be flexible when setting up the assay and meaning that samples could be accepted at various days and times of the week. The assay was optimised using whole blood rather than PBMCs. This contrasts with the CFSE assay which had been done previously, where PBMC preparation was required. In order to analyse the lymphocytes after whole blood cell culture, a lysing step was added, to lyse RBCs, allowing clearer analysis of lymphocytes. The optimal time for inserting this step was investigated as part of method development.

3.2 Flowchart of parameters investigated in this chapter



3.3 Antibody panel design for surface and intracellular staining, to identify proliferating T cells and incorporating anti-CD14 to remove monocytes

An antibody panel was designed to identify lymphocytes, CD4, CD8 T cells and to assess their proliferative response when stimulated and stained with Ki-67. This was done with assistance from BD Biosciences (Table 3.1). One potential issue with assessing proliferation of T cells is contamination with monocytes. The CFSE assay did not include a marker for monocytes, and it was felt that the addition of a marker to identify and remove monocytes from analysis would be an improvement. CD14, a marker of classical monocytes, was included, to ensure that monocytes

could be excluded from analysis when examining proliferating lymphocytes (Figure 3.1).

Table 3.1. Antibody panel design for the Ki-67 assay for lymphocyte proliferation

Marker/Antibody	Fluorochrome	Clone	Population identified
CD45	BV510	HI30	Lymphocytes
CD3	BV421	SK7	T cells
CD4	PE-Cy7	SK3	Helper T cells
CD8	Alexa Fluor 700	RPA-T8	Cytotoxic T cells
CD14	BB700	MϕP9	Monocytes
Ki-67	Alexa Fluor 647	B56	Proliferating lymphocytes

Table 3.1. Antibody panel design for optimal identification of lymphocytes, CD3 T cells and CD4 and CD8 T cells in unstimulated and stimulated whole blood. CD14 was added to remove monocytes and Ki-67 was used for intra-cellular staining.

3.4 Gating strategy to identify lymphocytes, CD3, CD4 and CD8 T cells

A gating strategy was designed to be similar to that which was used for the CFSE assay, which was being run at the time by the laboratory (Appendix 2). Lymphocytes were identified using forward and side scatter and then by CD45 positivity. Doublets were removed from analysis and CD3, CD4 and CD8 T cells were identified. This gating strategy was amended after initial experiments to include CD14 (Figure 3.1).

The addition of CD14 was seen as an improvement to the strategy, to exclude monocytes which can fall into the gate of proliferated lymphocytes. The highest percentage of monocytes was seen in cultures where the cells remained unstimulated and the percentage of monocytes present, differed between subjects (Figure 3.2). The highest percentage of monocytes seen in the healthy controls analysed was 6% with the lowest being <1%. This gating strategy was kept throughout all cell culture conditions in all subsequent experiments.

Once the cells of interest were identified using this strategy, the ability of the cells to express Ki-67, once proliferated was investigated.

Figure 3.1. A gating strategy designed to identify lymphocytes, CD3, CD4 and CD8 T cells and to exclude monocytes from analysis

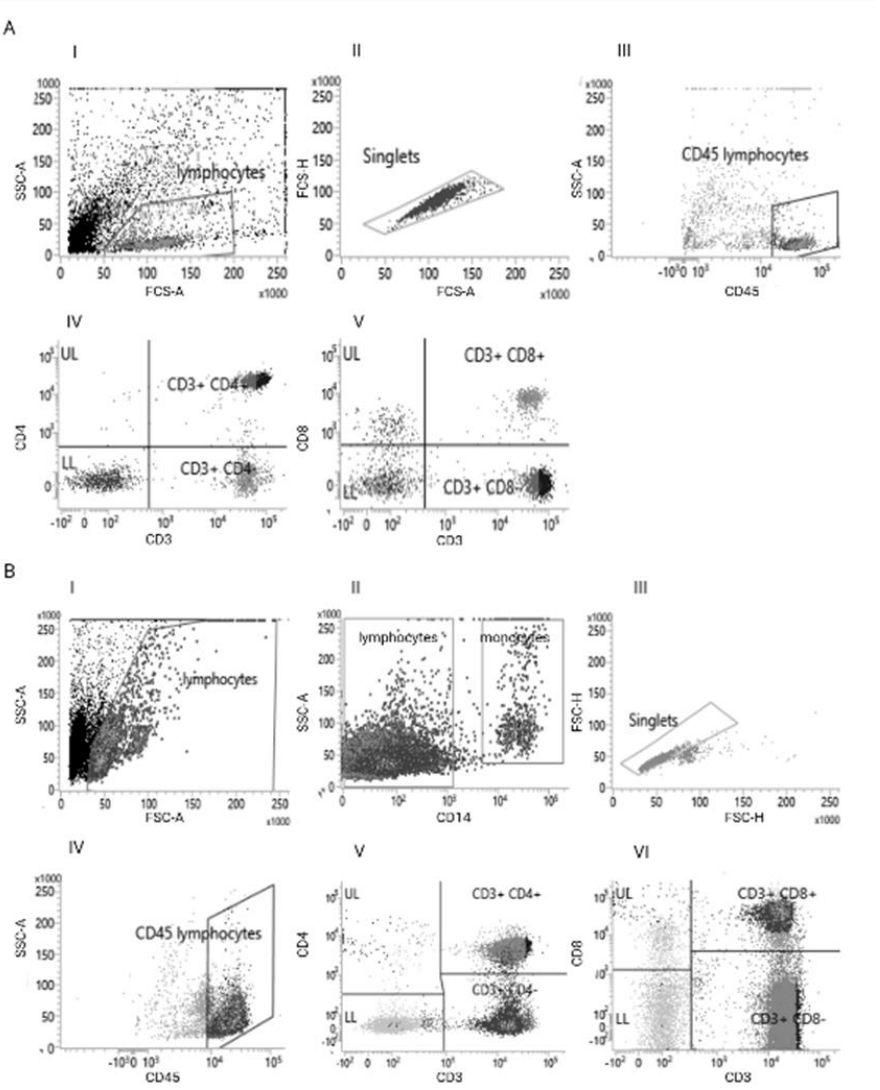


Figure 3.1. A gating strategy to identify lymphocytes, CD3, CD4 and CD8 T cells was employed. Whole blood was left unstimulated (shown) or stimulated with PHA or anti-CD3/anti-CD28 (data not shown). After 4 days of culture, RBC were lysed and stained using the antibodies shown. A gating strategy was designed.

Panel A: Prior to inclusion of anti-CD14 (I); lymphocytes were gated based on forward and side scatter, (II); gating through the lymphocyte gate, singlets were gated based on forward scatter area vs height, (III); using the singlet gate, CD45 positive lymphocytes were identified using side scatter vs CD45, (IV); using the CD45 gate, T cells positive for CD3 and CD4 were identified, (V); using the CD45 gate, T cells positive for CD3 and CD8 were identified.

Panel B: Anti-CD14 was added to remove monocytes (I); lymphocytes were gated based on forward and side scatter, (II); using the lymphocyte gate, CD14 negative cells were gated upon, to exclude monocytes from the analysis, (III); using the CD14 negative gate, singlets were gated upon, (IV); using the singlet gate, CD45 positive cells were identified using side scatter vs CD45, (V); using the CD45 gate, T cells positive for CD3 and CD4 were identified, (VI); using the CD45 gate, T cells positive for CD3 and CD8 were identified.

Figure 3.2 The percentage of monocytes seen after 4 days in cell culture were highest in unstimulated cultures and varied between subjects

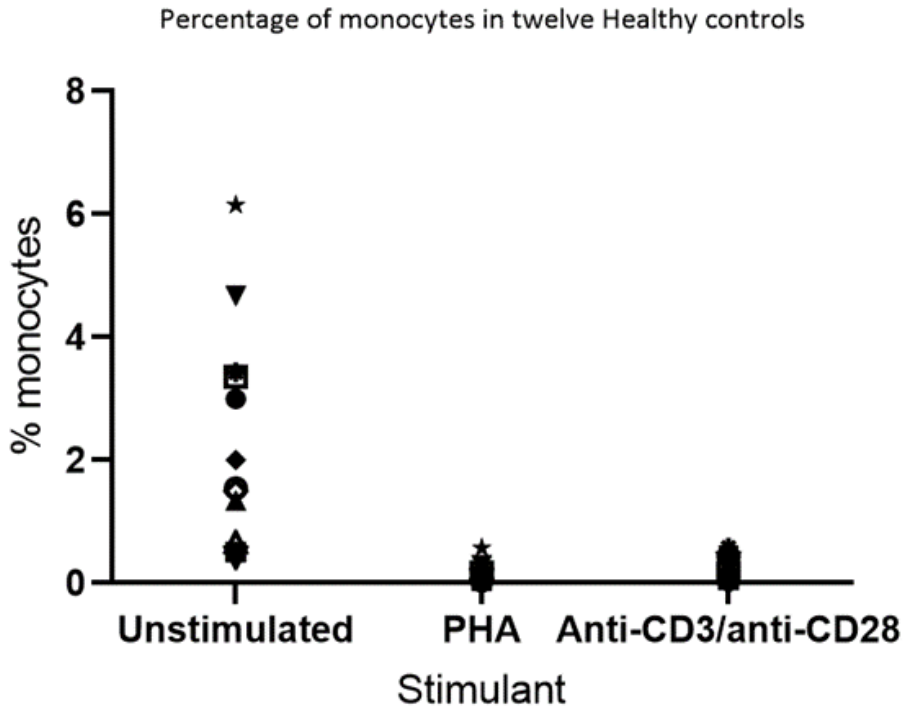


Figure 3.2. The percentage of monocytes seen after 4 days of cell culture was highest in unstimulated cell cultures and varied between subjects. Whole blood was left unstimulated or stimulated with PHA or anti-CD3/anti-CD28. After 4 days of culture, RBC were lysed and monocytes were identified using anti-CD14 and as shown in Figure 3.1, panel B, plot II. The percentage of monocytes in 12 healthy controls is shown as a percentage of total lymphocytes.

3.5 Lymphocytes stimulated with PHA or anti-CD3/anti-CD28 express Ki-67

Whole blood was stimulated with PHA or anti-CD3/anti-CD28 and some cells left unstimulated, with no added mitogen or antibody added. Cells were cultured and harvested on days 4, 5, 6, 7 and RBCs lysed. Cells were stained with surface antigens anti-CD45, anti-CD3, anti-CD4, anti-CD8 and anti-CD14. They were then fixed, permeabilised and stained with anti-Ki-67. Gates were set on unstimulated cells to examine the percentage of CD3 T cells which proliferated in response to PHA or anti-CD3/anti-CD28 (Figure 3.3).

Figure 3.3. T cells stimulated with PHA or anti-CD3/anti-CD28 express Ki-67, whereas unstimulated cells do not

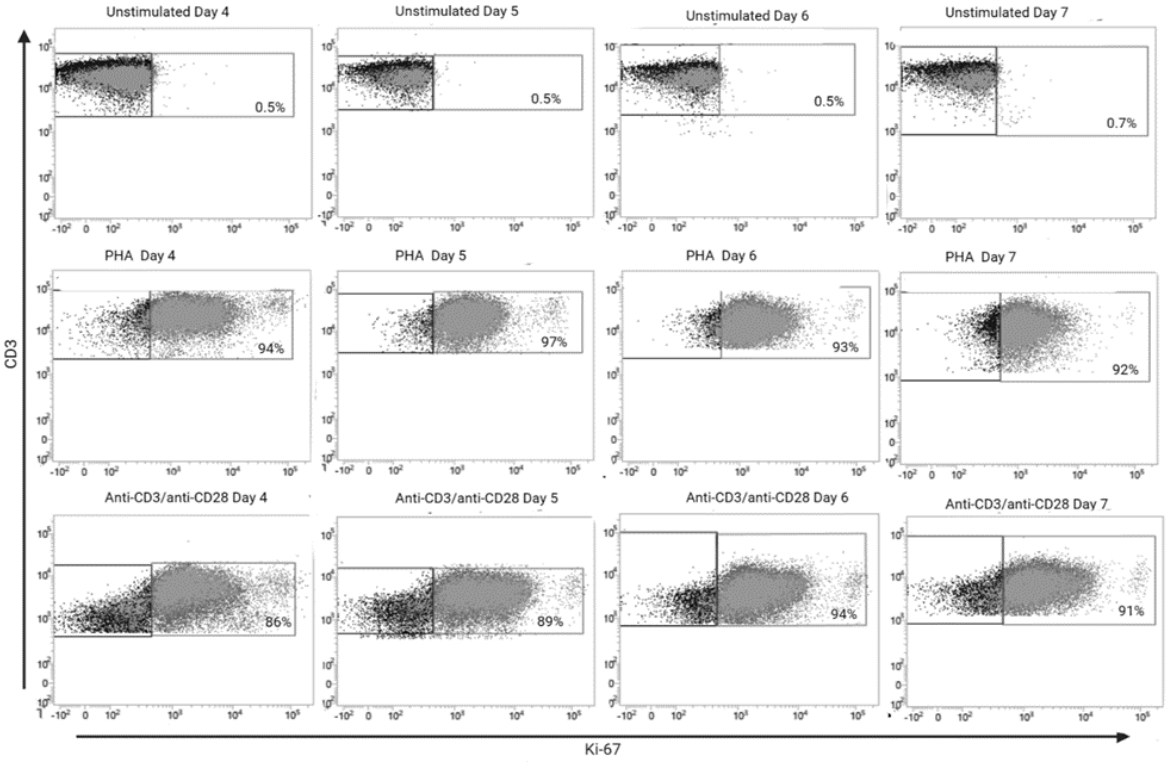


Figure 3.3. T cells stimulated with PHA or anti-CD3/anti-CD28 express Ki-67, whereas unstimulated cells do not. Whole blood was stimulated with PHA or left unstimulated and harvested at day 4, 5, 6 and 7. RBCs were lysed and cells stained for CD3 and fixed, permeabilised and stained intracellularly for Ki-67. Gates were set on unstimulated cells and the percentage of Ki-67 positive cells were examined at each timepoint. At least 20,000 CD3 T cells were acquired on FACSLyric flow cytometer on each day. The percentage of CD3 Ki-67 positive cells is shown.

Ki-67 expression was shown in cells which had been cultured for 4, 5, 6 and 7 days. The percentage of unstimulated CD3 T cells expressing Ki-67, was less than 1% on all days. The percentage of CD3 T cells which expressed Ki-67 after stimulation with PHA remained above 90% on days 4, 5, 6 and 7. The percentage of CD3 T cells expressing Ki-67 after stimulation with anti-CD3/anti-CD28 was between 86% - 97%, demonstrating that Ki-67 was expressed and could be detected in proliferating T cells.

The optimal day for harvesting cells was investigated by examining the percentage of CD45, CD3, CD4 and CD8 T cells positive for Ki-67 at each timepoint.

3.6 The percentage of Ki-67 positive cells does not appear to be significantly affected between 4 and 7 days of culture

The percentage of lymphocytes and T cells positive for Ki-67, were assessed after 4, 5, 6 and 7 days of culture with PHA or anti-CD3/anti-CD28 stimulation. Negative gates were set on unstimulated cells (Figure 3.4). In one of the healthy controls, the percentage of Ki-67 positive cells declined after 6 days of culture, however, this was not seen in the other healthy control analysed. Overall, the percentage of Ki-67 positive lymphocytes or T cells did not appear to decline between 4 and 7 days of culture.

Figure 3.4. The percentage of Ki-67 positive cells does not appear to be affected between 4 and 7 days of culture

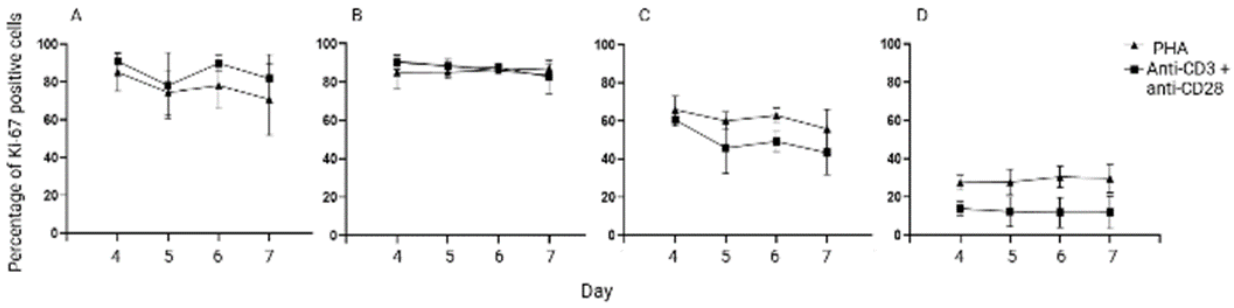


Figure 3.4. The percentage of Ki-67 positive cells does not appear to be significantly affected between 4 and 7 days of culture. Whole blood was stimulated with PHA or anti-CD3/anti-CD28 or left unstimulated and harvested at day 4, 5, 6 and 7. RBCs were lysed and cells stained for CD45, CD3, CD4 and CD8, then subsequently fixed, permeabilised and stained intracellularly for Ki-67. Gates were set on unstimulated cells and the percentage of Ki-67 positive analysed at each timepoint. **Panel A:** CD45 positive lymphocytes, **Panel B:** CD3 positive T cells, **Panel C:** CD4 positive T cells, **Panel D:** CD8 positive T cells. The mean and range of results from 2 subjects is shown.

3.7 The optimal time to lyse RBCs was after harvesting cells at the end of culture or prior to culture

Experiments were set up using whole blood, rather than separating PBMCs, in an attempt to develop a simpler method than the previous CFSE method. This meant that a step to lyse RBCs, needed to be introduced. The optimal stage at which to lyse RBCs was investigated, in order to get the least amount of cell debris and

optimal cellular proliferation and cell numbers for analysis. BD Pharmlyse, an ammonium chloride-based lysing reagent, was added to cells either prior to their culture for 4 days, just after they had been harvested at the end of culture, or after staining with antibodies and just before running the samples on the flow cytometer. The percentage of Ki-67 positive CD45 lymphocytes, CD3 T cells and CD4 and CD8 T cells, when stimulated with PHA or anti-CD3/anti-CD28, were examined under each lysing condition (Figure 3.5).

Figure 3.5. The percentage of Ki-67 positive cells was not affected by the lysing conditions of RBCs

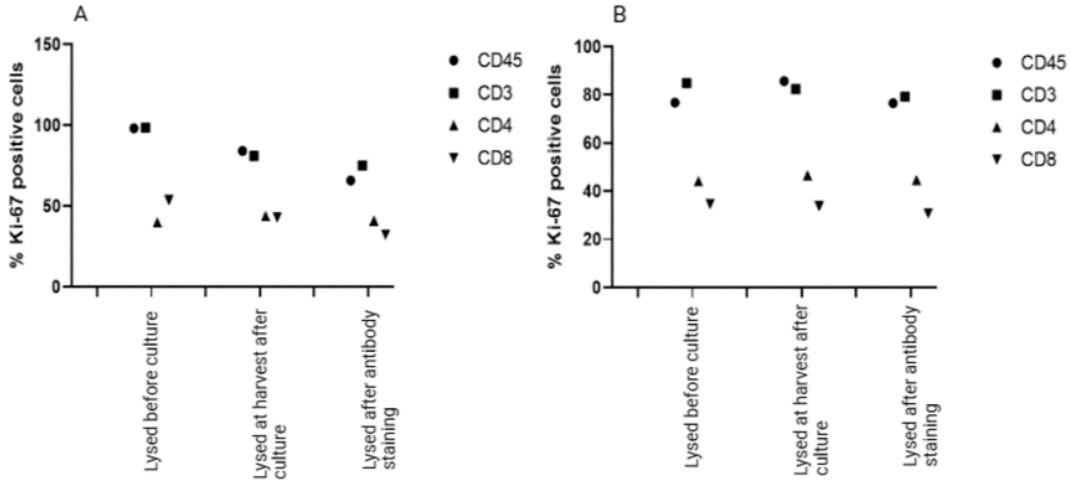


Figure 3.5. The percentage of Ki-67 positive cells was not affected by the lysing conditions of the RBCs. Whole blood was stimulated for 4 days with PHA **(Panel A)**, anti-CD3/anti-CD28 **(Panel B)**, or left unstimulated (not shown). Cells were stained with surface anti-CD45, CD14, CD3, CD4 and CD8. The cells were then fixed, permeabilised and stained with anti-Ki-67. The RBCs were lysed either prior to cell culture, at the time of harvesting the cells at day 4, or after antibody staining and before running on the flow cytometer. Gates were set on the unstimulated cells and the percentage of Ki-67 positive lymphocytes was examined, after stimulation. Percentages were expressed as a percentage of lymphocytes.

The percentage of Ki-67 positive cells, was not affected when RBCs were lysed prior to flow cytometric analysis. No difference was observed if RBCs were lysed before cultures were set up, after harvest or after antibody staining. This was seen in lymphocytes, and both CD4 and CD8 T cells. However, when analysing the number

of cells acquired for flow cytometry, in a given time, under different lysing conditions, the numbers were lower when the RBCs were lysed at the very end of antibody staining, than when they were lysed either before or after stimulating but before antibody staining (Figure 3.6). The aim was to acquire at least 10,000 CD3 T cells under all conditions. This was not achievable when the RBCs were lysed after antibody staining and before running on the flow cytometer, in cells which were unstimulated or stimulated with PHA. In all stimulation conditions, the number of cells able to be acquired was at least two-fold when the RBCs were lysed either before culture or at harvesting.

Figure 3.6. The number of cells acquired during flow cytometry was reduced in those cultures where the RBCs were lysed after antibody staining

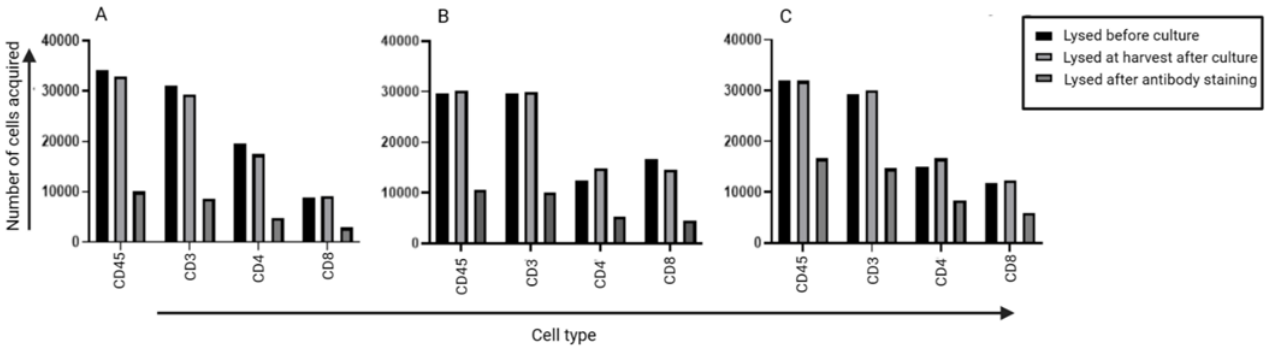


Figure 3.6. The number of cells acquired during flow cytometry was reduced in those cultures where the RBCs were lysed after antibody staining, compared to lysing prior to culture or when harvesting the cells after culture. Whole blood was stimulated for 4 days with PHA **(Panel B)**, anti-CD3/anti-CD28 **(Panel C)**, or left unstimulated **(Panel A)**. The RBCs were lysed either prior to cell culture, at the time of harvesting the cells at day 4, or after antibody staining and before running on the flow cytometer. Cells were stained with surface anti-CD45, CD14, CD3, CD4 and CD8. The cells were then fixed, permeabilised and stained with anti-Ki-67. The number of cells which were able to be acquired by flow cytometry was examined. The aim was to acquire greater than 10,000 CD3 T cells.

In order to see whether the lysing conditions affected the amount of debris which was visible when analysing the cells, the number of events captured in a debris gate was also examined under each of the lysing conditions. At least 10,000 CD3 T cells were acquired (Figure 3.7). The percentage of events in the debris gate was highest when RBCs were lysed after antibody staining.

Figure 3.7. The percentage of events in the debris gate was highest when RBCs were lysed after antibody staining

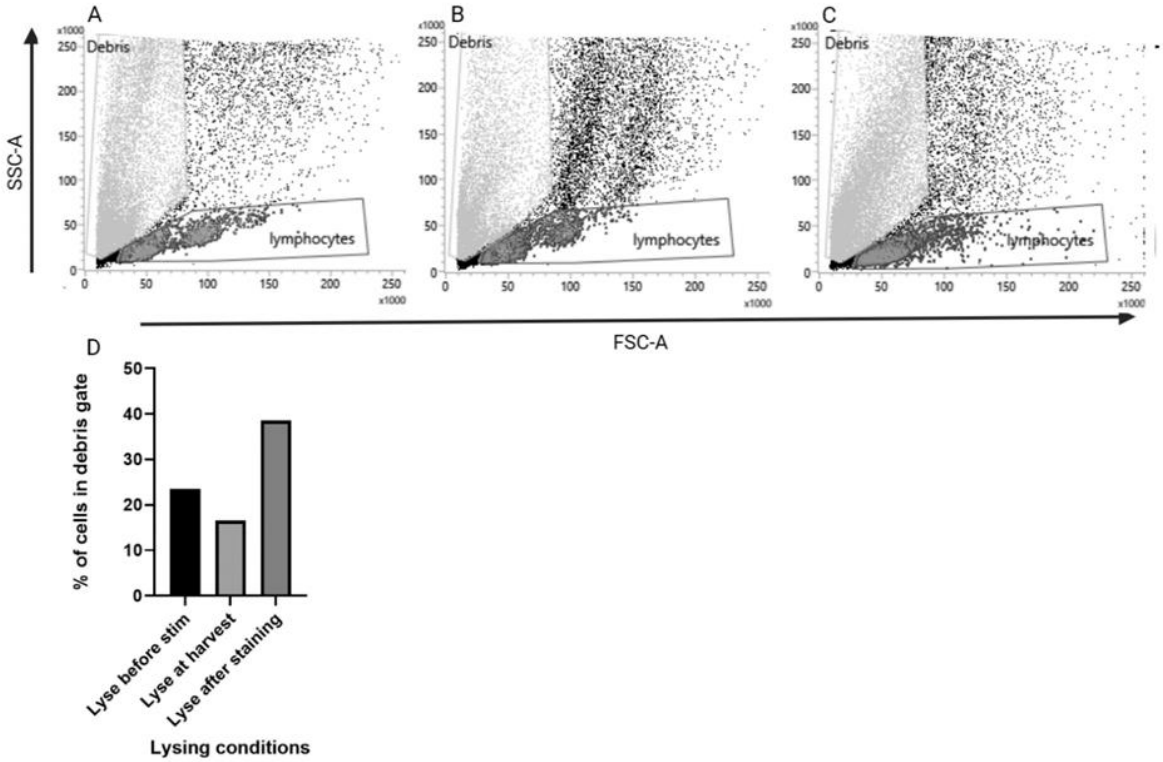


Figure 3.7. The percentage of events in the debris gate was highest when RBCs were lysed after antibody staining. Whole blood was stimulated for 4 days with PHA, anti-CD3 and anti-CD28, or left unstimulated. Cells were stained with surface anti-CD45, CD14, CD3 and CD8. Cells were then fixed and permeablised and stained with anti-Ki-67. The RBCs were lysed either prior to cell culture, at the time of harvesting the cells on day 4 or after antibody staining. A debris gate was placed to the left of the lymphocyte population. The percentage of events in the debris gate were analysed for unstimulated cells. **Panel A:** Unstimulated cells where RBCs were lysed before cell culture. **Panel B:** Unstimulated cells where RBCs were lysed at cell harvest, post cell culture. **Panel C:** Unstimulated cells where RBCs were lysed after antibody staining. **Panel D:** Graphical representation of the percentage of cells in the debris gate. At least 10,000 CD3 T cells were acquired.

3.8 Discussion

The aim of this section was to develop an assay to measure T cell proliferation using Ki-67. The hope was that this may replace the CFSE assay which was run by the laboratory at the time of starting of this project. An antibody panel, which was similar to the CFSE method was designed in order to identify proliferating

lymphocytes and T cells. The use of the bright fluorochromes, as recommended by BD, was to ensure that cell populations could be clearly distinguished from one another, even when the cells had proliferated. The gating strategy used, was similar to the CFSE gating strategy, as originally, a direct comparison was going to be made between the Ki-67 method and the CFSE method. Improvements were made including the use of a gate to exclude doublets and the introduction of a CD14 stain, to exclude monocytes. The potential for monocyte contamination within the gate for proliferated lymphocytes, was something which the laboratory was aware of during the use of the CFSE assay, however, a monocyte marker was never introduced. This meant that it was not known how many, if any, monocytes were potentially contaminating the lymphocyte gate. This could lead to incorrect interpretation of the proliferation results, with monocytes being categorised as non-proliferated lymphocytes. Upon investigation, the number of monocytes was found to be less than 1%, in cell cultures where the cells were stimulated with PHA or anti-CD3/anti-CD28 in the 12 healthy controls analysed, although higher in unstimulated cultures. The percentage of monocytes differed between subjects and therefore, the use of the CD14 stain to remove any potential monocyte contamination was viewed as a good addition to this protocol. Some patients or controls may have high numbers of monocytes, which could influence result interpretation.

Once the gating strategy was established, the ability of proliferating cells to express Ki-67 and the ability to detect this, was investigated. Whole blood was either left unstimulated or stimulated with PHA or anti-CD3/anti-CD28 to see whether Ki-67 could be detected in those cells which had proliferated. In those cultures which had been left unstimulated, a very low percentage of cells expressed Ki-67, typically less than 1%. In cells which had been stimulated with PHA, this rose to 80-90%, in CD3 T cells. This was consistent with work done by others in demonstrating that Ki-67 is expressed in proliferating cells (Gerdes et al., 1984; Soares et al., 2010). Soares et al., demonstrated that when stimulated with anti-CD3/anti-CD28, Ki-67 was detected in greater than 90% of CD4 T cells from whole blood cultures on days 3

and 4 (Soares et al., 2010). Gates were set on unstimulated cells throughout, to ensure consistency of gating. This strategy was used throughout this project.

A time course was examined to see which days might be optimal for analysis of proliferation. This was done on two separate occasions on two different HCs. One subject, demonstrated a decline in CD3 positive T cells, after day 6 of culture. However, this was not seen in the other HC. As this was only carried out on two HCs, further work, on a greater number of individuals, would be needed to establish whether there was a decline in any cell populations on days 5, 6 and 7 of culture. A time-course experiment undertaken by Soares et al, demonstrated that CD4 T cells expressing Ki-67 declined after day 4, although the level of decline appears to be variable between subjects, which is similar to our findings (Soares et al., 2010).

Day 4, was chosen as the best day for measuring proliferation to PHA and anti-CD3/anti-CD28. This meant that the Ki-67 method could be directly compared to ^{3}[H] thymidine incorporation at the referral laboratory to which lymphocyte proliferation assays were being sent, as these were also analysed on day 4 of culture. Also, when considering which days would be optimal for patient samples, arriving at our laboratory, harvesting the cells at day 4 would also work well. The PID clinic at the Royal London Hospital, is on a Wednesday and Thursday, which is where most of the requests for lymphocyte proliferation are generated, therefore samples could be set up on a Thursday and harvested on a Monday. However, if future work established that there was no difference between those harvested at day 4 or later days, samples could be harvested at later timepoints. This would give the laboratory greater flexibility in when cells could be harvested. For example, if cells were set up over a Bank Holiday weekend, cells could potentially be analysed on a Tuesday, rather than a Bank Holiday Monday. A greater number of time course experiments on HCs and patients would need to be carried out to establish if this would be possible.

In order to develop an assay which was as straightforward as possible for laboratory staff to set up, whole blood was used for cell culture, rather than PBMCs. This meant that a step to lyse RBCs needed to be included. RBCs outnumber mononuclear cells by around 600:1 in a whole blood sample and therefore many

more events would need to be acquired on the flow cytometer in order to analyse lymphocytes (Song et al., 2002). However, it was also important to establish that the lysing process did not hinder the proliferation of the cells or result in poor numbers for cell acquisition. An experiment was done to establish the optimal time for RBC lysis. Whole blood cultures were either lysed prior to going into culture, at the time of cell harvest but before the cells were stained with antibodies, or after culture and antibody staining, just before acquiring cells on the flow cytometer. The percentage of lymphocytes and T cells which expressed Ki-67 when stimulated with PHA or anti-CD3/anti-CD28 were examined under each lysing condition to ensure that there was no effect of lysing at different times on the cell populations. No difference was seen in the percentage of cells expressing Ki-67 when stimulated in any of the lysing conditions. However, when examining the number of cells which were able to be acquired under each condition, a difference was seen when lysing the RBCs after antibody staining and before running on the flow cytometer. When RBCS were lysed after antibody staining, the number of CD45 positive lymphocytes, overall CD3 T cells and CD4 and CD8 T cells were approximately half the number which could be acquired when lysing RBCs either before cell culture or at the time of cell harvest but prior to antibody staining. This may be an issue when analysing lymphocytes from patients with primary or secondary immune deficiencies as they may be lymphopenic or have poorly proliferating lymphocytes, making it more difficult to acquire enough events for reliable analysis. Also, if this method were to be used in examining the proliferation of antigen-specific T cells, which would occur at a much lower frequency than cells stimulated with mitogen or through the CD3 receptor, this would prove problematic as a larger number of lymphocytes would need to be acquired in order to see these rarer T cells. The amount of debris which was seen during flow cytometric analysis, was also around two-fold higher when RBCs were lysed after antibody staining. Having larger amounts of debris would mean it would take longer to acquire the required amount of lymphocytes for analysis and again, could be problematic in lymphopenic patients. It was decided that lysing the RBCs at the time of cell harvest and before antibody staining would be chosen, as cell acquisition or debris was not affected. Also when considering the practicalities of potentially introducing this test into routine use, having a test

which is as easy to set up as possible, when the sample arrives, is beneficial. Sample arrival times can be unpredictable throughout the day, however, harvesting the cells, lysing and staining could be planned for. This would allow maximum flexibility for setting up samples whenever they arrived.

In summary, the Ki-67 assay was optimised for use in healthy controls, by designing a suitable antibody panel and gating strategy, demonstrating the ability to detect Ki-67 in proliferating but not unstimulated cells and determining the optimal conditions for RBC lysing.

4. Validation of Ki-67 proliferation as a routine test

4.1 Introduction

This chapter describes the validation of Ki-67 as a potential routine test to be brought in-house, as an alternative to sending samples for lymphocyte proliferation to a referral laboratory. At the outset of this project, the routine method for examining lymphocyte proliferation in patients with primary and secondary immune deficiency, in our laboratory, was to use CFSE staining. This method involved performing a PBMC preparation, then labelling the cells with CFSE. CFSE is a fluorescein which crosses the cell membrane and binds to intracellular proteins (Weston and Parish, 1990). This changes the structure of the dye, causing it to fluoresce. Upon cellular division, the fluorescent dye is halved between the daughter cells, arising from that divided cell (Lyons and Parish, 1994). These dividing cells can be visualised by flow cytometry, allowing the progeny of a proliferating cell population to be followed (Quah and Parish, 2012). This method worked well, in providing detailed and robust measures of lymphocyte proliferation for several years, however, it was time-consuming and involved an experienced scientist spending most of a full day in setting up the assay (Appendix 1). During the COVID pandemic, the CFSE assay was stopped for several reasons, including reduced numbers of face-to-face appointments resulting in a lower number of patients having phlebotomy, and also staffing pressures in our laboratory. Instead, samples for lymphocyte proliferation were sent away to a referral laboratory as an alternative to the in-house assay. This referral laboratory uses the ^{3}[H] thymidine incorporation method for measuring lymphocyte proliferation. It exploits the incorporation of thymidine into DNA during its synthesis (Lyons and Parish, 1994). Peripheral blood mononuclear cells, or whole blood, is cultured, with the ^{3}[H] thymidine added during the final 4-7 hours. Incorporated radiolabel is measured by a liquid scintillation counter and the results reported as counts per minute (CPM) (Shahid, N. Internal SOP, GOSH).

Sending samples to a referral laboratory comes with additional sample transport costs and carries the risk of damage or loss of samples in transit. As increasing numbers of patients have now returned to face-to-face appointments, there is a greater demand for lymphocyte proliferation assays. Therefore, a straightforward in-house assay for lymphocyte proliferation in our laboratory may be warranted. When introducing a new test into a clinical laboratory, the test must be validated or verified to ensure it is appropriate for clinical use. Verification is used when a clinical assay is being evaluated and the manufacturers findings are verified by the laboratory, when the laboratory uses the assay in the same way as the manufacturer, without deviation from their method or scope of use (Dolphin, H. Internal policy. RLH). Validation is required for non-standard methods, such as laboratory designed in-house assays or where standard assays are used outside of their intended scope or when a previously validated assay has been modified (Dolphin, H. Internal policy. RLH). These requirements come from the standards to which clinical laboratories are inspected, ISO15189 and objective evidence must be provided in all cases (Dolphin, H. Internal policy. RLH). Some of the factors to consider when undertaking validation or verification work are shown in Table 4.1. The Ki-67 assay is a non-standard assay, meaning that a validation of the method would be required to conform to ISO15189 accreditation; however, as this is a functional assay, rather than a diagnostic assay, achieving several of the parameters which are listed for assay validation would need to be thought about differently, such as linearity, External Quality Assurance (EQA) and confirmation of analytical specificity (Dolphin, H. Internal policy. RLH). There are no established EQA schemes for lymphocyte proliferation assays, however, a sample exchange programme with another laboratory could be established to overcome this. Also, analytical specificity could be demonstrated in principle, by comparing T cell proliferation to the antigen in question with proliferation to a control antigen to which the to the subject is not immune. For example, using CMV peptide as the stimulant in a CMV IgG negative individual.

As part of the validation of the Ki-67 assay, reference ranges using HCs, intra- and inter-assay variation, and a comparison to the current method of ^{3}[H] thymidine incorporation were performed, in addition to examination of the correlation of the

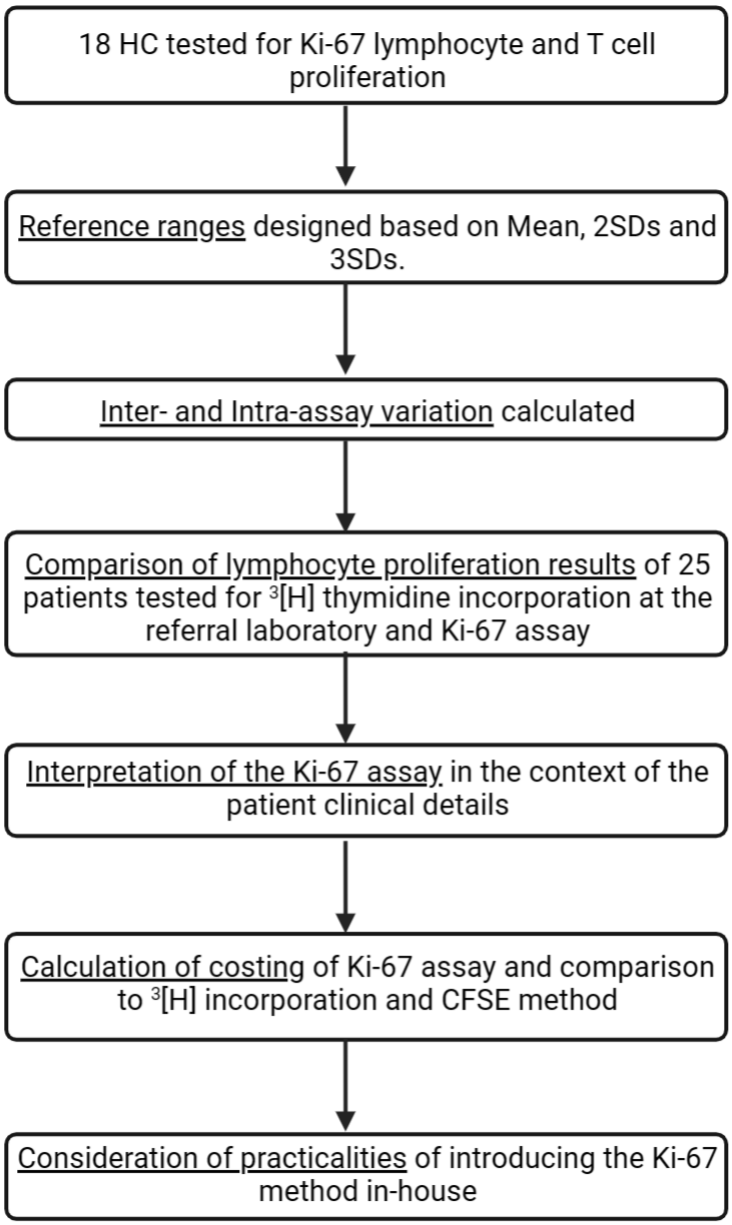
assay results with the patients' clinical picture. One of the original aims of this project was to compare the results of the Ki-67 assay with both the ^{3}[H] thymidine assay at the referral laboratory, but also the CFSE assay which was run in-house. However, as previously described, during the Covid-19 pandemic, far fewer patients were coming into hospital for face-to-face appointments. This was particularly marked in the cohort of patients for whom this test was indicated, namely those with primary and secondary immunodeficiency, who were advised to shield. In September 2021, the CFSE assay was discontinued. The small numbers of requests which were received were sent to the referral laboratory, therefore a comparison to the CFSE method was not possible, despite it being part of the original plan of this project. A comparison was done between the Ki-67 assay and the ^{3}[H] thymidine incorporation assay. Cost comparisons and the consideration of the practicalities of each assay were carried out, including the CFSE assay.

Table 4.1. Factors to consider when undertaking a method validation or verification of a new assay for introduction into a clinical laboratory

Assay performance characteristics	**Validation**	**Verification**
Inter- and intra-assay variation	X	X
Linearity	X	X
Limit of detection	X	
External Quality Assurance	X	X
Patient sample comparison with new method	X	
Confirmation of analytical specificity	X	
Reference ranges	X	X
Diagnostic sensitivity/specificity	X	X

Table 4.1. A table showing factors to consider when undertaking validation or verification of a new assay for introduction into a clinical laboratory. Adapted from Dolphin, H. ESEL.Policy.005. Validation and Verification in pathology, RLH.

4.2 Flowchart of parameters investigated in this chapter



4.3 Reference ranges were calculated on healthy controls for lymphocyte and T cell proliferation

Eighteen healthy controls were recruited either from the Immunology laboratory at the RLH or control samples which were sent to the laboratory alongside the patient samples. These comprised healthy male and female adults between the ages of 26

– 56. Some of the HCs were taken in the clinic at the same time as patient samples and their demographics are not known. These are healthy volunteers, unrelated to the patient and are usually laboratory or clinical staff.

Whole blood was stimulated for 4 days with PHA or anti-CD3/anti-CD28 or left unstimulated. The percentage of Ki-67 positive lymphocytes, CD3, CD8 and CD4 T cells were analysed, using the gating strategy outlined in Section 3.4.

A test of skewness was carried out to determine if the data for the percentage of T cells and lymphocytes positive for Ki-67 was normally distributed when examining those stimulated with PHA and anti-CD3/anti-CD28 (Table 4.2). The setting of reference ranges using the mean and standard deviations would not be appropriate, if the data were highly skewed.

Table 4.2. Skewness was calculated on data from 18 HCs to determine if the data was normally distributed

Stimulation conditions		**Skewness**
PHA	CD45 lymphocytes	-0.99
	CD3 T cells	-0.87
Anti-CD3/anti-CD28	CD45 lymphocytes	0.43
	CD3 T cells	0.01

Table 4.2. A test of skewness was carried out on data from 18 HCs. The percentage of Ki-67 positive CD45 lymphocytes and CD3 T cells when stimulated with PHA or anti-CD3/anti-CD28 were examined, and a test of skewness was performed to determine if the data was normally distributed.

Based on this test, the data was shown to be slightly negatively skewed for lymphocytes and T cells when stimulated with PHA, and the data were very nearly symmetrical when stimulated with anti-CD3/anti-CD28. Median and mean percentage of Ki-67 positive T cells for each cell population: CD45 lymphocytes, CD3, CD4 and CD8 T cells were compared to see if they were similar (Tables 4.3 and 4.4). The variation between the mean and median for each population was 0 – 3 percent, therefore, a reference range based on the mean and two or three SD from the mean could be calculated.

Reference ranges were calculated using mean value of Ki-67 positive cells for each cell population and two or three standard deviations (SD) either side of the mean (Tables 4.3 and 4.4). When analysing CD4 and CD8 T cells, the percentage of Ki-67 positive cells were examined in both CD4 and CD8 T cells as a percentage of lymphocytes and as a percentage of T cells. This was because the percentage of CD4 and CD8, particularly CD8 T cells which proliferated, as a percentage of lymphocytes, varied considerably between individuals, ranging from 7 – 32% of lymphocytes. In response to anti-CD3/anti-CD28, the lowest percentage of positive Ki-67 CD8 T cells when viewed as a percentage of lymphocytes was 7% (data not shown). When viewing this as a percentage of T cells, in the same patient, the percentage was 86%. When examining the proliferation of CD8 T cells as a percentage of T cells, the range between patients was 70 – 96%. A more meaningful reference range was able to be set, using CD8 T cells as a percentage of T cells, rather than lymphocytes.

Table 4.3. The mean, median, Standard deviation (SD) and reference ranges for Ki-67 lymphocytes and T cells, when stimulated with PHA, were calculated

Parameter (PHA)	Mean	Median	SD	2SDs	2SD Range	3SD Range
CD45	86	87	5.24	10.49	**77 - 99**	**70 - 100**
CD3 cells	85	87	5.94	11.87	**73 - 97**	**67 - 100**
CD4 T cells	52	50	7.63	15.27	**36 - 67**	**29 - 100**
CD4 T cells as a percentage of T cells	92	93	3.78	7.57	**84 - 99**	**81 - 100**
CD8 T cells	33	30	9.19	18.39	**15 - 51**	**5 - 60**
CD8 T cells as a percentage of T cells	91	90	3.90	7.79	**83 - 98**	**79 - 100**

Table 4.3. The mean, median, SD and reference ranges for Ki-67 positive lymphocytes and T cells were calculated. Whole blood was stimulated for 4 days with PHA or left unstimulated (data not shown). The percentage of Ki-67 positive lymphocytes, CD3, CD8 and CD4 T cells were analysed, in 18 HCs, using the gating strategy outlined in Section 3.4. To calculate CD4 T cells as a percentage of T cells: Ki-67 positive T cells were examined by gating on CD45 and then CD3 positive T cells and CD4 positivity, and the percentage of CD4+ Ki-67+ cells calculated. The same approach was taken for CD8+ Ki-67+ T cells to calculate CD8 T cell as a percentage of T cells.

Table 4.4. The mean, median, Standard deviation (SD) and reference ranges for Ki-67 lymphocytes and T cells, when stimulated with anti-CD3/anti-CD28, were calculated

Parameter (anti-CD3/anti-CD28)	Mean	Median	SD	2SDs	2SD Range	3SD Range
CD45	81	80	5.37	10.73	**70 - 91**	**64 - 100**
CD3 cells	79	79	5.10	10.20	**69 - 90**	**64 - 100**
CD4 T cells	60	58	9.52	19.10	**41 - 79**	**31 - 100**
CD4 T cells as a percentage of T cells	89	90	5.27	10.54	**78 - 99**	**73 - 100**
CD8 T cells	19	17	8.25	16.50	**3 - 36**	**0 - 45**
CD8 T cells as a percentage of T cells	84	83	6.22	12.44	**71 - 96**	**65 - 100**

Table 4.4. The mean, median, SD and reference ranges for Ki-67 positive lymphocytes and T cells were. Whole blood was stimulated for 4 days with anti-CD3/anti-CD28 or left unstimulated (data not shown). The percentage of Ki-67 positive lymphocytes, CD3, CD8 and CD4 T cells were analysed, from 18 HCs, using the gating strategy outlined in Section 3.4. To calculate CD4 T cells as a percentage of T cells: Ki-67 positive T cells were examined by gating on CD45 and then CD3 positive T cells and CD4 positivity, and the percentage of CD4+ Ki-67+ cells calculated. The same approach was taken for CD8+ Ki-67+ T cells to calculate CD8 T cell as a percentage of T cells.

Using a reference range with 2SDs from the mean gave the most stringent reference range and when using 3SDs from the mean, the reference range was wider. Both reference ranges were considered when interpreting results, detailed in Section 4.5.

Once reference ranges had been calculated, inter- and intra-assay variation were examined as part of the validation process, to see if these were acceptable for a functional assay.

4.4 Inter and Intra-assay variation was less than 20%

Inter-assay variation was calculated by running a sample from a single HC on three separate occasions. In brief, whole blood was stimulated with PHA or anti-CD3/anti-CD28 or some cells left unstimulated, with no added mitogen or antibodies. Cells were cultured and harvested on days 4 and RBCs lysed. Cells were stained with surface antigens anti-CD45, anti-CD3, anti-CD4, anti-CD8 and anti-CD14. They were

then fixed, permeabilised and stained with anti-Ki-67. Gates were set on unstimulated cells to examine the percentage of lymphocytes and T cells which proliferated in response to PHA or anti-CD3/anti-CD28.

The mean, SD and percentage Coefficient of Variation (CV) was calculated for cells when stimulated with PHA or anti-CD3/antiCD28 (Table 4.5). The inter-assay variation was less than 20%, with the highest %CV being 19.8% for CD8 T cells, as a percentage of lymphocytes, in response to anti-CD3/anti-CD28 stimulation. When examining CD8 T cells as a percentage of all T cells (CD3 positive) the inter-assay variation was around half, at 9%.

Table 4.5. The inter-assay variation was calculated by processing samples from the same HC on three separate occasions

PHA stimulation	**CD45**	**CD3**	**CD4**	**CD4 % of T cells**	**CD8**	**CD8 % of T cells**	**n**
Mean	83.3	81.4	56.1	93.75	23.8	93.32	3
SD	11.4	10.4	5.96	4.92	1.5	3.72	
%CV	**13.7**	**12.8**	**10.6**	**5.3**	**6.4**	**4.0**	

Anti-CD3/anti-CD28 stimulation	**CD45**	**CD3**	**CD4**	**CD4 % of T cells**	**CD8**	**CD8 % of T cells**	**n**
Mean	83.2	80.1	67.9	90.05	9.5	84.42	3
SD	7.9	6.7	11.6	7.30	1.9	8.28	
%CV	**9.5**	**8.4**	**17.0**	**8.10**	**19.8**	**9.81**	

Table 4.5. Inter-assay variation was below 20% in the percentage of lymphocytes and T cells positive for Ki-67. A sample from a single healthy control was processed on three separate occasions. The mean, SD and CV were calculated.

Intra-assay variation was calculated by running a sample from a single HC three times within the same experiment (Table 4.6). The intra-assay variation was less than 14%, with the highest %CV being 13.5% for CD8 T cells in response to anti-CD3/anti-CD28. When examining CD8 T cells as a percentage of all T cells (CD3 positive), the CV was lower, at 8%. This demonstrates that the percentage of CD8 T cells responding to stimulation differs, not only between HCs, as seen by the reference ranges, but also within individuals.

The lowest %CV was 1.2% for CD8 T cells, as percentage of all T cells, in response to PHA.

Table 4.6. The intra-assay variation was calculated by processing a sample from a single HC three times within the same experiment

PHA stimulation	CD45	CD3	CD4	CD4 % of T cells	CD8	CD8 % of T cells	n
Mean	90.8	93.4	45.8	93.55	44.8	93.84	3
SD	1.9	1.6	1.3	1.87	0.7	1.14	
%CV	**2.1**	**1.7**	**2.8**	**2.00**	**1.5**	**1.21**	

Anti-CD3/anti-CD28 stimulation	CD45	CD3	CD4	CD4 % of T cells	CD8	CD8 % of T cells	n
Mean	80.5	81.2	53.0	89.53	26.8	88.39	3
SD	2.3	2.7	1.9	0.67	3.6	7.00	
%CV	**2.8**	**3.3**	**3.5**	**0.75**	**13.5**	**7.92**	

Table 4.6. Intra-assay variation was below 14% in the percentage of lymphocytes positive for Ki-67. A sample from a single healthy control was processed three times in the same experiment. The mean, SD and CV were calculated.

4.5 Comparison of Ki-67 proliferation assay and ^{3}[H] thymidine incorporation shows some correlation

Eight HCs and 26 patient samples were tested by the Ki-67 method and in parallel with ^{3}[H] thymidine incorporation at the referral laboratory where samples are currently sent for lymphocyte proliferation. These patients were all being investigated for primary or secondary immune deficiency. All HC samples were classified as normal in both assays and were not included in subsequent analysis. One patient sample did not have any results available from the referral laboratory and was therefore excluded from analysis. Technical difficulties were encountered when processing one patient sample, when cells were stimulated with anti-CD3/CD28, therefore only the PHA results were included for this patient.
Results from the ^{3}[H] thymidine incorporation were classified as normal, impaired or absent, as reported by the reference laboratory and results reported as CPM. When considering how to interpret the results of the Ki-67 assay several different

approaches were taken. To have a similar classification system to the ^{3}[H] thymidine incorporation assay and the CFSE assay, results were classified as either absent, impaired, or normal. Patient clinical details were also considered when deciding upon which approach was the most appropriate:

Approach 1: Use the reference range of 2SDs of the mean and examine CD45 and CD3 T cells values only.

Approach 2: Use the reference range of 2SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the reference range for either PHA or anti-CD3/28.

Approach 3: Use a reference range of 3SDs from the mean and examine CD45 and CD3 T cells values only.

Approach 4: Use the reference range of 3SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the ref range for either PHA or anti-CD3/28.

Table 4.7. The classification of the results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared using Approach 1

PHA	Ki67		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	0	0
Impaired	0	0	0
Normal	1	8	16

Anti-CD3/anti-CD28	Ki67		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	1	1
Impaired	0	3	4
Normal	0	4	11

Table 4.7. Results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared for 25 patients, using the classification of normal, impaired or absent. For the Ki-67 assay, the reference range using 2SDs from the mean was used and only CD45 and CD3 T cell values were used.

Using approach 1, with PHA stimulation, 16 patients were classified as normal in both assays and 8 samples were deemed impaired in the Ki-67 assay, but normal in the ^{3}[H] thymidine incorporation assay. 1 patient had an absent PHA response in the Ki-67 assay but was classified as normal in the ^{3}[H] thymidine incorporation assay.

With anti-CD3/anti-CD28 stimulation, 11 patients were classified as normal in both assays and 3 patients were classified as impaired in both assays. One patient was classified as having an absent response in the ^{3}[H] thymidine incorporation assay but was deemed normal for Ki-67. Four patients with an impaired response in the ^{3}[H] thymidine incorporation assay, were classified as normal in the Ki-67 assay and one patient with an absent response in the ^{3}[H] thymidine incorporation assay, was classified as impaired in the Ki-67 assay. Four patients with a normal response in

the ^{3}[H] thymidine incorporation assay, were classified as impaired in the Ki-67 assay.

Table 4.8. The classification of the results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared using Approach 2

PHA	**Ki67**		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	0	0
Impaired	0	0	0
Normal	1	8	16

CD3/28	**Ki67**		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	1	1
Impaired	0	2	5
Normal	0	1	14

Table 4.8. Results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared for 25 patients, using the classification of normal, impaired or absent. For the Ki-67 assay, the reference range using 2SDs from the mean was used, calling it impaired if more than 3 parameters were below the reference range for either PHA or anti-CD3/28.

Using approach 2, with PHA stimulation, 16 patients were classified as normal in both assays and 8 samples were deemed impaired in the Ki-67 assay, but normal in the ^{3}[H] thymidine incorporation assay. 1 patient had an absent PHA response in the Ki-67 assay but was classified as normal in the ^{3}[H] thymidine incorporation assay.

For anti-CD3/anti-CD28 stimulation, 14 patients were classified as normal in both assays and 2 patients were classified as impaired in both assays. One patient was classified as having an absent response in the ^{3}[H] thymidine incorporation assay but was deemed normal for Ki-67. Five patients with an impaired response in the

^{3}[H] thymidine incorporation assay, were classified as normal in the Ki-67 assay and one patient with an absent response in the ^{3}[H] thymidine incorporation assay, was impaired in the Ki-67 assay. One patient with a normal response in the ^{3}[H] thymidine incorporation assay, were classified as impaired in the Ki-67 assay.

Table 4.9. The classification of the results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared using Approach 3

PHA	**Ki67**		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	0	0
Impaired	0	0	0
Normal	1	7	17

CD3/28	**Ki67**		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	1	1
Impaired	0	1	6
Normal	0	1	14

Table 4.9. Results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared for 25 patients, using the classification of normal, impaired or absent. For the Ki-67 assay, the refence range using 3SDs from the mean was used and only CD45 and CD3 T cells values were used.

Using approach 3, with PHA stimulation, 17 patients were classified as normal in both assays and 7 samples were deemed impaired in the Ki-67 assay, but normal in the ^{3}[H] thymidine incorporation assay. 1 patient had an absent PHA response in the Ki-67 assay but was classified as normal in the ^{3}[H] thymidine incorporation assay.

For anti-CD3/anti-CD28 stimulation, 14 patients were classified as normal in both assays and 1 patient was classified as impaired in both assays. 1 patient was classified as having an absent response in the ^{3}[H] thymidine incorporation assay

but was deemed normal for Ki-67. 6 patients with an impaired response in the ^{3}[H] thymidine incorporation assay, were classified as normal in the Ki-67 assay and 1 patient with an absent response in the ^{3}[H] thymidine incorporation assay, was impaired in the Ki-67 assay. One patient with a normal response in the ^{3}[H] thymidine incorporation assay, was classified as impaired in the Ki-67 assay.

Table 4.10. The classification of the results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared using Approach 4

PHA	Ki67		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	0	0
Impaired	0	0	0
Normal	1	7	17

CD3/28	Ki67		
Thymidine	**Absent**	**Impaired**	**Normal**
Absent	0	1	1
Impaired	0	0	7
Normal	0	2	13

Table 4.10. Results of the Ki-67 assay and the ^{3}[H] thymidine incorporation assay were compared for 25 patients, using the classification of normal, impaired or absent. For the Ki-67 assay, the reference range using 3SDs from the mean was used, calling it impaired if more than 3 parameters were below the reference range for either PHA or anti-CD3/28.

Using approach 4, with PHA stimulation, 17 patients were classified as normal in both assays and 7 samples were deemed impaired in the Ki-67 assay, but normal in the ^{3}[H] thymidine incorporation assay. One patient had an absent PHA response in the Ki-67 assay but was classified as normal in the ^{3}[H] thymidine incorporation assay.

For anti-CD3/anti-CD28 stimulation, 13 patients were classified as normal in both assays. One patient was classified as having an absent response in the ^{3}[H] thymidine incorporation assay but was deemed normal for Ki-67. Seven patients

with an impaired response in the ^{3}[H] thymidine incorporation assay, were classified as normal in the Ki-67 assay and one patient with an absent response in the ^{3}[H] thymidine incorporation assay, was impaired in the Ki-67 assay. Three patients with a normal response in the ^{3}[H] thymidine incorporation assay, were classified as impaired in the Ki-67 assay.

Some correlation between the Ki-67 method and ^{3}[H] thymidine incorporation was seen whichever approach was used for interpreting the Ki-67 method. Patient clinical details were examined in all cases to see if the assays correlated with the patient's clinical picture.

4.6 Interpretation of the Ki-67 results in the context of the patient clinical details

To gain further insight into the most appropriate way to interpret the Ki-67 results, the clinical details of the patients were reviewed. The patient's lymphocyte count was noted, as a low lymphocyte count can lead to a low proliferation response, in particular in the ^{3}[H] thymidine incorporation assay (Marits et al., 2014). This issue may not affect the Ki-67 assay to the same extent, as T cells can be visualised on the flow cytometer during the assay readout, in contrast, the ^{3}[H] thymidine incorporation assay does not have this benefit. Patient lymphocyte counts are often unknown at the time of assay set up. Whether the patient had a history of opportunistic infections was also noted. Opportunistic infections are those which very rarely occur in immunocompetent individuals but can occur in patients with impaired cellular immunity, particularly with a low CD4 T cell count, such as HIV. A history of opportunistic infections could be a sign that the patient has a definitive impairment in T cell immunity and therefore, would be likely to have an abnormal T cell proliferation assay. Other patient clinical details were also noted, in order to gain a better picture of whether there was a suggestion of impaired cellular immunity. All patients who were analysed are listed in Table 4.11, along with their clinical details, their ^{3}[H] thymidine incorporation results and Ki-67 assay results, for the different approaches of Ki-67 assay interpretation, to determine the method of interpretation that best matched patient’s clinical picture.

Table 4.11. Patient clinical details were correlated with their lymphocyte counts and the results of their ^{3}[H] thymidine incorporation assay and Ki-67 assay, as interpreted by the four approaches described

Patient	Lymphopenic	Opportunistic infections	Clinical details	Thymidine	App 1	App 2	App 3	App 4
1	No	No	1 case of VZV opthalmicus, normal vaccine responses	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
2	Yes	No	Poor T cell response to Covid, but antibodies present. Secondary ID, Waldenstroms macroglobulinemia	N PHA N CD3/28	N PHA I CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
3	Yes	No	CVID, on Ig replacement. Recurrent sino-pulmonary infections with bronchiectasis	N PHA A CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
4	No	No	Isolated low IgM, no infections	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
5	No	No	NTM, decreased IFN-γ production, bacterial infections, 2 previous pneumonias	N PHA I CD3/28 with CD3 only	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
6	Yes	No	Unknown	N PHA I CD3/28 with CD3 only	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
7	No	No	One episode of HSV-2 meningoencephalitis, no history of recurrent infections, Elsberg syndrome	N PHA I CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
8	Yes	No	Neutropenia, proliferation repeated March 23 = normal	N PHA I CD3/28	N PHA I CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
9	No	No	Borderline low IgM and Low IgG	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28

Patient	Lymphopenic	Opportunistic infections	Clinical details	Thymidine	App 1	App 2	App 3	App 4
10	No	No	Recurrent VZV shingles, borderline low IgG, good vaccine response	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
11	No	No	Alpha-1 antitrypsin deficiency	N PHA I CD3/28	I PHA I CD3/28	I PHA I CD3/28	N PHA N CD3/28	N PHA N CD3/28
12	Yes	No	Pan-hypogammaglobulinaemia, recurrent chest infections	N PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28
13	No	No	Recurrent history of fevers	N PHA N CD3/28	I PHA I CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28
14	No	No	One episode of a vesicular rash	N PHA N CD3/28	I PHA I CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28
15	No	No	Airway disease, asthma, recurrent LRT infections. Low IgM, previous low IgG but normalised.	N PHA N CD3/28	I PHA. Unable to analysed CD3/28 due to technical difficulties	I PHA	I PHA	I PHA
16	No	No	Cartilage-hair hypoplasia	N PHA, I CD3/28	I PHA I CD3/28	I PHA I CD3/28	I PHA I CD3/28	I PHA N CD3/28
17	No	No	Recurrent infections in the context of severe atopy, poor responses to polysaccharide vaccine, TH17 T cells normal	N PHA I CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
18	No	No	Chronic fatigue syndrome, autoimmune Pernicious anaemia	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28 CD3/28

Patient	Lymphopenic	Opportunistic infections	Clinical details	Thymidine	App 1	App 2	App 3	App 4
19	Low CD8 T cells	No	Hypogammaglobulinemia and probable CVID	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
20	Marginally low CD4 T cells	No	Secondary ID, Hodgkin's lymphoma, 3 episodes of HSV	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
21	No	No	Low IgG and IgM, CVID	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
22	Borderline low CD4	No	Low IgG, IgM, autoimmune haemolytic anaemia	N PHA N CD3/28	I PHA I CD3/28	I PHA I CD3/28	I PHA I CD3/28	I PHA I CD3/28
23	No	No	Recurrent presumed shingles	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28	N PHA N CD3/28
24	No	No	Pan-hypogammaglobulinemia and recurrent genital herpes	N PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28	I PHA N CD3/28
25	Yes	No	CF, long term steroid use, bronchiectasis	N PHA, A CD3/28	A PHA I CD3/28	A PHA I CD3/28	A PHA I CD3/28	A PHA I CD3/28

Table 4.11. Patients were listed alongside whether they were lymphopenic, whether they had a history of opportunistic infections, suggestive of a cellular defect, their clinical details and results of their ^{3}[H] thymidine incorporation and Ki-67 assay. (App = Approach, CF = Cystic fibrosis, CVID = Common Variable Immune Deficiency, NTM = Non tuberculous mycobacteria, HSV = Herpes simplex virus, LRT = Lower respiratory tract, N = Normal, I = Impaired, A = Absent). Approach 1: Use the reference range of 2SDs of the mean and examine CD45 and CD3 T cells values only. Approach 2: Use the reference range of 2SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the reference range for either PHA or anti-CD3/28. Approach 3: Use a reference range of 3SDs from the mean and examine CD45 and CD3 T cells values only. Approach 4: Use the reference range of 3SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the reference range for either PHA or antiCD3/anti-CD28.

4.7 Abnormal ^{3}[H] thymidine incorporation or Ki-67 results could not be attributed to low lymphocyte counts in all cases

To investigate whether abnormal results could be attributed to the patients' lymphopenia, those patients with low CD4 and/or CD8 counts were examined further to see if the lymphopenia corresponded with an impaired or absent result in ^{3}[H] thymidine incorporation and/or the Ki-67 assay (Table 4.12). Patients with low T cell numbers can demonstrate poor proliferative responses (Marits et al., 2014).

Table 4.12. Patient CD4 and CD8 counts and their lymphocyte proliferation results do not necessarily correlate

Patient	CD4 count (Range: 300 – 1400 x10^{6}/L)	CD8 count (Range: 200 – 900 x 10^{6}/L)	Thymidine	Ki-67
2	278	170	N PHA N CD3/28	N PHA N CD3/28 Apart from App 1 – I CD3/28
3	225	108	N PHA A CD3/28	N PHA N CD3/28
6	456	198	N PHA N CD3/28	N PHA N CD3/28
8	437	167	N PHA I CD3/28	Normal PHA N CD3/28 Apart from App 1 – I CD3/28
12	563	128	N PHA N CD3/28	I PHA N CD3/28
19	402	111	N PHA N CD3/28	N PHA N CD3/28
20	294	216	N PHA N CD3/28	N PHA N CD3/28
22	286	318	N PHA N CD3/28	I PHA I CD3/28
25	256	91	N PHA A CD3/28	A PHA I CD3/28

Table 4.12. Patients with a low CD4 and /or CD8 count were examined to see if either the ^{3}[H] thymidine incorporation or Ki-67 assay were abnormal (N = normal, I = Impaired, A = Absent, App = Approach).

Four patients with a low lymphocyte count had normal responses to PHA and anti-CD3/anti-CD28 in both the ^{3}[H] thymidine incorporation and the Ki-67 assay. These patients had only marginally low CD4 and or CD8 counts. Patient 3, had low CD4 and CD8 T cell numbers and demonstrated an absent CD3/CD28 ^{3}[H] thymidine incorporation result, however, the Ki-67 results were normal in all four approaches to data interpretation. Patient 8 had a low CD8 T cell count, and an impaired anti-CD3/CD28 ^{3}[H] thymidine incorporation result, which was normal when examined by the Ki-67 method, apart from Approach 1, the most stringent reference range. Patient 22 had an impaired PHA and anti-CD3/CD28 response by the Ki-67 method, but a normal ^{3}[H] thymidine incorporation result. This patient had only marginally low CD4 T cells and normal CD8 T cells. Patient 25 had both low CD4 and CD8 T cell counts and showed an absent PHA and impaired anti-CD3/28 response by Ki-67, but a normal ^{3}[H] thymidine incorporation result for PHA with an absent response to anti-CD3/anti-CD28.

These results suggest that lymphopenia may not be the only reason for abnormal results. Therefore, all patient results and clinical details for those patients with discrepant results were investigated in greater detail and are discussed in Section 4.10.

Having investigated the correlation of the Ki-67 method with the ^{3}[H] thymidine incorporation method and the patients' clinical picture, the cost and general practicalities of introducing this assay were examined.

4.8 The cost of doing Ki-67 in house is less than sending to referral laboratory for ^{3}[H] thymidine incorporation and the CFSE assay

To further investigate whether the Ki-67 method would be a suitable alternative for sending samples to a referral laboratory for investigation of lymphocyte proliferation, the cost of both assays was examined. The cost of the CFSE assay, which had been performed in our laboratory previously was also examined to compare the cost.

The cost of the ^{3}[H] thymidine incorporation assay at the referral lab was £291.33. This does not include courier costs. Samples need to be at the referral laboratory

within 24 hours of venesection. Depending on when in the day the samples arrive, there are several options to get the samples there on time. Samples are usually sent via the DX system, to arrive the next morning, which is calculated as £8.10 excluding VAT, per box sent. If this is not possible, a courier is used. The cost of a standard courier is £8.29 excluding VAT. The cost of a premium courier, which is sometimes used if the sample needs to be there on the same day is £12.40 excluding VAT. Therefore, the minimum cost of sending the sample to the referral laboratory for ^{3}[H] thymidine incorporation is £300.85 and the maximum cost if a courier is used is £306.21.

To compare costings, the cost of the Ki-67 assay was calculated based on the cost of reagents and the time taken for the assay to be carried out by the appropriate staff member. The hands-on time of each step in the assay was calculated (Figure 4.1).

Figure 4.1. The steps involved in carrying out the Ki-67 lymphocyte proliferation assay and the hands-on time for staff required

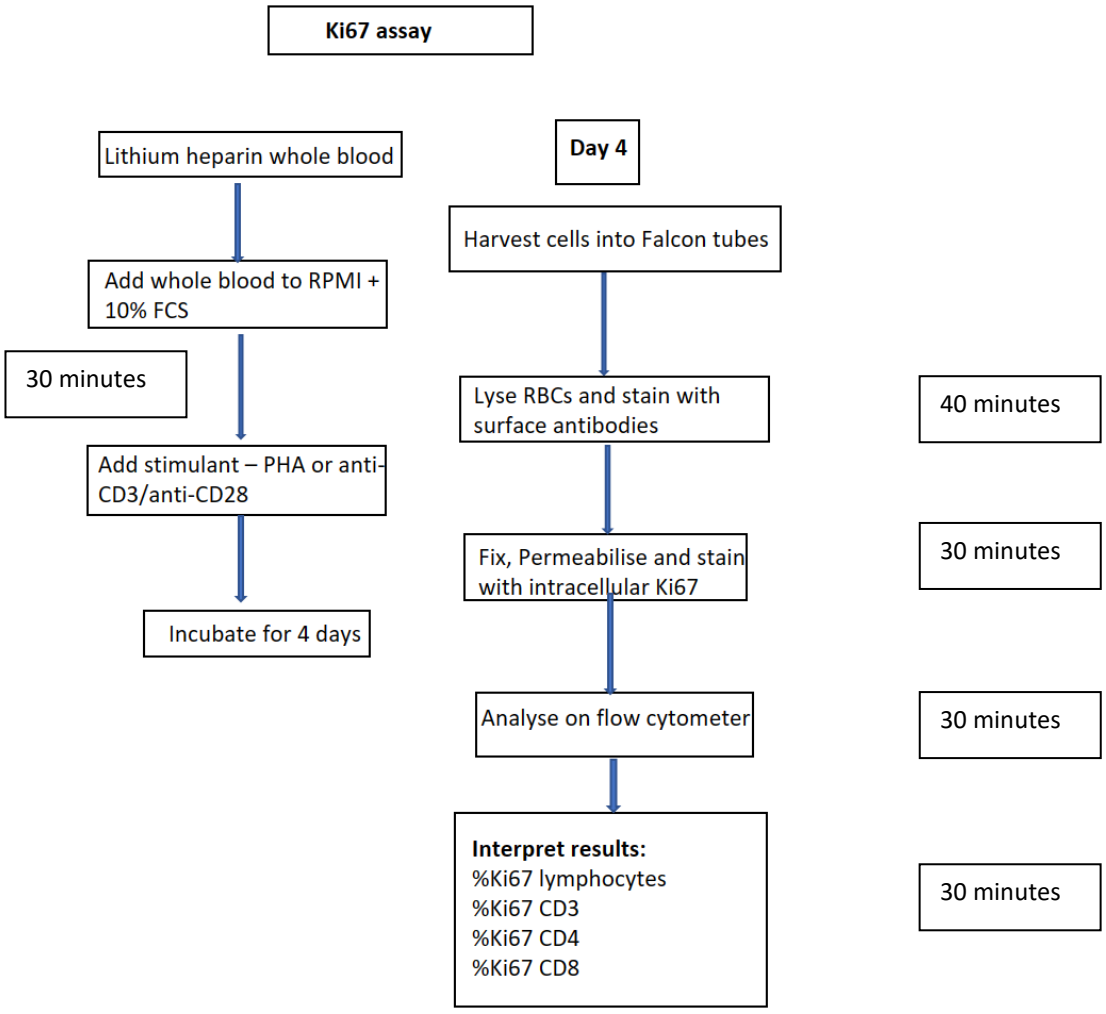


Figure 4.1. The steps involved in setting up the Ki-67 lymphocyte proliferation assay. The staff hands-on time was calculated by timing each step of the testing process, when testing a HC and a patient.

Once the hands-on time had been calculated, staffing costs were calculated. This was done using the New Day Rate for the relevant staff band (Appendix 3). Staffing (pay) and reagent (non-pay) costs were calculated (Appendix 4). The total pay cost was calculated as £71.47 and non-pay as £147.28. Therefore, the total cost of the Ki-67 assay for one HC and one patient was calculated as £218.75, excluding VAT. The FACsLyric flow cytometer is already in use in the department, meaning that new equipment would not need to be purchased. Bulk reagents for this analyser are already in use and are not included in this costing.

The cost of the CFSE assay, when it was done in our laboratory in 2019 cost £207.83, with non-pay being £71.07 and pay costs being £136.76. After adding a percentage to include the consumer price index, this would cost approximately £245.55.

Therefore, the cost of the Ki-67 assay is less than the cost of the ^{3}[H] thymidine incorporation assay at the referral laboratory and less than CFSE which had previously been carried out in our laboratory.

4.9 Practicalities of introducing the Ki-67 assay, including clinic timings and staff training would not be barriers to bringing the Ki-67 assay in-house

When considering the practical aspects of bringing the Ki-67 assay in-house, rather than sending the samples away, the main users of the assay were considered. Most requests for lymphocyte proliferation assays come from the Immunology clinicians from outpatient Immunology clinics. In our hospital, these take place on a Wednesday afternoon and Thursday morning. Therefore, samples from these clinics could be set up on a Thursday afternoon and harvested on a Monday. Any requests received throughout the week could also be set up to be harvested on Day 4. For example, samples arriving on a Monday could be harvested on a Thursday. Samples arriving on a Tuesday could be harvested on a Friday, and samples arriving on a Friday could be harvested on a Tuesday. As described in Section 3.8, further work could be done to establish whether harvesting the samples a day later would be a possibility to give greater flexibility on harvesting of cells.

Appropriate training would need to be given to staff members carrying out the assay. Suitable staff members could easily be identified as they had previously been carrying out the CFSE assay, and the method for the Ki-67 is much simpler. Therefore, training staff should not be a barrier to bringing in this assay.

Other considerations which would need to be addressed would be the writing of an SOP for trained staff members to follow and to devise a record of training and competence. How to report this assay within the limitations of the laboratory IT system would also need to be planned. Finally, the users of this assay would need to be contacted and the changes in testing procedure discussed, so that they were

aware of any change to the assay and the reporting of lymphocyte proliferation results.

4.10 Discussion

The aim of this section was to investigate whether the Ki-67 method was suitable to introduce as an in-house assay for the measurement of lymphocyte proliferation. HCs were used to investigate if the data was normally distributed and then reference ranges were calculated. The reference ranges were based on the mean and 2 or 3SDs from the mean. This was the same way that the CFSE assay had been analysed. Reference ranges were calculated for CD45 positive lymphocytes, CD3 T cells and CD4 and CD8 T cells. When examining the reference ranges for CD4 and CD8 T cells, the percentage of CD4 and CD8 T cells were examined both as a percentage of lymphocytes and as a percentage of T cells. This was because the number of CD8 T cells, as a percentage of lymphocytes, varied greatly between individuals, meaning that the reference range would be too wide and would not be a sensible measure of cell proliferation. This gave a lower degree of variation in the percentage of dividing cells and so this was used when comparing the results using the different approaches to interpretation. The main drawback to the calculation of the refence ranges was sample numbers. Eighteen HCs is a good starting point but ideally a reference range based on a larger number of samples would be drawn up. This could take place once the assay was in use in the laboratory, with a comment added to all reports stating that the test is still under development and that reference ranges may change in the future. All results should be interpreted with caution and in the context of clinical features.

As well as reference ranges, inter- and intra-assay variation were calculated as part of the validation of this assay. The inter-assay variation was less than 20%, with the highest %CV being 19.8% for CD8 T cells, as a percentage of lymphocytes, in response to anti-CD3/anti-CD28 stimulation. When examining CD8 T cells as a percentage of all T cells (CD3 positive), the inter-assay variation was around half, at 9%. In response to PHA the highest CV was 13.7%, for the percentage of Ki-67

positive lymphocytes. This is comparable to the inter-assay variation which was calculated for the CFSE assay when that assay was validated. For the CFSE assay, the highest CV was 17% when looking at the percentage of lymphocytes dividing in response to PHA, looking at one HC run on two separate occasions. The inter-assay variation of the CFSE assay in response to anti-CD3/anti-CD28 was not investigated. The inter-assay variation of the ^{3}[H] thymidine incorporation assay for PHA stimulation is calculated as 28% (Matthew Buckland, personal communication). Intra-assay variation was calculated by running a sample from the single HC three times within the same experiment. The intra-assay variation was less than 14%, with the highest %CV being 13.5% for CD8 T cells in response to anti-CD3/anti-CD28. When examining CD8 T cells as a percentage of all T cells (CD3 positive), the CV was lower, at 8%. This demonstrates that the percentage of CD8 T cells responding to stimulation differs not only among HCs, as seen by the reference ranges, but also within individuals. The CFSE assay showed an intra-assay CV of 6%, which calculated from one HC being run 3 times in the same experiment, in response to PHA only. When examining the Ki-67 assay intra-assay variation in response to PHA, the highest CV was 2.8%, which is less than the CFSE assay. No estimate of intra-assay variation was available for the ^{3}[H] thymidine incorporation assay for comparison.

When comparing the values of intra- and inter-assay variation to the literature, Soares et al., demonstrated intra-assay variation of up to 4.3% for CD4 T cells in the Ki-67 assay, and up to 3.2% for CD8 T cells, when stimulating with PHA for three days. This was calculated from three donors who were tested 5 times in a single experiment (Soares et al., 2010). No data was reported for stimulation with anti-CD3/anti-CD28. The highest intra-assay variation when stimulating with PHA in the Ki-67 assay in our hands, was 2.8%, for CD4 T cells. This is very similar to the data shown by Soares, et al. The intra- and inter-assay variation values for the Ki-67 assay would make this an appropriate test for clinical use. Inter and intra-assay variation for other manual laboratory assays can be 20%, and possibly higher for functional assays. This assay is well below this, demonstrating that this is a robust and consistent assay for measuring lymphocyte proliferation.

Twenty six patients and eight HC were processed for the ^{3}[H] thymidine incorporation assay at the referral laboratory and the Ki-67 assay at the RLH, in order to compare results. Results were compared based on their interpretation and classification of the response as being absent, impaired or normal. All HCs were deemed normal for both Ki-67 and the ^{3}[H] thymidine incorporation assay. Patients tested had a variety of clinical symptoms but were all being investigated for immune deficiencies. Clinical details were not available at the time of testing or interpretation but were gathered afterwards and correlated with results. Four different approaches were tried when considering the interpretation of the Ki-67 assay:

Approach 1: Use the reference range of 2SDs of the mean and examine CD45 and CD3 T cells values only.

Approach 2: Use the reference range of 2SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the reference range for either PHA or anti-CD3/28.

Approach 3: Use a reference range of 3SDs from the mean and examine CD45 and CD3 T cells values only.

Approach 4: Use the reference range of 3SDs of the mean and examine all parameters, calling it impaired if more than 3 parameters were below the ref range for either PHA or anti-CD3/28.

The previous in-house CFSE assay, had ranges calculated on fifty three HCs based on median and 2SDs. For the Ki-67 assay, the mean was used to calculate the ranges, as the median and mean were very similar, and the data appeared to be normally distributed with a minimal negative skew when examining cells proliferating to PHA (Ryu, 2011). The data for T cells stimulated with anti-CD3/anti-CD28 was deemed to be very nearly symmetrical (Gawali, 2021). Reference ranges looking at 2SDs and 3SDS were used to see if there was a difference between the two. Reference ranges using 2SDs may be too stringent and gave too many results which would be classified as impaired, which may not be clinically significant.

Overall, when comparing the Ki-67 assay with the ^{3}[H] thymidine incorporation assay, it was encouraging that so many of the results were concordant, 16/17 of 25 for PHA responses and 13-16 of 24 responses to anti-CD3/anti-CD28.

For PHA, when comparing the ^{3}[H] thymidine incorporation and the Ki-67 assay, approaches 1 and 2 gave the same results; 8 patients were deemed normal in the ^{3}[H] thymidine incorporation assay which were classified as impaired by Ki-67. Approaches 3 and 4 also gave the same results as each other, however, one patient (Patient 11) moved from being classified as impaired in the Ki-67 assay to being normal, when compared to approaches 1 and 2. This patient had alpha-1 anti-trypsin deficiency, a genetic disorder causing lung damage, which would account for their clinical picture of respiratory infections. This patient has no immune defect according to their clinical records. Therefore, approach 3 or 4 would best fit this patient's clinical picture. Patient 25 who had an absent PHA response in the Ki-67 assay but a normal response in the ^{3}[H] thymidine incorporation assay, was lymphopenic, with marginally low CD4 T and low CD8 T cells. When examining the ^{3}[H] thymidine incorporation assay results in more detail, the patient's highest CPM was 14,285 at 8.0μg/ml, with 665 CPM at 4.0 μg/ml and 3587 CPM at 2.0 μg/ml. The lowest control CPM were 64,123 which was much higher than the patient. The results for this patient in the ^{3}[H] thymidine incorporation assay demonstrated a large degree of variation between the PHA stimulated wells. This may be due to operator error or other inherent variation in the ^{3}[H] thymidine incorporation assay. Seven patients showed a marginally low PHA response in the Ki-67 assay compared to the ^{3}[H] thymidine incorporation assay when looking at all four approaches. A single experiment had five patients (Patients 1 – 5 in Table 4.13) where the PHA was marginally impaired compared to the ^{3}[H] thymidine incorporation method. This may have been due to technical issues on the day, rather than a genuine impairment, although the HC and one other patient on the same day had a normal response to PHA. The lot number of the PHA was checked and found to be the same as previous experiments. One other reason for the difference seen between these results is due to these patients showing hyper-proliferation with PHA stimulation and then cell exhaustion. This has been experienced by the referral laboratory and is a reason why 3 concentrations of PHA are used when stimulating

lymphocytes in the ^{3}[H] thymidine incorporation assay (Matthew Buckland, personal communication). Patients 13 and 14 both demonstrated the highest CPM at the lowest concentration of PHA tested, 2.0μg/ml. PHA in the Ki-67 assay was used at 10μg/ml and could contribute to the differences seen between the ^{3}[H] thymidine incorporation and Ki-67 methods. The patients who were tested here could be repeated with differing PHA concentrations to see if their results became normal with a lower PHA concentration. Future work could include using a range of PHA concentrations as routine, in the Ki-67 assay, such as 5μg/ml and 2.5μg/ml and an audit of results, to see if there are any significant differences in results in the same patient and whether this would be a useful addition to the method. This would however, increase the workload slightly, by introducing two more stimulation conditions and as a result, two more tubes of cells for antibody staining. The cost of the assay would also increase slightly.

PHA is the lectin of the red kidney bean and was first found to possess mitogenic ability by Nowell in 1960, changing the assumption that lymphocytes do not proliferate (Sharon and Lis, 2004). It was originally used to agglutinate and remove RBCs in the culture of lymphocytes but was discovered to have mitogenic ability (Nowell, 1960). There is natural variation in responses to PHA, such as in older people, with PHA responses being significantly reduced in healthy elderly people, over 70 years old with no significant health problems (Hallgren et al., 1988). None of the patients in this project were older than 70, however, natural variation in the response to PHA could contribute to the differences seen.

Patient 16, with an impaired response to PHA in the Ki-67 assay but normal in the ^{3}[H] thymidine incorporation assay, has a diagnosis of Cartilage-hair hypoplasia (CHH). CHH is an autosomal recessive disorder with a defect in cellular immunity and short-limbed dwarfism. Patients have distinctive sparse, fine hair (Spickett, 2013). Patients have been shown to have a decreased response to PHA in a number of cohorts (de la Fuente et al., 2011; Kooijman et al., 1997; Mäkitie, Kaitila and Savilahti, 1998). Kooijman et al, found that 69% of patients in a cohort of 35, had reduced lymphocyte proliferation in response to PHA (Kooijman et al., 1997). Therefore, the Ki-67 result may be more closely aligned with the patient's clinical picture, in this case. Using approaches 3 or 4 and interpreting the results in the

context of the patient's clinical details appears to be the best approach when interpreting the lymphocyte proliferation response in the Ki-67 assay.

Table 4.13. Discrepant results for PHA stimulation between the ^{3}[H] thymidine incorporation and Ki-67 assay were examined in more detail and compared to patient clinical details

Patient	Clinical details	Comments
11	Alpha 1 antitrypsin deficiency	Normal Ki-67 with approaches 3 and 4, normal ^{3}[H] thymidine
12	Pan-hypogammaglobulinemia, lymphopenia, recurrent chest infections	Marginally impaired Ki-67, normal ^{3}[H] thymidine
13	Recurrent history of fevers	Impaired Ki-67, normal ^{3}[H] thymidine
14	One episode of a vesicular rash	Impaired Ki-67, normal ^{3}[H] thymidine
15	Airway disease, asthma, recurrent LRT infections. Low IgM, previous low IgG which is now normal.	Impaired Ki-67, normal ^{3}[H] thymidine
16	Cartilage-hair hypoplasia	Impaired Ki-67, normal ^{3}[H] thymidine
22	Borderline lymphopenia, low IgG and IgM, autoimmune haemolytic anaemia.	Marginally low CD4 counts, marginally impaired Ki-67, normal ^{3}[H] thymidine
24	Pan- hypogammaglobulinemia and recurrent genital herpes	Impaired Ki-67, normal ^{3}[H] thymidine
25	CF, long-term steroid use, bronchiectasis	Absent PHA response in Ki-67 assay with only 10% of CD3 T cells proliferating.

Table 4.13. Patients with discrepant results between the Ki-67 and ^{3}[H] thymidine incorporation assay, when stimulated with PHA, were listed and clinical details investigated. (LRT = Lower respiratory tract, CF = Cystic fibrosis).

When T cells were stimulated with anti-CD3/anti-CD28, there appeared to be greater concordance between the ^{3}[H] thymidine incorporation and Ki-67 assay (Table 4.14). This may be due to stimulation with anti-CD3/anti-CD28 being a more physiological way of stimulating T cells.

Seven out of nine of the discrepant results were due to an impaired ^{3}[H] thymidine incorporation result, with the Ki-67 assay giving a normal result. Two patients had a normal ^{3}[H] thymidine incorporation result but an impaired Ki-67 assay. However, these results were classified as normal in approaches 3 and 4, using 3SDs as the reference ranges. Therefore approaches 3 and 4 may be the most appropriate way to interpret the Ki-67 results.

When considering other potential differences in the Ki-67 and ^{3}[H] thymidine incorporation and how this may lead to differences in results, the ^{3}[H] thymidine incorporation assay uses a CD28 cell supernatant and therefore the concentration is not known. The anti-CD28 used in the Ki-67 assay was a commercial antibody with a known concentration. Cellular supernatant could also contain cell debris which could contribute to proliferation through pathways other than the CD28 pathway. The ^{3}[H] thymidine incorporation assay is also performed by coating the tissue culture plate with the anti-CD3/anti-CD28. Differences in coating could contribute to variability in results.

Table 4.14. Discrepant results for anti-CD3/anti-CD28 stimulation between the ^{3}[H] thymidine incorporation and Ki-67 assay were examined in more detail and compared to patient clinical details

Patient	Clinical details	Comments
3	Lymphopenic, CVID on Ig replacement with sinopulmonary infections. No history of opportunistic infections.	Normal Ki-67, absent ^{3}[H] thymidine
7	One episode of HSV-2 meningoencephalitis, no history of recurrent infections, Elsberg syndrome	Normal Ki-67, impaired ^{3}[H] thymidine
8	Neutropenia, ^{3}[H] thymidine incorporation repeated March 2023 - normal	Normal Ki-67, apart from approach 1, impaired ^{3}[H] thymidine
13	Recurrent history of fevers	Normal Ki-67, apart from approach 1, impaired ^{3}[H] thymidine
14	One episode of a vesicular rash	Normal Ki-67, apart from approach 1, impaired ^{3}[H] thymidine
16	Cartilage-hair hypoplasia	Normal Ki-67 in approach 4 only, impaired ^{3}[H] thymidine
17	Recurrent infections in the context of severe atopy, poor responses to polysaccharide vaccine, TH17 T cells normal	Normal Ki-67, impaired ^{3}[H] thymidine
22	Borderline lymphopenia, low IgG and IgM, autoimmune haemolytic anaemia.	Impaired Ki-67 in approaches 1 and 2, normal ^{3}[H] thymidine
25	CF, long-term steroid use, bronchiectasis	Impaired Ki-67 in approaches 1 and 2, normal ^{3}[H] thymidine

Table 4.14. Patients with discrepant results between the Ki-67 and ^{3}[H] thymidine incorporation assay, when stimulated with anti-CD3/anti-CD28, were listed and clinical details investigated. (CVID = Common Variable Immune Deficiency, HSV = Herpes simplex virus, CF = Cystic fibrosis)

When comparing the results obtained in this project with the literature, Lastovicka et al, compared the Ki-67 assay with ^{3}[H] thymidine incorporation and the CFSE assay (Lastovicka, Rataj and Bartunkova, 2016). They found that the CFSE assay gave lower values, of approximately 10%, compared to both the Ki-67 assay and ^{3}[H] thymidine incorporation, which they attributed to the toxicity of CFSE on the proliferating cells. Good correlation was found between the Ki-67 and ^{3}[H] thymidine incorporation assays. There are some differences in their methodology compared to that used in this project. Firstly, PBMCs were used throughout, whereas whole blood was used in this project. Secondly, the cut-off used when comparing the Ki-67 assay with ^{3}[H] thymidine incorporation was also different. They used a Stimulation index (SI) when reporting the ^{3}[H] thymidine incorporation assay, using the PHA stimulated/unstimulated CPM. When comparing their results, they correlated an SI of 50, which they state is commonly used for interpreting ^{3}[H] thymidine incorporation results, with 40% of Ki-67 positive cells (Lastovicka, Rataj and Bartunkova, 2016). This is a different approach to that reported by the referral laboratory for ^{3}[H] thymidine incorporation results, which only reports CPM. Thirdly, the data was analysed after 72 hours of stimulation whereas, in this project 96 hours was used, as this afforded direct comparison between the ^{3}[H] thymidine incorporation and Ki-67 assays. Therefore, there may be differences in the results of their method comparison, and those of this project. Also, only data for PHA stimulated T cells were discussed in this paper, responses to anti-CD3/anti-CD28 were not examined.

Other differences when comparing ^{3}[H] thymidine incorporation results with those of the Ki-67 assay is that they are measuring different parts of the cell cycle. ^{3}[H] thymidine incorporation looks at cells in the S phase of proliferation, during the last few hours of the cell culture, after the thymidine has been added (Lastovicka, Rataj and Bartunkova, 2016). Ki-67 is expressed in proliferating cells during G1, G2 and S phases and may therefore, be a more sensitive assay for detecting defects in proliferation (Gerdes et al., 1984; Lastovicka, Rataj and Bartunkova, 2016). Other technical contributors to variability and thereby potential differences in results could be differences in the detector calibration of the scintillation counter. This needs to be adjusted for isotope decay and can contribute to variability of the assay

(Matthew Buckland, personal communication). Additional disadvantages of the ^{3}[H] thymidine incorporation assay are the cost of the equipment used, the health and safety aspects of the radioactivity involved and the maintenance of the cell harvester. This must be carefully maintained, due to its propensity to become colonised with fungal growth. Also, in the Ki-67 assay T cells can be identified and therefore if there are low T cell numbers this will become apparent during flow cytometeric interpretation of the results, as lymphocytes and T cells are stained in this method. This is not possible in the ^{3}[H] thymidine incorporation method, therefore low T cell counts could lead to a low result. The Ki-67 assay has the additional quality assessment of knowing if there is T cell lymphopenia within the assay.

When considering the cost of the Ki-67 assay compared to both the ^{3}[H] thymidine incorporation and CFSE assays, the cost of the Ki-67 was less than the other two assays. This is in contrast to Lastovicka et al, who found, the CFSE assay was the most economical, with Ki-67 and ^{3}[H] thymidine incorporation being 'near-equal cost' (Lastovicka, Rataj and Bartunkova, 2016). The method used by this group to calculate this and final costs are not shown. The analysis in this project shows the ^{3}[H] thymidine incorporation assay to be the most expensive, followed by the CFSE assay, with the Ki-67 assay coming in at the most economical. The caveats to this are that the cost of the CFSE assay was calculated using a 'top-down' method, and the Ki-67 assay was calculated using a 'bottom-up' approach (Chris Scott, personal communication). Also, as the costing for the CFSE assay was done several years ago before the assay was discontinued, a percentage was added, to account for price-rises since that time. This is an approximation and not completely accurate. The main advantage of using the Ki-67 assay over the CFSE assay is the amount of staff hands-on time required during set up of the assay and also for result interpretation (Figure 4.2). This is substantially decreased in the Ki-67 assay (Figure 4.1). The complexity of the Ki-67 assay could be simplified further by just staining for anti-CD45 and anti-CD3, and not staining for CD4 and CD8 T cells. This would decrease the cost further and the complexity of the harvesting and staining procedure on day 4 of the assay. The different approaches to interpretation of results were identical between approach 3 which only used CD45 and CD3 positive Ki-67 results, and

approach 4 using the other parameters for PHA stimulation and very similar in response to anti-CD3/anti-CD28, meaning that only staining for CD45 and CD3 could be used in most cases and the option of examining CD4 and CD8 responses, could be given to clinicians looking to investigate subtle T cell defects in more detail.

Figure 4.2. The steps involved in setting up the CFSE assay

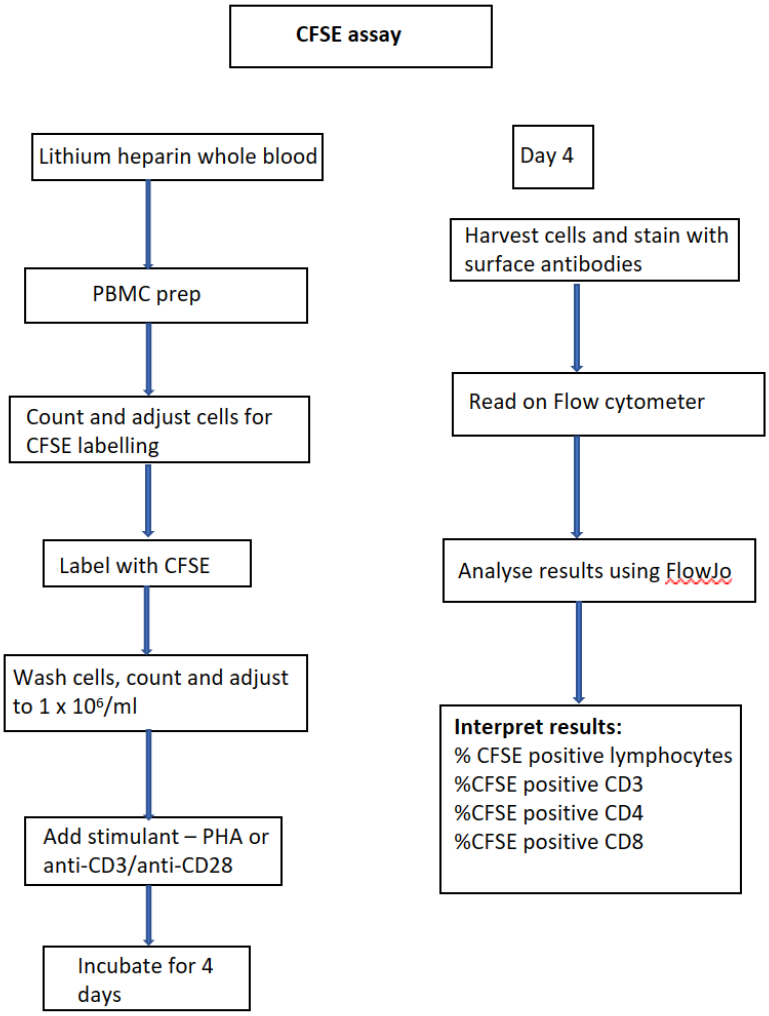


Figure 4.2. The steps involved in setting up the CFSE lymphocyte proliferation assay. The set up time takes one whole day.

When considering other practical challenges to the introduction of the Ki-67 assay, no other significant barriers were identified. Staff training, using the existing flow cytometer and Information Technology (IT) issues could all be overcome and would be significantly easier than when the CFSE assay was used. It would also allow staff

who were experienced in the CFSE assay to continue to use those skills. This would contribute to greater staff morale and retention. Many assays in a clinical laboratory are automated and the inclusion of some manual assays for skilled staff would keep their interest in the role.

In conclusion, the Ki-67 assay would be suitable to use as a routine test to measure lymphocyte proliferation in a clinical immunology laboratory. The inter- and intra-assay variation was good and comparable to those in the literature and other assays for lymphocyte proliferation in our experience. Results of the Ki-67 compared well with the ^{3}[H] thymidine incorporation assay and differences seen would be acceptable, given that this assay should always be interpreted in conjunction with the patient's clinical picture and in conjunction with other assays in the investigation of immune deficiency.

5. Investigating the use of the Ki-67 assay to assess VZV-specific T cell responses

5.1 Introduction

As it had been established that the Ki-67 assay could be implemented for clinical use in measuring lymphocyte proliferation to mitogen (PHA) and through the T cell receptor (anti-CD3/CD28), the question was asked as to whether it was also suitable for examining the antigen-specific responses of VZV-specific T cells.

VZV is a virus causing the common childhood illness, chicken pox. The virus becomes latent after primary infection, in cranial nerves and dorsal root ganglia without causing clinical symptoms in immune competent individuals (Weinberg, A. and Levin, 2010). There is a higher risk of viral reactivation, causing shingles, or HZ, in older individuals as well as individuals with an impaired immune system. Older adults have a higher morbidity associated with HZ, which can lead to post-herpetic neuralgia and a reduction in quality of life (Sadaoka et al., 2008; Weinberg et al., 2017). Those individuals with impaired immunity, can develop overwhelming varicella, leading to hepatitis, pneumonitis and multi-organ failure. Cellular immunity is essential in the response to VZV, and the presence of memory T cells are thought to be responsible for the avoidance of viral re-activation. Despite the importance of cellular immunity in the response to VZV, anti-VZV antibodies, IgG and IgM, are measured to assess immunity to VZV. VZV antibody levels do not always correlate with protection against viral re-activation or disease severity (Gershon and Steinberg, 1981; Sadaoka et al., 2008; Webster et al., 1989). Therefore, the measurement of VZV-specific T cell proliferation may be a better measure of protection.

In the PID clinic at the RLH, there are a significant minority of patients with recurrent or severe HZ. Little is known about cellular responses to VZV and therefore having a measure of T cell immunity would contribute to knowledge of this response. It may also give an alternative measure of protection against VZV re-

activation. Prior to the advent of the recombinant VZV vaccine, the measurement of T cell responses to VZV could also have been used as an indication of whether the live vaccine was safe to be given to immunocompromised individuals, such as those awaiting stem cell transplants.

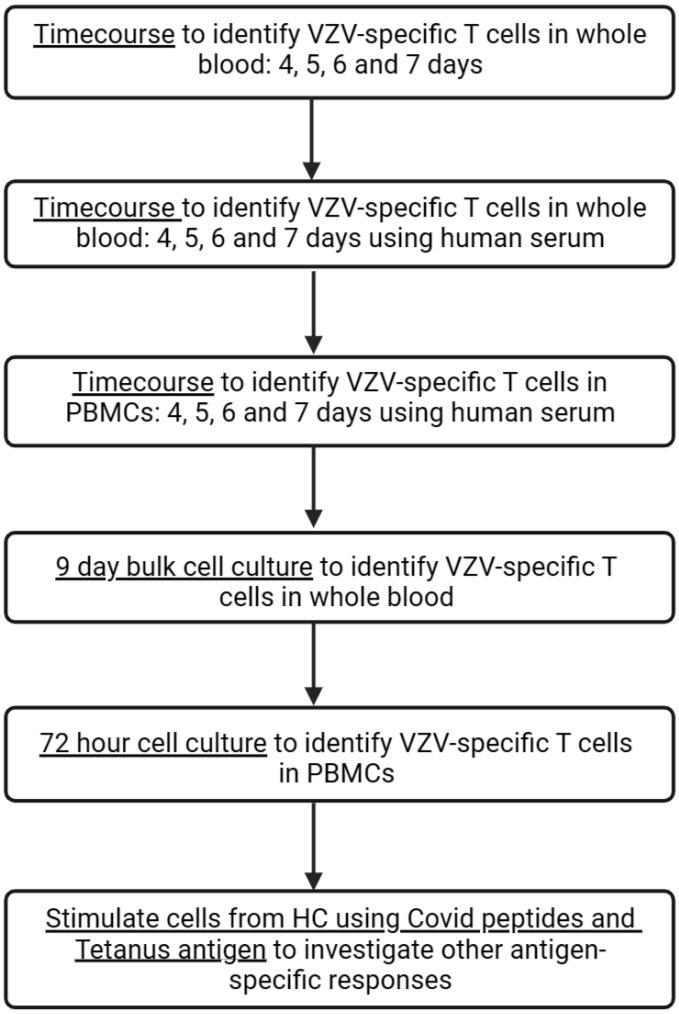
In this project, a number of different cell culture conditions were used to investigate the ideal conditions for identification of VZV-specific T cells. Two HCs were used who were less than 30 years old and had a history of chicken pox in childhood. Both were positive for VZV IgG antibodies, as measured in the Virology department of the RLH. Initially, whole blood was used for culturing lymphocytes as this is easier to set up as a routine assay. The preparation of PBMCs entails additional steps to the assay set up. The timings for carrying out antigen-specific T cell stimulation vary in the literature (Haredy et al., 2019; Li et al., 2012; Vukmanovic-Stejic et al., 2015). Timecourse experiments were planned to optimise identification of VZV-specific T cells. Different culture media supplementation was also tried. FCS was used in initial experiments, as this had been used in the previous Ki-67 experiments. Normal human serum was also tried as a culture media supplement, to see whether having a more physiological culture supplementation was conducive to identifying antigen-specific T cells.

Haredy et al., used a whole blood, bulk culture method of T cell stimulation in order to identify VZV-specific T cells, and to overcome the difficulties associated with detecting low numbers of antigen specific T cells (Haredy et al., 2019). These culture conditions were recreated to see if they were applicable to the Ki-67 method. Also, Vukmanovic-Stejic et al., demonstrated the prescence of Ki-67 positive VZV-specific T cells after culturing PBMCs for 72 hours (Vukmanovic-Stejic et al., 2015). These culture conditions were also recreated. Unfortunately, due to the Covid-19 pandemic, many patients did not attend the hospital for face-to-face appointments and therefore, the opportunity to test patients pre- and post-VZV vaccination was not forthcoming.

The VZV peptide used in these experiments were against immediate-early protein 63 (IE63). This protein is thought to be important in the cellular immune response to VZV, including in latency (Jacquet et al., 2002). It has been used in lymphocyte

proliferation assays in mice, who had been immunised with an IE63 fusion protein, as measured by ^{3}[H] thymidine incorporation (Jacquet et al., 2002). Matthias et al., used a peptide pool covering IE63 to assess T cell responses, using IFN-γ production as a readout, in patients diagnosed with multiple sclerosis (MS) who had been treated with fingolimod, to investigate the differences in T cell response of patients to VZV with or without treatment (Mathias et al., 2016). Patients treated with fingolimod have a higher incidence of HZ (Calabresi, Radue and Goodin, 2014). They examined T cell responses in patients with MS who were treated with fingolimod and found that two patients who developed HZ after 6 months of treatment, had no detectable T cell response to VZV/IE63, whereas patients who did not develop HZ demonstrated a detectable cellular response (Mathias et al., 2016). Patients who did not develop HZ also did not demonstrate a T cell response to VZV lysate, indicating the importance of IE63 in the cellular VZV response. All patients were positive for VZV antibodies, demonstrating that the measurement of cellular responses may be a better indicator of protection against HZ than antibody responses. The presence of anti-IE63 antibodies in serum in individuals with a history of VZV infection was shown by Sadzot-Delvaux et al., (Sadzot-Delvaux, Arvin and Rentier, 1998). They also demonstrated the ability of purified CD8 T cells to lyse IE63-expressing transfected cells to the same extent as IE62, a known immunogenic VZV protein, further illustrating the importance of IE63 in the immune response to VZV (Sadzot-Delvaux, Arvin and Rentier, 1998).

5.2 Flowchart of parameters investigated in this chapter



5.3 VZV-specific T cells were not detected when stimulating whole blood for 4, 5, 6 or 7 days

To investigate whether VZV-specific T cells could be identified in healthy individuals, different cell culture conditions were set up. A timecourse experiment was set up to determine the optimal day for VZV-specific T cells identification. Whole blood was stimulated with PHA, anti-CD3/anti-CD28, VZV peptides diluted in dimethylsulfoxide (DMSO), DMSO alone and some cells left unstimulated. Cells were cultured and harvested on days 4, 5, 6, 7 and RBCs lysed. FCS was used to supplement the media for cell culture. Cells were stained with surface antigens anti-CD45, anti-CD3, anti-CD4, anti-CD8 and anti-CD14, as described in sections 3.3 and 3.4. They were then fixed, permeabilised and stained with anti-Ki-67. Gates

were set on unstimulated cells to examine the percentage of CD3 T cells which had proliferated in response to VZV peptide (Figure 5.1). Cells stimulated with PHA and anti-CD3/anti-CD28 were used as technical controls (data not shown). The HC used in this experiment was less than thirty years old with a history of childhood VZV infection. They also were positive for VZV IgG antibodies with levels >150 mIU/ml, demonstrating evidence of humoral immunity to VZV.

Figure 5.1. The percentage of Ki-67 positive T cells was no different in unstimulated, VZV stimulated or DMSO T cells, on days 4, 5, 6 or 7

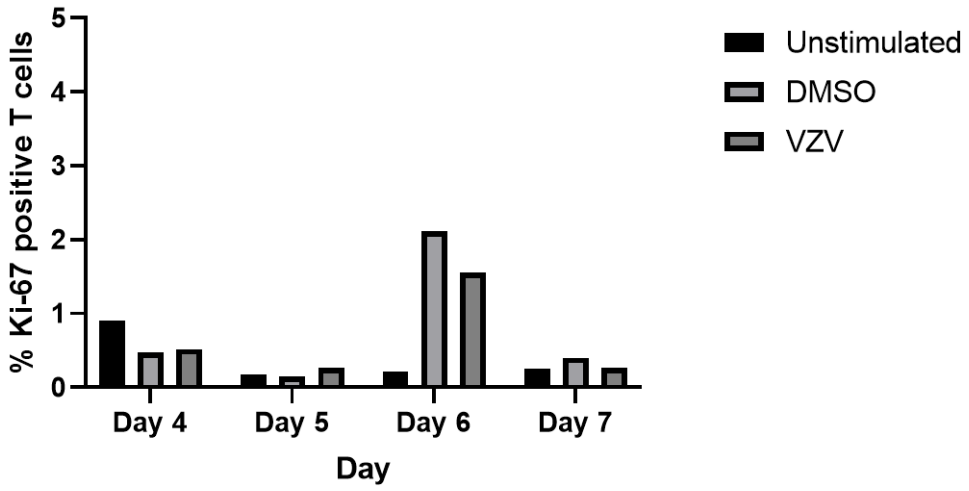


Figure 5.1. Whole blood from a HC was stimulated with PHA, anti-CD3/anti-CD28, VZV-specific peptides, DMSO or left unstimulated. Cells were harvested and RBC lysed on days 4, 5, 6 and 7. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of Ki-67 positive CD3 T cells when VZV peptides were added or DMSO, were compared with unstimulated cells. PHA stimulated Ki-67 T cells were greater than 70% (data not shown). At least 10,000 CD3 T cells were analysed.

At each timepoint, there was no noticeable difference between the number of Ki-67 positive T cells in those cell cultures which had VZV peptides added, DMSO added or left unstimulated. PHA stimulated cells in the same experiment demonstrated at least 70% of T cells being Ki-67 positive as seen in previous experiments, see Section 3.5.

To examine the effect of changing culture conditions on detection of VZV-specific T cells, normal human serum was used instead of FCS to supplement the cell culture medium. Whole blood was used and was stimulated with PHA, anti-CD3/anti-CD28,

VZV peptides or DMSO and some cells left unstimulated. Cells were cultured and harvested on days 4, 5, 6, 7 and RBCs lysed. DMSO was used as a control (Figure 5.2). Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8, before fixing, permeabilization and intracellular staining with anti-Ki-67. The percentage of Ki-67 positive T cells were examined at each timepoint (Figure 5.2).

At each timepoint, there was no noticeable difference between the number of Ki-67 positive T cells in those cell cultures which had VZV peptides added, DMSO added or left unstimulated. PHA stimulated cells in the same experiment demonstrated at least 70% of T cells being Ki-67 positive (data not shown).

Figure 5.2. Supplementing the culture medium with normal human serum does not lead to an increase of Ki-67 positive T cells in response to VZV stimulation

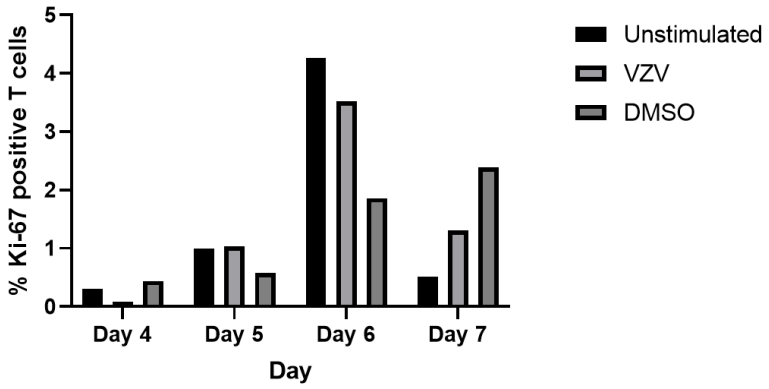


Figure 5.2. Whole blood from a HC was stimulated with PHA, anti-CD3/anti-CD28, VZV-specific peptides, DMSO or left unstimulated. Cells were harvested and RBC lysed on days 4, 5, 6 and 7. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of CD3 T cells were compared between unstimulated, VZV stimulated and those with DMSO. PHA stimulated Ki-67 positive T cells were greater than 60% (data not shown). 3000 – 10,000 T cells were analysed.

5.4 VZV-specific T cells were not detected when stimulating PBMCs for 4, 5, 6 or 7 days

PBMCs were used rather than whole blood to enumerate VZV-specific T cells in cell culture rather than whole blood. PBMCs were prepared by diluting whole blood in RPMI and layering over Lymphoprep. After centrifugation, the mononuclear cell

layer was removed and washed twice before re-suspending at 1 x 10^6/ml. Cells were re-suspended in culture medium containing 10% normal human serum and plated into sterile 96 well plates. Cells were left unstimulated, stimulated with PHA, anti-CD3/anti-CD28, VZV peptide or DMSO and cells harvested on days 4, 5, 6 and 7. After harvesting the cells, PBS was added to each well and left for 30 minutes to remove any adherent cells from the base of the well. This was then harvested and added to the previously harvested cells. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8, before fixing, permeabilisation and intracellular staining with anti-Ki-67. The percentage of Ki-67 positive T cells were examined at each timepoint (Figure 5.3).

At each timepoint, there was no noticeable difference between the number of Ki-67 positive T cells in those cell cultures which had VZV peptides added, DMSO added or left unstimulated.

Figure 5.3. The percentage of Ki-67 positive T cells was no different in unstimulated, VZV stimulated or DMSO T cells, on days 4, 5, 6 or 7 when examining PBMCs rather than whole blood

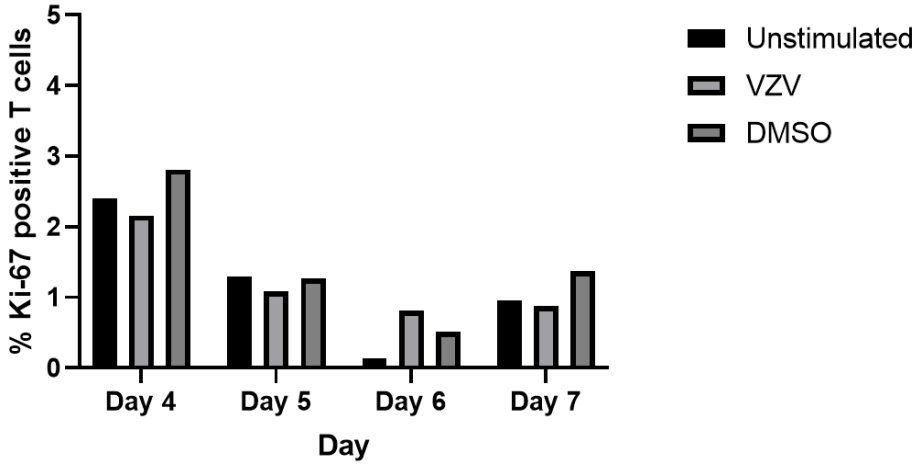


Figure 5.3. PBMCs from a HC were stimulated with PHA, anti-CD3/anti-CD28, VZV-specific peptides, DMSO or left unstimulated. Cells were harvested on days 4, 5, 6 and 7. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of CD3 T cells were compared between unstimulated, VZV stimulated and those with DMSO. PHA stimulated Ki-67 positive T cells were greater than 60% (data not shown). At least 2000 T cells were analysed.

5.5 VZV-specific T cells were not identified in bulk whole blood cultures harvested after 9 days

Haredy et al., used a whole blood method of T cell stimulation in order to identify VZV-specific T cells, and to overcome the difficulties associated with detecting low numbers of antigen specific T cells (Haredy et al., 2019). In this paper, bulk culture conditions were set up using 1ml of patient blood for each stimulation condition and cells were stimulated with a VZV infected cells extract or an uninfected cell line extract. $CD3^{+}CD4^{high}$ T cells with CD45RO and CCR7 (memory phenotype) positivity were identified only in cell cultures stimulated with VZV antigen and were at their highest frequency on day 9 (Haredy et al., 2019). This population was also found to express intracellular granzyme B, as a marker of cytotoxic T cell function. The same culture conditions were applied to the Ki-67 method, to examine if VZV-specific T cells could be identified. 1ml of HC blood was diluted with 9ml complete media, supplemented with 10% FCS, in accordance with the paper. Cells were left unstimulated, stimulated with PHA, VZV peptides or DMSO added. Cells were cultured in sterile 50ml Falcon tubes with the lids loosely fitted at an incline. After 9 days the Falcon tubes were centrifuged and RBCs lysed. After washing once, cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8, before fixing, permeabilisation and intracellular staining with anti-Ki-67. The percentage of Ki-67 positive T cells were examined (Figure 5.4).

Figure 5.4. There is no difference in the percentage of Ki-67 positive T cells when whole blood is stimulated in a bulk culture for 9 days with VZV peptides, DMSO control or left unstimulated

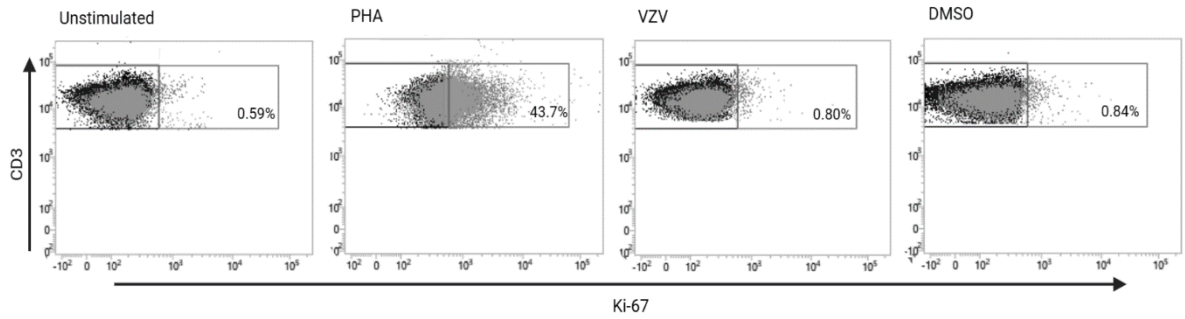


Figure 5.4. Whole blood was cultured in a 10ml bulk culture for 9 days in complete media and stimulated with PHA, VZV-specific peptides, DMSO or left unstimulated. Cells were harvested on days 9 and RBCs lysed. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8, followed by intracellular staining with anti-Ki-67. The percentage of CD3 T cells were compared between unstimulated, VZV stimulated and those with DMSO. At least 20,000 T cells were analysed in each condition.

A greater overall number of T cells could be analysed using this method, however, no difference in the number of Ki-67 positive T cells was seen between unstimulated cells, those with DMSO added and those stimulated with VZV peptides.

5.6 No significant increase in VZV-specific T cells were seen when stimulating PBMCs for 72 hours

Vukmanovic-Stejic et al. successfully demonstrated the presence of VZV-specific T cells after 72 hours of stimulation with VZV lysate in young and old HCs (Vukmanovic-Stejic et al., 2015). These were detected by ^{3}[H] thymidine incorporation after 6 days of culture and using Ki-67 after 3 days. The culture conditions used in this paper were recreated, however, the same VZV peptides which had been used throughout this project were used, as we did not have access to VZV lysate. PBMCs were prepared by diluting whole blood in RPMI and layering over Lymphoprep. After centrifugation, the mononuclear cell layer was removed and washed twice before counting the cells and re-suspending them at 1 x 10^{6}/ml. Cells were re-suspended in culture medium containing 10% FCS and plated into sterile 24 well plates, 1ml per well. Cells were left unstimulated, or stimulated with PHA, VZV peptide or DMSO and cells harvested after 72 hours. After harvesting the cells, PBS was added to each well and left for 30 minutes to remove any adherent cells from the base of the well. This was then harvested and added to the cells which had previously been harvested. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8, before fixing, permeabilisation and intracellular staining with anti-Ki-67. The percentage of Ki-67 positive T cells were examined (Figure 5.5).

Figure 5.5. There is a small increase in the percentage of Ki-67 positive T cells when PBMCs are stimulated for 72hours with VZV peptides, compared to DMSO control or left unstimulated

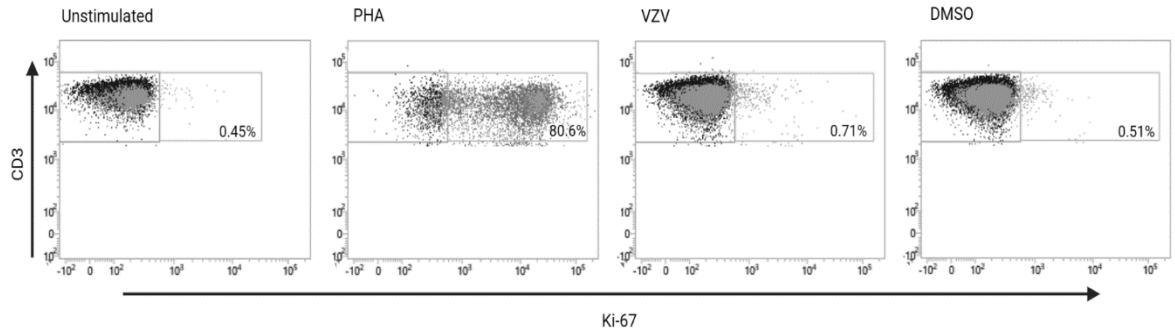


Figure 5.5. PBMCs from a HC were stimulated with PHA, anti-CD3/anti-CD28, VZV-specific peptides, DMSO or left unstimulated. Cells were harvested after 72 hours. Cells were surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of CD3 T cells were compared between unstimulated, VZV stimulated and those with DMSO. At least 2000 T cells were analysed.

The percentage of Ki-67 positive T cells was slightly higher in those cells stimulated with VZV peptides compared to those with only DMSO added. However, as this experiment was only carried out on one HC, the significance of this result is unclear.

5.7 The Ki-67 method of lymphocyte proliferation was able to identify antigen-specific T cells in a HC with recent Covid-19 infection and when stimulating with Tetanus toxoid (TT) antigen

In order to determine whether the Ki-67 method of lymphocyte proliferation was able to detect antigen-specific T cells, a HC with Covid-19 infection 10 weeks previously was tested. PBMCs were separated from whole blood and washed twice before re-suspending in 4ml RPMI supplemented with 10% normal human serum. Cells were stimulated with Covid peptides Spike (S), Nucleocapsid (N) and Membrane (M) at a final concentration of 1, 0.5 and 0.25μg/ml in 96 well plates. These concentrations were taken from the internal SOP for detecting Covid-19 proliferation in the referral laboratory and their published method (Awuah et al., 2022). After 4 days of culture, cells were harvested and washed once with PBS before surface staining with anti-CD45, anti-CD3, anti-CD14, anti-CD4 and anti-CD8, and fixing, permeabilization and intracellular staining with anti-Ki-67. The

percentage of Ki-67 positive T cells were examined (Figure 5.6). Samples were run in parallel at GOSH immunology laboratory for Covid T cell proliferation using ^{3}[H] thymidine incorporation. The subject had Covid infection 10 weeks prior to being tested.

Figure 5.6. Stimulation of PBMCs from a recently infected HC with Covid peptides shows an increase in Ki-67 positive T cells compared to unstimulated cells

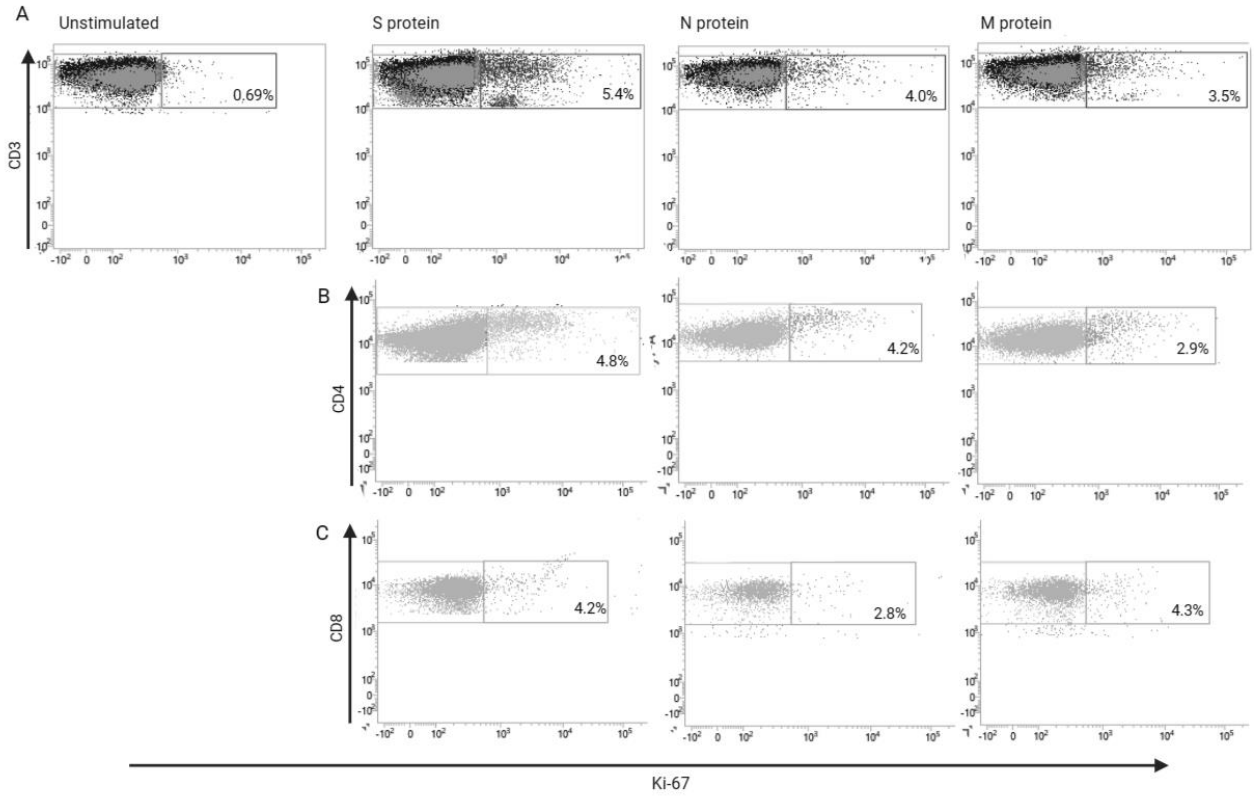


Figure 5.6. PBMCs from a HC who had recent Covid-19 infection were stimulated with Covid peptides Spike (S), Nucleocapsid (N) and Membrane (M), PHA or left unstimulated for 4 days. Cells were then harvested, and surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of CD3, CD4 and CD8 T cells were compared between cells which were unstimulated and those stimulated with COVID peptides. Data shown for peptide concentration of 1μg/ml. At least 20,000 T cells were analysed. **A:** Percentage of Ki-67 positive T cells. **B:** Percentage of Ki-67 positive CD4 T cells, **C:** Percentage of Ki-67 positive CD8 T cells.

A higher percentage of Ki-67 positive T cells were seen in those cells stimulated with Covid-19 peptides than those left unstimulated, with the highest percentage being 5.2% with Spike protein. A proliferative response in both CD4 and CD8 T cells, could be detected to COVID-19 peptides. The same HC was also tested at GOSH for Covid-19 peptide stimulation using the same assay set up and using ^{3}[H] thymidine

incorporation as the readout (Appendix 5). These results showed that the HC proliferated in response to all three Covid peptides, with the highest proliferation seen in response to S protein. This is consistent with the results seen in the Ki-67 assay and demonstrates that the Ki-67 method of lymphocyte proliferation can be used in measuring antigen-specific responses.

Stimulation of lymphocytes with TT antigen was also undertaken. PBMCs from a HC were separated from whole blood and washed twice before re-suspending at 1 x 10^6/ml in RPMI supplemented with 10% normal human serum. Cells were stimulated with TT antigen at a final concentration of 10μg/ml in a 96 well plate. After 7 days of culture, cells were harvested and washed once with PBS before surface staining with anti-CD45, anti-CD3, anti-CD14, anti-CD4 and anti-CD8, before fixing, permeabilization and intracellular staining with anti-Ki-67. The percentage of Ki-67 positive T cells were examined (Figure 5.7). The HC had TT IgG antibodies tested in the Immunology department at RLH and had a tetanus IgG level of 2.4 IU/ml, which is consistent with long term protection.

Figure 5.7. Stimulation of PBMCs with Tetanus toxoid antigen showed an increase in Ki-67 positive T cells compared to cells left unstimulated

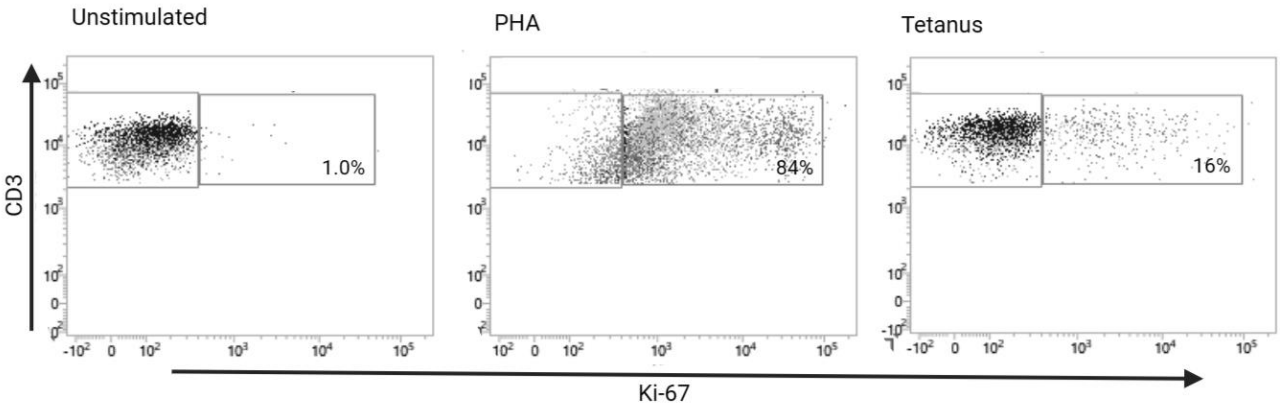


Figure 5.7. PBMCs from a HC were stimulated with Tetanus toxoid antigen, PHA or left unstimulated for 7 days. Cells were then harvested and surface stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8. Cells were also stained with intracellular anti-Ki-67. The percentage of CD3, CD4 and CD8 T cells were compared between cells which were unstimulated and those stimulated with TT. Data for CD4 and CD8 T cells not shown as the cell numbers acquired were less than 2000. At least 2000 T cells were analysed.

A higher percentage of Ki-67 positive T cells were seen in those cultures stimulated with TT, compared to cells which were left unstimulated, demonstrating that antigen-specific T cells can be detected by the Ki-67 method in our hands.

5.8 Discussion

The aim of this chapter was to investigate the suitability of the Ki-67 method of lymphocyte proliferation to measure antigen-specific T cells responses, namely to VZV peptides. In order to investigate this, several different methods were attempted. These were either adapted from our own laboratory experience of measuring antigen-specific T cells or by attempting the recreate cell culture conditions from the literature.

Cell culture conditions using whole blood was attempted first. As this was being investigated as a routine clinical test, the easiest method of set up was sought. A timecourse from day 4 to day 7 was set up, to investigate whether VZV-specific T cells could be detected on any day. Supplementing the cell culture media with FCS or normal human serum was tried. FCS had been used up to this point in the project and so was used first. It has also been used by others when examining antigen-specific T cells (Haredy et al., 2019; Li et al., 2012). Human serum had also been used, as an alternative to FCS in our own laboratory when examining antigen-specific T cells, and by others (Asanuma et al., 2000; Awuah et al., 2022; Vukmanovic-Stejic et al., 2015). The use of normal human serum in cell culture more closely resembles the usual physiological conditions in which human lymphocytes grow and proliferate, which may be optimal when detecting rarer events, such as antigen specific T cells. It may also reduce noise from stimulation with irrelevant xenotypic calf antigens (Honarvar et al., 2013). However, when using whole blood and supplementing the media with FCS or normal human serum, no difference was seen in the number of Ki-67 positive T cells compared to those cells which were unstimulated or those which had DMSO added, as a background control. Separated PBMCs were also tried in the same timecourse experiment, with normal human serum used to supplement the media. This also did not show any

difference between unstimulated cells, those with DMSO added and those with VZV peptides.

Two other culture conditions were tried after consulting the literature. The first being a bulk culture using whole blood and media supplemented with FCS (Haredy et al., 2019). Haredy et al., were able to demonstrate the presence of $CD3^{+}CD4^{high}$ CD45RO positive T cells in cell cultures stimulated with VZV cell lysate, which were not present in mock-antigen stimulated cultures. They recognised the advantage of using whole blood cultures over PBMC separation in that the additional separation steps add complexity to the method and the use of whole blood establishes 'more natural conditions that retain all blood components and maintain cells in their in vivo ratios' (Haredy et al., 2019). They identified day 9 as having the highest number of $CD3^{+}CD4^{high}$ CD45RO positive T cells, therefore day 9 was chosen in this project. However, no increase in Ki-67 positive VZV-specific T cells was seen in cell cultures stimulated with VZV-peptides compared to unstimulated or in those cultures with DMSO added as a dilution control for the VZV-peptides.

Vukmanovic-Stejic et al., successfully demonstrated the presence of VZV-specific T cells after 72 hours of stimulation with VZV lysate in young and old HCs (Vukmanovic-Stejic et al., 2015). PBMCs and media supplemented with normal human serum, rather than FCS was used and Ki-67 as the readout for lymphocyte proliferation. In the one experiment which was done in this project, there was a slight increase in the percentage of Ki-67 positive T cells compared to unstimulated or with DMSO added, 0.71%, 0.45% and 0.51% respectively. This does not appear to be a significant increase, and the percentage of Ki-67 positive T cells is very small. However, when comparing this data to that from Vukmanovic-Stejic et al., and others, the small numbers detected are similar. They report the mean percentage of Ki-67 positive CD4 T cells in young adults as 0.9%, however, the percentage was pulled up by five subjects who had Ki-67 positive CD4 T cells ranging from 1.6% - 5%. The remaining 15 HCs had percentages of Ki-67 positivity of 0.5% or less (Vukmanovic-Stejic et al., 2015). The low frequency of VZV-specific T cells is consistent with current knowledge of VZV T cell immunity (Asanuma et al., 2000; Weinberg and Levin, 2010).

The findings that VZV specific T cells are infrequent in peripheral blood are in keeping with work by Asanuma et al. (Asanuma et al., 2000; Haredy et al., 2019). They compared the frequency of memory T cells against VZV, HSV and CMV in adults with natural immunity to infection and after vaccination. Mean frequency of CD4 T cells specific to VZV were 0.11% in VZV-immune donors, compared to CD4 T cells specific to CMV, which were mean of 1.21%, as measured by IFN-γ detection. The same increase in T cell specific to CMV were seen when looking at TNF-α as a marker of antigen-specific T cells. CD8 T cell responses were not detected using intracellular cytokine measurement to VZV or HSV, when compared to unstimulated control cell cultures, but were detected in cell cultures stimulated with CMV antigen (Asanuma et al., 2000). Simonetti et al., found that the frequency of antigen-specific CD8 T cells in mice after vaccination with an inactivated adenoviral vaccine, using Ki-67 and DNA staining, were three-fold lower in peripheral blood than spleen and eleven-fold lower than lymph nodes (Simonetti et al., 2019). This agrees with other findings that some antigen-specific T cells may be difficult to detect in peripheral blood.

In the case of this project, no detectable VZV specific T cell proliferation was seen, in young HCs with a history of childhood VZV infection, however, when looking at Covid or Tetanus specific T cells, a greater response was seen. This suggests that the Ki-67 assay is able to detect antigen-specific T cells, in keeping with other publications (Cellerai et al., 2007; Li et al., 2012; Simonetti et al., 2021). However, VZV-specific T cells may not be detected, or in too small numbers, to be reliably detected by this method. When comparing the method employed in this project to those in the literature, there are a number of differences. Cytokine production is often used as a readout of the T cell response and may be more sensitive than the Ki-67 method. Also, VZV cell lysates are also used, rather than peptides in a number of studies. These could also contain other forms of cell debris which may increase cellular proliferation, compared to specific peptides.

Given the low frequencies of Ki-67 positive T cells, in VZV-immune HCs, it would be challenging to distinguish an abnormal result from a normal result. One way this could potentially be used to measure a T cell response to VZV would be to examine

VZV- specific Ki-67 positive T cells pre- and post-vaccination and see if that produced an increase in antigen specific T cells. When investigating primary and secondary immune deficiency, antibody responses to protein and polysaccharide antigens are investigated by measuring IgG antibodies to these antigens pre-vaccination and then if low, the patient is vaccinated and antibody levels re-measured 4-6 weeks post vaccination. This is seen as a surrogate marker of humoral immunity. The same principle may be possible in patients who have recurrent HZ. Although determining what is a good response may be difficult, given the low numbers of detectable VZV specific T cells in HCs with no history of recurrent shingles. Asanuma et al, examined T cell proliferation using ^{3}[H] thymidine incorporation, in a cohort of young and older adults prior to and 6 week after vaccination with the live attenuated VZV vaccine. The SI of those in the young adult group (aged 18-40) showed no significant difference in proliferation after vaccination or IFN-γ release, whereas the older adults showed a significant increase in SI and IFN-γ release after vaccination. This may indicate that measuring T cell proliferation to VZV antigen post vaccination may be a good way to measure T cell responses to vaccination in the older population. Further investigation of post-vaccine responses was not possible during this project. This was due to time constraints and the lack of patients being vaccinated against VZV in our patient cohort. Many patients did not come into hospital for appointments due to the Covid-19 pandemic. Further work could look at whether this is a useful way of measuring T cell responses to VZV vaccination.

Given the difficulty in detecting Ki-67 positive T cells in response to VZV-antigen, two other antigen-specific proliferation assays were performed to investigate whether the lack of detection of VZV-specific Ki-67 positive T cells was due to the Ki-67 method or due to the choice of antigen. A HC with recent Covid-19 infection had a lymphocyte proliferation test done by Ki-67 and also at the referral laboratory using ^{3}[H] thymidine incorporation. This was an established and published method at the referral laboratory (Awuah et al., 2022). A significant rise in CD4 and CD8 T cells which were Ki-67 positive were seen above the unstimulated background levels. Comparable results were seen in the ^{3}[H] thymidine incorporation assay,

which demonstrated an increase in lymphocyte proliferation, consistent with past Covid-19 infection (Appendix 5). A further lymphocyte proliferation experiment, using the Ki-67 method, was carried out using TT antigen and stimulating PBMCs from a HC for 7 days. An increase of Ki-67 positive T cells was seen in those cultures with TT added compared to unstimulated T cells. These results were consistent with Tetanus IgG antibody levels in this subject, of 2.4 IU/ml, associated with long term protection against tetanus.

This project has demonstrated that Ki-67 method for lymphocyte proliferation can be used to measure antigen-specific responses to Covid antigens and TT. Further work on this may be warranted if there is a clinical need for these assays. This could include widening the repertoire of antigens tested to include measuring proliferation to CMV, for example. CMV is a significant cause of mortality and morbidity in patients with primary and secondary immune deficiency (Emery, 2001., Godsell et., 2021). CMV-specific T cells have also been found at higher frequencies than VZV-specific T cells and may therefore, be easier to detect (Borysiewicz et al., 1983). This is discussed in further detail in Section 6.

The Ki-67 method, in our hands, does not appear appropriate to routinely measure VZV-specific T cells in HCs, due to the very low starting numbers of VZV-specific T cells. There may be a role for measuring VZV-specific T cells in the same individual pre- and post-vaccination to demonstrate a response, in the same way that antigen-specific IgG antibodies are used routinely to assess B cell function when investigating immune deficiencies.

6. Discussion

6.1 Aims of the project

The aims of this project were:

- to evaluate and introduce Ki-67 as a measurement of lymphocyte function in a clinical immunology laboratory
- to use this method to examine responses to mitogens and antigens, namely VZV
- T cell proliferation to VZV antigen will be examined:
    - in patients with primary and secondary immune deficiency,
    - patients with recurrent or severe VZV
    - before and after HZ vaccination

6.2 Summary of the project

The Ki-67 method for lymphocyte proliferation was designed and implemented. This involved the design of a suitable antibody panel and gating strategy on the flow cytometer, to identify both proliferating CD4 and CD8 T cells. Ki-67 positivity was compared in unstimulated cells and those cells stimulated with PHA or anti-CD3/anti-CD28 to ensure that Ki-67 expression could be detected in proliferating lymphocytes. Once this was established, the optimal day for stimulation and harvesting was identified. This was in conjunction with considering what had been done previously in the laboratory using the CFSE assay and the ^{3}[H] thymidine incorporation assay, at the referral laboratory, to which the Ki-67 assay was being compared. When considering which day was optimal for measuring proliferation in this assay, the clinic days of the PID service at the RLH were also taken into account. This was to ensure that if this test was introduced routinely, that the days on which it could be set up and harvested would be suitable for the needs of the users. A whole blood set up was used, not only to address practical issues with assay set up but also in consideration of other factors, discussed in Section 6.3.

In practical terms, the use of whole blood assays would minimise the staff hands-on time and give the laboratory greater flexibility in when the assay could be set up. Samples can arrive towards the end of the working day and a short and simple assay set up time would be advantageous. As whole blood was used, RBCs were lysed to ensure optimal lymphocyte numbers and flow plots. The best time to do this was investigated. By the end of method development, a simple assay was developed which would be suitable for use in a routine clinical laboratory. Once the method had been established, the assay was run on HCs to establish reference ranges. Several approaches were explored. Reference ranges using the mean and 3SDs gave the most clinically relevant results. This was established by examining the patient clinical details alongside their Ki-67 and ^{3}[H] thymidine incorporation results and is discussed in detail in Section 4.10. Some patients who had marginally abnormal Ki-67 results when using ranges based on 2SDs, were classified as normal, when using ranges based on 3SDs. This classification was more in keeping with their clinical picture.

Alongside this, samples for the Ki-67 lymphocyte proliferation assay were set up in parallel with the ^{3}[H] thymidine incorporation assay for lymphocyte proliferation performed at a referral laboratory. The ^{3}[H] thymidine incorporation assay is currently used to measure lymphocyte proliferation in the patients at the RLH Immunology department, after the CFSE assay for lymphocyte proliferation was discontinued in September 2021. This was due to a reduction in the numbers of patients coming into hospital during the Covid-19 pandemic and concurrent staff shortages. Concordance between the ^{3}[H] thymidine incorporation and Ki-67 assay was good, 17 of 25 for PHA responses and 15 of 24 responses to anti-CD3/anti-CD28, using 3SD reference ranges. The discrepancies seen between results could be attributed to many factors, including operator error, sample handling, differences in what each method measures, concentration of reagents and interpretation. These are discussed in detail in Section 4.10. Any test result for lymphocyte proliferation would not be taken in isolation but also in the context of clinical picture and other tests to investigate primary or secondary immune deficiency.

Once it had been established that this method was a viable option to bring in house, as a means of measuring lymphocyte proliferation, further work was done to

determine if the method could be used for detection of proliferating antigen-specific T cells, namely after stimulation with VZV-peptides. Various methods were tried to identify proliferating VZV specific T cells, including whole blood cultures, stimulation of PBMCs, using media supplemented with FCS or normal human serum. Cell culture for 4, 5, 6 and 7 days were examined to see if an increase in Ki-67 positive T cells could be seen in those cultures stimulated with VZV peptides. No difference in the number of Ki-67 positive T cells could be detected in those cultures stimulated with VZV peptides compared to unstimulated cells or those where DMSO had been added at the same concentration as the VZV peptides. Two other methods were tried, after consulting the literature (Haredy et al., 2019; Vukmanovic-Stejic et al., 2015). A bulk 9-day whole blood culture, which did not yield an increase in Ki-67 positive T cells in those cells stimulated with VZV peptide. Also, a 72-hour stimulation of PBMCs was tested which may have given a small increase in the percentage of Ki-67 positive T cells in the VZV stimulated cultures. However, as the small increase was seen in a HC with positive VZV IgG antibodies and a childhood history of VZV, the percentage would be too low to compare to a patient, who may be having recurrent or severe HZ. This 72 hour stimulation also gave good proliferation of PHA stimulated cells which showed clearer discrimination between unstimulated and stimulated T cell populations (Figure 5.5). Antigen specific stimulation with Covid and Tetanus peptides was also undertaken to see if a robust antigen-specific T cell response could be detected using the Ki-67 method. Higher numbers of antigen-specific T cells were detectable in a HC using either Covid or Tetanus peptides, which could potentially be used to compare patient and control responses. Stimulation with TT gave the highest percentage of Ki-67 positive T cells (16%). This led to the conclusion that the Ki-67 assay could be used to identify antigen-specific T cells but that there may be particular challenges when examining VZV-specific T cells, compared to other antigens. This is discussed in Section 6.3.

6.3 Critique of the project methodology and difficulties encountered

This project was looking to evaluate an alternative method of measuring lymphocyte proliferation in a clinical laboratory. As T cell proliferation assays are functional assays, many factors can cause variation in results. This has previously been discussed in Section 4.10. Comparing two different methods of lymphocyte proliferation would always make for a challenging project, due to the nature of these tests and the large amount of variation within them. These are also only one part of the investigation of patients with immune deficiency, and so should always be considered within the context of other tests and the patient's clinical picture. It is well known that patients undergoing long term treatment with steroids, or other immunosuppressive therapies, can demonstrate impaired or absent lymphocyte proliferation results. This is relevant in patient 25 in this project, who had absent responses to anti-CD3/anti-CD28 and normal PHA responses as reported in the ^{3}[H] thymidine assay and absent PHA responses and impaired anti-CD3/anti-CD28 responses in the Ki-67 assay. However, as previously discussed in Section 4.10, the ^{3}[H] thymidine responses in two out of three PHA concentrations were low, not showing a clear dose-response curve, which is more in keeping with the patient clinical picture and the Ki-67 results. The effect of immunosuppressive therapies on lymphocyte proliferation results may be of relevance to other patients, with impaired proliferation responses, both in this project and going forward. These factors were not always known when interpreting results during this project, although patient clinical details were obtained after carrying out the assay, to aid interpretation.

Many of the difficulties encountered were due to access to patients. During the Covid-19 pandemic, very few patients came into hospital for face-to-face appointments, therefore getting any blood for evaluation purposes was limited. Many appointments were conducted by telephone during this time, meaning patients did not come into hospital for routine phlebotomy appointments. Patients with primary and secondary immune deficiency were deemed particularly vulnerable during the Covid-19 pandemic and advised to shield, exacerbating the lack of patient access during this project.

In this project both whole blood and PBMCs methods were used. The use of whole blood assays allows cells of the immune system to remain in 'their natural environment in in vivo proportions' (Damsgaard et al., 2009), whereas the process of isolating PBMCs may affect the viability of the cells and their function by removing key components of the immune system such as cytokines, granulocytes and other growth factors which may affect the ability of T cells to proliferate in vitro (Damsgaard et al., 2009). De Groote et al., found that monocyte concentrations were significantly lower in PBMC preparations when compared to diluted whole blood (De Groote et al., 1992). This is pertinent to this project, in particular when considering that Ki-67 positive cells responding to VZV peptide were being identified. Having fewer monocytes in PBMC preparations could mean that less antigen presentation was taking place in culture and therefore decreasing the chances of detecting VZV-specific T cells.

The decision to examine VZV-specific T cell responses brought its own challenges. Several authors have reported difficulties in identifying VZV-specific T cells in peripheral blood (Asanuma et al., 2000; Haredy et al., 2019; Hickling, Borysiewicz and Sissons, 1987). This is a problem that was also encountered during this project, whereby several different methods of cell culture found no detectable increase in Ki-67 positive T cells when cells were stimulated with VZV peptides compared to unstimulated, or at best a very small difference was seen. However, the Ki-67 method was able to detect an increase in Ki-67 positive T cells when stimulating cell cultures with Covid or TT peptides. The reasons for this could be due to methodological reasons or more probably associated with the nature of the latency of VZV infection.

One reason for this may be the history of the healthy volunteers selected to examine T cell responses to VZV. Both HCs tested here were below 30 years of age, had primary VZV infection during childhood and were positive for VZV IgG antibodies, however there was a large time lag between these individuals having VZV primary infection in childhood, and the measurement of T cell responses. In contrast the HC who was tested for proliferation responses to Covid antigens, had a recent Covid infection, leading to a higher antigen-specific T cells and higher

likelihood of Covid-specific T cells circulating in peripheral blood, rather than sequestered in lymphoid tissue, making them easier to detect in peripheral blood. Hickling et al, were only able to detect VZV-specific T cells in four out of six VZV IgG seropositive individuals by using autologous VZV-infected fibroblasts to stimulate T cells (Hickling, Borysiewicz and Sissons, 1987). Testing individuals with recent infection or a recent HZ vaccination, would have increased the likelihood of detecting Ki-67 positive T cells in response to VZV antigen.

Other potential reasons why Ki-67 positive T cells could not be detected in response to VZV peptides, could be due to the nature of the peptide used. The IE63 peptide was used as it was available and designed for lymphocyte proliferation assays, as stated by the manufacturer. As previously discussed, IE63 is a protein important in the cellular response to VZV and in viral latency (Jacquet et al., 2002; Mathias et al., 2016; Sadzot-Delvaux, Arvin and Rentier, 1998). Sadzot et al., used this peptide to examine stimulation responses in VZV seropositive individuals by examining SI in ^{3}[H] thymidine incorporation assays (Sadzot-Delvaux, Arvin and Rentier, 1998). The overall SI for individuals stimulated with IE63 was lower than when stimulated with whole VZV antigen, with 40% of subjects tested demonstrating a SI of 2, which was equal to the non-immune individuals (Sadzot-Delvaux, Arvin and Rentier, 1998). This demonstrates that this peptide may not induce proliferative responses in all individuals and to an overall lower level, in those subjects which did respond. This may have contributed to the fact that Ki-67 positive T cells in response to VZV may not have been detected in this project.

The IE63 peptide used in this project was dissolved in DMSO, as per the manufacturer's instructions. DMSO is known to be toxic to cells, however this was used at a concentration of less than 0.1% of volume to avoid toxicity. A control stimulation with DMSO alone was included to ensure that the DMSO was not causing excess cell death or contributing to background proliferation. The TT antigens used were diluted in PBS. This is one technical difference between the different antigen stimulations used which, although unlikely, may have contributed to not detecting VZV specific T cells, but the ability to detect TT and Covid specific T cells.

The nature of VZV latency and the location of antigen-specific T cells may also be reasons why T cells proliferating in response to VZV peptides may not have been readily detectable in this project. Primary VZV infection manifests as chicken pox, with the virus going on to lie dormant in dorsal root ganglia without clinical symptoms (Weinberg and Levin, 2010). If viral reactivation does occur, this is manifested in a skin rash, typical of HZ or shingles. Several groups have commented on the difficulty in measuring VZV specific T cells and this may be due to the frequency of circulating memory T cells. Hickling et al., found a precursor cell frequency ranging from 11 per 10^6 in an asymptomatic carrier to 63 per 10^6 T cells in subjects with recent HZ infection (Hickling, Borysiewicz and Sissons, 1987). They concluded that the virus-specific T cells 'may be present in the peripheral blood of normal individuals seropositive for VZV but at a frequency lower than that for other non-neuronal sites of latency' (Hickling, Borysiewicz and Sissons, 1987). They were unable to detect these T cells in two of six seropositive HCs tested. These findings are supported by Borysiewicz et al., who estimated that the frequency of human CMV specific T cells ranged from 50-200 per 10^6 T cells, which is potentially five times higher than VZV-specific T cell frequencies (Borysiewicz et al., 1983). Similarly, Asanuma er al., also reported that the frequency of VZV-specific T cells in blood compared with CMV-specific T cells was much lower and slightly lower than HSV-specific T cells (Asanuma et al., 2000). They concluded that different patterns of latency may account for this difference, as CMV persists in the monocyte lineage, it may require greater immune surveillance than VZV or HSV, which persist in the dorsal root ganglia and may be easier to contain.

Significantly increased numbers of VZV-specific CD4+ T cells were found in skin compared to blood of older and younger adults, by Vukmanovic-Stejic et al., therefore, the difficulty in detecting VZV-specific T cells in blood may be due to the fact that they have relocated to the skin (Vukmanovic-Stejic et al., 2015). Similarly, Patel et al., also examined VZV-specific T cells in the blood and skin of HCs over 70 years of age, 2-6 months after vaccination. Although the percentage of IFN-γ producing T cells in response to stimulation with VZV lysate increased in blood, after vaccination, the percentage rose from <0.1 – 1.5% (Patel et al., 2018). This is

still a small number of VZV-specific T cells, particularly since these responses are being measured a short time post-vaccination, where the number of VZV-specific T cells would be boosted. Therefore, although a few groups have demonstrated the presence of VZV-specific T cells in blood, the numbers are small, and have been shown to be less abundant than other herpes virus-specific T cells (Borysiewicz et al., 1983; Hickling, Borysiewicz and Sissons, 1987). Due to the very low numbers of VZV-specific T cells reported to be in circulation, this presents a technical challenge in terms of detecting these rare cells by flow cytometry. The frequency of circulating VZV-specific T cells are comparable to the detection of other rare events using flow cytometry, for example, the detection of cells in minimal residual disease (MRD), in patients being treated for haematological malignancy. Clinical assays for MRD have been developed to reliably detect 0.01% of cells. In these assays, 500,000 – 1 million cells must be acquired in order to reliably detect MRD, with at least 50 cells required for accurate quantitation of the population of malignant cells (Craig and Foon, 2008; Farren, T., Internal SOP, RLH). This represents a challenge for testing as large numbers of cells need to be acquired in order to obtain reliable identification and quantification of such small cell numbers. In the Ki-67 assay, cells are lost during cell culture, and during the permeabilisation and fixing and washing steps, post-cell culture. Therefore, for this assay to reliably detect small populations of VZV-specific T cells, a greater number of cells may need to be set up for culture and optimisation of the steps post-culture may help to ensure enough cells are present to reliably detect rare events. Additionally, the acquisition of large cell numbers means increased time spent acquiring results on the flow cytometer. This may have repercussions on other routine clinical work being carried out on the flow cytometer. Other potential technical challenges presented by rare event flow cytometry, is ensuring that there is no carryover between tubes run on the flow cytometer (Craig and Foon, 2008). The addition of wash steps between tubes can be included to ensure carryover is avoided.

The healthy control subjects used in this project were different from those used in the published literature, which may partly explain the difference in detecting VZV-specific T cells. Several groups examined antigen-specific responses in individuals

who had been vaccinated, which was not the case in this project (Patel et al., 2018; Trannoy et al., 2000). The ages of the individuals tested may also have affected the ability of the Ki-67 assay to detect VZV-specific T cells. In the two methods replicated from the literature in Sections 5.5 and 5.6, Haredy et al., optimised their assay for measuring VZV-specific T cells in whole blood in young adults aged 18-45 (Haredy et al., 2019). This is a younger age range than the adults used in this project, although they were both under 30 years old. Also, the method to detect VZV-specific T cells was different. Haredy et al., used flow cytometry to identify CD4 high CD45RO T cells and examined their intracellular granzyme B, as a marker of their cytotoxic abilities (Haredy et al., 2019). This may be a more sensitive method than using whole blood to detect antigen specific T cells as it enriches the memory cell population. CD45RO staining could be incorporated into the Ki-67 method in future to help identify memory T cells, by adding this marker to the antibody panel.

The other method which was replicated in this project was that undertaken by Vukmanovic-Stejic et al., who examined Ki-67 positive T cells in HCs grouped into young (<40 years old), middle aged (40-60) and old (>60) (Vukmanovic-Stejic et al., 2015). This group were able to detect Ki-67 positive T cells in response to stimulation with VZV antigen but the responses in old and young ranged from 0 – 5% of CD4 T cells with a mean of <1%. Ki-67 positive CD4 T cells were only detected above the mean in 5 of 20 individuals in the young age group, with the majority of individuals having counts of <0.5% of CD4 T cells (Vukmanovic-Stejic et al., 2015). Therefore, the findings in the one HC in this project may well be in keeping with the findings of this group as the individual tested may have belonged to the group of HCs with detectable Ki-67 positive CD4 T cells below 0.5%. Further work looking at a greater number of individuals would help to confirm this.

The introduction of the recombinant VZV vaccine for severely immunocompromised individuals in 2021, has made the VZV part of this project less clinically relevant. In immunocompromised patients where it is unclear if the live-attenuated VZV vaccine can be given safely, the recombinant vaccine could be given instead, negating the need for investigation to help ensure safety. However, as

discussed in Section 5.8, the method could still potentially be used pre- and post-vaccination to measure a patient's cellular response to vaccination.

In this project, T cells were gated using CD45 and CD3 positivity. During T cell activation the CD3 complex becomes phosphorylated leading to signal transduction and T cell activation. CD3 expression is known to become downregulated upon T cell activation, in order to regulate the immune response and prevent T cell exhaustion (Cantrell, Davies and Crumpton, 1985). This may have an impact on the gating of T cells during CD3 downregulation. Although this was not particularly marked in T cells stimulated with PHA or antigen specific stimuli in this project, this is something what would need to be taken into account when analysing results. Review of results by a scientist experienced in flow cytometry would need to be included in the analysis to ensure cells which should be included in the CD3 gate were present and not being missed due to CD3 downregulation. One other potential complication of using CD3 as a T cell marker could be that when stimulating T cells through the T cell receptor, using anti-CD3 as a stimulant, as in this project, this could potentially mask the binding of the antibody used to stain the T cells, leading to further downregulation of CD3 when staining T cells (Li et al., 2005). Downregulation of surface CD3 was noted in this project when cells had been stimulated with anti-CD3/anti-CD28, although this was addressed when gating the cells. The possibility of using anti-CD45, for the discrimination of the relevant cell populations, on its own, rather than alongside anti-CD3, could be explored for future analysis of these results, as this would include the cells of interest without complicating the analysis of results.

Results in this project were analysed by examining the percentage of T cells which proliferated in response to stimulation, this is consistent with the literature in this field (Gerdes et al., 1984; Li et al., 2012; Lopez et al., 1991; Soares et al., 2010). When analysing flow cytometry results, mean fluorescence intensity (MFI) can also be quantified to examine the expression of surface and intracellular markers. It is defined as the mean of the fluorescence intensity of that fluorescence channel or gated population. Analysing the data using MFI in this project could have been an alternative method of data analysis, rather than examining the percentage of cells

which had upregulated Ki-67. The MFI of T cells after stimulation could be compared to unstimulated cells. These results may however, be skewed in patients who have a small number of T cells which proliferate but the vast majority do not. Therefore, the number of cells which were Ki-67 positive may need to be examined in conjunction with MFI. The potential clinical significance of this would need to be corelated with the patient's clinical picture.

A cell viability dye, such as a fixable viability stain, would have been a useful addition to this project. As the cells in this project are being fixed and permeabilised, a traditional viability dye such as 7AAD or propidium iodide would not be suitable. Fixable viability stains bind amines on the cell-surface and intracellular amines, which are in higher concentration in dead cells. Therefore, dead cells demonstrate a higher fluorescence intensity than live cells when stained with the viability dye. Cells can then go on to be fixed and permeabilised without loss of staining. After stimulation, some cells proliferate but some cells undergo cell death and therefore having a measure of what proportion of cells have undergone cells death would have been useful, as these cells could be have been excluded from further analysis. Also, the inclusion of a fixation and permeabilisation step in this assay would lead to an increase in cell debris, compared to surface antibody staining alone. Therefore, a viability dye would be particularly useful. This would be included in future work.

During this project an assay was developed which can be used routinely as a measure of lymphocyte proliferation, as a viable alternative to ^{3}[H] thymidine incorporation. The assay could also be used to examine antigen-specific T cell responses, which could be utilised by the laboratory in a number of clinical situations, which is further discussed in Section 6.6.

6.4 Limitations of this project

One of the major limitations of this project is sample numbers. 18 HCs were used to establish normal ranges and ideally many more would be tested to give a more reliable and realistic range. As discussed in Section 4.10, this could be continued

during further development of this assay. Other limitations include the inability to use patients who had been vaccinated with VZV vaccine to demonstrate the ability to determine Ki-67 positive T cells. Due to the timing of this project, much of this work was carried out during the Covid pandemic, meaning that access to patients was limited. This was particularly true of those patients with immune deficiency as they had been instructed to shield and only leave the house when absolutely necessary.

A further limitation of this project was to only use one source of VZV peptide for attempting to measure VZV-specific T cell responses. This peptide mix covered an important protein in the cellular response to VZV, IE63 (Jacquet et al., 2002). It was also designed for use in experiments looking at T cell proliferation to VZV, however, the manufacturers recommended the measurement of IFN-γ as the most straightforward readout of cellular proliferation, rather than the measurement of proliferation using Ki-67 or other readouts. In the absence of time constraints, further work could have been taken to investigate other ways of stimulating T cells using VZV, such as those used in the literature, for example, VZV cell lysate (Irwin et al., 1998; Levin et al., 2003; Patel et al., 2018; Vukmanovic-Stejic et al., 2015).

This project examines the use of Ki-67 for the identification and quantitation of VZV-specific T cells. This is just one way of measuring T cell responses to VZV. Other tests which could be used include ELISPOT testing for IFN-γ, MHC tetramers or intradermal skin testing. ELISPOT is a sensitive technique which directly quantitates IFN-γ producing T cells in response to antigen and mitogen and has been used by many groups to examine VZV-specific T cell responses, particularly in response to vaccination, but also in the context of patients on immunosuppressive therapy post-transplant and in patients with primary VZV infection (Cassaniti et al., 2021; Laing et al., 2014; Malavige et al., 2008; Sadaoka et al., 2008). Intra-cellular flow cytometry to detect IFN-γ producing T cells in response to antigen could also be used and has been utilised in examining antigen specific T cell responses to VZV (Hata et al., 2002; Laing et al., 2014; Patterson-Bartlett et al., 2007). Intracellular cytokine staining is used in combination with surface staining for T cells, using CD3, CD4 and CD8 and activation markers, such as CD69 (Hata et al., 2002; Laing et al.,

2014). Both ELIPSPOT and intra-cellular cytokine staining would be potential alternative methods to measure cellular immunity to VZV in a routine clinical laboratory.

Another method to measure antigen-specific T cells is using MHC tetramers. These are complexes composed of four MHC molecules, associated with a specific peptide and bound to a fluorochrome, enabling them to be detected by flow cytometry. Malavige et al., used MHC Class II tetramers against VZV proteins gE and IE63 to examine VZV specific T cell response in patients with acute VZV (Malavige et al., 2008). Tetramer positive CD4 T cells were found to have an activated phenotype, including expression of skin homing receptors, compared to the overall CD4 T cell population (Malavige et al., 2008). MHC tetramers are expensive and therefore may not be an appropriate test to introduce into routine clinical use.

As well as in vitro testing, intradermal skin testing with varicella antigen has long been used to measure cellular immunity. VZV skin test antigen is injected intradermally into the subject's arm and a reaction, in the form of erythema at the site is measured 24-48 hours later (Sadaoka et al., 2008, Somekh et al, 2001). Sadaoka et al., reported that intradermal skin testing results correlated with ELISPOT results but not with VZV IgG antibody measurement, and concluded that VZV skin testing is a valid test for the measurement of cellular immunity to VZV (Sadaoka et al., 2008). Although intradermal skin testing is straightforward to carry out, it must be performed by appropriately trained clinical staff and may not be appropriate for all patients, for example, those with dermatographism or those with widespread eczema. It would also involve a patient making two trips to the hospital to have the test and then reading the result.

6.5 Contribution of this work to clinical practice

Currently, when investigating primary or secondary immune deficiency, a measure of lymphocyte function is carried out. The main method used in clinical laboratories is ^{3}[H] thymidine incorporation which has several disadvantages, discussed in Section 4.10. There was a need in the Immunology laboratory at the RLH to

investigate the possibilities of different methods for measuring lymphocyte proliferation. The CFSE method which was done previously at the RLH and was in place when this project was started, was a robust and reliable method but was extremely time consuming, requiring a large amount of staff hands on time. Due to this there was not much flexibility in when the assay could be set up. It was only run on a Thursday or Friday. This did fit in well with PID clinic days, however, if samples came on other days of the week, then these would need to be sent to the referral laboratory. This led to inconsistency in the service provided. There was a need to consolidate the service and look for alternatives. The Ki-67 method for lymphocyte proliferation could be a method to replace the current process, as it has good inter and intra-assay variation and is a simple method. It brings the expertise for running lymphocyte proliferation tests back in house using a method which is less time consuming and cheaper than the previous CFSE method, and the current process which involves sending samples to a referral laboratory. The risk of losing samples in transit is removed alongside the challenge of getting the samples to the referral laboratory within the specified timeframe.

In terms of wider clinical practice, clinical immunology laboratories may be looking towards alternatives to ^{3}[H] thymidine incorporation as a method of lymphocyte proliferation. This would be to avoid the use of radioactivity and to avoid buying new pieces of equipment when old scintillation counters and plate harvesters need to be replaced. The Ki-67 method is done using a flow cytometer which most clinical immunology laboratories already own and have expertise in using. Also, in terms of clinical utility, the Ki-67 method can give more detailed information on the different populations of T cells and their ability to proliferate, which may give further clues as to the patients' cellular defect. This may help to identify more subtle defects in cellular immunity which may be welcome in some clinical settings. For example, in patients who clinically demonstrate a cellular immune deficiency but have a normal overall proliferative response to mitogen or antigen. Being able to identify whether there is a subtle defect in the patient CD4 or CD8 T cell response, which is being masked by the overall T cell response, may give further information on the defect in that particular patient. Having spoken informally to

other clinicians, there is interest in an alternative method for lymphocyte proliferation, potentially as confirmation of results seen using ^{3}[H] thymidine incorporation, or when the clinical significance of results is unclear.

As discussed in Section 5.8, the ability of the Ki-67 assay to be used to measure a patient's antigen-specific T cell response was partially successful. There are a number of patients with inborn errors of immunity and acquired immunodeficiencies associated with severe VZV infection and viral reactivation, for whom such an assay may be useful. These include patients with inborn defects in T and/or NK cells, innate immunity and acquired defects in these pathways (Ansari et al., 2021). Inborn errors of immunity associated with disseminated VZV infection include T and NK cell defects such as SCID, due to mutations in *RAG1*, *RAG2* and *IL-7R* genes and patients with *GATA2* and *MCM4* mutations, who have low NK cell numbers and impaired NK mediated cytotoxicity (Ansari et al., 2021). Some CVID patients have also been reported to have opportunistic infections, including severe HZ (Cunningham-Rundles and Bodian, 1999). Other inborn defects in T cell mediated immunity are associated with severe VZV infection such as WAS, due to impaired immune synapse formation and DOCK-2 deficiency, due to low T cell numbers and impaired T cell responses to mitogen (Ansari et al., 2021). Patients with inborn defects in the innate immune response, such as the IFN signalling pathway, have also been shown to develop severe VZV or HZ (Gerada et al., 2020). These include patients with gain-of-function or loss-of-function mutations in STAT-1, and STAT-3 mutations in patients with autosomal dominant hyper IgE syndrome (Ansari et al., 2021). IFN signalling is vital in the immune response to VZV and disruption of the IFN pathway is a known mechanism of VZV viral evasion, as described in Section 1.5. Defects in proteins which sense RNA and DNA, such as TLR-3, and its downstream signalling molecules and RNA polymerase 3, have been associated with severe VZV or childhood HZ, indicating the importance of these proteins in the innate immune response to VZV (Ansari et al., 2021).

Acquired defects in immunity are also associated with severe VZV or HZ, including HIV patients and those post-HSCT (Groot et al., 2017; Sadaoka et al., 2008; Weinberg and Levin, 2010). Some patients with autoantibodies against IFNs and

other cytokines, were shown to have severe VZV or HZ (Ansari et al., 2021; Bayat et al., 2015). Bayat et al., demonstrated the presence of high levels of anti-cytokine antibodies, including to IFN-α, IFN-γ, IL-6 and GM-CSF, in some HZ patients with post-herpetic neuralgia, whereas no or low levels of anti-cytokine antibodies were seen patients with HZ without post-herpetic neuralgia (Bayat et al., 2015). Ansari et al., described a patient with Brown-Sequard syndrome with VZV central nervous system vasculitis who had neutralising IgG antibodies to IFN-α in his serum and high levels of IgG to VZV in his cerebrospinal fluid (Ansari et al., 2021). Taken together, these findings demonstrate the importance of these cytokines in controlling the severity of HZ and VZV. Having a measure of T cell function to VZV could be useful in a wide variety of patients with either inborn or acquired errors of immunity.

Much method development work was done to try to identify VZV-specific Ki-67 positive T cells in healthy controls. However, this proved challenging and in most cases detection of Ki-67 positive T cells in response to VZV peptides was not forthcoming. One method may have demonstrated a small increase in VZV-specific Ki-67 positive T cells in those cells cultured in the presence of VZV peptides. However, as the number was so low, this could not reliably be compared to an abnormal response. As discussed, one way to utilise this for clinical use could be to use this measurement pre- and several weeks post-vaccination with HZ vaccine, to measure a response. This could be used in those patients who have recurrent HZ, to measure if they have a T cell response post vaccination. This type of method is already used routinely to assess patient antibody responses to protein and polysaccharide antigens both before and after vaccination.

One of the hopes for this project was to use the Ki-67 method to identify patients with a defective cellular response to VZV and to help stratify those who may be at risk of developing HZ. Due to the challenging nature of identifying Ki-67 positive T cells in response to VZV peptides, this would not be possible, given that HCs had such low detectable numbers. Also, since the conception of this project, the vaccination landscape has changed for HZ. There is now a recombinant vaccine which is safer for those patients with severe immune deficiency. Before the introduction of the recombinant vaccine, having a correlate of cellular function to

VZV may have been helpful in deciding whether giving the live attenuated HZ vaccine was safe. This is no longer required as the recombinant vaccine could be given instead. However, the Ki-67 method was shown in this project to be suitable for measuring antigen-specific T cells to Covid and TT peptides. This may be a useful method to have within the laboratory, to measure recall antigen responses to these particular antigens or others. The Ki-67 method could also be used to look at other measures of T cell function, as discussed in the next section.

6.6 Suggestions for future work

Some of the suggestions for future work have already been mentioned in the discussion sections of the results chapters.

This project has developed a set of normal ranges from 18 HCs. Further work would be undertaken to increase the numbers and thereby increase the validity of the ranges. As discussed, this could be undertaken alongside the Ki-67 test being introduced into the laboratory with service users being made aware that the test was still in development and that reference ranges may change in future. A test of lymphocyte function would always be done alongside a patient's clinical picture and other tests for the investigation of primary or secondary immune deficiency, therefore this approach would be acceptable.

Some of the discrepancies seen between T cell proliferation to PHA in patients tested by the Ki-67 method and the ^{3}[H] thymidine incorporation method, may be due to differences in the concentration of PHA used, as well as the reasons already discussed in Section 4.10. The referral laboratory have experience of lymphocyte hyper-proliferation with PHA stimulation and then exhaustion, and therefore three concentrations of PHA are used. Some patients examined in this project, demonstrated their highest CPM in the ^{3}[H] thymidine incorporation assay at the lowest concentration of PHA. Future work could include using a range of PHA concentrations as routine in the Ki-67 assay, such as 10μg/ml, 5μg/ml and 2.5μg/ml and an audit of the results to identify any significant differences in results in the same patient and whether this would be a useful addition to the method. The

addition of these extra stimulation conditions would increase the workload slightly as well as the cost of the assay but would not be prohibitive.

When considering the optimal timepoint to measure Ki-67 positive T cells in response to PHA and anti-CD3/anti-CD28, further work would be done to examine proliferation at 72 hours of stimulation. When examining VZV responses at 72 hours, it was noted that the response of T cells to PHA looked to give clearer discrimination between populations of T cells which had proliferated and those which had not. Therefore, going forward, this would be a potential time point to consider when measuring patient and HC responses to stimulation, as it may provide further discrimination between patients with normal responses and those who may have an impaired response.

Further work to look at VZV-specific T cells, would be to investigate the potential of using the Ki-67 assay to investigate a patient's VZV-specific T cell response pre- and 6 weeks post-HZ vaccination. This could be used in patients with recurrent severe HZ infection. This was not investigated during this project due to challenges faced in the identification of Ki-67 positive T cells after stimulation with VZV peptides. Also, due to the Covid-19 pandemic, many patients did not come to face-to-face appointments and routine vaccinations, such as those for HZ, did not take place as usual. The number of patients who suffer from recurrent HZ are small and so it would take time to recruit enough patients for this, and they would need to be followed up to see if any VZV-specific T cell responses correlated with protection against further episodes of HZ. IgG responses to TT, Haemophilus influenzae and Pneumococcus are already used to examine patient antibody responses to these antigens as a measure of B cell function. The measure of VZV-specific T cells could be used in a similar way.

Another group of patients which my benefit from an assay to measure VZV-specific T cell function, could be patients treated Janus Kinase (JAK) inhibitors. An increasing number of patients are being treated with JAK inhibitors as modulators of the immune response. JAKs are tyrosine kinases which phosphorylate and activate STAT proteins, regulating the transcription of their downstream genes. encoding many cytokines important in the immune response, including IFNs, IL-4, IL-5, IL-13 and IL-

6 (Georas et al., 2021). JAK inhibitors are currently approved for use in patients with inflammatory disorders including, rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis and atopic dermatitis (Medicines and Healthcare products Regulatory Agency, 2023). However, there are significant safety concerns in patients taking these medicines, due to their immunosuppressive nature. These include, increased risk of venous thromboembolism, cardiovascular disease, malignancy and opportunistic and respiratory infections (Georas et al., 2021). Pertinent to this project is the increased risk of viral reactivation, particularly of HZ, in patients taking JAK inhibitors and the increased risk of disseminated disease (Curtis et al., 2016; Georas et al., 2021; Matucci et al., 2021). The National Institute for Health and Care Excellence (NICE) advises that patients receive all recommended vaccines, including prophylactic VZV vaccination prior to the commencement of treatment (National Institute for Health and Care excellence, 2023). Recently, there has been discussion around the use of JAK inhibitors in the treatment of severe asthma and there are several clinical trials evaluating the use of JAK inhibitors in this group of patients. Studies have examined the efficacy of both oral and inhaled JAK inhibitors in these patients (Georas et al., 2021). The investigation of patients VZV-specific T cell response before treatment and when treated with oral vs inhaled JAK inhibitors would make for interesting further work and could become useful in making a decision to treat patients who have not responded to vaccination with prophylactic anti-viral medications.

The work in this project has demonstrated that the Ki-67 assay is suitable for measuring antigen-specific T cells, such as Covid and TT, in keeping with other studies (Cellerai et a., 2007; Li et al., 2021; Simonetti et al., 2021). Further work could be done to use these antigens as measures of T cell responses in HCs and patients with clinical signs of a defect in their cellular responses to these antigens. Stimulation with TT gave the highest percentage of Ki-67 positive T cells in this project, and therefore, this stimulus could be used further to investigate a patient's antigen specific responses. The repertoire of antigens investigated could also be increased to include Cytomegalovirus (CMV) or others, depending on clinical need.

CMV is a well characterised cause of morbidity and mortality in the setting of both primary and secondary T cell immune deficiencies (Emery, 2001; Godsell et al., 2021). These include Severe Combined Immune Deficiency (SCID), Wiskott Aldridge syndrome, Omens and Common Variable Immunodeficiency (CVID). Also patients with secondary causes of immune deficiency, such as AIDS and those receiving immune suppression, post stem cell or solid organ transplant, can suffer from invasive CMV disease (Godsell et al., 2021). Current measurement of immunity to CMV utilises antibody serology or viral load which may not always correlate clinically with immune competence, and may be falsely positive in patients receiving immunoglobulin replacement. Tests examining T cell function in response to CMV may be a more relevant marker of cellular immune competence to the virus (Godsell et al., 2021). The Ki-67 assay could be utilised for this purpose.

The Ki-67 method could also be extended for use in the clinical setting of T cell mediated delayed-type hypersensitivity. The Allergy team at the RLH, occasionally request lymphocyte transformation tests (LTT). This test is used in patients for the investigation of delayed-type drug reactions in patients who have had severe, systemic allergic reactions, such as Stevens-Johnson's syndrome and Drug reaction with Eosinophilia and systemic symptoms (DRESS), where the use of patch testing to investigate their drug reactions is contra-indicated (Kano et al., 2007). PBMCs are separated and cultured with the drugs suspected of causing the reaction alongside, unstimulated cells, PHA and TT as an antigen-specific control. Proliferation is measured as a readout of T cell response to the drug in question. Currently in the Immunology laboratory at the RLH, these are done using the CFSE method of measuring lymphocyte proliferation, on a research basis. As discussed previously, this method is labour intensive and complex. Using the Ki-67 method as a measure of drug-specific T cell proliferation could be a way of simplifying this assay set up, making it more amenable to routine use. Therefore, the Ki-67 assay, established in this project, could be used outside the scope of investigating immune deficiency, into other clinical areas of the Immunology laboratory.

6.7 Conclusion

In conclusion, this project fulfilled the majority of its aims. An assay for lymphocyte proliferation, using Ki-67 as a readout was developed and its suitability for use in a routine clinical immunology laboratory was demonstrated. This project also showed that the Ki-67 method for lymphocyte proliferation could be used to measure antigen-specific T cell responses, which could potentially be used clinically, for antigens such as Covid and TT. However, the measurement of VZV-specific T cell responses in patients may need a different approach, to that proposed in this project. The scope of use of the Ki-67 method can be widened in the Immunology laboratory at the RLH, and used for other clinical applications, such as the LTT. This project demonstrates a viable alternative assay for the measurement of lymphocyte proliferation which could be utilised widely by clinical laboratories with access to a flow cytometer, for routine use, and for further development into other clinical applications.

7. References

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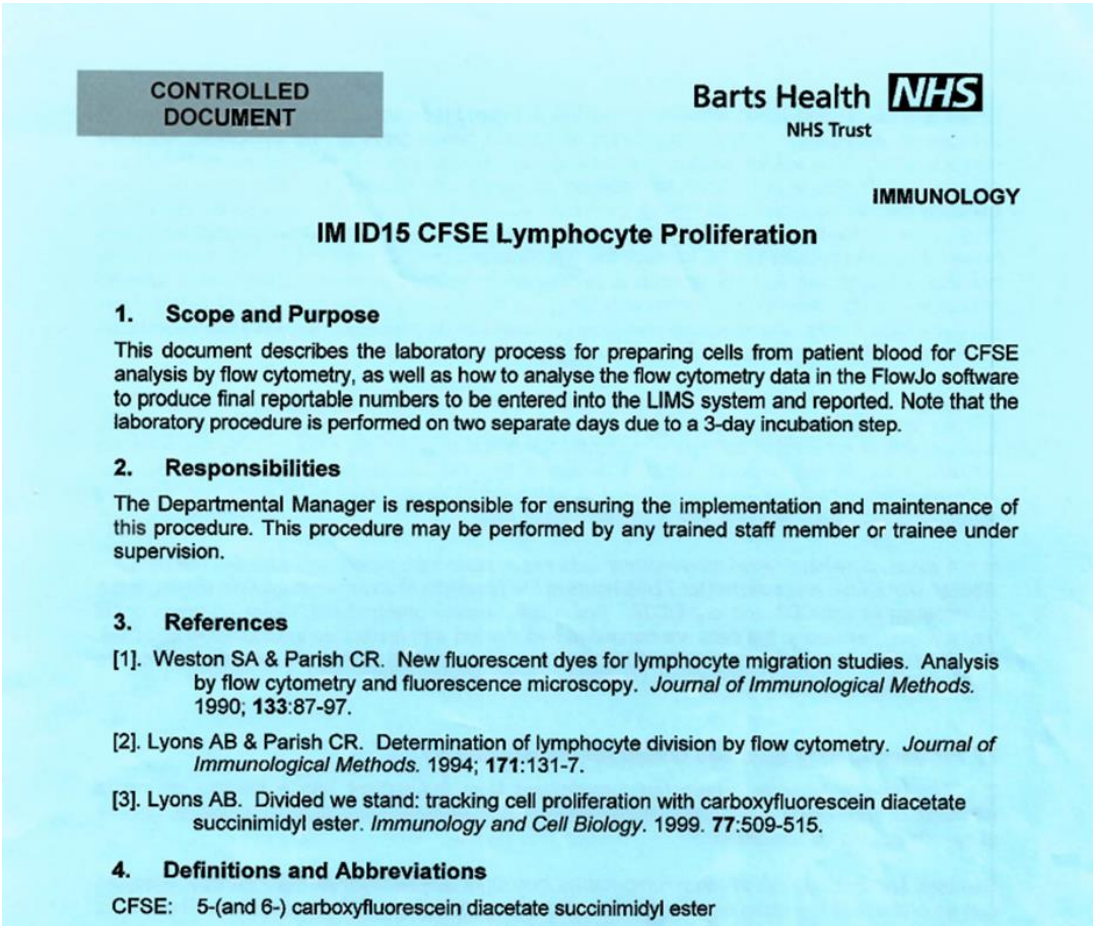
Appendix

Appendix 1. Excerpts of CFSE lymphocyte proliferation SOP

The documents shown below are taken from the CFSE lymphocyte proliferation SOP which was in use at the Royal London Hospital Immunology Department from 2006 until 2021. The SOP in its entirety is 24 pages long, including assay set up, antibody staining, data analysis and reporting. This was a highly complex assay.



CONTROLLED DOCUMENT

Barts Health NHS
NHS Trust

IMMUNOLOGY

IM ID15 CFSE Lymphocyte Proliferation

1. Scope and Purpose

This document describes the laboratory process for preparing cells from patient blood for CFSE analysis by flow cytometry, as well as how to analyse the flow cytometry data in the FlowJo software to produce final reportable numbers to be entered into the LIMS system and reported. Note that the laboratory procedure is performed on two separate days due to a 3-day incubation step.

2. Responsibilities

The Departmental Manager is responsible for ensuring the implementation and maintenance of this procedure. This procedure may be performed by any trained staff member or trainee under supervision.

3. References

[1]. Weston SA & Parish CR. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. *Journal of Immunological Methods.* 1990; **133**:87-97.

[2]. Lyons AB & Parish CR. Determination of lymphocyte division by flow cytometry. *Journal of Immunological Methods.* 1994; **171**:131-7.

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4. Definitions and Abbreviations

CFSE: 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester

6. Principle

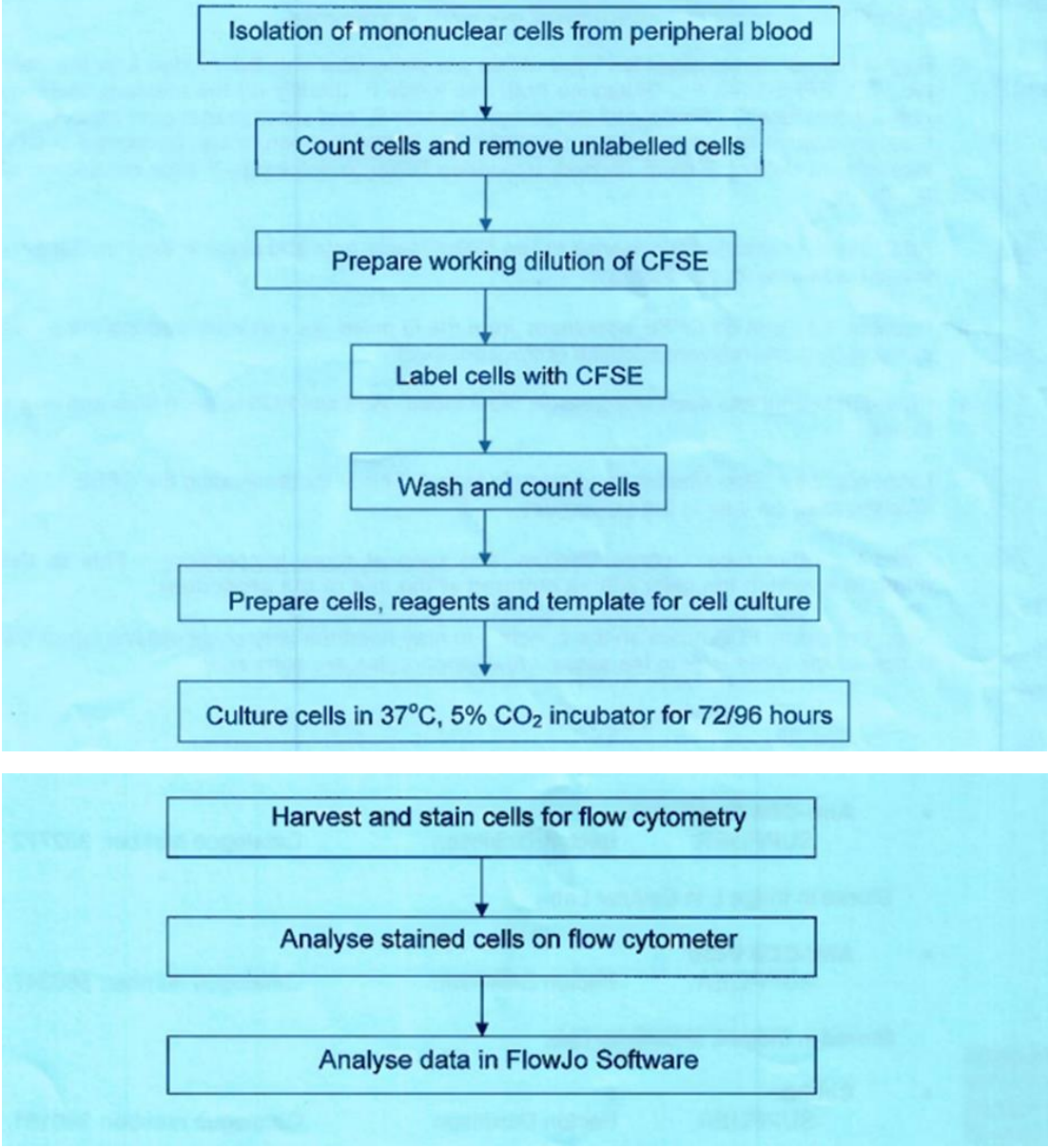
The most commonly used test to measure the response of lymphocytes to an antigen or mitogen was to use 3[H] thymidine incorporation, which had several limitations, including the use of radioisotopes. Patients with subtle cellular immune defects may not be identified and may benefit from a more detailed test of lymphocyte function. CFSE is a way of achieving this.

CFSE is a fluorescein which diffuses across the cell membrane and fluoresces after cleavage by intracellular esterases. It covalently binds to internal components of the cytoplasm, such as intracellular proteins, via it's succinimidyl ester group, changing the structure of the molecule and causing it to fluoresce [1]. When the labelled cell undergoes division, the fluorescein is halved between the two daughter cells arising from the divided cell [2]. CFSE is excited by the 488nm argon laser, so the labelled cells can then be visualised by flow cytometry, allowing the progeny of a proliferating cell population to be followed through several rounds of division [3]. This method does not use radioisotopes and the readout is performed on a flow cytometer. Due to this, labelled antibodies can be used in conjunction with CFSE to identify the dividing populations, in this case, CD4 and CD8 T cells, and activation marker expression can be measured, giving further information on the ability of the lymphocytes to become fully and appropriately activated.

An absent or poor response of lymphocytes to mitogen or antigen may support a diagnosis of primary immune deficiency. This assay may help to identify patients which may have a subtle immune defect accounting for their symptoms, but which may not be picked up by the tests we currently have at our disposal. Also it is hoped that the use of this assay will improve our understanding of complex immunodeficiencies which would be expected to translate into improved disease classification and ultimately improved patient care.

In this assay, peripheral blood mononuclear cells are isolated from patient and control. The cells are labelled with CFSE and cultured for 72/96 hours in the presence of phytohaemaglutinin (PHA), and a combination of anti-CD3 and anti-CD28. Some cells remain unstimulated. After 72 hours or 96 hours where necessary, the cells are harvested and stained with antibodies to CD3, CD4 and CD8. The results are then acquired on the FACSLyric flow cytometer and analysis is carried out using the FlowJo software package.

8.4. Summary of procedure - ensure cell count is adequate for CFSE and check if tested previously. Note results and any issues previously.



This excerpt shows a summary of the steps involved in the entire CFSE assay, including assay set up, cell harvesting and staining and data analysis.

Data Analysis - Checking gating strategy.

8.16. Data Analysis - Checking gating strategy.

76. Check the lymphocyte gate on each sample by double clicking on the first sample, reviewing/adjusting gate and then clicking on arrow at the top of the plot to go to the next sample. (X-axis=FSC-A, Y-axis=SSC-A).
    First check that the blank sample shows no sign of CFSE autofluorescence by double clicking on the lymphocyte gate and viewing as a CFSE histogram.
    N.B. Ensure that there is a tight gate around the unstimulated CFSE labelled lymphocyte population, as including debris in the gate can interfere with the analysis. Dead cells retain some CFSE.

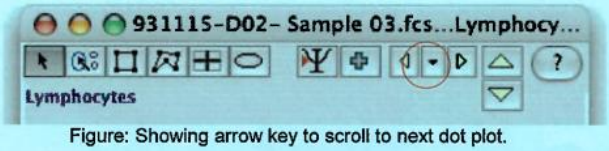


Figure: Showing arrow key to scroll to next dot plot.

77. Then check the CD3, CD4(PerCP) and CD8(V450) gates on each sample. Open a stim lymphs dot plot by double clicking on the sample. Double click on the lymphocyte population within the gate, change the plot X-axis to <APC-A>:CD3 and Y-axis to SSC-A and ensure that the gate is over the CD3+ population. Ensure that any monocytes are excluded from the gate. Do this on all stimulated samples using the arrow key (see figure above).

78. Check the CD4(PerCP+) and CD8(PerCP-) gates by double clicking on the CD3+ population. Ensure the plot has <FITC-A>:CFSE on X-axis and <PerCP-Cy5.5-A>:CD4 on Y-axis. **Ensure that the gate includes those cells on the X-axis.**

79. Do the same for the CD8(V450) gates by ensuring the plot has <FITC-A>:CFSE on the X-axis, and <Pacific Blue-A>:CD8 on the Y-axis.

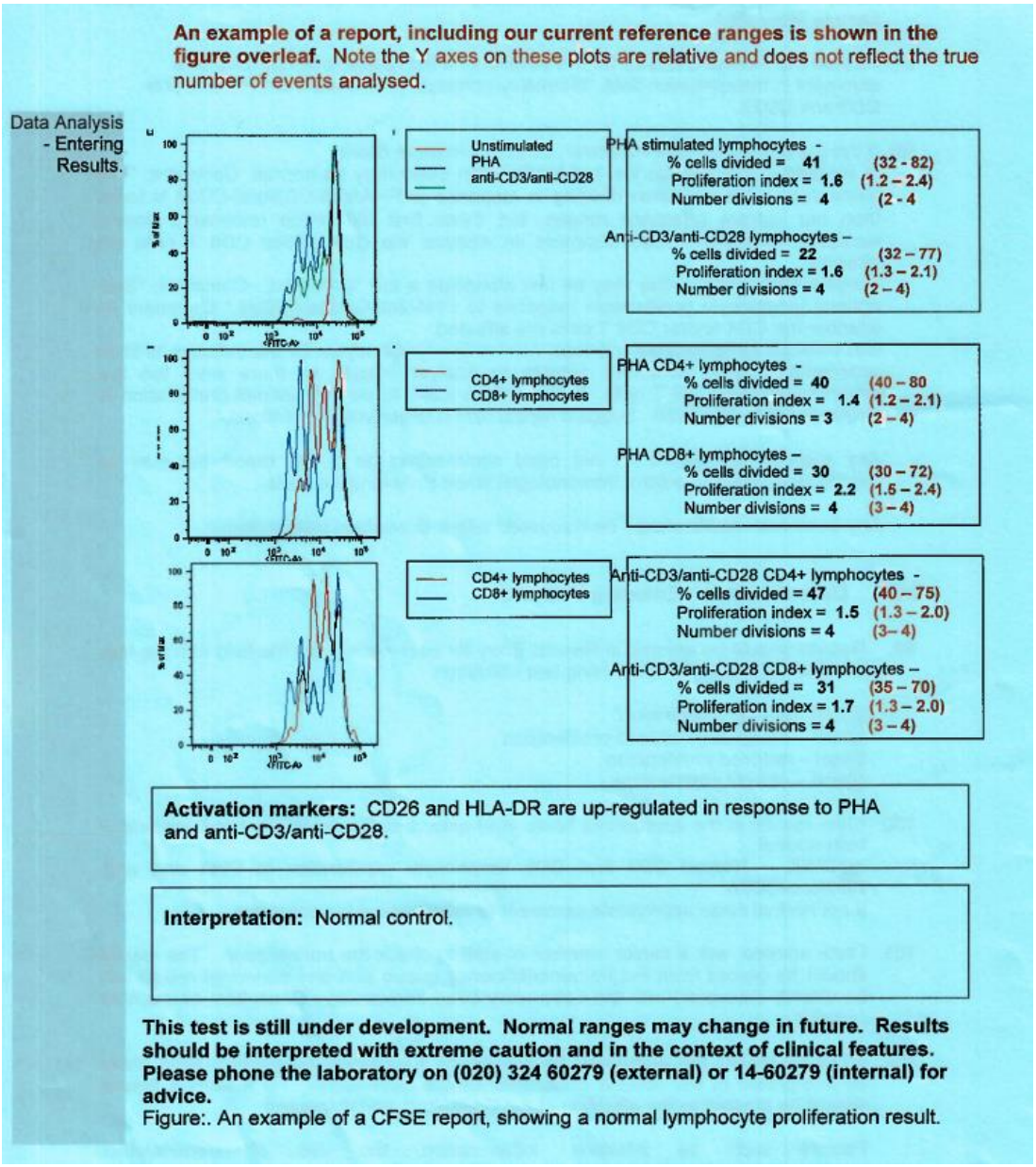
80. Check that the gates are appropriate in each sample using the arrow key.

Data Analysis - Using the proliferation platform.

8.17. Data Analysis - Using the proliferation platform.

81. For each stimulated sample, the proliferation data is worked out on CD3 T lymphocytes, and CD4(PerCP+) and CD8(PerCP-) T cells using the Proliferation Platform. Click on the data to be analysed, e.g. PHA stim CD3 and select Platform (top tool bar) → Proliferation. The data will be shown as in figure 2. (The reason we define CD8 as PerCP- is because the reference range was developed using this gate. The CD8 V450 is of use where there is debate as to whether the CD8 cells are in fact CD8 cells.)

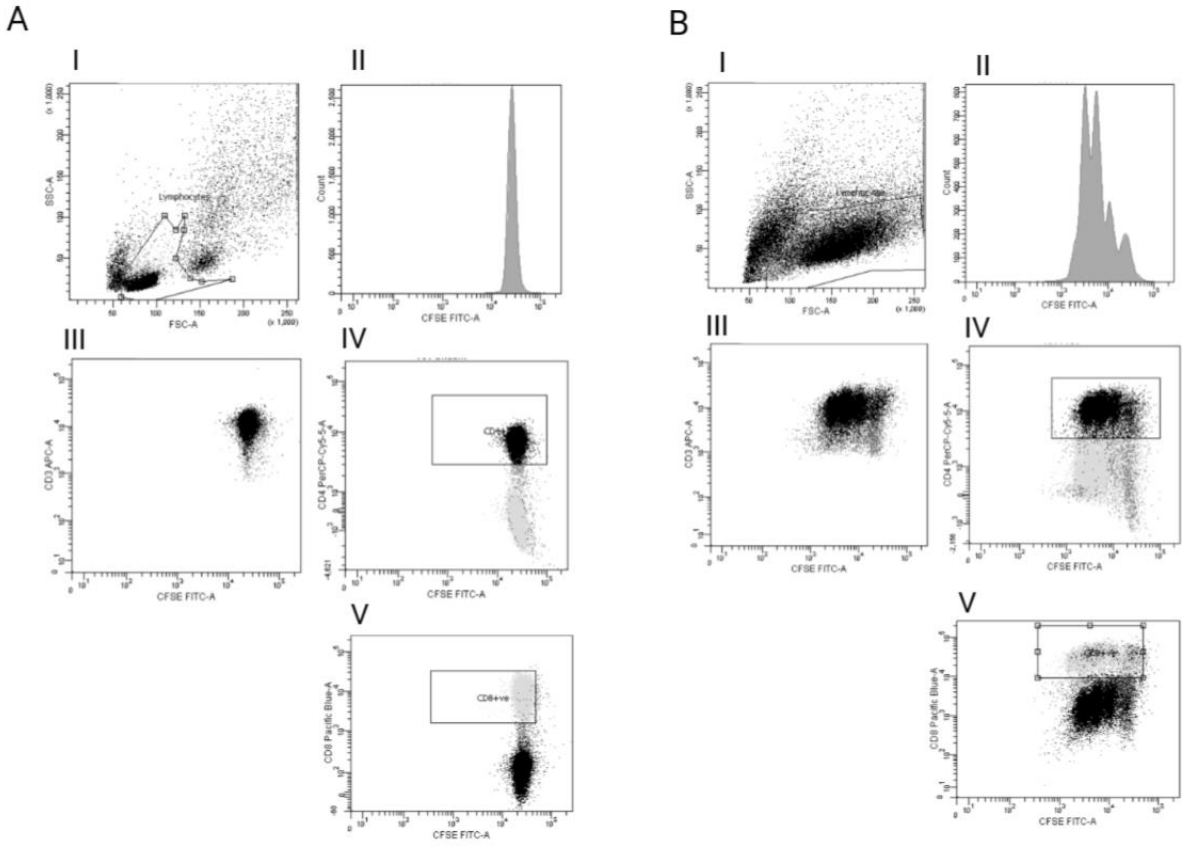
This excerpt shows some of the steps involved in the data analysis of the CFSE assay. The proliferation platform in the FlowJo software (Becton Dickinson, UK) was used. Several steps were required before generating a report of the results.



An example of the report created for the CFSE assay, taken from the CFSE SOP.

Appendix 2. Gating strategy for the CFSE proliferation assay

The gating strategy used for the CFSE proliferation assay. This assay was performed at the Royal London Hospital prior to September 2021. The method for this assay is detailed in Appendix 1.



Panel A: Unstimulated PBMCs from a HC. (I) lymphocytes were gated based on forward and side scatter, (II) gating through the lymphocyte gate, CFSE level was shown (III) using the lymphocyte gate CFSE positive, CD3 positive T cells were identified, (IV) gating on CD3 positivity, CFSE positive, CD4 positive T cells were identified, (V) using CD3 gate, CFSE positive CD8 positive T cells were identified.
Panel B: PHA stimulated PBMCs from a HC. (I) lymphocytes were gated based on forward and side scatter,, (II) gating through the lymphocyte gate, CFSE level was shown (III) using the lymphocyte gate CFSE positive, CD3 positive T cells were identified, (IV) gating on CD3 positivity, CFSE positive, CD4 positive T cells were identified, (V) using CD3 gate, CFSE positive CD8 positive T cells were identified.

Appendix 3. The rate of pay for staff at Barts Health Trust

The New Day rate from Barts Health NHS Trust was used to calculate the staffing costs for each band of staff member throughout the Ki-67 assay. This is the staffing pay rates per hour for each staff band.

		Subs Total Bank Pay Rates				
	Old Day Rate	**New Day Rate**	**Old Sat/Night**	**New Night/Sat**	Old Sun/BH	**New Sunday/BH**
Band 2	£11.50	£12.89	£14.72	£16.50	£19.21	£21.53
Band 3	£12.38	£13.87	£15.37	£17.23	£19.51	£21.86
Band 4	£12.67	£14.20	£16.48	£18.47	£20.28	£22.73
Band 5	£15.72	£17.62	£19.25	£21.57	£23.69	£26.55
Band 5 S	£20.50	£21.68	£25.00	£26.00	£29.00	£29.00
Band 6	£17.35	£22.22	£22.56	£28.89	£27.76	£35.55
Band 6 S	£21.61	£25.66	£26.26	£31.86	£31.68	£35.55
Band 7	£20.31	£27.03	£26.41	£31.86	£32.50	£36.42
Band 7 S	£23.70	£28.00	£29.76	£33.35	£36.62	£41.04
Band 8a	£24.88	£32.00	£31.93	£32.00	£39.30	£32.00
Band 8b	£29.06	£32.57	£37.54	£32.57	£46.20	£32.57
Band 8c	£33.80	£37.88	£43.90	£37.88	£54.03	£37.88
Band 8d	£40.08	£44.92	£52.11	£44.92	£64.13	£44.92

Appendix 4. Staffing and reagent costs for the Ki-67 assay

Staffing (pay) and reagent (non-pay) costs were calculated for the Ki-67 assay. The staff cost for each stage of the Ki-67 was calculated using the New Day Rate (Appendix 3) for Band 6, 7 and 8c. The time taken for validation was calculated as 15 minutes for a Band 8c Clinical Scientist.

Staff hands-on time	**Set up**	**Day 4**	**Interpretation**	**Validation**
Band 6	£11.11	£11.11		
Band 7			£14.00	
Band 8c				£9.47

Total staffing costs = £71.47

Reagent costs were calculated using the price of the reagent and calculating the cost for one Healthy control (HC) and one patient.

Reagent	**Per item**	**Cost per test (HC + patient)**
RPMI	£5.32	£5.32
FCS	£459.18	£23.10
PHA	£25.26	£0.06
Anti-CD3	£65.12	£6.26
Anti-CD28	£65.12	£6.26
Pharmlyse	£36.78	£1.76
Intrastain	£261.27	£15.67

Antibodies

Reagent	**Per item**	**Cost per test (HC + patient)**
CD3 BV421	£201.55	£12.12
CD14 BB770	£255.93	£15.30
Ki-67 Alexa Fluor 647	152.25	£18.30
CD8 Alexa Fluor 700	£111.65	£13.38
CD4 PE-Cy 7	£150.08	£9.00
CD45 BV510	£218.95	£13.08
Brilliant stain buffer	£66.70	£3.96

Appendix 5. Lymphocyte proliferation results for PBMCs stimulated with Covid peptides

Lymphocyte proliferation result, using the ^{3}[H] thymidine incorporation assay at GOSH. PBMCs were stimulated with Covid peptides on a Healthy control (HC) 10 weeks post COVID-19 infection. Cells were also stimulated with PHA as proliferation control and some cells left unstimulated. The Ki-67 assay was carried out on the same HC at the same time and is described in section 5.7.

COVID T cell proliferation (Final result)

Component	Value	Ref. Range
PHA patient 4 ug/ml	81,634	MEAN CPM
COVID patient BKG 0 ug/ml	279	MEAN CPM
COVID S patient 0 ug/ml	3,428	MEAN CPM
COVID M patient 0 ug/ml	1,305	MEAN CPM
COVID N patient 0 ug/ml	1,500	MEAN CPM
Resulting Lab: GOSHLAB		

Comments:
Normal PHA response.
Patient proliferates to all three COVID peptides tested (spike, membrane and nucleocapsid). Consistent with past COVID infection.

Appendix 6. Overview of the Doctorate in Clinical Sciences (DClinSci)

The DClinSci is undertaken as part of the Higher Specialist Scientific Training (HSST) programme. This is a five-year programme comprising a work-based and an academic element. The work-based element involves the collation of evidence for an online portfolio, covering a variety of competencies.
The academic element is comprised of three components:

Section A: Leadership and professional development
Section B: Specialist Scientist Clinical programme
Section C: Research, Development and Innovation

Section A Leadership and Professional development (120 credits)

This section is completed and assessed by the Alliance Manchester Business School and comprises five taught modules. The assignments which were done for each module, the word count and marks are shown in the table below.

Assignment	**Word count**	**Mark**
A1-1: Construct a personal model of professionalism underpinned by one or more recognised models of professionalism and using a range of tools of critical reflective practice.	2691	58%
A1-2: Drawing on what you have learned about social mobilising theory, construct a compelling and evidence-based 'public narrative' for a proposed change or improvement in a service that your department or organisation provides. The narrative should take cognisance of NHS governance requirements in relation to approval and funding for changes or developments of services.	3260	75%
A2-1: Critically evaluate two models of leadership and how these relate to your specialism.	3253	75%
A2-2: Critically analyse your wider work environment through the lens of collective leadership approaches providing examples of opportunities or barriers to promoting your organisation's values and mission. Using the pledge that you have given at Day 3, write an action plan that outlines what you need to do to implement your pledge.	3264	65%
A3-1: We are asking you to consider and reflect on 3 key areas: Key learning from your working life, learning associated with the Programme, personal learning from outside of work. Reflect on one or two key issues in each are. We are looking	1562	65%

for you to be able to extract the learning and consider what actions you will take in the future.		
A3-2: Who am I? Knowing and reflecting on self	4099	82%
A4-1: Understand ("Grasp the current condition" of) your Quality Improvement process and plan a first test (Experiment).	2496	68%
A4-2: Experiments and critique of a recent Quality Improvement project in my department.	2469	58%
A5-1: Group presentation on an outline proposal to conduct a research study into Hospital Trust Board Effectiveness.	15 minute presentation	75%
A5-2: Review and critique an example or case study of innovation in the healthcare system, drawing appropriately on both the relevant research literature and on the literature, ideas and concepts of research management and knowledge mobilisation.	3133	62%
	Total word count: 26,227	

The final marks for each module are shown in Appendix 7.

Section B: Specialist Scientific Clinical Programme (150 credits).

For Clinical Scientists in Life Sciences, this comprises of Royal College of Pathologists Fellowship (FRCPath) Examinations Part I and II. The results of these examinations can be found in Appendices 8 and 9 repectively.

Section C: Research, Development and Innovation (270credits).

Section C assessment comprises:

- A literature review of approximately 4000 words. This was completed in March 2020 and was assessed as 'excellent'.
- A lay presentation (explain the basis of your project to a lay audience). This was completed in July 2020.
- An Innovation proposal of no more than 5 A4 pages, where the trainee must conceive an innovation within their healthcare science discipline that has potential to make a positive contribution to service delivery or patient experience or patient outcomes or health economics, or any other aspect of healthcare. This was accepted and is included as Appendix 10.
- The research project. The aim of the project is to 'improve health and health outcomes'.

References:

MAHSE website: DClinSci-Life-Sciences-Programme-Overview.pdf (mahse.co.uk). Accessed: 21/06/2023

Appendix 7. Summary of marks obtained on PGDip Leadership and Management in the Healthcare Sciences

The University of Manchester
Alliance Manchester Business School

PGDip Leadership & Management in the Healthcare Sciences
Unit marks ratified by Chair's Action, January 2022

Trainee name: Elizabeth Walker
Student ID: **10272247**
Award: PG Credit

Unit	Unit Title	Mark	Credits
BMAN73511	Unit A1 Professionalism and Professional Development in the Healthcare Environment	68% **Pass**	**30**
BMAN73522	Unit A2 Theoretical Foundations of Leadership	70% **Pass**	**20**
BMAN73531	Unit A3 Personal and Professional Development to Enhance Performance	77% **Pass**	**30**
BMAN73542	Unit A4 Leadership and Quality Improvement in the Clinical and Scientific Environment	63% **Pass**	**20**
BMAN73550	Unit A5 Research and Innovation in Health and Social Care	65% **Pass**	**20**
			120 / 120

Appendix 8. Certificate showing successful completion of the FRCPath Part I examination in Immunology

The Royal College of Pathologists

By these letters make it known that

Elizabeth Ursula Walker

having undertaken the required training and
after having passed the Part One examination in

Immunology

has been awarded

Diplomateship of
The Royal College of Pathologists

In witness whereof the Seal of the College and the signatures of the proper Officers have been affixed this thirteenth day of February 2020

LONDON

President *Registrar* *Member of Council*

Appendix 9. Letter to demonstrate the successful completion of FRCPath Part II examinations

The Royal College of Pathologists
Pathology: the science behind the cure

College Reference Number: **20006819**
Candidate Number: **489**

Elizabeth Ursula Walker

19 November 2021

Dear Mrs Walker

FRCPath Part 2 Oral Examination in Immunology – Autumn 2021

I am pleased to inform you that you have satisfied the Examiners in the Part 2 Examination.

However, as you are aware, you are not yet eligible to become a Fellow of The Royal College of Pathologists as your Part 2 Project has not yet been approved.

We look forward to receiving the project in due course. If you have any queries about your project, please contact exams@rcpath.org.

Congratulations on your success in this examination.

Yours sincerely

Dr Sanjiv Manek
Clinical Director of Examinations

Appendix 10. Innovation Proposal

Change of method and testing algorithm for investigating patients for Connective Tissue diseases from Anti-nuclear (ANA) testing to Connective Tissue Disease (CTD) screen

Executive Summary

Anti-nuclear antibody (ANA) testing is used to aid the diagnosis of connective tissue disease. In the Immunology laboratory at the Royal London Hospital, ANA testing is currently done by indirect immunofluorescence (IIF) using an automated slide processor, but relying on staff to manually read slides and enter results, followed by a second check. This process is at risk of error due to its manual nature with a large amount of staff hands-on time. This proposal seeks to change ANA testing by IIF, to Connective tissue disease (CTD) screening using an automated Elia method, for the vast majority of ANA requests. This would simplify the testing algorithm when investigating patients for CTDs. Other potential advantages of this include: reduced staff hands-on time, reduced risk of error, reduced turnaround time and false positive results, thereby benefiting patients. Potential barriers to this include current workload on the CTD analyser, de-skilling of staff and concerns from our service users. These will be addressed in this proposal.

Introduction

The Immunology laboratory at the Royal London Hospital covers Immunology testing for Barts Health Trust, local GPs, and many external hospitals in East London and Essex. A recent merger into the East and South East London (ESEL) partnership has meant that the work-load for the laboratory has increased. Streamlining of testing pathways, without a loss in quality, is one approach in helping the laboratory to absorb this increase.

ANA testing is commonly used in Immunology laboratories to aid the diagnosis of CTDs, such as Systemic Lupus erythematosus (SLE), Sjogren's syndrome and systemic sclerosis (Spikett, 2013). ANAs are tested using HEp-2 cell cultures as a substrate. These cells are in various stages of the cell cycle, enabling the identification of different patterns in positive ANAs. Certain patterns show disease associations, for example, a homogeneous pattern is associated with SLE, and a nucleolar pattern can be associated with systemic sclerosis (ANA SOP, Barts Health NHS Trust, 2021). These are identified by pattern recognition using a fluorescence microscope. Currently, the laboratory tests for ANAs using this method. The AP16 is an automated IIF slide processor, used for several tests in the autoimmune serology section of the laboratory. Serum samples and test substrates (slides) are loaded and tested in batches (AP16 Immunofluorescence analyser, Barts Health NHS Trust, 2020). However, mounting of slides, reading, second checking, entering and checking results onto the worklist are all done manually. Due to this, there is an increased capacity for error, although, mitigations are taken at each stage to minimize this. It also requires a large amount of staff hands-on time, due to the various steps required to read and enter patient results. Slide reading is inherently subjective, relying on skilled operators to identify clinically relevant patterns. If the ANA is positive, a series of other tests may be added. These include an Extractable Nuclear Antigen (ENA) screen, an ELISA, including a mix of antigens. If this is

positive, further ENA characterization takes place, using a blot method. An anti-double stranded DNA antibody (dsDNA) may also be tested, depending on clinical need. dsDNAs are routinely tested using the Elia method and new positive results confirmed by IIF. This additional testing adds to the complexity of the testing algorithm for these antigens, potentially lengthening turnaround times (TAT). See Appendix 1 for a visual representation of the testing algorithm for ANA and CTD testing.

Aim of this proposal

The aim of this proposal is to consider the consequences of a change from the IIF method of testing, to using CTD testing on the Phadia 250 analyser. The Phadia 250 is a continuous-loading, random-test-access machine, which is set up for a number of different tests at the start of the day and samples continuously loaded (Phadia 250 SOP, Barts Health NHS Trust, 2021). The solid-phase test substrate is bound to the bottom of a polystyrene well. The analyte, patient serum or plasma, is diluted and transferred onto the test substrate, before the addition of an enzyme-conjugated antibody, followed by a colourless development solution which is transformed into a fluorescent product by the reaction of the enzyme in the conjugate. The amount of fluorescence is proportional to the amount of analyte bound to the test substrate (Phadia 250 SOP, Barts Health NHS Trust, 2021). The CTD screen uses a mix of antigens to aid in the diagnosis of different types of connective tissue Connective Tissue Disease, namely: Ro, La, U1-RNP, Sm, Jo-1, SCL-70, PCNA, Centromere, Ribosomal P, U3-Fibrillarin, RNA Polymerase, Mi-2, PM-Scl and dsDNA. See Appendix 2 for a table showing the disease associations of these antigens.

Strategic fit

With the additional workload anticipated for the formalised ESEL pathology network, and joining of Lewisham and Greenwich NHS Trust, this proposal sits within, not just the Trust strategy, but also NHS England Long Term plan of 2019 and the subsequent Diagnostics: Recovery and Renewal report (4). See Appendix 3 detailing how this proposal fits into the Trust Strategy.

Outline of the case for need

The number of tests being carried out by the Immunology laboratory at the Royal London Hospital has increased gradually since 2011/12, only dipping temporarily during the first few months of the Coronavirus pandemic in 2020, see Appendix 4. Due to the formation of the ESEL pathology network the laboratory has also taken on work from Homerton University Hospital (HUH), Newham Hospital and Lewisham and Greenwich NHS Trusts (LGNT). 2021/22 activity increased by 50% from 2020/21 and by 4% from 2019/20. Further increase in workload is expected, with the acquisition of allergy testing from HUH in February 2023 and further incorporation of Immunology work from LGNT anticipated in the medium term. This requires change within the laboratory to successfully absorb this workload. A streamlining of some test workflows is one way in which the laboratory can fulfill this. The Immunology laboratory currently consistently receives over 1500 ANA requests per month,

which is an increase from 2021, see Appendix 5. There is also the expectation that this will increase, due to incorporation of LGTN work in the future.

Option appraisal

Option 1: Do nothing. With the knowledge that workload is due to increase, due to further consolidation of work through the ESEL Pathology Partnership, this would increase the workload and pressure on staff and lead to a fall in the ability of these staff to use their time for other aspects of their jobs, such as further validation of new assays and quality improvement work. This could potentially lead to an increase in TAT for this test, leading to poorer patient care.

Option 2 (preferred option): To change the method for testing for connective tissue diseases from using ANA to CTD screen. This would be done in conjunction with our service users, particularly the Rheumatology department and GPs.

Option 3: Look at alternative technologies for slide reading, such as automated slide readers. This would not address the issue of increased workload completely.

Benefits of proposal implementation

Reduction in staff hands-on time: There are currently several manual steps involved using the ANA method. The slides, samples and reagents are loaded onto the AP16 analyser where it dilutes the samples, and processes them onto slides. The operator must then mount the slides and read them on the fluorescence microscope. Once read by the operator, a second person second reads the slides to ensure agreement. Once this is complete the operator enters the results manually onto the laboratory LIMS system. The transcription is then checked by a second, more senior staff member, before the results are validated and transferred to the patient record. See Appendix 6 for a comparison of the hands-on time required to set up the ANA slides verses the CTD screen. As can be seen from this comparison, by moving the ANA to the CTD screen, 95 minutes of staff hands-on time can be saved per run of samples, freeing up staff to undertake other duties.

Reduction in errors: The process of ANA slide screening is subjective and therefore errors can be made during interpretation of ANA patterns. Also, by entering results manually, rather than using an automated interface, this process has a greater potential for error. This is despite processes being put in place to minimize this risk, such as second reading of slides and second checking the manual transcription of results. By moving this assay to the CTD screen, the samples are barcode scanned when loading on the analyser and results are a numerical value, not subject to manual interpretation. Results are validated by senior staff on the analyser itself and results go back to the laboratory LIMS system automatically, negating the need for second checking of results.

Lower staff grades able to set up the test: Currently, the staff grades which set up ANA testing and read slides are Band 5 or 6 Biomedical Scientists. Those second screening and transcription checking the slides are Band 7 or 8, as reading and interpreting slides are a highly skilled activity. This uses senior staff time running routine work when they could be using their time to greater effect in the strategic direction of the laboratory. In contrast, the

Phadia 250 is currently run by Band 4 – 6 Biomedical scientists. One of the laboratory's strategic goals is to enable more Band 4 scientists to run the automated analysers, freeing up more senior scientists to validate and undertake other roles in the laboratory, therefore this change would fit into the strategic goals of the laboratory.

Reduction in TAT: The current stated TAT of ANAs is 10 days, due to positive results requiring add-on tests. These consist of an ENA screen. This is done twice weekly and if this is positive, further ENA characterization takes place in the form of an ENA blot. A dsDNA may also be added on. This consists of Elia run on the Phadia 250 and if this is a new positive, a confirmatory IIF is done. Appendix 1 shows a flow chart of testing for these assays.

Reduction in cost per test: See Financial analysis.

Reduction in number of false positive ANA results: The process of screening for CTD by ANA is subjective and while this is a sensitive assay, it is not specific (Steiner et al., 2017). There can be weak positive ANAs with negative ENA and DNA results, in older people, those with infection and in the general population (Spikett, 2013). This can lead to unnecessary referral to Rheumatology. Currently, 70% of our ANA tests are negative, 20% are positive for ANA with a negative ENA and dsDNA and 10% have a defined clinical specificity (Chris Scott, personal communication). This means that there may be patients who have a positive ANA with no evidence of CTD whose test results give them undue concern and a potential unnecessary referral to secondary care. By changing to a more specific test, which utilizes specific recombinant antigens rather than an ANA pattern, these false positive results could be reduced, leading to a reduction in unnecessary patient concern over these test results and fewer inappropriate referrals to Rheumatology which would benefit the Trust overall. Clinical audit would be undertaken once this change has been implemented to verify this.

<u>Potential barriers to implementing this change:</u>

Capacity on Phadia 250 analysers: The Phadia 250 is already used for a wide number of autoimmune tests. The addition of CTD screening would increase this further. The capacity of this analyser may be reached, causing a backlog leading to delays in reporting out results. However, this could be replaced by a Phadia 2500, a larger model of analyser, which has far greater capacity and is already being considered as part of future planning for the laboratory.

Unusual ANA patterns would be missed: Patient antibodies to antigens which are not in the CTD screen would be missed by switching from ANA to CTD. These may result in unusual patterns on the ANA screen which would prompt further testing. However, these patterns may not be clinically relevant and the reporting of them may add to confusion about the patients' diagnosis. Furthermore, ANA testing would still be retained in house, due to requests from referral laboratories, and so any patient where ANA testing may aid their diagnosis, could still have it. This would be done upon individual request by their clinician.

De-skilling of staff: One of the benefits of this change would be that lower grade staff could run this test, freeing up the higher grades for other tasks, however, reduce the number of staff able to screen ANA slides. This is an important and sought-after skill in Immunology. This may impact staff morale, in believing that they are being replaced by automation and feeling like their role is being dumbed-down. If this change were to go ahead, this would have to be carefully managed and communicated. However, one counterargument to this, would be that staff reading slides after this change, would be likely to see more positive results, due to them being sent as confirmatory tests, rather than screening out the vast majority of negative results.

Alienation of stakeholders: Our current users would be consulted as part of this proposal and the advantages and disadvantages of this proposal discussed. Stakeholders are used to the current system of testing and reporting and may be reluctant to change. Engagement with stakeholders would include meeting with our main user groups, Rheumatology and GPs to discuss the impact of this change and address any concerns.

Quality Impact

The change in testing from ANA to CTD screen will result in the following increases in quality:

- Reduction in TAT, without comprising on clinical utility (Robier et al., 2015)
- Reduction in errors due to increased automation
- Freeing up staff time for further quality improvement work by reducing hands-on time
- Aiding in future proofing the department to be able to sufficiently respond to further acquisition of work due to the ESEL Pathology Partnership.

Financial Analysis

Appendix 7 shows the staffing cost per typical assay run for the ANA and CTD tests. This was calculated by using the minutes of hands-on time described in Appendix 6 and using the Trust Day rate for staff shown in Appendix 8. The cost is shown per typical run of samples done on a day. The minimum staffing cost per ANA run with a Band 5 setting up, slide reading and entering, Band 7 second screening and Band 8a authorising is £57.98. The minimum staffing cost per run of CTD samples with a Band 4 setting up and a Band 7 authorising is £18.70. Reagent costs per test for ANA and CTD are £3.25 and £3.55 respectively. Despite the reagents being slightly more expensive for the CTD screen, this would be outweighed by the much-reduced staffing costs, leaving the cost cheaper overall. Appendix 9 shows an example of the annual cost saving of bringing in this change.

The Phadia 250 analyser is already in use in the laboratory, meaning that new equipment would not need to be purchased. Reagents to carry out CTD testing on the Phadia 250 would need to be purchased, but would be offset by the reduction in reagents required for ANA testing. All of the bulk reagents for this analyser are already in use.

Summary and Key benefits of proposal

The implementation of this proposal will result in the below benefits:

- Improved turnaround times for testing
- Reduced staff hands-on time, particularly for Senior staff, Band 7 and above, enabling staff to undertake other tasks and reducing the cost of this assay
- Reduction in potential error and improvement in consistency of reporting
- Reduction in false positive ANA results, leading to fewer unnecessary referrals to secondary care, thereby benefitting the Trust as a whole
- Alignment to the departmental strategic plan of upskilling Band 4, 5 and 6 staff
- Alignment to the NHS Long Term Plan (2019) and Diagnostics: Recover and Renewal Report (2020)
- Alignment to the Trusts' Strategic plan

The implementation of this proposal would bring about benefits to laboratory staff, stakeholders and patients by reducing TATs, potentially decreasing unnecessary referrals to secondary care and undue concern from patients due to false positive ANAs.

References

IM A06 Anti-Nuclear Antibodies (ANA). (2021). Barts Health Trust Standard Operating Procedure.

IM E55 AP16 Immunofluorescence Processor. (2021). Barts Health Standard Operating Procedure.

IM_E052 Phadia 250. (2021). Barts Health Standard Operating Procedure.

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Robier, C., Amouzadeh-Ghadikolai, O., Stettin, M. and Reicht, G. (2015). ,'Comparison of the clinical utility of the Elia CTD Screen to indirect immunofluorescence on Hep-2 cells.' *Clinical Chemistry and Laboratory Medicine.* 54(8), pp: 1365-1370

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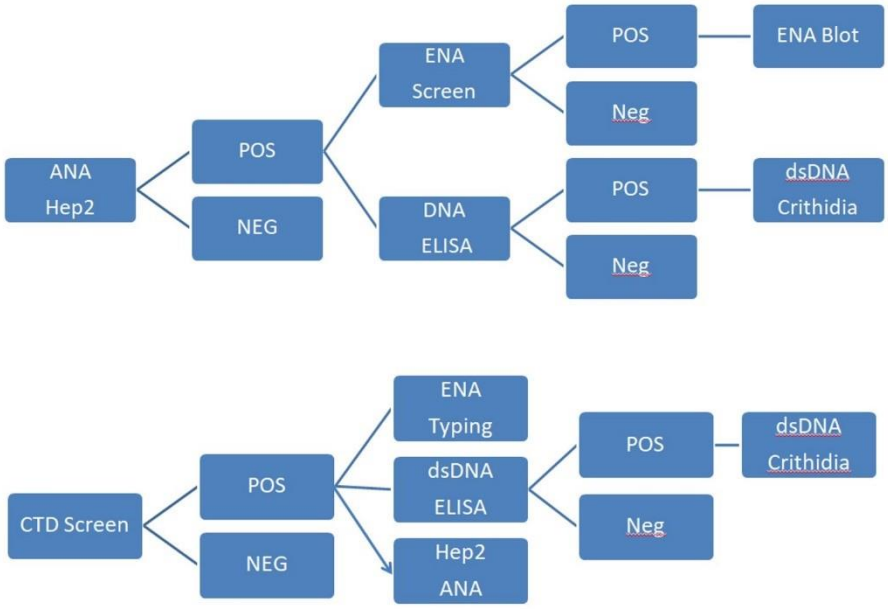
Steiner, G., Perkmann, T., Horn, T. And Kiener, H.P. (2017). ,'An ANA screening assay (ELIA CTD Screen) containing multiple antigens increases the sensitivity and specificity of ana testing by indirect immunofluorescence.' *Annals of the Rheumatic Diseases.* 76 (Suppl 2), pp.1414.

The NHS Long Term Plan. (2019). Available at: NHS Long Term Plan » The NHS Long Term Plan. Accessed: 25/10/2022.

Appendix

Appendix 1. Visual representation of testing algorithm for ANA verses CTD screen testing. ANA testing pathway has more complexity. The need for an ENA screen is removed by the implementation of the CTD screen, as typing can be done straightaway. The Hep2 ANA would not necessarily need to be done for every positive CTD screen. This would be discussed with our stakeholders, particularly the Rheumatology department to understand if this was needed.



Appendix 2. Table showing antigens included in the CTD screen, alongside their associated connective tissue disease

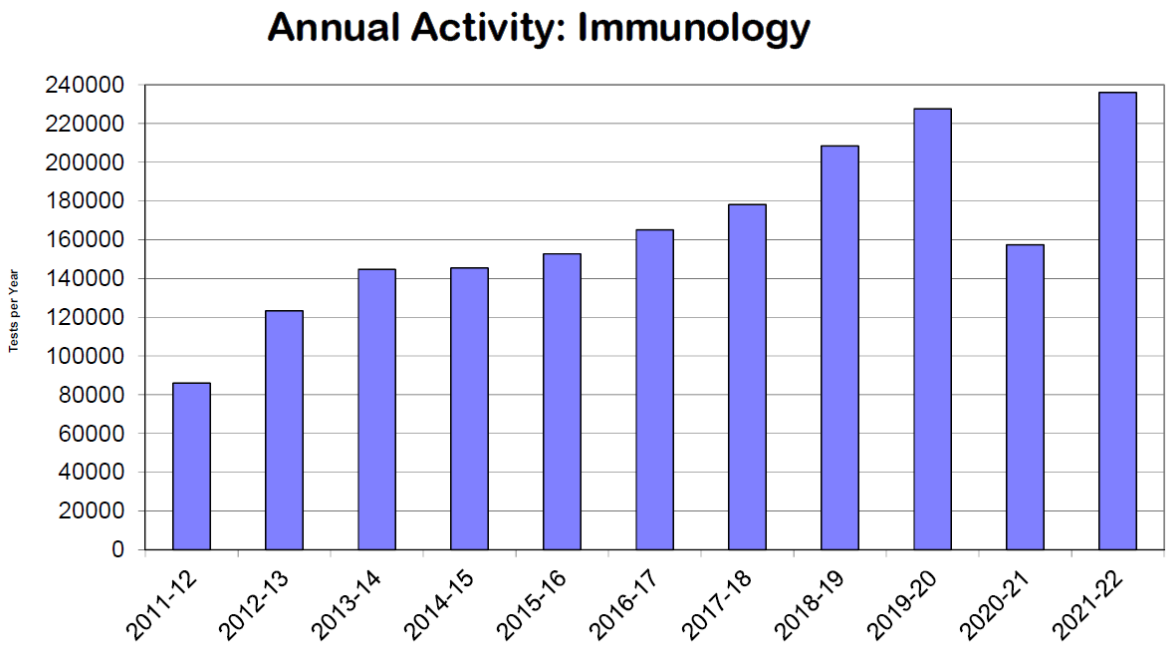
Antigen	Disease association
Ro	SLE, Sjogrens disease
La	Sjogrens disease
U1-RNP	SLE, Mixed connective tissue disease
Sm	SLE
Jo-1	Myositis, Anti-synthetase syndrome
Scl-70	Systemic sclerosis
PCNA	SLE
Centromere	Limited scleroderma
Ribosomal P	Neuro-psychiatric SLE
U3-Fibrillarin	Systemic sclerosis
RNA Polymerase	Systemic sclerosis
Mi-2	Myositis
PM-Scl	Polymyositis-scleroderma overlap
dsDNA	SLE

Appendix 3. Description of how this proposal fits into the Trust Strategy

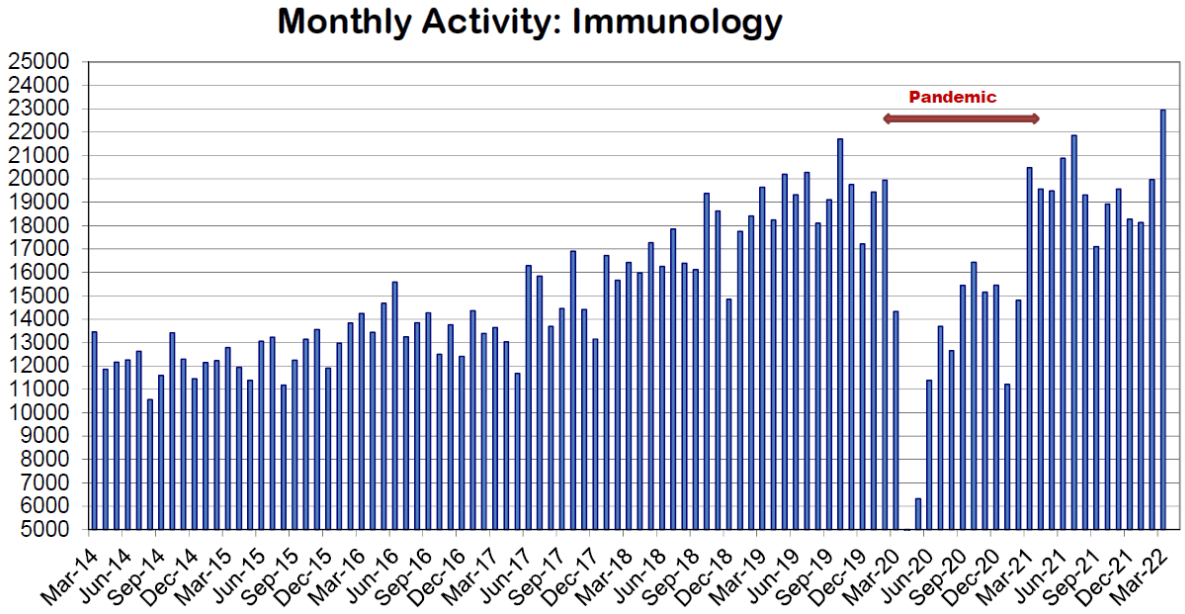
- **SA01 - Safe and compassionate care**
  - Reduction in turnaround times, aiding the timely diagnosis of connective tissue diseases
  - Potentially reducing the number of false positive ANA results, leading to a reduction of unnecessary referrals to Rheumatology.
- **SA02- Efficient and effective services**
  - Reducing subjectivity in the reading of ANA slides
  - Reducing laboratory staff hands-on time by removing the need to read a vast number of slides as well as reducing errors in the transcription of results.
- **SA03 – Service Transformation**
  - Enabling the laboratory to take on an increased workload due to the ESEL partnership by reducing staff hands-on time and reducing turnaround times.
  - Increase the use of automation to provide a robust pathology service.
- **SA04 - Improving our infrastructure**
  - Future-proofing the service against further predicted increases and complexity in workload.
- **SA06 – Developing our people**
  - Band 4 Associate Practitioners will be upskilled to be able to run this test on the Phadia 250. This will enable those Biomedical Scientists on higher bands which had previously been reading slides and entering results to be freed up to undertake more complex or quality improvement work.
  - Senior staff who previously second screened and checked this assay will be freed up to undertake more complex and quality improvement and quality management tasks.
- **SA07 - Reducing variation and improving productivity**
  - Subjectivity of slide reading will be removed and variation in results will decrease due to increased automation.
  - Increased automation will improve laboratory productivity by increasing turnaround times for this assay and it's associated assays, and freeing up staff time to enable them to work on other aspects of the service.
- **Better research and education**
  - Clinical audit will be undertaken using the data obtained from CTD testing alongside ANA testing, to verify the benefits of moving from a patten-based approach to an antigen-specific approach. The expectation is that this would decrease the numbers of patients which may have a positive ANA but negative ENA on further testing.

Appendix 4. Annual activity of testing in the Immunology laboratory, broken down by month in graph 2.

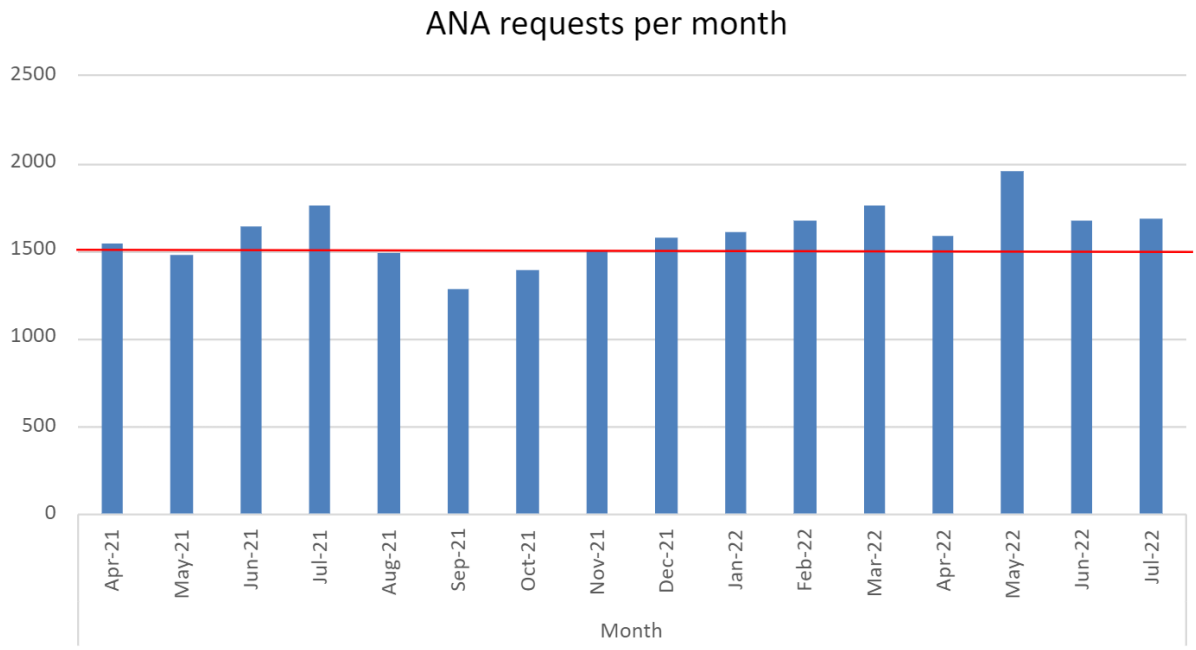
Graph 1. Annual activity of all tests in the Immunology laboratory.



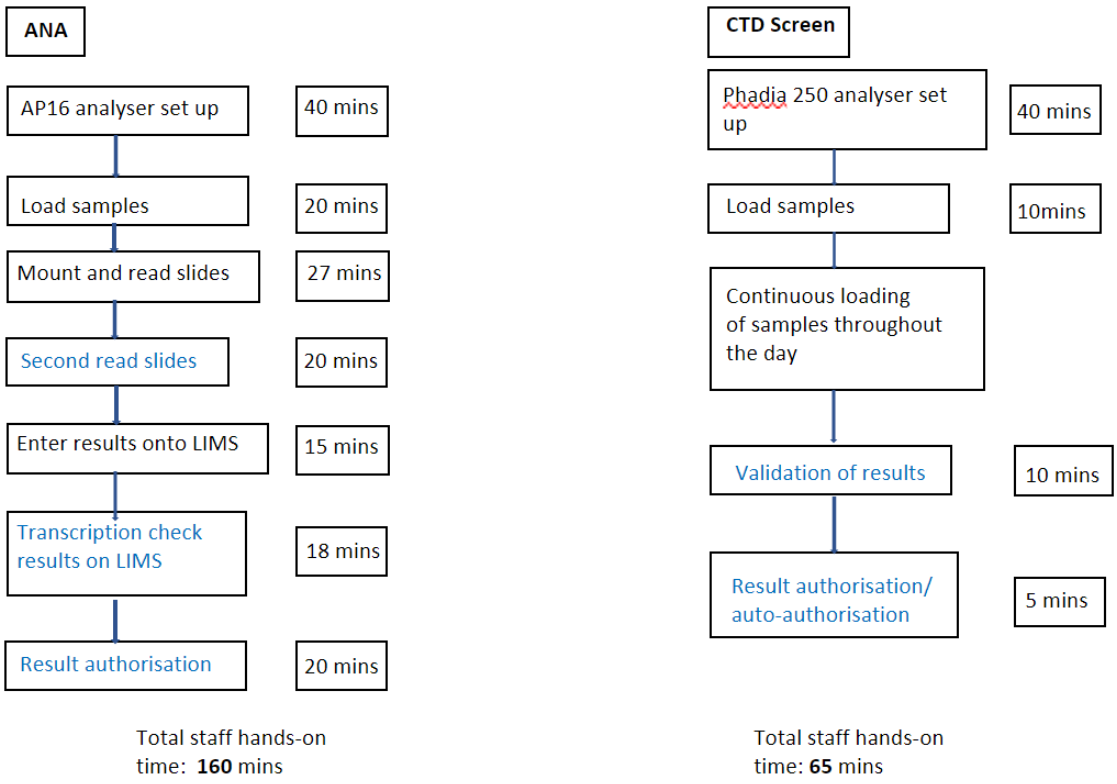
Graph 2. Annual activity of all tests, broken down by month.



Appendix 5. Number of ANA tests being done each month between April 2021 and July 2022.



Appendix 6. Flow chart showing the steps for the ANA verses the CTD screen.
The staff hands-on time for each step is show. In minutes (mins). The steps shown in blue, represent those undertaken by a senior staff member, Band 7 or above.



Appendix 7. Table showing staff costs per run of ANA and CTD screen.

	Staff hands on time	Set up	Slide read and enter	Second screen and transcription check	Authorise/ validate
ANA	**Band 5**	20.44	9.34		
	Band 6s	29.77	13.60		
	Band 7s			£17.64	
	Band 8a			20.16	10.56
CTD					
	Band 4	£11.70			
	Band 5	£21.22			
	Band 6s	£21.29			
	Band 7				£7.00
	Band 8a				£8.00

Appendix 8. Table showing hourly staff rate used in financial analysis. The New Day Rate was used.

		Subs Total Bank Pay Rates				
	Old Day Rate	**New Day Rate**	**Old Sat/Night**	**New Night/Sat**	Old Sun/BH	**New Sunday/BH**
Band 2	£11.50	£12.89	£14.72	£16.50	£19.21	£21.53
Band 3	£12.38	£13.87	£15.37	£17.23	£19.51	£21.86
Band 4	£12.67	£14.20	£16.48	£18.47	£20.28	£22.73
Band 5	£15.72	£17.62	£19.25	£21.57	£23.69	£26.55
Band 5 S	£20.50	£21.68	£25.00	£26.00	£29.00	£29.00
Band 6	£17.35	£22.22	£22.56	£28.89	£27.76	£35.55
Band 6 S	£21.61	£25.66	£26.26	£31.86	£31.68	£35.55
Band 7	£20.31	£27.03	£26.41	£31.86	£32.50	£36.42
Band 7 S	£23.70	£28.00	£29.76	£33.35	£36.62	£41.04
Band 8a	£24.88	£32.00	£31.93	£32.00	£39.30	£32.00
Band 8b	£29.06	£32.57	£37.54	£32.57	£46.20	£32.57
Band 8c	£33.80	£37.88	£43.90	£37.88	£54.03	£37.88
Band 8d	£40.08	£44.92	£52.11	£44.92	£64.13	£44.92

Appendix 9. Example calculation done for the annual savings which could be achieved by bringing in this change.

Current	Activity	Pay	Non-Pay	Total Cost
ANA	12680	£57.98	£3.25	£776,396.40
Saving				**£776,396.40**

Proposed*	Activity	Pay	Non-Pay	Total Cost
ANA	1268	£57.98	£3.25	£77,639.64
CTD	12680	£18.70	£3.55	£282,130.00
Saving				**£359,769.64**

*** Assuming 10% positivity**

Annual Saving **£416,626.76**