Development of strategies for manufacturing consistent T-cell populations

Development of Strategies for Manufacturing Consistent T-cell Populations

by

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Abstract

With an aging population, cancer is becoming increasingly prevalent in society. Statistics now suggest that half of all people will be diagnosed with cancer in their lifetime. In recent years there has been major progress in immuno-oncology, particularly in T-cell based therapies. The main advancement in this field has been the development of Chimeric Antigen Receptor (CAR) T-cell therapies, which use genetically reprogrammed T-cells' natural cytotoxic pathways to induce apoptosis in target cells.

There has been a large amount of work focussed on identifying T-cell population characteristics which can aid in the manufacture and efficacy of the therapy. In particular, the balance of subpopulations which allow improved viral transduction in culture, and improved efficacy and persistence on administering the therapy. However, there was limited evidence of work investigating the impact of the T-cell culture process and the balance of these subpopulations. This research sought to improve the definition of T-cell culture to improve confidence in predicting the output material as well as identifying conditions which may promote preferred populations.

Input material, delaying IL-2 supplementation, and concentrated incubation prior to standard culture was analysed for their impact on T-cell culture. Viable cell count and flow cytometry were used identify the impact of these elements on culture growth and phenotypic profile. In addition, a novel phenotypic analytical method was developed in response to limitations with the common method observed in the field. The novel method utilised algorithmic analysis of phenotypic data to reduce user bias, whilst introducing optimal phenotypic resolution of analysis.

Analysis of input material and CD4/CD8 cell balance appeared to show that there were limited differences in behaviour of the subpopulations, with primary negative responses seen in cultures that had undergone a greater level of manipulation. Delaying the supplementation of IL-2 into culture appeared to have a positive effect compared to immediate supplementation, however the evidence suggested that this was potentially no better than cultures not supplemented with additional IL-2. Investigation of concentrated incubation prior to seeding into culture had variable responses using different donors. This work proved inconclusive and requires further investigation, however the evidence suggested that the process may induce greater growth in cultures and increased maturation of subpopulations. It was also possible to observe across the breadth of the experimentation that an increase in proliferation of T-cell cultures correlated with an increased phenotypic shift to Effector Memory phenotype populations.

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List of Abbreviations

Abbreviation	Explanation
ALL	Acute lymphoblastic leukaemia
APC(s)	Antigen presenting cell(s)
BSA	Bovine Serum Albumin
BSC	Biological Safety Cabinet
CAR	Chimeric Antigen Receptor
CM	Central Memory
DAPI	4’,6-diamidino-2-phenylindole
DD	Double depleted
DLBCL	Diffuse large B-cell lymphoma
DoE	Design of Experiment
E	Effector
E2N	Effect 2 Noise ratio
EDTA	Ethylenediaminetetraacetic acid
EM	Effector Memory
FACS	Fluorescence Activated Cell Sorter
FBS	Foetal Bovine Serum
FME	Full medium exchange
FSA	Forward scatter area
HER2	Human Epidermal Growth Factor Receptor 2
HLA	Human Leukocyte Antigen
HTA	Human Tissue Authority
IC	Incubation Control
MFI	Median fluorescence intensity

MHC	Major histocompatibility complex
MNCs	Mononuclear Cells
N	Naïve
NoI	Node(s) of Interest
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PMTs	Photomultiplier tubes
RPMI	Roswell Park Memorial Institute
scFv	Single chain variable fragment
SLOs	Secondary Lymphoid Organs
SPADE	Spanning-tree Progression Analysis of Density-normalised Events
SPC	Standard Protocol Control
SSA	Side scatter area
SWIFT	Scalable Weighted Iterative Flow-clustering Technique
TCR	T-cell receptor
TILs	Tumour Infiltrating Lymphocytes
TNN	Target Number of Nodes

1 Introduction

1.1 Introduction to stratified medicine and CAR T-cell therapies

Over time, as the scientific body of knowledge grows, so does the field of medicine[1]. Medicine encompasses an expansive range of topics covering all elements of human health[2,3]. Improvements in the understanding of disease pathology, as well as the mechanisms behind treatments, means that medicine is constantly evolving in an effort to improve both the efficacy and availability of treatments. Historically, observation of the protective effects of cowpox on smallpox lead to vaccination, discovery of Human Leukocyte Antigen (HLA) matching led to reduced rejection in organ transplants and in-depth analysis of DNA has unlocked the ability to mass produce products such as insulin in *E. coli*[4–14].

As the understanding of disease and the human response has evolved, so has the appreciation for the variability of individual to influence that response[15,16]. In particular, historically treatments have been developed on the understanding that all individuals will respond similarly[17]. The primary concern in initial development of a treatment is that it is safe and efficacious[18–21]. However, once initial development is complete it would be possible to examine the responses and analyse patterns to identify trends. Tailoring of treatments to accommodate specific subpopulations of patients based on characteristics is known as personalised or stratified medicine[15–17].

Stratified medicine is defined by the medical research council as "identifying subgroups of patients with distinct mechanisms of disease, or particular responses to treatments" making it possible to "identify and develop treatments that are effective for particular groups of patients [*ensuring*] that the right patient gets the right treatment at the right time"[22]. However, for a stratified approach to a treatment to be developed it must first be justified[23,24]. The process of therapy stratification takes both time and money, so for it to take place there needs to be a substantial improvement on patients' quality of life through stratification[24–26]. This calculation is likely to be made ahead of time based on the cost of the work involved, the prevalence of the disease and the applicability of the final product[24].

This approach has existed for a long time in treatments such as blood transfusions, where it was identified that giving patients blood of the same blood type reduced further issues and improved recovery[10,27,28]. Similarly, it has been used in HLA typing to reduce the level of organ rejection in transplant patients, increasing the quality and length of life of the patient and limiting the number of unsuccessful transplants[29–33]. With the advent of genotypic analysis, it then became possible to measure if response was due to specific markers, such as in the case of Herceptin. Approximately 1 in 5 breast cancer cases in the UK are Human Epidermal Growth Factor Receptor 2 (HER2) positive,

meaning that they will respond to treatment with trastuzumab (brand name Herceptin)[34,35]. Herceptin was brought in as a treatment for patients with high expressing HER2 and was able to reduce recurrence by 52% in conjunction with chemotherapy[36]. However, in the field of cancer drug development, this is one of the few success stories. The heterogenous nature of tumours often means that stratified treatments are not 100% effective, and initial positive results overcome or superseded by non-responsive clones[16].

One branch of therapies that falls under stratified medicine is autologous cell therapies[37,38]. Autologous cell therapies utilise the patient's own cells to treat the disease, as opposed to allogenic cell therapies, using cells from other donors. Autologous therapies can consist of procedures as simple as transferring a sample from one site in the body to another, such as in skin grafts, involve a greater level of manipulation, such expanding the population and inducing phenotypic change in autologous chondrocyte implantation, or involve manipulation at the genetic level, such as the use of viral vectors to induce Chimeric Antigen Receptor (CAR) expression on T-cells in CAR T-cell therapy manufacture. CAR T-cell therapies are a relatively new autologous therapy, with the first commercial products, Yescarta (Kite Pharma) and Kymriah (Novartis), only approved by the FDA in 2017. In England the therapy is currently used to treat certain B cell cancers and involves genetic manipulation of T-cells to express CARs. These CARs can bind to target proteins on the cancer cells and induce apoptosis by utilising the natural cytotoxic pathways available to T-cells.

1.2 History of CAR T-cell therapies, the current state of the art and manufacturing challenges

T-cells are lymphocytes that have differentiated from hematopoietic stem cells (HSCs) and form part of the adaptive immune system (Fig 1.1). There are two main subpopulations of T-cells, CD4 expressing Helper T-cells and CD8 expressing Cytotoxic T-cells. The protein expressed informs their role in the immune system, and expression of these proteins on mature T-cells is mutually exclusive. After HSCs' initial differentiation into a common lymphoid progenitor cells, those which are to become T-cells migrate to the thymus for development, with 'thymus' being the origin of the T-cell name. Whilst in the Thymus, the cells undergo a maturation and selection process which induces the expression of the T-cell receptor (TCR), a multiprotein complex that enables binding to target antigens in an immune response, as well as specificity for the target antigen (Fig 1.2). The level of specificity the TCR affords the T-cell is akin to that of an antibody. The external domains of the TCR can be grouped into variable and constant regions, with the constant region being comprised of CD3 proteins. The internal domains of the TCR propagate signals on binding of the target antigen to induce an immune response. In addition to the TCR, this process will also induce the expression of both CD4 and CD8, proteins which help in the target binding process and dictate the role of the cells.

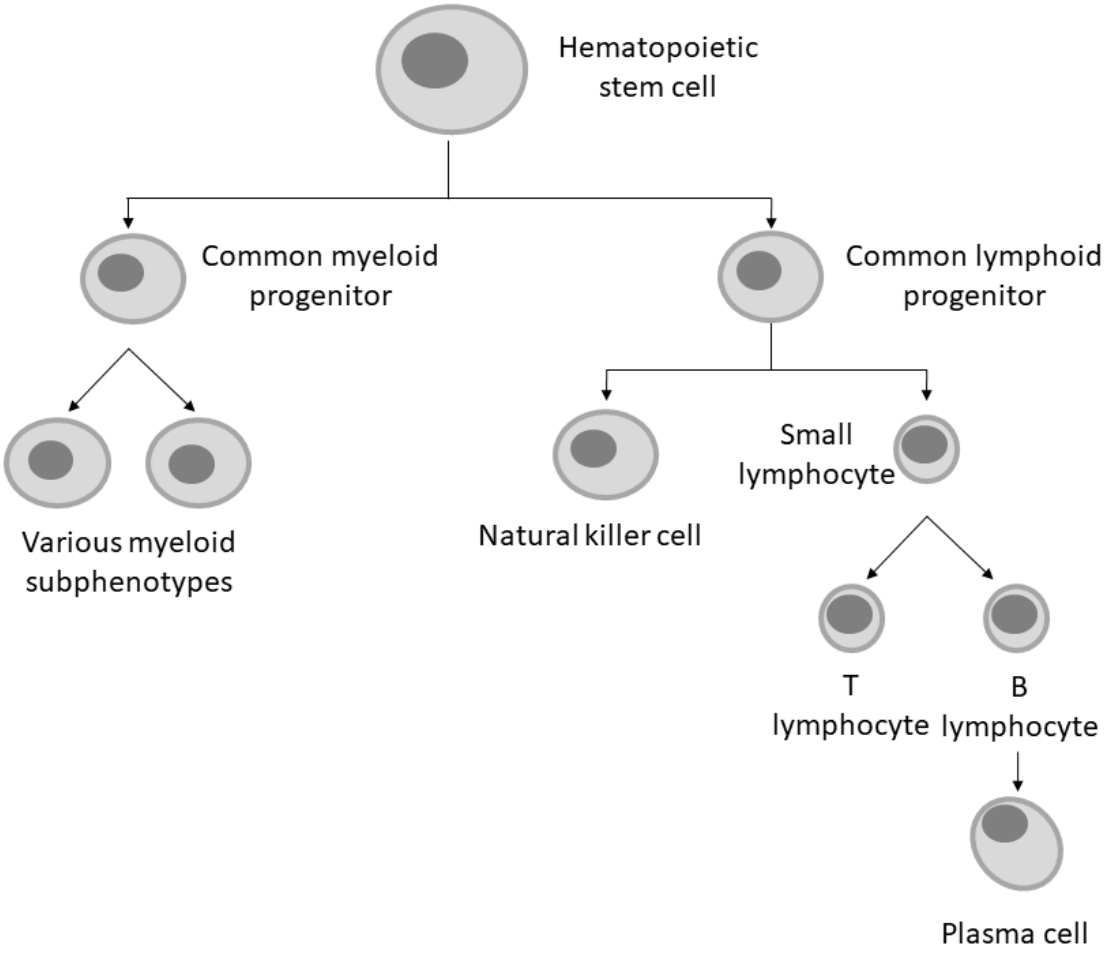


Figure 1.1. Diagram of blood cell development in the Hematopoietic system. The diagram shows how hematopoietic stem cells go through stages of being common lymphoid progenitor and small lymphocyte cells before becoming T-cells. *Adapted from https://en.wikipedia.org/wiki/Hematopoietic_stem_cell#/media/File:Hematopoiesis_simple.svg*

A trait of the TCR is that it can only detect antigens presented in a major histocompatibility complex (MHC) protein of another cell. MHC are proteins that are able to present short peptide chains to T cells. There are two classes of MHC, class I is expressed on nearly all nucleated cells, whilst MHC class II is only expressed by professional antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells. Cells will regularly take short chains of proteins being produced within the cells for presentation in MHC class I, allowing the cell to give a snapshot of what is currently undergoing manufacture in the cell. Meanwhile, MHC class II is used by APCs to present short chains of pathogens that they have endocytosed as part of their immune response[39,40].

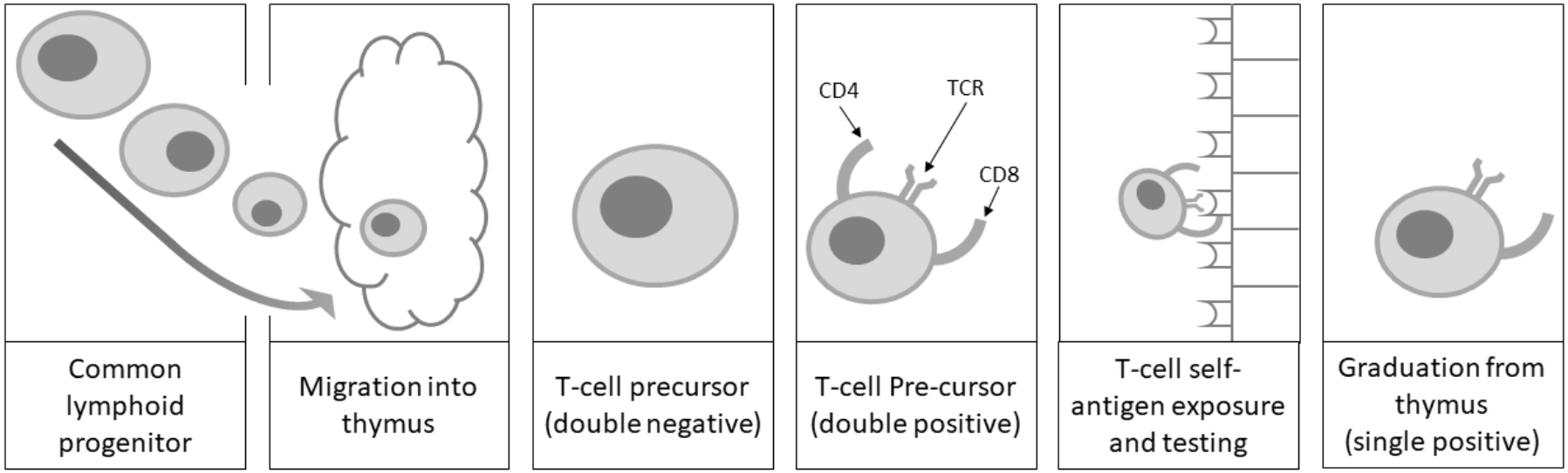


Figure 1.2. Pathway for T-cell genesis. Common lymphoid progenitors selected to become T-cells migrate to the thymus, where they become T-cell precursors and upregulate expression of CD4, CD8 and their T-cell receptor (TCR). After testing response to self-peptides, T-cells will be pushed down a pathway of expressing only CD4 or CD8, and can graduate from the thymus to perform their immune function.

Once the functional TCR is expressed, T-cells then migrate to another area of the thymus and are exposed to self-antigens presented on MHC proteins of both classes. If the TCR is unable to interact with either of the MHC classes it will receive an apoptotic signal, however if interaction is possible this signal will not be given. The TCR is able to bind to antigens presented on both MHC class I and II, however CD4 and CD8 will only bind to a specific class, with CD4 only binding MHC class II, and CD8 only binding MHC class I. Within the thymus, once the T-cell has bound to its target antigen either CD4 or CD8 will bind based on the MHC class the peptide is presented in, and this will induce a change in the cell. On binding to MHC class I, cells will downregulate the expression of CD4 and become cytotoxic CD8+ T-cells, whilst if they bind to MHC class II they will instead downregulate CD8 and become CD4+ cells, either Helper T-cells or Regulatory T-cells. In the next stage of selection of T-cells, they migrate to another area of the thymus and are again exposed to self-antigens in MHC proteins. If the T-cell binds with too high an affinity for the self-antigen they will be given an apoptotic signal, as it is indicative of cells that could produce an autoimmune response[39,40].

At this point T-cells will migrate out of the thymus as 'graduated' T-cells. As part of the selection process the T-cells will have become CD4+ or CD8+, with expression of the proteins being mutually exclusive. These markers are representative of their roles in the body, as highlighted by their MHC based selection in the thymus. CD4+ cells are able to view antigens presented by MHC class II, expressed on antigen presenting cells. The role of Helper T-cells in immune response, as suggested by the name 'Helper', is predominantly as a second stimulus for activating other immune cells such as B-cells, Cytotoxic T-cells and macrophages. Meanwhile, regulatory T-cells are stimulated in the same manner, but as with their specificity for self-antigens their response is immunosuppressant to reduce autoimmune activity. In both instances these cells achieve their role through secretion of cytokines. Helper T-cells and Regulatory T-cells have different cytokine profiles, and there are further subgroups of Helper T-cells which have different functions in immune response which require different profiles of cytokine production. CD8+ cells are able to view antigens presented on MHC class II, expressed on nearly all nucleated cells in the body. The role of these Cytotoxic T-cells is to monitor cells of the body for unusual activity. This activity could be the expression of incorrect proteins in tumour cells, or presentation of foreign antigens in virus infected cells. On stimulation, Cytotoxic T-cells induce apoptosis in the target cell through the release of perforins to form pores in the target cell membrane, and granzymes which enter through these pores and induce the apoptosis. Through this specific targeting process, T-cells are able to remove the infected or faulty cells whilst theoretically leaving other surrounding cells intact and unaffected[39,40].

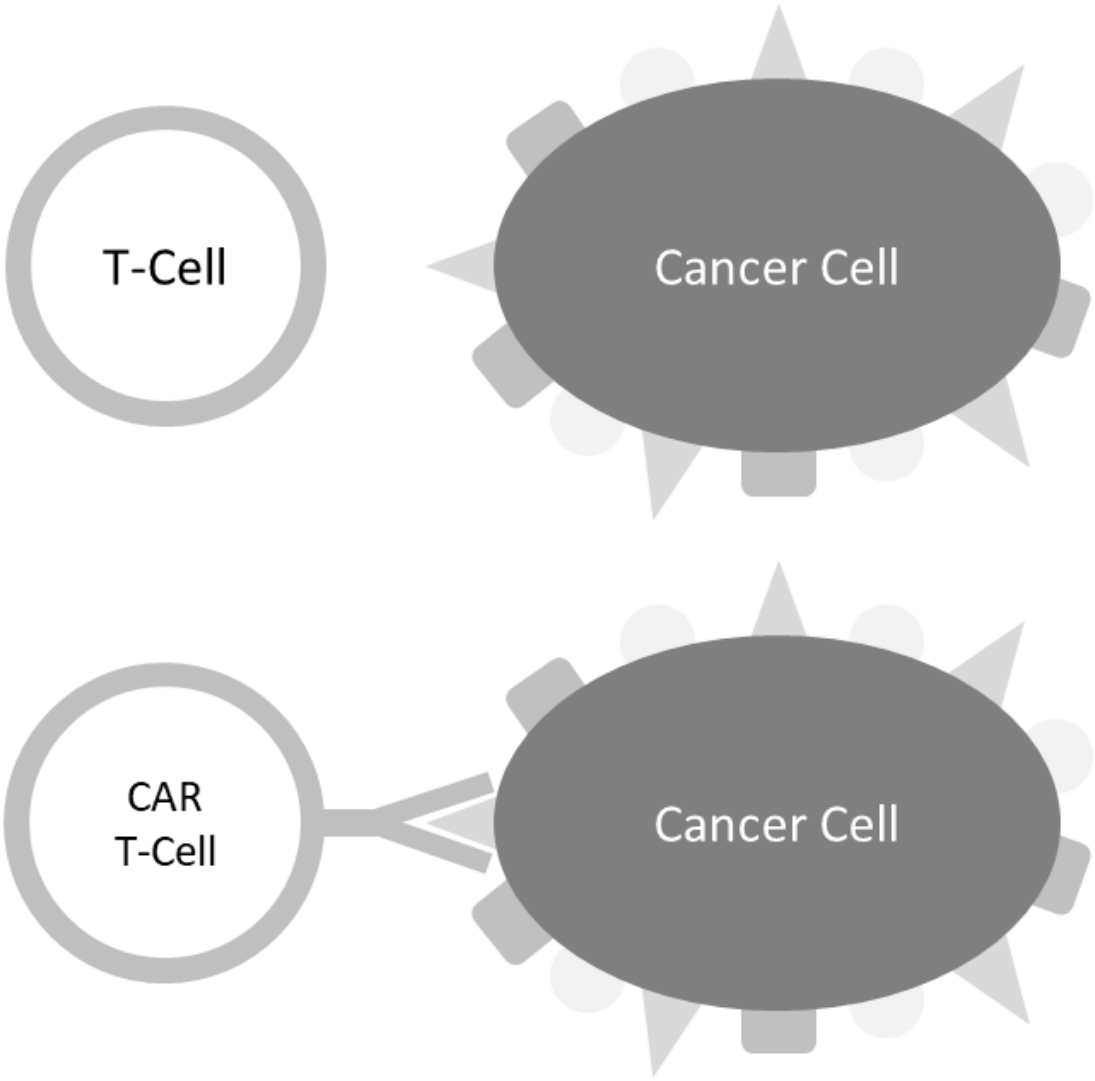


Figure 1.3. Expression of Chimeric Antigen Receptor (CAR) on T-cell surface allows detection of and response to cancerous cells. The diagram shows how the T-cell's reliance on antigen presentation in MHC proteins can reduce the ability to identify cancerous cells when MHC is downregulated. Genetic reprogramming to express CARs, with their direct binding domain, restores reactivity to cells.

Diagram adapted from https://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/immunotherapy/types/CAR-T-cell-therapy

Cancer T-cell therapies have used the cytotoxic abilities of CD8+ cells to induce apoptosis in target cancerous populations[41,42]. The first example of using T-cells as a cancer treatment was Tumour Infiltrating Lymphocytes (TILs)[43–45]. Tumours will often create an immunosuppressant environment, making normal immune response difficult, even when T-cells reactive to the tumour antigens are present[46,47]. TILs are generated through harvesting of tumour material which contains T-cells

responsive to the tumour antigen[48,49]. These cells are then cultured and repeatedly stimulated to generate a large enough population of the target T-cells that they can produce a substantial effect on the tumour despite the immunosuppressant nature of the environment[45,48–51]. Although this process works in theory, it does not have a short production time, and is also reliant on there being a suitably large population of T-cells responsive to the tumour in the initial harvest[45,52].

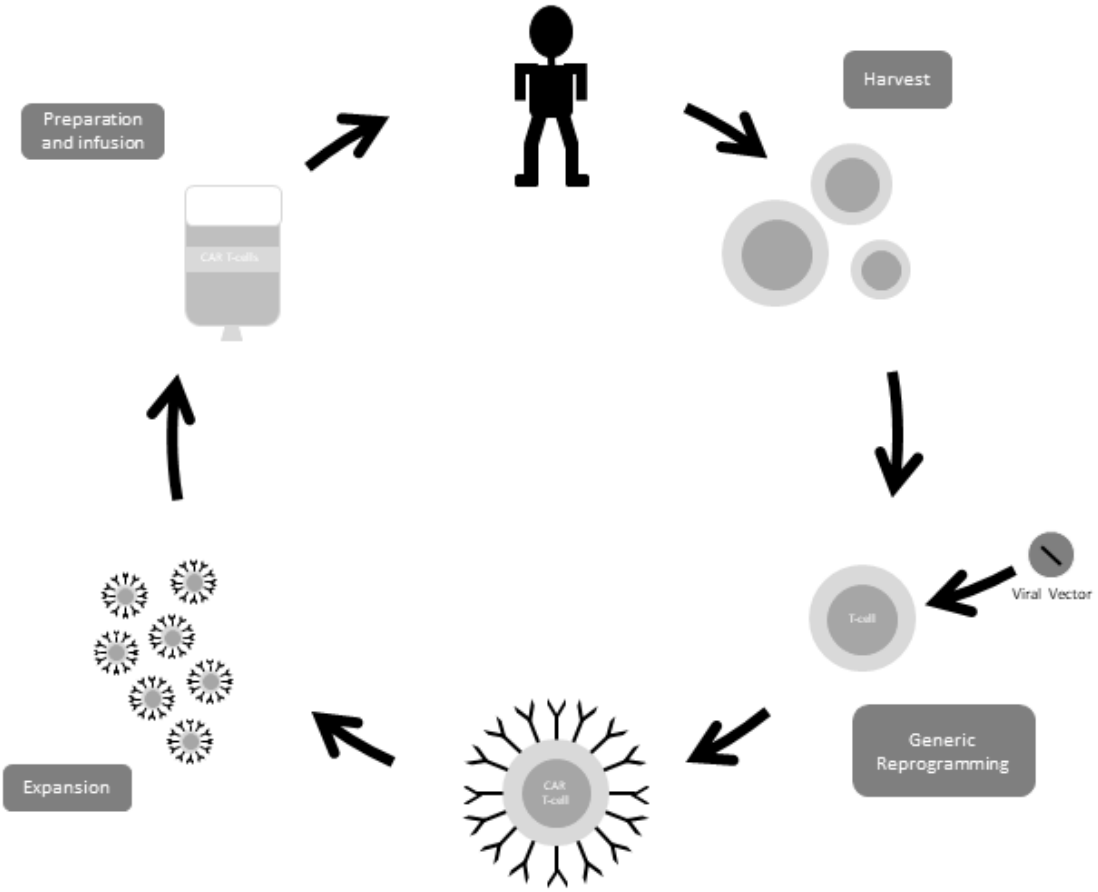


Figure 1.4. Manufacturing process of CAR T-cell therapies. The begins with harvesting of material from the donor, usually by apheresis. This is then purified and cultured for a short period before being genetically reprogrammed. After transduction the cells are expanded to the required therapeutic dose. The CAR T-cells are then concentrated and prepared for infusion back in to the patient.

Adapted from https://bioprocessintl.com/manufacturing/cell-therapies/challenges-and-opportunities-in-car-t-cell-manufacturing/

To overcome the limitations of harvesting tumour samples and relying on there being a high concentration of responsive T-cells, engineered TCR T-cells were developed. Engineered TCR T-cells

are generated through genetic programming of T-cells to express a specific TCR[53,54]. The TCR encoded is specifically designed to target a protein on the target cell surface, as opposed to relying on harvesting these cells in the TIL process. Manufacture of the therapy involves harvesting of the donor's T-cells and expanding them in culture. The cells are then genetically reprogrammed to display the engineered TCR and transplanted back into the patient[53,54]. The process shows improvements over the TIL process in many regards. The process is not reliant on harvesting tumour material, with it being possible to harvest T-cells from peripheral blood. In addition, TILs rely on repeat stimulation with tumour cells to ensure that the tumour reactive T-cells are the predominant population, however with genetic reprogramming a heterogenous population of T-cells will present the engineered TCR, subject to transduction efficiency[53–55]. As both of these processes require the presentation of target proteins, they are reliant on the presentation of MHC on the cell surface. One of the characteristics of cancer cells is the downregulation of surface proteins, including MHC, effectively making them invisible to the immune system[56–58].

The response to this limitation was the generation of CAR T-cells. These cells are manufactured in a similar fashion to engineered TCR T-cells, with T-cells harvested from the patient and genetically modified to express the CAR, before reimplantation in the patient (Fig 1.4). CARs differ from engineered TCRs and TILs in that they are not reliant on MHC presentation of their antigen (Fig. 1.3). CARs consist of an extracellular domain for antigen recognition and an intracellular domain for T-cell stimulation (Fig. 1.5). The extracellular domain includes a single chain variable fragment (scFv) that can recognise and bind to a target antigen, and a hinge region that allows flexibility and in interaction and reduces the spatial requirements for response[59–61]. The evolution of CAR T-cells has seen the CAR intracellular domain change with each generation. The first generation of CAR T-cells only had a CD3 signalling motif on the intracellular domain[41,61]. CD3 is the primary stimulus for T-cell response *in vivo*, however it also requires a costimulatory signal for persistence[62]. As such, in the second generation of CARs a second signalling motif was included in the intracellular domain, usually the secondary stimulant signals CD28 or 4-BB, and third generation CARs then added another signalling motif to increase persistence further[41,61,62].

Currently CAR T-cell therapies have had a large amount of success with B-cell cancers. There are several reasons for this, for example CD19 is a B-cell specific marker which is not downregulated in cancerous cells[63–65]. This means that it is possible to target these cells even when they are cancerous and induce apoptosis. In addition, as they are a liquid cancer these cells are easier to target than a solid tumour which can restrict access to all cells, as well as produce an immunosuppressant microenvironment which may limit response. There are two products released on the market that have approval for use by the FDA and the NHS, Tisagenlecleucel (Kymriah; Novartis) and

axicabtagene ciloleucel (Yescarta; Kite Pharma), with Lisocabtagene maraleucel (Liso-cel; Bristol-Mysers Squibb) currently going through clinical trial (TRANSCEND-NHL-001, ClinicalTrials.gov Identifier NCT02631044). Clinical trials listed in Table 1.1 highlight some of the ongoing clinical trials using CAR T-cell therapies.

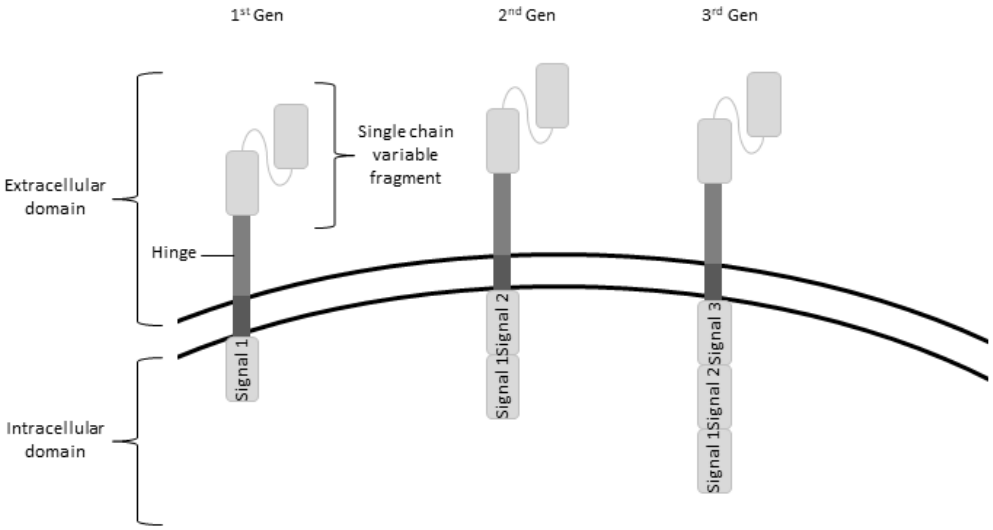


Figure 1.5. Evolution of CARs through each generation. New generations of CARs have been developed as evidence has identified areas for improvement. The extracellular region of the CAR has stayed consistent through generations. It comprises of a single chain variable fragment to recognise the target antigen, and a hinge region to facilitate high capacity clustering and reduce spacial restrictions. The intracellular domain of the first generation CARs only contained one signalling domain, CD3. Activation of T-cells via only CD3 does not convey persistence and therefore the second generation CARs included a second signalling domain which comprised of either CD28 or 4-1BB. Further development has increased the number of signalling domains in an effort to increase persistence and efficacy. Third signals used have included CD28, 4-1BB, ICOS, CD27 and OX40.
Adapted from "Chimeric Antigen Receptor Modified T-Cells for B-Cell Malignancies: An Advance in Cellular Therapy"; Wang, Klichinsky and Schuster, ; 2016

There has been evidence to suggest that in the manufacturing and therapeutic efficacy of CAR T-cell therapies Naïve (N) and Central Memory (CM) subpopulations of T-cells are preferable. In particular, *in vivo* studies have shown that these populations are more effective in conferring target cell response[66–70]. This is suggested to be due in part to the proliferative capacity of these cells, which normally reside in Secondary Lymphoid Organs (SLOs), and their capacity for persistence in the subject after reimplantation, as opposed to Effector (E) and Effector Memory (EM) subpopulations, which are usually found at the site of infection and resident in tissue respectively, and have a

comparatively limited persistence[66]. The greater proliferative capacity of N and CM populations means that in at transduction, a greater proportion of these subpopulations is likely to result in a greater concentration of CAR expressing T-cells in the final product. Meanwhile, at therapy transfusion the greater proliferative capacity and persistence means that these cells can produce an appropriately scaled, targeted attack, and increases the capability to maintain that response for an extended period of time.

Table 1.1. Sample of current clinical trials using CAR T-Cells.

Trial name	Recruitment stage	Trial summary
ZUMA-1[1]	Active, not recruiting	Comparing CAR T-cell treatment axicabtagene ciloleucel with the standard second-line treatment for people with DLBCL. Testing whether is it more effective than standard second-line treatment for people with relapsed or refractory DLBCL
JULIET[2]	Active, not recruiting	Efficacy and safety of CTL019 in adult patients with relapsed or refractory DLBCL
ELIANA[3]	Recruiting	Efficacy and safety of CTL019 in paediatric patients with relapsed or refractory B-cell ALL
TRANCEND NHL-001[4]	Recruiting	Safety, pharmacokinetic and anti-tumour activity of modified T-cells (JCAR017) administered to adult patients with relapsed od refractory B-cell Non-Hodgkins lymphoma.
COBALT[5]	Recruiting	The purpose of this study is to administer novel CD19 specific CAR T-cells to patients with relapsed or resistant DLBCL to assess the safety and efficacy of this strategy as a bridge to allogenic transplantation.

1. *ClinicalTrials.gov Identifier: NCT02348216*
2. *ClinicalTrials.gov Identifier: NCT02445248*
3. *ClinicalTrials.gov Identifier: NCT02435849*
4. *ClinicalTrials.gov Identifier: NCT02631044*
5. *ClinicalTrials.gov Identifier: NCT02431988*

There has been also been investigation into the effects of CD4+ and CD8+ population balance on the efficacy of therapy in manufacture and transplantation. Cytotoxic CD8+ cells are able to induce apoptosis in their target cells directly, and this makes them appear to be a suitable population to target for improved efficacy of therapy. However, helper CD4 cells are predominantly known for their cytokine producing capacity, and their ability to stimulate responses in B-cells and other cytotoxic T-cells. There is no evidence that the balance of the CD4:CD8 populations has a substantial or significant impact on the manufacturing process of CAR T-cell therapies. Furthermore, there is little research into the impact of CD4:CD8 balance on therapy efficacy, and the evidence available is not conclusive on the matter[66,69,71]. The main conclusion of these investigations is that the evidence

on CD4:CD8 balance is inconclusive, but both CD4+ and CD8+ populations should be included as part of the therapeutic dose manufactured, rather than limiting it to just the CD8+ population[72–74].

Therefore, as well as investigating the possibility of manufacturing consistent T-cell populations, or controlling culture conditions to dictate population outputs, this research had a greater focus on identifying culture conditions which would select preferentially for CD4+ and CD8+ N and CM subpopulations. Such findings could be translated from a research setting to a manufacturing process to improve transduction and therapy efficacy.

1.3 History of flow cytometry and phenotypic analysis

Flow cytometry is a common analytical tool in modern day cell culture analysis. The process involves using fluidics to analyse a sample by concentrating the cells into a single stream which is then passed through a detector to analyse characteristics of the cells. In the context of this research, flow cytometry was sometimes used to analyse the viable cell counts of samples, and extensively used to analyse their phenotypic qualities of culture samples. Modern flow cytometry equipment can use laser light detection to measure cell characteristics, however original flow cytometers used an electrical current.

Flow cytometry was developed in the 1950s as a response to the slow throughput of static cytometry when processing samples in the wake of atomic bombs dropped in WWII[75]. The original device involved a channel with an aperture and an electrical current running from one side of the aperture to the other. It was observed that cells were not as conductive as the flow buffer they were suspended in, and as they passed through the aperture the electrical resistance would increase, decreasing the current passing between electrodes. By monitoring the resistance recorded through the aperture it was possible to count the number of cells and compare their relative size in a sample[76].

Over time, the process was improved to sort cells based on their size using this principle, before advancing to cell sorting based on cell fluorescence[77]. A patent for the first Fluorescence Activated Cell Sorter (FACS) was awarded to Stanford University in 1972, and by 1974, Becton Dickinson had released the first commercial instrument, the FACS-II. Earlier fluorescence based FACS analysers would differentiate cells based on their autofluorescence. The development of methods by which monoclonal antibodies could be produced against specific targets and labelled with fluorophores was used to shepherd in a new era of characteristic analysis in cytometry[78–80]. The use of different fluorophores and multiple lasers resulted in flow cytometers which were able to accommodate ever increasing panels of stains to analyse simultaneously for cell phenotyping[78–80].

Currently there are many analysers that are able to assess cells' size and granularity, as well as measure labelling for multiple targets. The flow cytometer that was used in the course of this research, a BD FACSCanto II, had the capability of analysing response in up to 8 channels at the same time, however some analysers with as many as 5 lasers can analyse up to 50 channels simultaneously. Increasing the number of channels available allows for a greater range of cell markers to be analysed simultaneously, however it also necessitates a more complex set up as each channel needs to be individually configured, and an increased level of compensation in each channel as it receives spillover from multiple channels fluorophores[80–82].

1.4 Selection of a suitable panel for T-cell phenotypic analysis

There are many ways in which cells in culture can be analysed; growth, size, phenotype, metabolic activity, proteome and genome of cells can all be quantified to measure the state of a cell. The most common analyses of T-cells in culture are the proliferation and phenotype[72,83–86]. In experimental conditions the former highlights the ability of variables to induce a greater or lesser level of growth in culture, whilst the latter analyses the impact on the balance of different T-cell subpopulations. Measurement of viable cell count is possible using flow cytometry. Cell populations can be gated from other particles based on size, and there are several dyes such as 4',6-diamidino-2-phenylindole (DAPI; Fisher) and Fixable Viability Stain 520 (Becton Dickinson) that can be used to stain non-viable cells to highlight the viable cells[80].

Whilst assessment of viability only requires a single fluorescent stain, phenotypic analysis of cells can require a larger number of stains for analysing different cell markers depending on the complexity of the target cell identity. This is certainly true of T-cells, with a range of cell surface and internal markers available to analyse the state and functional phenotype of a cell. Phenotypic analysis of cultures was a key element of the research and it was important to develop a panel that would be accurate and have sufficient detail to define the subpopulations required.

The first purpose of the analytical panel is to have the capacity to identify T-cells in a heterogenous population. The TCR is comprised of variable protein binding regions that give T-cells their specificity for activation. The TCR complex is only expressed on the surface of T-cells and is therefore highly specific for T-cells. Due to the variable nature of the exposure portion of the α and β chains, or in 5-10% of instances γ and δ, that comprise the TCR they are not a suitable target for fluorescent antibody staining. The TCR proteins only have a short cytoplasmic tail, and therefore sit within the TCR complex in order to effectively propagate the signal through the cell membrane to activate signalling pathways[40]. As well as the TCR, the TCR complex also consists of CD3 subunits CD3δ, CD3γ, CD3ε and CD3ζ[40]. CD3 proteins expressed as part of the TCR complex have both a stable protein

shape and are more exposed on the cell surface[40]. T-cells are not able to 'graduate' from the thymus without expression of the TCR, and with CD3 being an integral part of the TCR complex it is therefore highly indicative of T-cells. As such, CD3 provides a suitable target to identify T-cells. The evidence also shows that CD3 is used extremely often in the identification of T-cells for others in a heterogenous population[84,87].

The TCR lends specificity to T-cells as well as propagating an activation signal, however in order to avoid accidental stimulation a costimulatory signal is required to fully activate the cell[40]. CD28 acts as the costimulatory receptor on T-cells and is able to bind to 2 different ligands expressed on the surface of APCs; CD86 is a constitutively expressed protein on the surface of APCs while CD80 is upregulated after activation in order to increase signalling and promote activation of cells[40]. Activation of CD28 by a CD80 or CD86 ligand results in a cascade of signalling which ends with a protein called PLC-γ being phosphorylated and activated after interacting with LAT. Downstream, this signal results in an upregulation in IL-2 gene transcription, producing a cytokine which promotes proliferation and differentiation of Naïve T-cells into Effector (E) T-cells[40,88,89]. Cells that do not express CD28 can become functionally inactivated or are anergic, recognising their target antigen but not producing a response to it[90–93].

T-cells constitutively produce IL-2 and express a moderate affinity IL-2 receptor on the cell surface comprised of an IL-2 receptor β and γ chain, even when not activated[40]. Activation of this receptor induces proliferation in the cells and IL-2 is used as a growth factor for T-cells in culture, whilst high concentrations of IL-2 are able to induce activation[94,95]. However, on activation expression of an IL-2 receptor α chain (CD25) is upregulated[40]. The α chain combines with the β and γ chains and substantially increases the affinity with which the receptor binds and responds to IL-2[40,96–99]. In this state the receptor is capable of stimulating proliferation even in relatively low levels of the ligand, promoting clonal expansion and ensuring that an effective response is raised against the target cell(s)[40,99]. Because of the direct correlation between activation and upregulation of CD25, it would be a suitable analytical marker for 'Effector' T-cells in T-cell populations.

In addition to CD3, the proteins CD4 and CD8 are also indicative of T-cells. CD4 expression on a T-cell's surface is indicative of helper or regulatory function in the body, with the former helping in activation of other cells and the latter working to reduce the instances of unnecessary autoimmune function[40,100,101]. CD8 expression is indicative of cytotoxic T-cells, which can induce apoptosis in cells presenting their target antigen[102–104]. In the initial stages of T-cell development in the thymus, T-cells express neither CD4 nor CD8 in a state referred to as 'double negative'. However, as the cells progress down this differentiation path the cells begin to express both CD4 and CD8 on their surface, referred to as 'double positive'. Finally, prior to leaving the thymus T-cells go through a process of

antigen exposure. This process ensures that the cell will sufficiently respond to induce activation when being exposed to their target antigen, but not react so much that it would induce an autoimmune reaction[39,40]. As part of this process the T-cell will either bind to cells expressing MHC class I or MHC class II which will stimulate the downregulation of either CD4 or CD8 respectively, rendering their expression on the T-cell surface mutually exclusive[39,40]. Not progressing to double expression and then single expression will result in T-cells that are unable to 'graduate' from the thymus and they will undergo apoptosis. As a result, CD4 or CD8 expression is indicative of T-cell function *in vivo*, and in conjunction they should represent all T-cells within a population. There was also a substantial number of examples where these markers were used in literature, using them to identify the two subpopulations of cells in culture[72,105–108]. As previously mentioned, there is currently no consensus on whether there is a preference in the balance of CD4:CD8 populations in either manufacture or therapeutic dose. However, in being able to monitor these subpopulations it may be possible to identify differences in their responses to experimental variables and help to improve the knowledge in this area for CAR T-cell therapy manufacture.

In addition to Helper/Regulatory and Cytotoxic, T-cells are also commonly split into maturity phenotypes based on their experience with their target antigen and ability to locate to the SLOs[39,40,68,109]. These maturity phenotypes appear in both CD4+ and CD8+ subpopulations and have the same marker profile in each instance. CD45 is a protein that can be indicative of T-cells like CD3 and CD4 & CD8, however their greater analytical power comes from their isoform expression. On binding of the TCR to its target protein, CD45 associates with the target cell MHC proteins and clusters the protein around the TCR complex. This collects essential components of activation, bound to the cytoplasmic domain, around the TCR and helps to facilitate activation. Prior to exposure to their target antigen, T-cells express the isoform CD45RA on their cell surface, therefore indicative of immature subpopulations[39,40]. On activation, T-cells upregulate the expression of CD45RO and downregulate CD45RA. CD45RO is able to more easily associate with the target MHC proteins, therefore improving signalling and the potential response[39,40].

The subpopulations which home to SLOs are present within both the immature and mature T-cell subpopulations. In immature populations, Naïve cells reside predominantly in the SLOs, waiting to be shown their target antigen by an APC[39,40,89]. For this to occur, the cells need to be able to migrate to these areas, which they do with the help of CD62L and CCR7[39,40,110]. Both of these proteins bind to targets expressed in the vasculature near the SLOs, which facilitates the rolling and slowing of cells along the vessel wall before being able to enter the organs. However, once a T-cell has been activated it needs to move away from the SLO towards the site of infection. Retaining CD62L and CCR7 would reduce the efficacy of response at the target site by inhibiting effective migration to the

target site. It could also increase the chance of an adverse response in the SLOs[111,112]. As such Effector phenotype cells downregulate these 'homing' markers to direct their way to the site of the infection. This is similar in the mature subpopulations, with Central Memory and Effector Memory (EM). Effector Memory cells reside in tissues, serving as a first line of mature T-cell defence against pathogens. In order to prevent them from homing to the SLOs and to stay in the peripheral tissues Effector Memory cells downregulate CD62L and CCR7 expression[39,40,113,114]. Meanwhile, Central Memory cells reside in the SLOs to be activated through exposure to antigen presenting cells, before undergoing high proliferation and migration to the target stimulus[39,40,110,113,114].

These markers (CD45RA/CD45RO and CCR7/CD62L) are essential for defining maturity phenotypes of T-cell cultures. It is understood that these markers are not used in release criteria for CAR T-cell therapeutic product, however as mentioned in section 1.2, a higher proportion of Naïve and Central Memory phenotype cells is preferable through both manufacture and infusion of the therapy into the patient[66–71]. Therefore, the inclusion of markers that can define maturity subpopulations will play an important role in understanding the impact of experimental variables on target subpopulations and will help to define culture techniques that select for them.

In addition to the surface markers of T-cells, the cytokine profile was considered for analysis of T-cells. The production of cytokines could be indicative of certain subpopulations, such as IL-9, IL-22 and IL-10 for certain subgroups of CD4+ T-cells[40,115–117], or of cell state, such as IFN-γ and TNF-α representing activation in CD8+ T-cells[118–121]. However, analysis of these markers would require permeabilization of the membrane for labelling, and it is understood that these cytokines are neither monitored during manufacture of the therapy, or used as release criteria for the product. Considering the frequency and volume of phenotypic analysis to be undertaken, it was considered that the additional time and work that would be needed to assess these markers would not be balanced by result of doing so. As such, no cytokines were used as phenotypic markers over the course of this research. This contributed to the reason to not distinguish between the multiple CD4+ T-cell populations.

Table 1.2. Expression of common T-cell markers in different maturity subpopulations.

Marker	Naïve	Effector	Effector Memory	Central Memory
CD3	+	+	+	+
CD4	Present dependent on phenotype			
CD8	Present dependent on phenotype			
CD25	-	+	-	-
CD28	+	+	+	+
CD45RA	+	+	-	-
CD45RO	-	+/- (some upregulation but not highly expressed)	+	+
CD62L	+	-	-	+
CCR7	+	-	-	+

The flow cytometer to be used for phenotypic analysis was a BD FACSCanto II. This analyser can accommodate analysis of up to 8 channels simultaneously. The markers mentioned so far total 9, and therefore it would not have been possible to run them all in one panel. It was considered that it would be possible to run analysis of T-cell cultures with two panels, however as with the cytokine markers it was considered that the additional work required outweighed the benefit of performing two analyses. As well as being necessary to remove a marker to meet the requirements of the equipment, it was also understood that using a panel at the full capacity of the analyser would require a high level of compensation in the analyser configuration which may introduce noise into the data[81]. The decision was taken to target a 5 marker panel for analysis.

Analysis of the markers highlighted a redundancy in those discussed. Both CD62L and CCR7 are markers of cells homing to SLOs, present on the Naïve and Central Memory cells. The two proteins play slightly different roles in the process of migrating cells to their destination, potentially warranting individual investigation[39,40]. However, with both conveying the same functional property of the T-cell it was not necessary to include both for an effective analysis. CD62L has shown to be more effected by cryopreservation and density centrifugation, and although the expression does recover, this could have caused issues with phenotypic assessment in the period after culture preparation[122–124]. CCR7 was understood to be more constitutively expressed, but with the caveat of having a relatively low expression level on the T-cell surface[125]. It was concluded that the issues with

low expression could be overcome by the use of a brighter fluorophore, whilst the loss of phenotypic data in early culture would be more of a hinderance. Therefore, CCR7 was retained as a potential marker, whilst CD62L was removed. It was similarly decided that CD28 would be removed from the panel for a similar reason. Although CD28 is a marker of T-cells, both CD3 and CD4/CD8 are also able to do so. In addition, CD3 was observed to be more commonly used as a single marker for T-cells than CD28[66,126–129].

Understanding the activation level of T-cells would have contributed to the analysis of experimental effects, potentially highlighting conditions which stimulated response quicker or greater than others. With activation inducing maturation of T-cell populations, it was considered that this may also help to identify conditions that stimulated a greater level of maturation. However, as CD25 is only transiently upregulated as part of activation[39,40] this would not be a lasting marker of maturity in cell populations. CD45RO is also a marker of maturity in T-cell populations, upregulated on activation and retained on the cell surface. It was therefore concluded that CD25 was not an essential marker to monitor and was also removed from consideration.

Of the 6 markers remaining, CD4, CD8 and CCR7 were considered essential. CD4 and CD8 described functional subpopulation variation and would be important in understanding any variable effects of any experimental variables on the helper/regulatory and cytotoxic populations. Meanwhile CCR7 was one of the markers which contributed to the definition of T-cell maturity subpopulations[83,113]. As target populations of cells in CAR T-cell therapy manufacture had been linked to these populations it was necessary to retain for identification of maturity subgroups in culture. Of the markers remaining, CD45RA and CD45RO were understood to have the same functional difference, however with opposite levels of expression. Meanwhile, the only function of CD3 on the panel would be to identify T-cells. It was suggested that having clarity on the expression of CD45RA and CD45RO, especially within effector populations, may provide clarification in the transition period of expression between the two proteins. It was also observed, that any CD3 expressing cell in culture would also be expressing CD4 or CD8. In the absence of CD3 as a marker it would therefore be possible to identify T-cells in culture using CD4 and CD8. As such it was concluded that CD3 would be removed from the panel and the final analytical markers would be CD4, CD8, CD45RA, CD45RO and CCR7. This panel would be able to identify T-cells within a heterogenous population, as well as give the insight into maturity subpopulations required to make meaningful conclusions about experimental variables on populations of interest in the context of the manufacturing and therapeutic process.

After selection of the marker panel it was necessary assign fluorophores. With markers having different expression levels on T-cell and fluorophores having different relative fluorescence it was

important to ensure that these were combined in a manner to achieve the clearest output from the analysis, preferably associating brighter fluorophores with less expressed markers. The fluorophore selection is outlined in 2.7.1.1.

1.5 Sources of T-cells, their availability and suitability

To perform experiments on T-cell populations, it was first necessary to source the culture material. In CAR T-cell therapy manufacture the cells are generally harvested from the patient using apheresis[66,72,130–133]. This process extracts the buffy coat fraction of whole blood and returns the remaining components to the donor. Only removing the required fraction puts a reduced strain on the patient and reduces processing time in the clinical setting[134–136]. For research purposes there were several options for the sourcing of T-cell material. Assessment of the T-cell sources was based on their availability and their suitability.

The first source of T-cells was whole blood. Fresh whole blood is available for purchase in the UK and the total volume required in each instance could be supplied by a single donor. The variability of donor material prompted consideration of using a pool of material from several donors to observe a more homogenised response to stimuli. However, this was rejected in favour of single donor cultures which would be more representative of the clinical manufacturing method[83,137,138]. In addition, it was not clear what the impact of introducing CD8+ cells from multiple donors would be. From whole blood it would be possible to use density centrifugation to isolate the buffy coat fraction of whole blood, which is comprised primarily of Peripheral Blood Mononuclear Cells (PBMCs). This would mimic the isolation of this fraction using apheresis, and from here T-cells could be isolated where necessary, or the isolated product placed straight into culture. In addition, similar to the clinical process this product would not undergo cryopreservation, which could have had negative effects on the T-cell populations. However, it was also understood that this source of T-cells was likely to require a greater level of processing, increasing the time and steps the material would go through before it got to culture.

An alternative source of T-cell material was cryopreserved PBMCs. These cells had already undergone the buffy coat isolation process and cryopreserved prior to transportation. One of the drawbacks of the cryopreservation process was an unknown effect on T-cells; the impact of cryopreservation on CD62L expression in T-cells highlights that there could be negative consequences to the process[122–124]. The pre-isolated T-cells would require a reduced level of processing before being able to transfer to culture which could help reduce any negative effects that this may cause by excess handling. The use of cryopreserved cells also had the benefit of being

analysed for cell count prior to the freezing process. With the quantity of cells available known, experimental design could be optimised to the available resources to achieve maximum output.

The final source of T-cells considered was cord blood derived Mononuclear Cells (MNCs). MNCs would be isolated by density centrifugation to obtain the buffy coat, as with the whole blood. This source of cells was considered due to the availability of leftover product from colleagues' work. Although these cells presented the opportunity for access to MNC material regularly, this source was not ideal. Cord derived MNCs are not used in any observed clinical manufacture of CAR T-cell therapies[83,137,138]. Therefore, culture with these cells would not be reasonably representative of clinical work. In addition, evidence suggested that cord derived T-cells can produce unusual phenotypes[139], which may be indicative of atypical behaviour compared to circulating T-cells. Furthermore, cord blood is a resource that is not in high supply, relying on individuals donating the material after giving birth. The availability of this product may not be the same in other circumstances as it is here, therefore not making it a suitable source of T-cell material in this research.

Another element of the source material was the consideration of whether to include cells from multiple donors within experiments. This would enable comparison of responses to the same experimental variables from multiple donors. Donor variability is a commonly acknowledged element of primary tissue culture and this research is focussed on identifying culture techniques that promote target subpopulations without impacting negatively on proliferation regardless of donor material. However, this benefit has to be measured against the increased volume of work to be performed by increasing the number of conditions. It was predicted that the magnitude of the experiments that would be undertaken would not accommodate the additional cultures, especially as the requirement to have within donor replicates for each condition would further increase the workload.

In summary, analysis of potential T-cell sources showed that cord blood derived MNCs were not a suitable source of T-cells moving forwards. There were benefits and drawbacks to the other sources, fresh whole blood or cryopreserved PBMCs. The greater level of processing required of the whole blood was balanced by the product being in a more similar condition to that used clinically, not having undergone cryopreservation. However, cryopreserved PBMCs were more of a known quantity, with approximate cell numbers known ahead of time, and the material requiring fewer processing steps into culture. Ultimately, the desire for a product that had not undergone cryopreservation meant that fresh whole blood was selected over cryopreserved PBMCs. In addition, it was anticipated that in following the clinical process the concluded outputs of each experiment would be directly relatable to the clinical work ongoing.

1.6 Commonality and divergence of T-cell culture methods

Across the breadth of current T-cell research there is a range of culture processes that are being used. Within these there is some strong commonality, as well as variation introduced with parameters from inclusion of different stimulants to isolation technique. Analysis of these was performed to help define a culture process to be used for T-cell culture in the presented research.

Initial investigations sought to identify the different methods and levels of isolation prior to culture. The source material in every example analysed was whole blood or apheresis product. In nearly all cases the buffy coat of mononuclear cells was isolated using density gradient separation with buffers such as Ficoll-Paque[105,108,126,127,129,131,132,140,141] (Fisher) and Lymphoprep[107,128,133] (Dakewei). However, there were some instances where T-cell populations were isolated from the buffy coat. These included CD4+ and CD8+ subpopulations isolated directly from whole blood[62,106] and T-cell populations isolated through negative selection of T-cells[105,128,129,142], or positive targeting of CD3 and CD28 antigens[130]. Where PBMCs were the seeded material, T-cells were selected through culture techniques, rather than through specific removal of non T-cells[105,106,108,128,131]. This was done primarily through stimulation of T-cells by targeting CD3 and CD28 proteins expressed on the cell surface and inducing activation, which would increase proliferation in T-cell subpopulations. There were limited examples of T-cells being purified by CD3 targeted stimulation[66,126,127], however nearly all examples analysed utilised the CD3/CD28 stimulation. It was suggested that the reason CD3 targeted isolation was not common was that the CD3 protein binding sites would be occupied or blocked to the stimulants, causing inefficiency in the stimulation and reducing the level of proliferation observed in the cultures.

Analysis of media used for T-cell culture showed that the most common basal medium was RPMI 1640[62,105–107,126,127,133,141,142]. There were also number of examples using X-VIVO15 as the basal medium[108,132,140], and occasional references to TexMACS[143,144], SuperCulture L500[128] and ImmunoCult-XFT[131], all of which are optimised for culture of T-cells in a serum-free environment. Despite this optimisation there were several examples of X-VIVO being supplemented with serum. It was observed that the basal medium was supplemented with Foetal Bovine Serum (FBS) in some circumstances[132,140], whilst in others it was supplemented with human AB serum[86,108]. The supplementation of FBS into culture introduces animal derived elements, however the addition of human AB serum means that the basal medium would remain xeno-free, free from animal derived products. The development of serum-free media is constantly ongoing to reduce the reliance on traditional sera such as FBS, as well as the variability that they introduce, and would potentially have appropriate applications in CAR T-cell therapy manufacture[145–147]. However, the scope of this research did not cover the testing or development of serum-free medium, and therefore RPMI 1640

was selected as the basal medium for T-cell culture. In addition, RPMI was supplemented with 10% FBS as observed nearly universally[62,105–107,126,127,133,141,142], as well as 2mM Glutamine as an energy source for cells in culture[106,127].

In investigating the culture protocol, several growth factors were identified that could be used to stimulated growth in T-cell cultures. Analysis of potential T-cell markers in phenotypic analysis (1.4) had identified IL-2 as a growth factor that stimulates both proliferation and maturation in T-cell cultures. The modal concentration of IL-2 returned from the investigation was 100 UI/mL, with the concentrations observed ranging from 1000 to 40 UI/mL. In addition to IL-2, IL-7 and IL-15 were often observed in the literature[129,132,140,143,148,149]. Both growth factors promote activation and proliferation in T-cells, with IL-15 also having a particular modality that inhibits apoptosis in memory T-cells in the absence of the target antigen. Although it was considered that additional growth factors had the potential to induce alternate responses in T-cell populations, it was concluded that increasing the number of growth factors could also unnecessarily increase the number of experimental variables and introduce confounding factors. Furthermore, although IL-15 played some role in sustaining memory cells, all three growth factors had similar impacts on T-cells in culture. IL-2 was selected due to the commonality of its use in the literature, and in accordance with the evidence seen would be supplemented at 100 UI/mL.

In addition to growth factors, supplements which stimulate the CD3 and CD28 activation pathways were often added to culture. This was most commonly achieved through the supplementation of anti CD3/CD28 magnetic beads into culture[105,108,129,131–133,140], however it was also achieved using anti-CD3 antibodies[66,126,127]. CD3/CD28 bead products were available from multiple suppliers. There did not appear to be any functional variation between bead products, and therefore Miltenyi Biotech Anti-CD3/CD28 beads were selected based on financial impact.

1.7 Summary of introduction

There is currently an abundance of research and interest around CAR T-cell therapies. Over the last 5 years two CAR T-cell therapies have gone through FDA and NHS approval, and a multitude of clinical trials have been undertaken to assess their safety and efficacy. They have had a considerable impact in the field of B-cell cancer treatment, and the potential future applications with solid tumours could see them improve prognosis for a range of other cancers.

Analysis of the available literature highlighted several factors which appeared to benefit CAR T-cell therapies with regards to viral transduction in the manufacturing stage, and efficacy and persistence on treatment, particularly around subpopulation balance. There did not appear to be substantial work looking at the impact of culture methods on the relative proportions of these subpopulations,

or whether it would be possible to predict the outcome of a culture based on the starting material. As such, the aim of this research is to identify what impacts the input material of T-cell culture may have on the growth and phenotypic profile of the resultant T-cell populations, and whether controlling culture conditions can influence populations to a desired outcome.

2 Methods and materials

This chapter outlines the standard processes and procedures utilised across the experimental period, as well as the items and materials used to implement them.

2.1 Source cell material

Two main sources and suppliers were used to provide the raw cell material for the experiments described below. Initial investigation suggested the necessity of fresh human whole blood from which PBMCs and then T-cells should be isolated. However, further work moved on to rely on cryopreserved PBMCs as the initial input material. In any instance where a 'single donor' is mentioned, they can be considered as different and separate from any other 'single donor' unless specifically mentioned as being the same as a previous instance.

2.1.1 Fresh human whole blood

After some initial issues identifying sources for fresh human whole blood, Cambridge Bioscience was selected to supply the material required in this form.

The whole blood was collected from healthy donors with Na-Heparin anticoagulant and delivered within 24 hours of donation. 495mL whole blood was requested per order, delivered in 55 9mL aliquots. This was due to the collection method and not a specification to the supplier.

2.1.2 Cryopreserved PBMC aliquots

For frozen PBMC aliquots, AXOL Bioscience was selected as the supplier. These cells were provided as 2mL aliquots with an advertised cell count of ~$1x10^8$ cells. AXOL Bioscience isolated the cells using density gradient separation and were then suspended in CS10 (Sigma, cat no. C2874) for cryopreservation. On receipt, the cell vials were placed into cryostorage until required.

2.1.3 Human Tissue Authority compliance

In addition to usual laboratory risk assessment, the material above also came under jurisdiction of the Human Tissue Authority (HTA). The HTA monitor collection, use and disposal of primary material. All material was approved and documented in accordance with the regulations set out by Loughborough University and the Centre for Biological Engineering.

2.2 Thawing

A pre-existing research group protocol was used for thawing of frozen cell aliquots. Prior to thawing standard culture medium was placed into a water bath (Grant Instruments, Sub Aqua 26 Plus) at 37°C for 20 minutes. 10 mL standard culture medium was also set aside to be kept at room temperature.

Cells were thawed from cryopreservation by placing in a water bath at 37°C for 2 minutes or until the point at which all liquid had thawed, which ever was soonest. 500 µL of room temperature medium was transferred to the cryovial with a 1000 µL micropipette and gently mixed by pipetting up and down 4-6 times. The cells were then transferred to a 15 mL centrifuge tube, and the cryovial was then washed with 1 mL standard culture medium at room temperature to minimise cell loss.

The centrifuge tube was then supplemented with sufficient room temperature medium so that the total volume was 10 mL with a Pipetboy pipette controller (Integra, PIPETBOY acu 2) and 10 mL Strippette. The cell suspension was then centrifuged for 10 minutes at 300G (Sigma centrifuge, 3-15). The supernatant was gently removed using a Pipetboy pipette controller and a 10 mL Strippette, and was replaced by 10 mL warmed standard culture medium using a fresh Stripette. The pellet was then resuspended by gently pipetting medium up and down 4-6 times. At this point the cell suspension was analysed for viable cell count prior to seeding.

2.3 Isolation

In all experiments the starting material consisted of PBMCs or subpopulations isolated, positively or negatively, from PBMC populations. Furthermore, the T-cell subpopulation isolation methods outlined below required PBMCs as the initial material. Therefore, it was necessary, when starting with whole blood, to first isolate the buffy coat before further isolation or experimentation could take place.

2.3.1 PBMCs/buffy coat

The buffy coat is the fraction of cells that contain most of the white blood cells and platelets. It was isolated from the whole blood material receipt. The protocol for isolation of the buffy coat was provided by a member of the research group who had previously worked with the process.

2.3.1.1 Preparation

Initially the blood was diluted 1:1 with Phosphate Buffered Saline (PBS) (Fisher, cat no. 10398999). Whole blood vials were pooled by pouring into a sterile container sufficient in size to hold the final volume. The vials were then each rinsed with 1mL PBS, which was then transferred to the container using a 1000 µL pipette. The remaining volume of PBS required to create a 1:1 mix was added to finish the whole blood dilution. The dilute whole blood was then layered onto Ficoll Paque plus density gradient buffer (Fisher, cat no. 11778538) using a Pipetboy pipette controller set to the lowest ejection rate with a 25mL Stripette. This was done gently to reduce or eliminate mixing of the whole blood with the buffer, as this would reduce its effectiveness. The volume of diluted blood added was monitored to ensure that the volume layered on top did not exceed twice the volume of buffer.

2.3.1.2 Centrifugation

The aliquots of diluted blood and density gradient buffer were then carefully transferred and loaded into a centrifuge. The centrifuge was programmed to run at 600G for 30 minutes, with no brakes on during the deceleration period. This was to limit the mixing of the sample with either the buffer of the plasma layer through forced deceleration or braking. The samples were then carefully removed from the centrifuge and prepared for buffy coat harvesting.

2.3.1.3 Harvesting

Ficoll Paque density gradient buffer separates the buffy coat into 3 fractions in descending density: whole blood and neutrophils, buffy coat and plasma. The buffer sits between the buffy coat and the whole blood fraction. Pasteur Pipettes were used to gently extract the buffy coat layer from the centrifuged samples, taking care to reduce the level of buffer and plasma collected.

2.3.1.4 Washing

The harvested volume required washing to remove contaminants such as platelets and Ficoll Paque. Samples were topped up with PBS to 50mL total volume and were centrifuged at 300G for 10 minutes. The supernatant was removed via Pipetboy pipette controller and 50mL Strippette.

The cell pellet was resuspended in 20mL PBS buffer and was then centrifuged for 8 minutes at 100G. After centrifugation the samples were retrieved, and supernatant removed and discarded. 10mL culture medium was added to the pellet and was resuspended by pipetting up and down 4-6 times with a pipetteboy pipette controller set to low flow and a 10mL Strippette. The cells were then passed through a 30µm filter, removing any large debris before being prepared for a viable cell count and preparation for the next stage of experimentation where necessary.

2.3.2 T-cell isolation

Investigation of isolation targets necessitated varying isolation methods. Miltenyi Biotech positive isolation kits were used to isolate subpopulations, and the protocol provided by the manufacturer was followed as described below.

2.3.2.1 CD3 targeted isolation

CD3 MicroBeads were used to isolate CD3 expressing T-cells from the buffy coat suspension. Prior to isolation, cell suspensions were counted to ascertain to number of cells. The buffy coat samples were then centrifuged at 300G for 10 minutes, and the supernatant was removed using a Pipetboy pipette controller and 10mL Strippette.

The pellet was then resuspended in 80 µL of isolation buffer per $1x10^7$ cells and gently mixed using an appropriately sized pipette. CD3 MicroBeads (Miltenyi Biotech, cat no. 130-050-101) were mixed thoroughly to ensure suspension, and 20 µL beads were added per $1x10^7$ cells. The suspension was

mixed well and transferred to a fridge for 15 minutes. Cell/bead suspensions were diluted with 1.5 mL isolation buffer per $1x10^7$ cells and centrifuged for 10 minutes at 300G to wash. The supernatant was removed using a 1000 µL pipette and 500 µL isolation buffer was added to the pellet. This was resuspended by gently pipetting up and down 4-6 times.

An LS MACS Column (Miltenyi Biotech, cat no. 130-042-401) was then prepared by placing in a QuadroMACS Separator (Miltenyi Biotech, cat no. 130-090-976) and rinsed with 3mL isolation buffer. A fresh 15 mL centrifuge tube (Fisher, cat no. 11849650) was placed below the column and the cell suspension was added to the LS column. The unlabelled cells were washed from the column by washing the column with 3 mL isolation buffer 3 times. The unlabelled cells were then removed and a new centrifuge tube was placed to collect the effluent.

The column was removed from the QuadroMACS Separator, ensuring that the outlet remained over the collection tube at all points. 5mL isolation buffer was added to the column and the supplied column plunger was applied and depressed to flush out the cells into the collection tube.

The isolated cells were centrifuged for 10 minutes at 300G. Next, they were extracted and the supernatant was removed with a Pipetboy controller and 5mL Strippette. 10mL culture medium was added to the cells using a 10mL Strippette, before using it to resuspend the cells by drawing them up and down 4-6 times. Cells were then seeded into culture according to the experimental requirements.

2.3.2.2 CD4/CD8 targeted isolation

At points it was necessary to isolate T-cells by targeting CD4 and/or CD8. The different methods used to isolate T-cells based on these markers are outlined below.

2.3.2.2.1 Coincubation

As described in 1.3.2.1, however due to a product that targeted both CD4 and CD8 not being commercially available, it was necessary to incubate with two types of bead which each targeted one of the markers. As such, on resuspension of the cells 60 µL was added per $1x10^7$ cells, rather than 80 µL, and 20 µL of each bead type, CD4 MicroBeads (Miltenyi Biotech, cat no. 130-045-101) and CD8 MicroBeads (Miltenyi Biotech, cat no. 130-045-201), was supplemented subsequently.

2.3.2.2.2 Two step

As described in 1.3.2.1 except the initial incubation is with CD8 MicroBeads. Furthermore, the unlabelled cells were centrifuged and at 300G for 10 minutes before following the same steps from resuspension, with labelling being done by the CD4 MicroBeads.

Once the target cells had each undergone centrifugation as part of the washing process, 5mL was added to each using a Pipetboy pipette controller and a 5 mL Strippette. The two isolated cell populations were then combined using a Pipetboy pipette controller and 5 mL Strippette.

2.3.2.2.3 Individual isolation

If an individual subpopulation was required, isolation occurred as in 1.3.2.1, however the CD3 MicroBead was replaced by that of the desired target.

2.4 Cell culture

PBMC and T-cell culture were performed as described below. All cell work, medium and reagent formations were performed in a class II Biological Safety Cabinet (BSC).

2.4.1 Culture vessels

All culture was carried out in 6 well plates (Fisher, cat no. 10088612). The wells have a maximal operating volume of 2.9mL, culture volume was set at either 2 or 3mL based on the experiment. The plates were not agitated during culture, and therefore at all times cells were in static culture

2.4.2 Medium and buffer formulation

2.4.2.1 Culture medium

Supplementation of growth factor and CD3/CD28 Dynabeads varied based on experiment, however the standard culture medium remained consistent throughout. This medium comprised of Roswell Park Memorial Institute 1640 (RPMI) (Fisher, cat no. 31870074) base medium, supplemented with 10% FBS (Fisher, cat no. 11533387) and 2mM Glutamine (Fisher, cat no. 11500626). This medium will henceforth be referred to as standard medium. The medium composition was based from what was previously used to culture suspension cells within the research team, however investigation showed that this was sufficiently common in the literature as well to have confidence[106,107,127,133].

Standard medium was prewarmed in a water bath (Grant Instruments, Sub Aqua 26 Plus) at 37 °C for 20 minutes prior to use.

2.4.2.2 Isolation and flow buffer

The buffer solution used for both isolation and flow cytometry was kept consistent across all experiments. The buffer was based in PBS, supplemented with 0.5% Bovine Serum Albumin (BSA) (Fisher, cat no. 11423164) and 2mM Ethylenediaminetetraacetic acid (EDTA) (Sigma, cat no. 324504). The buffer was used as standard by the research team for isolation and flow cytometry prior to initiation of the research. This buffer was referred to as both isolation buffer and flow buffer.

2.4.3 IL-2

IL-2 is a key growth factor of T-cells [108,150–152]. It was supplemented into culture at a concentration of 100 UI/mL to promote proliferation in addition to the endogenous IL-2 generated by the T-cells. Supplementation occurred throughout unless otherwise stated.

Premium grade lyophilised IL-2 was purchased from Miltenyi Biotchech (Miltenyi Biotech, cat no. 130-097-746). The lyophilised growth factor was aseptically split into 100 µg aliquots, and reconstituted ahead of freezing, in accordance with the manufacturers supplied methods.

The IL-2 aliquot was initially reconstituted by adding 1mL sterile deionised water with a 1000 µL micropipette and gently pipetting up and down 4-6 times. This sample was then further diluted with 4mL growth factor buffer (PBS, 0.1% BSA), added using a 1000 µL micropipette. After pipetting up and down 4-6 times, this was then split into five 1mL aliquots. The aliquots at this point could be further processed to produce aliquots suitable for supplementation into culture or frozen to be processed further at a later date.

For further processing, the 1 mL aliquot was diluted with 9 mL growth factor buffer, added using a Pipetboy pipet controller and 10mL Stripette. It was then separated out into 100 µL aliquots using a 300 µL micropipette. This final dilution factor of 50 gave a concentration of growth factor at 2 µg/mL. The provided material stated that IL-2 was calibrated as having a biological activity of $\geq 5 \times 10^6$ UI/mL, which therefore gave the final aliquots an activity of 1×10^4 UI/mL.

As such, for every 10 µL of aliquoted IL-2 supplemented per mL standard medium, the IL-2 concentration was increased by 100 UI/mL. In cultures where the medium was supplemented with the standard IL-2 concentration, 100 UI/mL, 10 µL IL-2 was supplemented per mL medium.

2.4.4 Human T-Activator CD3/CD28 Dynabeads

Human T-Activator CD3/CD28 Dynabeads (Fisher, cat no. 11131D), hereafter referred to as CD3/CD28 Dynabeads, were used in culture to activate T-cells and stimulate proliferation. The beads were used according to the manufacturers recommended process as outlined below. The beads were stored in a storage buffer at 4×10^7 beads/mL and required washing prior to supplementation into culture. The manufacturer recommends the beads are used at 1:1 bead to cells and were used in this concentration unless otherwise stated.

To perform washing, the vials where initially vortexed (SciQuip Vortex Mixer) for ~30 seconds to resuspend beads in the solution as they are prone to settling. After this, a suitable volume for the culture requirements was taken with an appropriate size micropipette and transferred to a lobind Eppendorf tube (Fisher, cat no. 10708704) of appropriate size. 1mL isolation buffer was then added to the tube, which was then vortexed for ~30 seconds. The tube was placed onto a magnet rack

(Fisher, cat no. 10625463) for 1 minute to allow the beads to focus around the magnet. After one minute the supernatant was removed using a 1000 µL micropipette, and the tube was removed from the magnet rack. The same volume of standard medium as the volume of Dynabeads initially taken was added to the beads using an appropriately sized micropipette and mixed by gently pipetting up and down 4-6 times.

The relevant volume of prepared CD3/CD28 Dynabeads was then transferred into culture(s). Some preparations required the resuspension volume to be lower to account for a lower final volume, stating specifically where it occurred.

2.4.5 Standard culture

2.4.5.1 Seeding

After isolation and/or washing as necessary, cells were counted to calculate the volume required to seed at the correct density outlined in the experiment. The volume of IL-2 and CD3/CD28 Dynabeads to be added to culture was also calculated so that the volume of standard medium to add could be obtained. A suitable number of IL-2 aliquots were removed from the freezer and placed into the biological safety cabinet to thaw. The required volume of cells was then transferred to the necessary number of wells, and supplemented with the calculated volume of standard medium required. Cultures were then supplemented with the appropriate volume of CD3/CD28 Dynabeads, and finally the required volume of IL-2. All liquid transfer was completed with appropriate size micropipettes. The cultures were then gently mixed by pipetting up and down 4-6 times with a 1000 µL micropipette.

2.4.5.2 Full Medium Exchange

Due to the suspended nature of T-cell/PBMC culture it is not possible to remove exhausted medium from culture wells without potentially losing some cells. The following exchange process was provided by the research group for exchange of suspension cell culture media. Cultures were mixed using a 1000 µL micropipette 4-6 times to resuspend cells and, where supplemented, CD3/CD28 Dynabeads in the culture. The contents of each well was then transferred to an individual 15 mL centrifuge tube (Fisher, cat no. 11849650) which were centrifuged at 300G for 10 minutes. Meanwhile the required volume of IL-2 was removed from freezer and placed into the safety cabinet for thawing. When centrifugation was complete, the supernatant was gently removed using a Pipetboy pipette controller and 5 mL Strippette. 3 mL standard medium was added to each tube using a Pipetboy pipette controller and 5 mL Strippette, and gently mixed by pipetting up and down 4-6 times. The contents of the tube were then transferred using the Strippette to the well from which they were previously obtained. Finally, wells were supplemented with the required volume of IL-2 using an appropriately sized micropipette.

2.4.6 Sampling for count/stain

When sampling cultures for viable cell count or phenotypic analysis, cultures were gently mixed by pipetting up and down 4-6 times with a 1000 µL micropipette. A suitable sized micropipette was used to take the required sample of cells.

2.5 Flow cytometry

Flow cytometry was a key element of this research and time and care was taken to ensure the correct implementation of the process.

2.5.1 Introduction

Flow cytometry is one of the most common methods of cell characterisation used. The process involves the passing of a single file stream of cells past a detector, which can then be used to identify properties of the cell. Data from cells within a single sample can then be combined to identify patterns and trends, which allow for further analysis of effects and dynamics downstream.

In addition to the cell's interaction with the detection element, cells can also be labelled for identification of certain characteristics. In laser light flow cytometry, the most common form is antibody conjugated fluorophores. This allows for targeted labelling of cells with excitable molecules which can be detected to define the level of expression on or in the cell. The use of multiple lasers, optical filters and detectors can facilitate the use of a panel of antibody conjugated fluorophores, targeting multiple proteins. These data can then be used to identify specific phenotypic subpopulations within samples, and with effective panel design it is possible to get high quality, informative data.

2.5.2 Equipment used

The equipment used throughout was a BD FACSCanto II flow cytometer.

2.5.3 Fluidics

One of the most important elements of a flow cytometer is the fluidics system. This system facilitates both the movement of the sample within the system and the concentration of the sample into a single line.

The fluidics system acquires the sample based on preset parameters and moves it into a central nozzle surrounded by a sheathing fluid. The direction of the fluid towards the decreasing diameter lumen causes hydrodynamic focussing and organises the cells into a single cell stream through the Bernoulli effect [153]. The laminar flow in the system at this point reduces the chance of the sample and sheath fluid mixing.

2.5.4 Lasers

The FACSCanto II uses lasers to detect and categorise cells. The configuration and number of lasers can vary substantially across devices, and even within the CD FACSCanto II range it is also possible to have different configurations of lasers and optics.

The BD FACSCanto II used had a 4-2-2 configuration. It was configured with 3 lasers, a blue 488nm, red 633nm and violet 405nm laser. The lasers are configured so that they do not interact with the cells simultaneously, which allows the collection of response from the individual lasers of each cell. The 4-2-2 connotation refers to the ability to detect 4, 2 and 2 scattered wavelengths as a response to these lasers respectively.

Within the flow cytometer, the lasers emit light towards the interrogation point, the point at which cells interact with the laser light, and the resultant scatter is monitored. Scatter can be measured in two directions; forward scatter measures the size of cells, while the side scatter can be indicative of other cell characteristics such as the granularity of the cell[154]. Both forward scatter and side scatter within this device are measured using the blue 488nm laser. The forward scatter is measured by a forward scatter detector, while the side scatter is detected as part of the blue laser detection optics.

Labelled analysis of cells worked by absorption of the light from one of the lasers by a fluorophore. This put the fluorophore in an excited state, and in returning to a ground state the energy was emitted as light which could be picked up by detectors. Although fluorophores were specifically designed to respond in this manner, cells could also give some level of response in this manner also.

2.5.5 Optical filters and detectors

After passing through each laser, the side scattered light is routed to the collection optics for each laser. The collection optics consisted of photomultiplier tubes (PMTs) and filters. The collection optics for the blue laser consisted of 5 filters, and 5 PMTs, whilst the collection optics for the red and violet lasers consisted of two filters and two PMTs.

The filters are used to distribute the light amongst the detectors correctly, thus facilitating the analysis of multiple wavelengths from one laser. The filters within the collection optics have a threshold wavelength, anything about this will be transmitted through the filter, and below will be reflected. The positioning of these filters results in light above the threshold being able to pass through to a PMT behind, whilst the remaining light moves on. With these in place it is possible to register emission of light at multiple wavelengths through excitation with a single laser.

The photomultiplier tubes present in the collection optics detect light which can pass through the filters to them. On stimulation the PMTs convert the light into an electronic signal, which is then

amplified by the onboard electronics and converted into a digital signal. The level of amplification applied to the electronic signal is dictated by the voltage applied to the channel.

The voltage applied to a channel is a user defined parameter and requires optimisation to achieve the most informative data. As the set voltage is an amplification of the signal, increasing the voltage can increase the separation between cell populations that are expressing and non-expressing of the target protein. However, setting this too high can cause the signal to move beyond the range of registration.

In addition, increasing the voltage can amplify noise in the system. For instance a poor response, caused by a dim fluorophore labelling a less densely expressed protein, would require a greater level of amplification, and could result in issues discerning true responses from the noise. This can be negated mostly by effective planning of the labelling panel used, targeting brighter fluorophores to less highly expressed targets and vice versa.

2.5.6 Compensation

The fluorophores used in flow cytometry work through excitation of the molecule, followed by the release of energy as light when returning to the ground state.

Although each fluorophore has a peak absorption and emission level, which informs the target wavelength for detection, there can also be some overlap with detectors of other wavelengths. The overspill generated has the potential to induce false positives in off target detectors.In order to account for this, the level of overspill from one channel into another can be calculated and used to adjust the response in the non-target channel. This is known as compensation.

The compensation process involved the incubation of each stain independently with compensation beads (Miltenyi Biotech, cat no. 130-104-693). The stained beads were then analysed using the compensation protocol on the flow cytometer to measure the level of overspill, so that the output of the non-target channels can be adjusted accordingly in a full analysis scenario.

2.6 Counting

Analysis of viable cell counts was regularly undertaken on cell samples and experimental cultures. Over the course of the research two different methods were used to analyse samples. One method utilised flow cytometry using the FACSCanto II, the other utilised the Beckman Coulter Vi-CELL XR cell viability analyser.

2.6.1 Canto

Analysis of the viable cell count was possible using the FACSCanto II and the viability stain 4’,6-diamidino-2-phenylindole (DAPI) (Fisher, cat no. 10184322).

2.6.1.1 Sample preparation

2.6.1.1.1 Sampling

Each culture was sampled 10 µL into an individual well on a 96 well plate using a 20 µL micropipette. These wells were then supplemented with 90 µL flow buffer using a 300 µL micropipette.

2.6.1.1.2 DAPI

Samples were then supplemented with 100 µL DAPI, a fluorescent stain that binds to effectively to adenine-thiamine regions of DNA. DAPI is cell membrane permeable and over time would label all cells. However, in low concentrations permeability is low in cells with intact membranes, such as live cells. Dead or dying cells have a more permeable membrane, allowing DAPI to bind to the adenine-thymine regions. As a result, it is possible to identify viable cells, by using DAPI as a marker for non-viable cells.

2.6.1.2 Mechanical settings

The mechanical settings for running samples on the flow cytometer included the voltage setting for the forward and side scatter, and 5 parameters which defined the onboard sampling process.

The voltage for forward scatter was set at 200, whilst the side scatter voltage was set at 450.

The 5 onboard sampling parameters were the sample volume, mixing volume, mixing speed, no. of mixes and wash volume. The sample volume was set to 150 µL, with a mixing volume and speed of 100 µL and 200 µL respectively. The no. of mixes was set at 5, and the wash volume was set at 200 µL.

2.6.1.3 Data analysis

After data acquisition, the flow cytometry data were used to identify the viable cells within the sample. First the DAPI positive cells were gated out of the selection. Next any events that did not fit within a reasonably cellular profile, such as CD3/CD28 Dynabeads, were also removed.

2.6.2 Vi-cell

Analysis of viable cell counts was also performed by using the Beckman Coulter Vi-CELL XR cell viability analyser. The analyser was connected to a Sartorious ambr 250 automated cell culture platform, which could be utilised for its process automation.

2.6.2.1 Sample preparation

For individual cell counts, 50 µL of cells were sampled using a 300 µL micropipette and diluted 1/10 with flow buffer using a 1000 µL micropipette, and directly transferred to the sample cup of the analyser. For a high number of counts, 50 µL of samples was transferred to an individual well of a 24 well plate using a 300 µL micropipette. These samples were then diluted 1/10 using a 1000 µL

micropipette, and the plate was placed into the ambr system. A program was then set to run which would sample wells as the user defined, and allowed automated counting of culture samples.

2.6.2.2 Staining and analysis

Once added to analyser, either manually or mechanically, the samples are then drawn up into the Vi-CELL and mixed with Trypan Blue. Trypan blue works using the same principle as DAPI. The samples are then photographed as they pass through an imaging area, and the Vi-CELL algorithmically assesses which cells are viable and which cells are not and will generate a total cell count, viable cell count and viability percentage.

2.7 Phenotypic analysis

Understanding the impact of culture conditions on T-cell subpopulations was a key element of the research carried out. As a result, phenotypic analysis was regularly performed on cell cultures within experiments.

2.7.1 Introduction

Cell counting processes are numerous and generally well established. However, the broad range of markers and fluorophores available, as well as the varying configuration of flow cytometers, means that setting up a new analytical panel remains a complex task.

2.7.1.1 Phenotypic panel

Initial work focussed on selecting suitable markers for phenotypic analysis of T-cell cultures. This investigation, shown previously, concluded with the selection of 5 markers, CD4, CD8, CCR7, CD45RA and CD45RO

The maximum level of expression on the cell surface of a marker varies depending on the protein, with some markers being expressed substantially more than others. In addition, the different levels of excitation of fluorophores results in intensities of light emission on returning to ground state. Combining a fluorophore with a low level of light emission, a dim fluorophore, with a marker with low levels of expression on the cell surface, would require a higher voltage when analysing via flow cytometry to separate positive and negative samples. This amplification of low resolution signal would also increase the signal of noise in the system and may create difficulty or confusion in analysis of the genuine protein expression on the cell surface.

Consequently, the expression densities of the selected T-cell markers were obtained[125] and the relative fluorescence collected from manufacturer literature. Efforts were taken to combine high expressing surface proteins with dimmer fluorophores and vice versa where possible. The resultant antibody panel is displayed in Table 1, and was used throughout for phenotypic analysis of cultures.

Target protein	Dye	Cytometer channel	Relative Fluorescence	Expression Density
CD4	PerCP-Vio 700	PerCP	75	36400
CD8	VioGreen	V500	12.7	65500
CCR7	VioBlue	V450	302	2000
CD45RA	PeVio-615	PE-Cy7	309	33400
CD45RO	APC-Vio770	APC-Cy7	52.8	12600

Table 2.1. Fluorophores conjugated to the antibodies used in labelling for phenotypic analysis, as well as the relative fluorescence of the fluorophore and the expression density of the target marker.

All antibodies used were Miltenyi Biotech REAfinity antibodies, selected due to the superior consistency and purity courtesy of their recombinant manufacturing process [155,156].

2.7.2 Antibody titration

Antibodies have a high affinity for their target antigen, preferentially binding the target over other proteins. However, in high concentrations all available targets will be quickly saturated, with excess antibodies binding to lower affinity targets, which can generate false positives in flow cytometry data. Conversely, a low concentration of antibody would reduce the response of high expressing cells which may lead to difficulty in separating expressing and non-expressing populations. As antibody labelling was a core process for monitoring culture outputs, it was important to be able to accurately and consistently report marker expression levels. It was therefore necessary to analyse concentrations of each antibody to identify optimal separation between expressing and non-expressing populations.

A suggested concentration is provided by the manufacturer. However, fluorophore response and detection can be affected by configurational differences, environmental variation and cell type marker expression. To optimise population separation within this research, analytical panel antibodies were titrated to identify the best concentration of each antibody.

2.7.2.1 Titration process

The process of titration involves incubating cells that are both positive and negative for the target expression with a decreasing concentration of the antibody to identify the optimal separation. To generate the positive and negative populations to perform titration, it would have been possible to isolate cell populations using MicroBeads targeted to the staining marker in the same manner as CD3 cells had been isolated. However, this process would have been labour and resource intensive, and it would have been difficult to conclusively state that residual labelling beads were not affecting results. Compensation beads were used in place of cells to provide a positive and negative response to the

stains. Due to the recombinant nature of the REAfinity antibodies [155,156] it was possible to use a single vial of positive and negative compensation beads to titrate all 5 antibody stains.

The manufacturers recommended concentration was used as the basis for concentrations tested in the titration process. Each stain analysed with positive and negative compensation bead populations at the recommended concentration, twice the recommended concentration, and then at dilution factors of 2, 4, 8 and 10.

2.7.2.2 Separation index

After running the different antibody concentration, the samples were analysed to calculate the separation index. Separation index is a ratio of separation between positive and negative populations and can be used to identify the optimal concentration for each stain. The flow cytometry data was analysed to identify the positive and negative populations, and the median fluorescence intensity (MFI) of each was calculated. The separation index was then calculated using the following formula:

$$\frac{\text{Positive population MFI} - \text{Negative population MFI}}{(84^{th}\text{ percentile Negative population} - \text{Negative population MFI})/0.995}$$

Analysis of the separation indexes showed some variation in optimal concentrations between antibodies. Both the CD8 and CD45RA stains were optimally diluted at 1/4 the recommended concentration, whilst the CCR7 and CD45RO stains were optimally diluted at 1/2 the recommended concentration and the CD4 stain at 1/10. It should be noted that the pattern of data for the CD45RO stain appears to suggest that 1/4 dilution may have been optimal, were it not for data that appeared anomalously low (data not shown).

In the interest of simplicity, the decision was taken to use a common dilution across all stains. Assessment across all data lead to the conclusion that 1/4 was the most appropriate dilution factor for all stains relative to the standard concentration.

2.7.2.3 Acknowledgement

This work was predominantly carried out by Lydia Beeken under the supervision of the author as part of a placement within the Centre for Biological Engineering.

2.7.3 Staining process

The staining process for phenotypic analysis was as follows. Staining was either performed in 1.5 mL Eppendorf tubes (Fisher, cat no. 10509691) or in a 96 deep well plate (Fisher, cat no. 10089910).

2.7.3.1 Culture sampling

20 µL was sampled from each culture and transferred to either an individual Eppendorf tube or an individual well in a 96 deep well plate using a 20 µL micropipette. In both circumstances these samples were then supplemented with 155 µL flow buffer using a 300 µL micropipette.

2.7.3.2 Manufacture of staining mix

The recommended volume of antibody to add to samples for phenotypic analysis was 10 µL per 100 µL. This was the same across all 5 stains. Therefore, in using 1/4 of the recommended stain concentration it was necessary to supplement 2.5 µL per 100 µL. With a target staining volume of 200 µL, this meant that for each staining sample, 5 µL of each stain was to be added.

There was a concern that the low volume that was to be transferred to each culture could induce variation in the staining, as slight variation could have large differences on the concentration added. This was further compounded by the requirement of supplementation of 5 stains. The process was adapted to pool sufficient volumes of all 5 antibody stains to supplement all samples being analysed.

The greater volume required to transfer 5 stains simultaneously would reduce the likelihood of proportionally large variation brought in through pipetting. Using a central pool would also reduce the likelihood of variation in the relative proportions of antibodies. Creating a central pool of antibody had a positive effect on reducing the level of inter- and intravariation between the supplemented stains. (5 X no. of samples to be tested) + 10% µL of each stain was transferred to an Eppendorf tube using an appropriately sized micropipette. 25 µL of the pool was then supplemented into each sample using a 20 µL micropipette (2x12.5 manipulations).

2.7.3.3 Incubation

Once supplemented with the stain, samples were covered to protect from light and incubated at 4°C for 10 minutes.

2.7.3.4 Washing

After incubation the stained samples were washed before they could be analysed. The two staining vessels required different washing techniques as described below.

2.7.3.4.1 Eppendorf individual samples

1 mL flow buffer was transferred to each Eppendorf tube using a 1000 µL micropipette. The tubes were then centrifuged using a microfuge (Sigma 1-14 Microfuge) at 300 G for 10 minutes. Supernatant was then gently removed using a 1000 µL micropipette and 200 µL of fresh flow buffer was added to the cell pellet using a 300 µL micropipette. The pellet was then gently resuspended by pipetting up and down 4-6 times using the 300 µL micropipette.

2.7.3.4.2 96 deep well plates

After incubation, flow buffer was supplemented into each sample well to fill it, approximately 2 mL, using a 1000 µL micropipette.

The plate was then centrifuged at 300 G for 10 minutes and the wash supernatant was removed by quickly inverting the plate over a sink to remove the supernatant whilst maintaining the integrity of the pellet.

200 µL flow buffer was then added to wells using a 300 µL micropipette and the pellet was resuspended by gently pipetting 4-6 times

2.7.3.5 Plating

Once resuspended, each sample was transferred to a separate well of a 96 well plate (Fisher, cat no. 10088612). Once fully prepared the plate was inserted into the FACSCanto II ready for analysis.

2.7.4 FACSCanto settings

The FACSCasnto settings for running phenotypic analysis samples were as follows

2.7.4.1 Voltages

The voltages for each channel were set as displayed in table 2.

Cytometer channel	Voltage
V450	406
APC-Cy7	549
PE-Cy7	624
PerCP	553
V500	480

Table 2.2. Voltages applied to channels used for collection of phenotypic data.

2.7.4.2 Mechanical settings

Mechanical settings were as for cell counting, described in section 2.6.1.2.

2.7.4.3 Compensation

Compensation values were calculated as in table 3 following and were applied across all phenotypic analysis throughout.

Detection channel	-% Detection channel	Spectral overlap
V500	V450	27.22
V450	FITC	7.03
APC-Cy7	FITC	0.84
PE-Cy7	FITC	4.33
PerCP	FITC	5.6
V500	FITC	12.1
V450	APC-Cy7	0.2
FITC	APC-Cy7	0.44
PE-Cy7	APC-Cy7	4.97
PerCP	APC-Cy7	0.6
V500	APC-Cy7	0.54
FITC	PE-Cy7	0.07
APC-Cy7	PE-Cy7	3.61
PerCP	PE-Cy7	3.56
APC-Cy7	PerCP	3.11
PE-Cy7	PerCP	42.98
V450	V500	5.32
FITC	V500	0.19
PE-Cy7	V500	0.05
PerCP	V500	0.08

Table 2.3. Level of compensation applied to channels based on analysis of spectral overlap.

2.7.5 Phentoypic data processing

Having collected the phenotypic data, it was necessary to use software to process the raw information, translating it into meaningful outputs and identifying experimental impacts.

2.7.5.1 Gating and population identification

The processing of the phenotypic data to apply gates and identify subpopulation proportions, as described in 4.3.1.2.1, was performed using FlowJo software.

2.7.5.2 Statistical analysis

Statistical analysis data produced from gating and subpopulation identification was performed using Minitab. This software was also used to assist in the set up of experiments using a Design of Experiments methodology.

3 Investigating the impact of the state of input material on growth and phenotypic response of T-cell populations in culture

3.1 Introduction

With the emergence of T-cell based cancer therapies, such as CAR T-cell therapies, there was a renewed interest in the field of T-cells and how best to optimise therapeutic product that could be delivered to the patient[41,137]. There has been a great deal of investigation into optimal transfection rates and efficacy of treatment[41,83,130,137], however there has been less investigation into the culture of T-cells. More specifically, there is limited evidence of how the input state of material could impact cultures. Understanding the impact of material input state on T-cell cultures could have lasting practical implications for the T-cell therapy manufacture process as well as assisting research into improving the previously mentioned elements.

One way in which input material may have such a substantial impact on the culture of T-cell populations is the different cytokine profiles of T-cell subpopulations. There are multiple subpopulations of T-cell cultures which have different roles and responses *in vivo* The different roles of these subpopulations require the production of different combinations of cytokines depending on their function, which could have a varying impact on the culture. Expression of either CD4 or CD8 on the surface of a T-cell is an indicator of the specialisation of the cell. CD4 expression on a T-cell indicates that it is either a Helper T-cell or Regulatory T-cell. Helper and Regulatory T-cells have relatively opposing roles in the body, which may therefore impact cultures differently depending on their concentration. Helper T-cells help to promote immune responses on activation, which is done through the upregulation of cytokines[39,40,100,101]. These cells predominantly interact with B-cells, however their cytokine production has an effect on a large array of cells, including themselves. Furthermore, there is a range of subpopulations of Helper T-cells that are targeted for different roles within immune response, and therefore different subpopulations of Helper T-cells have a range of different cytokine production profiles[40,101,116]. The role of Regulatory T-cells meanwhile is to limit autoimmune responses. This subpopulation of T-cells reduces the immune response in an area on activation through cytokines and attempts to inhibit response of the immune system to self antigens[39,40,100,101].

CD8 expressing T-cells are also known as Cytotoxic T-cells. The function of Cytotoxic T-cells in the body is to monitor host cells and induce apoptosis in those which are performing irregularly, whether it be through unregulated growth, or viral production[102–104]. These cells are particularly important in CAR T-cell therapies as they are the primary method by which apoptosis is induced in the target cells. These cells are able to induce apoptosis in target populations through exocytosis of

perforins and granzymes in their close proximity which disrupts the membrane and allows the granzymes to induce apoptosis[40,41,103]. The cytokinetic profile of Cytotoxic T-cells is generally smaller than that of the Helper T-cells, depending on the Helper T-cell subpopulations, but still has impacts on themselves and the cells around them[41,130,137].

In addition to the CD4 and CD8 subpopulations, T-cells can also be categorised into maturity subpopulations. These subpopulations are defined by expression of CD45RA, CD45RO and CCR7, and the same subpopulations occur in both CD4 and CD8 subpopulations. Both CD45RA and CD45RO are maturity markers; CD45RA is indicative of naivety in cells that have not yet been exposed to their target antigen, whilst CD45RO is upregulated in cells that have been exposed to their target antigen and are therefore classed as experienced or mature. CCR7 protein expression in T-cells is indicative of cells that will home to SLOs[157]. *In vivo* Naïve subpopulation cells (CD45RA+CD45RO-CCR7+) have not been exposed to their target antigen and have a high proliferative capacity to respond to activation[39,40]. Meanwhile, Effector subpopulation cells (CD45RA+CD45RO-CCR7-) are cells that have been exposed to their target antigen and are active. *In vivo* this means that they are in the process of mounting a response to their target. When the stimuli that maintain the Effector phenotype are removed, the majority of these cells will die by apoptosis[39,158]. However, some of the cells will become one of two mature phenotypes, Effector Memory (CD45RA-CD45RO+CCR7-) or Central Memory (CD45RA-CD45RO+CCR7+). The difference in CCR7 expression in these two subpopulations indicates their different roles *in vivo* with Effector Memory cells remaining in residence in tissues, while Central Memory cells return to the SLOs. Effector Memory cells are quick to respond and mount an early defence against pathogens or damaged cells in tissue but have a lesser proliferative capacity, while Central Memory cells have a slower response to their target proteins but have a greater proliferative capacity for supplementing the Effector Memory response[114,159,160].

T-cells have evolved so that *in vivo* exposure to their target antigen will induce responses as a reaction to a perceived threat, including proliferation and phenotypic changes depending on the subpopulation[101,103]. Analysis of activation identified key proteins which allowed the development of a method to artificially stimulate T-cells through the targeted binding of the CD3 and CD28 proteins, the primary and secondary activation signals[39,40]. There are a number of commercially available products capable of stimulating T-cells in this manner, with one such product being CD3/CD28 Dynabeads (Fisher). Upon activation, T-cells also upregulate the production of cytokine IL-2 and expression of surface protein CD25. IL-2 is a key growth factor of T-cells, promoting both proliferation and maturation in all subpopulations, while CD25 is a subunit of the IL-2 receptor capable of increasing the affinity of binding to the IL-2 receptor 100-fold[161,162]. As a result of the

proliferation stimulating properties of IL-2 on T-cells populations, the growth factor has become an essential element of T-cell culture. In most T-cell culture processes both IL-2 and CD3/CD28 Dynabeads, or other stimulating element, are supplemented into culture at seeding, and therefore form a part of the input material for the culture process. With stimulation increasing the endogenous IL-2 production and expression of the high affinity receptor, it is possible to see how a greater level of stimulation may influence the response of cultures differently to lesser stimulation. *In vivo*this difference would equate to a higher number of cells expressing the target antigen, which would then induce a greater response from T-cell populations.

The investigation into the impact of the state of input material was predominantly aimed at the initial stage in CAR T-cell manufacture, the expansion step prior to transfection. This expansion period is used to increase the concentration of T-cells to an acceptable level prior to transduction with viral vectors[41,130,137,138]. As a result, the success of this process from a purely proliferative standpoint can be measured by viable cell counts in cultures. Evidence also suggests that higher concentrations of certain maturity subpopulations, specifically Naïve and Central Memory, would increase the efficacy of therapy products *in vivo*[66–70]. Therefore, as well as measuring the proliferation of cultures, there was also a desire to monitor the impact of input material on the phenotypic profile of cultures over this period and identify conditions that may increase the concentration of the target subpopulations.

3.1.1 Measurable outputs of T-cell cultures

The two main outputs of T-cell cultures to monitor in the context of T-cell therapy manufacture are growth rate and phenotypic profile[72,85,86,163]. Understanding and improving definitions of the relationship between the input material state and these outputs could help to inform culture processes. Understanding these relationships, could make it possible to better predict the outcome of these cultures from their input state, or allow tailoring of culture conditions to achieve an optimal output. In addition, this information may make it possible to direct cultures toward certain phenotypic lineages or induce more optimal growth by manipulating the input material.

The two experimental outputs needed to be considered in combination when analysing the outcomes of T-cell cultures. It would have been of little benefit to develop a culture protocol that produces purely or predominantly 'homing' subpopulations (CCR7+) that showed little to no proliferation, and vice versa. The growth of cultures was to be monitored through analysis of viable cell counts from samples at regular intervals, while phenotypic assessment was executed through flow cytometry analysis of cells stained with the marker panel developed in 1.4.

3.1.2 Impact of isolation targets and process on growth and phenotypic profile in culture

The initial stage of T-cell culture was preparation of the start material. This preparation for culture of T-cells can either start with whole blood or with apheresis product collected from the donor. In the case of apheresis product, the buffy coat is extracted from the donor's blood without having to harvest other fractions such as red blood cells or plasma[66,72,134]. From this, T-cell cultures can be initiated either from buffy coats, where non T-cell PBMCs are not sustained in culture and undergo apoptosis, or from purified T-cell samples. There is no explicit need to isolate T-cells from a buffy coat to effectively culture T-cell populations; T-cells are naturally purified through the culture process as the conditions that support T-cells do not generally support the other populations present in PBMCs. Initial investigation appeared to show that isolation was a common practice in T-cell culture[62,105,106,129,132,142], and in trying to remain as comparable to the field as possible it was concluded that T-cell isolation was an important area of T-cell culture to analyse.

Focussing on isolation of T-cells from apheresis product prior to seeding, the main method of isolation identified in the literature used CD3/CD28 Dynabeads[41,130]. Through targeted binding of the CD3 protein, a specific marker of T-cells, CD3/CD28 Dynabeads are able to specifically target T-cells in heterogenous populations. In addition, the binding of CD3 and CD28 proteins induces activation in T-cell populations, which promotes greater proliferation in cultures as well as phenotypic change[130,132,133]. As a result, the process is an efficient, dual impact method of isolating pure T-cell populations and stimulating growth in preparation for seeding into culture. However, stimulation of T-cell cultures was one of the elements of T-cell input material to be tested. There was an initial desire to avoid activation in the isolation process to limit confounding factors. It was understood that activation would reduce the concentration of Naïve cells, one of the target subpopulations in the initial stage of CAR T-cell manufacture, through maturation of T-cell populations. This could have skewed the perceived effects of other experimental variables being tested. As a result, the decision was taken to investigate other methods for purification of T-cell populations for isolation of the target primary material without activation.

CD3 was already known to be a highly specific marker of T-cells, and a suitable target for purifying T-cells. However, the relationship between CD3 and the TCR, and therefore the activation of T-cells, meant that this isolation target would only be suitable in this research if it were not to induce activation. Advice was received that appeared to highlight a method that would acceptably fit these criteria. In addition to CD3, investigation highlighted the ubiquity of either CD4 or CD8 on T-cell surfaces. Expression of these proteins is mutually exclusive but targeting both markers would take into account all T-cells in the sample. CD3 and CD4/CD8 targeted isolation were tested to assess for impacts of the isolation method on the growth of the culture and the presentation of phenotypic

profile. It was predicted that as both methods theoretically isolate the same populations, isolation of T-cells through a non-activating CD3 target on the TCR should generate similar data to the cultures isolated using CD4/CD8 targeting.

3.1.3 Variation of seeding material to induce specific outputs

Due to different subpopulations of T-cells having different roles in immune response, and therefore different cytokine profiles, it was predicted that they may have different impacts in culture. Understanding the roles that these subpopulations play in culture, both in their independent behaviour and their influence on other subpopulations, could identify relationships that will help to direct T-cell cultures to target outputs. As previously mentioned, T-cell populations can be subdivided based on maturity phenotypes (Naïve, Effector, Effector Memory and Central Memory) as well as their CD4/CD8 expression. Different maturity subpopulations of T-cells will respond differently to the same stimulus in culture through increased or decreased proliferation[69,89,108]. However, it was predicted that due to the different cytokine expression profiles of CD4+ and CD8+ populations, the balance of these subpopulations would have a greater influence on culture performance.

In order to investigate what impact the balance of CD4+ and CD8+ subpopulations had on the performance of cultures, it was first necessary to test the performance of CD4+ and CD8+ subpopulations individually. Culturing the different subpopulations in isolation from each other would enable analysis of their growth patterns and phenotypic response. Improving the understanding of how CD4+ and CD8+ cells respond independently in culture would give a more informed perspective when analysing the results of culturing both subpopulations together. The main stimulants of proliferation in the culture of T-cells are IL-2 and activation through stimulation of the CD3 and CD28 proteins[130,160,164]. CD3/CD28 targeting stimulants were not to be added to the culture, as mentioned in 3.1.1, and IL-2 would be supplemented into culture; it was therefore anticipated that CD4+ and CD8+ subpopulation cells would grow at a similar rate. In order to understand the relationship between these two subpopulations they would also be cultured together at a set ratio. It was speculated that where PBMCs were used as the seeding material, non T-cell PBMCs may also have an effect on T-cell populations. It was not clear if the effect would be positive or negative, however as an element of potential input material there was a desire to measure the effect it had on target populations. As a result, as well as independently and in combination, CD4+ and CD8+ subpopulations were each cultured with the non T-cell PBMCs recovered from the T-cell isolation process to identify the effect of other cell types.

3.2 Analysing the effects of stimulation of T-cell cultures

3.2.1 T-cell source materials

The following work was completed using isolated material from whole blood provided by Cambridge Bioscience as described in 2.1.1 and frozen PBMC aliquots provided by Axol Bioscience as described in 2.1.2. The instances in which each source was used are described in the specific circumstances below.

3.2.2 T-cell isolation methods

3.2.2.1 Isolation of buffy coat from whole blood

Where relevant, the isolation of the buffy coat fraction from whole blood was performed as specified in 2.3.1.

3.2.2.2 Isolation of T-cells from buffy coat

Where relevant, isolation of T-cells or T-cell subpopulations was performed according to the various processes outlined in 2.3.2.

3.2.3 T-Cell culture methods

Seeding material was prepared as described in 2.2 and 2.3 as relevant and cultures were set up and maintained according to the processes set out in 2.4.1-5 other than specific parameters listed in 3.2.5-11.

3.2.4 Sampling of T-cell cultures for viable cell counts and phenotypic analysis

Viable cell count analysis was carried out as described in 2.6, with the specific methods below detailing which process was used. Phenotypic analysis was carried out according to processes in 2.7. Timing of sampling was dependent on experiment and is detailed in the specific sections below.

3.2.5 Specific materials and methods used in assessing the impact of T-cell isolation methods

For testing the impact of isolation methods on T-cell populations, whole blood from a single donor was used as the source material. Once the buffy coat was isolated it was divided equally into three and each aliquot was then subject to one of three isolation processes: CD3 targeted isolation (as outlined in 2.3.2.1), CD4/CD8 co-incubated isolation (as outlined in 2.3.2.2.1) and CD4/CD8 two step isolation (as outlined in 2.3.2.2.2).

Cultures were seeded at $3x10^5$ cells/mL and supplemented with IL-2, but not CD3/CD28 Dynabeads. Cultures were measured for viable cell count at 48, 72, 144 and 216 hours. Phenotypic measurements were taken at 72 and 216 hours, however at 72 hours cultures were only stained for

CD4 and CD8 expression. Viable cell count analysis was carried out using the FACSCanto II as outlined in 2.6.1.

3.2.6 Specific materials and methods used to analyse the performance of CD4+ and CD8+ isolated and depleted cultures

Analysis of CD4+ and CD8+ targeted and depleted cultures used whole blood from 2 different donors supplied by Cambridge Bioscience as the source material. Initial investigation into depleted and targeted cultures was performed on blood from one single donor, however work investigating the role of non T-cell PBMCs as supportive in culture was performed with blood sampled from a different donor. Once the buffy coat was isolated from the whole blood, CD4+ and CD8+ populations were isolated from the buffy coat according to 2.3.2.2.3. The pure CD4+ and CD8+ populations were cultured as well as CD4+ and CD8+ depleted cultures that were generated as a result of the targeted isolation. PBMCs were also cultured to compare the performance of non-isolated cultures.

Cultures were seeded at a target density of $5x10^5$ cells/mL and supplemented with IL-2, but not CD3/CD28 Dynabeads. Cultures were sampled at 24, 48, 72 and 144 hours for analysis of viable cell count. After 144 hours, due to substantial loss of viable cells in all conditions, cultures were reseeded to a target density of $5x10^5$ cells/mL where possible. Cultures were continued in 6 well plates, and the possibility of reseeding was measured by the ability to concentrate a sample to $5x10^5$ cells/mL within the working volume of the well (1.9-2.9 mL). It was possible to reseed all cultures except the CD8 targeted condition. The subsequent cultures were sampled for cell counts at 168, 192, 264 and 312 hours. Viable cell count analysis was carried out using the FACSCanto II as outlined in 2.6.1.

3.2.7 Specific materials and methods used to compare performance of CD4+ and CD8+ cell populations cultured independently, in combination with each other, and each in combination with non T-cell PBMCS

For investigation into various subpopulation combinations, whole blood from a single donor, provided by Cambridge Bioscience, was used as the source material. Buffy coat was initially isolated from whole blood as described in 2.3.1. CD4+ and CD8+ subpopulations were then isolated using the methods described in 2.3.2.2.3. To generate the non T-cell PBMC populations, samples had both T-cell subpopulations isolated from them in a two-step fashion. These populations were referred to as double depleted (DD).

Cultures were seeded at a target density of $5x10^4$ cells/mL and supplemented with IL-2 but not CD3/CD28 Dynabeads. Where different subpopulations were cultured together the total seeding

density was $5x10^4$ cells/mL, and therefore each subpopulation contributed $2.5x10^4$ cells/mL to the seeding material. Cultures were sampled at 16, 89, 92, 110, 130, 139, 158 and 177 hours for viable cell count analysis. Viable cell count analysis was carried out using the FACSCanto II as outlined in 2.6.1.

3.2.8 Specific materials and methods used to analyse the impact of variable CD4+:CD8+ T-cell populations in culture

The source material for investigation into the impact of variable CD4+:CD8+ ratios on T-cell culture was whole blood from a single donor, provided by Cambridge Bioscience. The buffy coat was initially isolated from the whole blood, as outlined in 2.3.1, before isolation of CD4+ and CD8+ subpopulations, detailed in 2.3.2.2.3.

Test conditions involved the seeding of T-cell populations at $5x10^4$, $1x10^5$ and $5x10^5$ cells/mL with CD4:CD8 ratios of 3:1, 1:1 and 1:3. CD4+ and CD8+ subpopulation cells were seeded at the required concentration into each well, and the cultures were supplemented with IL-2, but not CD3/CD28 Dynabeads. Cultures were sampled at 89, 93, 145, 161, 168, 237, 260, 277 and 293 hours for analysis of viable cell count. Viable cell count analysis was carried out using the FACSCanto II as outlined in 2.6.1.

3.2.9 Specific materials and methods used to assess performance of T-cell populations with delayed supplementation of CD3/CD28 Dynabeads

Whole blood, provided by Cambridge Bioscience from a single donor, was the source material for investigation of delayed CD3/CD28 Dynabead supplementation. The buffy coat was isolated from whole blood as outlined in 2.3.1.

The PBMCs were then seeded at a target density of $1x10^6$ cells/mL and supplemented with IL-2 but no CD3/CD28 Dynabeads. However, due to a calculation error the cultures were actually seeded at $1x10^7$ cells/mL. When this was realised at 72 hours, the culture was diluted to reduce the culture concentration to the sustainable level of the intended seeding density. Cultures were sampled for viable cell count analysis at 72, 144, 192, 264, 312 and 336 hours.

After 336 hours, the cultures were reset to a target seeding density of $5x10^5$ cells/mL and supplemented with an appropriate concentration of CD3/CD28 Dynabeads as outlined in 2.4.4. The cultures were then sampled for viable cell count at 408, 456, 480 and 504 hours. Viable cell count analysis was carried out using the FACSCanto II as outlined in 2.6.1.

3.2.10 Specific materials and methods used to determine the effects of supplementing CD3/CD28 Dynabeads, with and without IL-2, into T-cell cultures, in comparison to no supplementation

The source material for initial investigation into the impact of CD3/CD28 Dynabeads on T-cell culture was frozen PBMC aliquots from Axol Bioscience, all from a single donor. Vials were thawed and prepared as outlined in 2.2 and seeded into culture at a target density of $1x10^6$ cells/mL. Depending on condition, cultures were supplemented with either CD3/CD28 Dynabeads or CD3/CD28 Dynabeads and IL-2. There was a further condition which received no additional supplementation.

All cultures were sampled at 48 and 120 hours for analysis of viable cell count. After 120 hours it was decided not to continue the non-supplemented culture, and therefore only the supplemented cultures were sampled at 196 hours for viable cell count. Viable cell count was assessed using the Vi-cell cell analyser, as outlined in 2.6.2. Cultures were also sampled for phenotypic analysis at 120 hours.

3.2.11 Specific materials and methods used to compare the proliferative and phenotypic response of T-cell cultures to supplementation of CD3/CD28 Dynabeads and IL-2 independently and in combination

For analysis of the culture of T-cells supplemented with IL-2 and/or CD3/CD28 Dynabeads, frozen PBMCs from a single donor were provided by Axol Bioscience. Samples were thawed and prepared as described in 2.2. Depending on condition, cells were seeded at a target density of $1x10^6$ cells/mL or $2x10^6$ cells/mL. Cultures were then supplemented with either CD3/CD28 Dynabeads or IL-2 or both.

Cultures were sampled at 24, 72, 96, 120, 144 and 168 hours for analysis of viable cell count. The assessment was carried out on a Vi-cell cell analyser as in 2.6.2. Cultures were also sampled for phenotypic analysis at 0, 72 and 120 hours.

3.3 Results

3.3.1 Analysis of different isolations targets for T-cell isolation on proliferation and phenotypic profile

Initial investigation looked into the potential impacts of T-cell isolation on cultures. Three different isolation methods were compared with one isolation targeting CD3 and the remaining two targeting CD4 and CD8 as a co-incubation and as a two-step process. After isolation of PBMCs from whole blood, T-cells were then isolated using these markers and seeded in to culture. Cultures were sampled for analysis of viable cell counts at intervals specified in 3.2.5. Comparing these counts over

time shows a substantial difference between the two targets, with those isolated by CD3 showing substantially higher growth (Fig. 3.1).

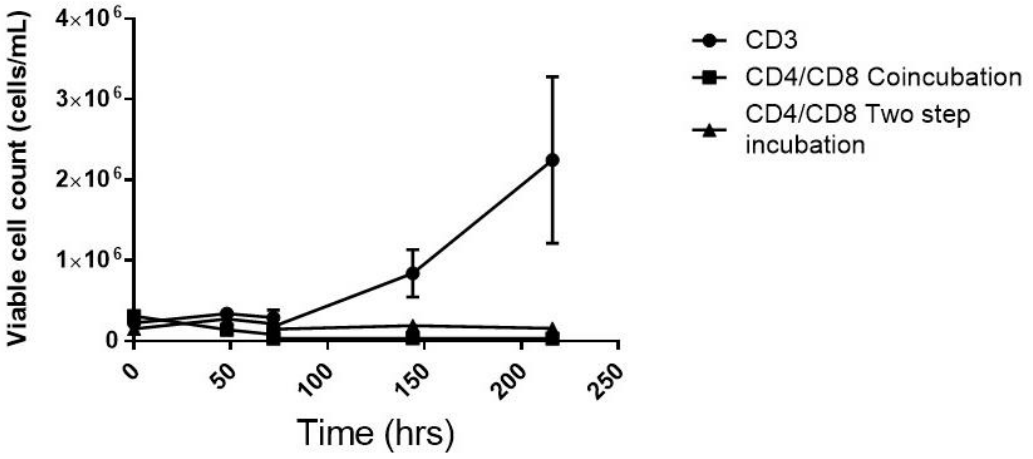


Figure 3.1. Analysis of three methods of T-cell isolation show that the CD3 targeted selection induces that greatest proliferation in culture. After an initial lag period of 72 hours cell growth is seen in the populations targeted with CD3 isolation beads, increasing at a steady rate until 216 hours. The cultures selected through targeting CD4 and CD8, both the simultaneous and two step isolations, showed no substantial growth over time but remained relatively stable through the course of the experiment.

From 0 to 72 hours it is possible to see that the CD3 targeted and two step CD8/CD4 targeted cultures perform similarly, showing a slight increase to 48 hours, and a slight reduction in viable cell count to 72 hours. Meanwhile, the concurrent CD4/CD8 isolated cultures show a slightly higher but comparable starting density, followed by a consistent decline until 72 hours and a more gradual decline until 216 hours. From 72 hours it is possible to see the two step CD8/CD4 isolated condition showed a gradual decline until 216 hours similar to that seen in the concurrent CD4/CD8 isolated cultures. In comparison, from 72 hours it was possible to see substantial growth to 144 hours and then on to 216 hours as well. These data show clear differences between the two isolation targets, with the CD3 isolated cultures growing substantially in comparison to the CD4/CD8 isolated cultures regardless of method. It is not clear why the CD3 targeted cultures grew so efficiently in comparison to the CD4/CD8 targeted cultures, however it does bring in to question the assertion that the antiCD3 MicroBeads will not induce activation in the target cells.

At 72 and 216 hours, samples were taken to analyse for CD4+ and CD8+ subpopulations in cultures. The data showed that there were similarities in the behaviour of the CD3 targeted cultures and the CD4/CD8 concurrent targeted cultures. Analysis of the phenotypic data at 72 hours shows that the CD3 targeted cultures have both the highest CD4+ subpopulation proportion, at ~40%, and the lowest CD8+ subpopulation proportion, at ~50% (Fig. 3.2). The CD4/CD8 concurrently isolated culture showed ~30% and ~65%, whilst the CD8/CD4 two step isolated cultures showed ~10% and 85% subpopulation proportions of CD4+ and CD8+ cells respectively. It is possible to see that the

subpopulation proportions do not cumulatively represent the whole samples. As the proportions were calculated from a gated subpopulation of live cells, the additional proportion is made up of non T-cell PBMCs. It is likely that as part of the isolation process the cells were somehow retained into the culture material. This could have been because they remained in the filtration column rather than passing through prior to extraction of the target material, or could have been sue to non-specific binding of the MicroBeads.

The justification, as mentioned previously, for isolating CD8+ T-cells first in the two step CD8/CD4 isolation process is that it was thought that the subpopulation that was isolated second may respond negatively to the additional manipulation. It is possible to see here that in comparison to the concurrent incubation CD4/CD8 targeted cultures, the two step cultures showed a higher relative proportion of CD8+ T cells (Fig 3.2B). This suggests that the premise of the hypothesis was correct and that in this experiment the second isolation has a negative effect on the CD4+ subpopulation cells (Fig 3.2A).

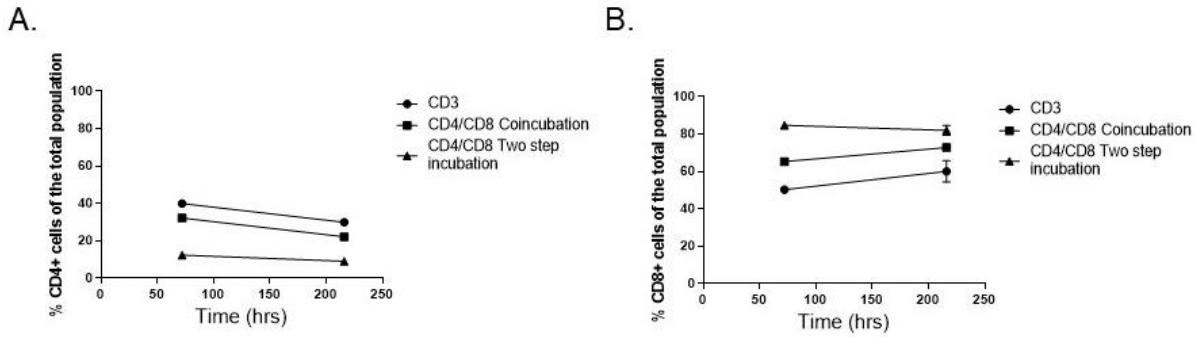


Figure 3.2. Phenotypic data shows that overtime the proportion of CD8+ cells decreased, while CD4+ cells generally increased. (A) Graph shows the percentage of CD4 positive cells at day 3 and day 9 of culture; in all isolation methods the number of CD4+ cells reduced over time as a proportion of the population. (B) Graph shows the percentage of CD8 positive cells at day 3 and day 9 of culture. CD8+ cells increase as a proportion of the total population over time in the CD3 and simultaneous CD4 and CD8 isolated cells, whereas a minor drop in the two step CD4 and CD8 isolated cells was observed.

Comparing the relative proportions of CD4+ and CD8+ subpopulations at 72 hours and 216 hours it is possible to see that in both the CD3 targeted and CD4/CD8 concurrent targeted cultures the relative CD4+ subpopulation proportion reduces by ~10% and the CD8+ subpopulation proportion increases by the same amount. In the CD8/CD4 two step cultures, it is possible to see a very slight drop in both the CD4+ and CD8+ relative subpopulation proportions. It was unusual to see both subpopulation proportions drop, especially as it implied a relative increase in non T-cell populations. Comparing this to the viable cell count data, the CD4/CD8 concurrent isolated cultures showed a sustained decrease in viable cell counts. It is plausible that rather than seeing an increase in the non T-cell PBMC subpopulation proportion due to their growth, the effects seen are due to the T-cell subpopulations being more sensitive to the negative conditions and decreasing in proportion. Comparison of the event plots of cultures at 72 and 216 hours on a CD4/CD8 axis shows that the

trend in change of the CD4+ and CD8+ populations in cultures isolated on CD3 and CD4/8 coincubation are relatively comparable. It was particularly noteworthy to see these differently isolated cultures show a similar trend despite their drastically different levels of proliferation. The common trend in the proportional variation could imply a similarity in the T-cell subpopulations that were isolated, despite the different targets. If this were the case it would suggest that as well as the isolation target influencing proliferation (Fig. 3.1), the two-step incubation process was also negatively effecting CD4+ populations in culture.

The samples taken for phenotypic analysis at 216 hours were stained not only for CD4 and CD8, but also for CD45RO and CCR7. Using these markers, it was possible to analyse the maturity phenotype subpopulations (Naïve, Effector, Effector Memory and Central Memory) present in the cultures. Analysis of the CD4/CD8 isolated cultures showed that there were negligible differences in the phenotypic presentation between those that were isolated concurrently and the two step isolation (data not shown). With these data, rather than compare all three isolation techniques, the comparison was be carried out between those cultures isolated through targeting CD3, and those targeting CD4 and CD8. Comparison of the event plot data immediately suggests that the two isolation targets induce different phenotypic responses in their respective T-cell populations (Fig. 3.3). Observing the range and level of CCR7 and CD45RO expression shows that between the differently isolated cells these is relatively low. However, 2D analysis of these plots shows substantial differences in the way events are distributed within this space.

The CD4/CD8 isolated cells appear to show 3 main clusters in the data (Fig. 3.3Bii). The densest cluster is a CCR7+CD45RO- population, which would appear to fit in to the Naïve phenotype definition. There are two other clusters of similar greatest density, the one of which is a CCR7-CD45RO- cluster, which aligns with the phenotypic definition for Effector phenotype cells. The final cluster in the CD4/CD8 isolation event plot has a high CD45RO expression, showing that it is a mature population. The minimum level of CCR7 expression for this cluster is at a similar level to the minimum CCR7 expression in the Effector cluster, showing that this cluster has some level of Effector Memory presence. However, the maximum level of CCR7 expression reaches beyond that of the Effector subpopulation and shows a similar level of expression to that of the lower end of the Naïve cluster events. This implies that there are some 'homing' events present in the cluster, and therefore the presence of Central Memory phenotype cells. Considering these elements, it would appear that this is an Effector Memory cluster of cells, that also contains cells which are transitioning to Central Memory Phenotype, however without dynamic data this is hard to confirm.

To better understand the roles that CD4+ and CD8+ subpopulations play in culture and respond to experimental variables, the CD4+ and CD8+ subpopulations were gated and plotted using CD45RO

and CCR7 to identify if there were any subpopulation related responses. Analysis of the CD4/CD8 isolated CD4+ subpopulation shows, similar to the total cell plot, that the Naïve cluster is the densest (Fig. 3.3Cii). The CD4+ subpopulation also presents a cluster of Effector Memory cells, however this appears less dense than in the total cell plot, and also has a substantially lower maximum CCR7 expression. There appears to also be a slight clustering of cells that are CCR7+CD45RO+, suggesting that they are Central Memory phenotype cells. These events are of relatively low density and would not appear to have a substantial impact on the total cell plots. In addition, it is possible to see that the CD4+ subpopulation does not show an Effector phenotype cluster, implying that the presence of this cluster in the total cell plot is substantially due to the CD8+ subpopulation.

Analysis of the CD8+ subpopulation shows, as with the CD4+ subpopulation and the total cell, that the densest cluster is the Naïve subpopulation (Fig. 3.3Cii). This plot also shows a large of concentration of events with a relatively low CCR7 expression level across the full range of CD45RO expression observed. This distribution of events is most dense at either end, however there is only a slight reduction in density in the middle. These events appear to represent an Effector and Effector Memory cluster, with cells transitioning between the two. The denser areas correlate quite strongly with the Effector and Effector Memory clusters that were defined in the total cell plot. In addition, it is possible to see that the CD45RO+ events show a level of CCR7 expression that is on average above that of the CD4+ Effector Memory subpopulation, but below that of the CD4+ Central Memory subpopulation.

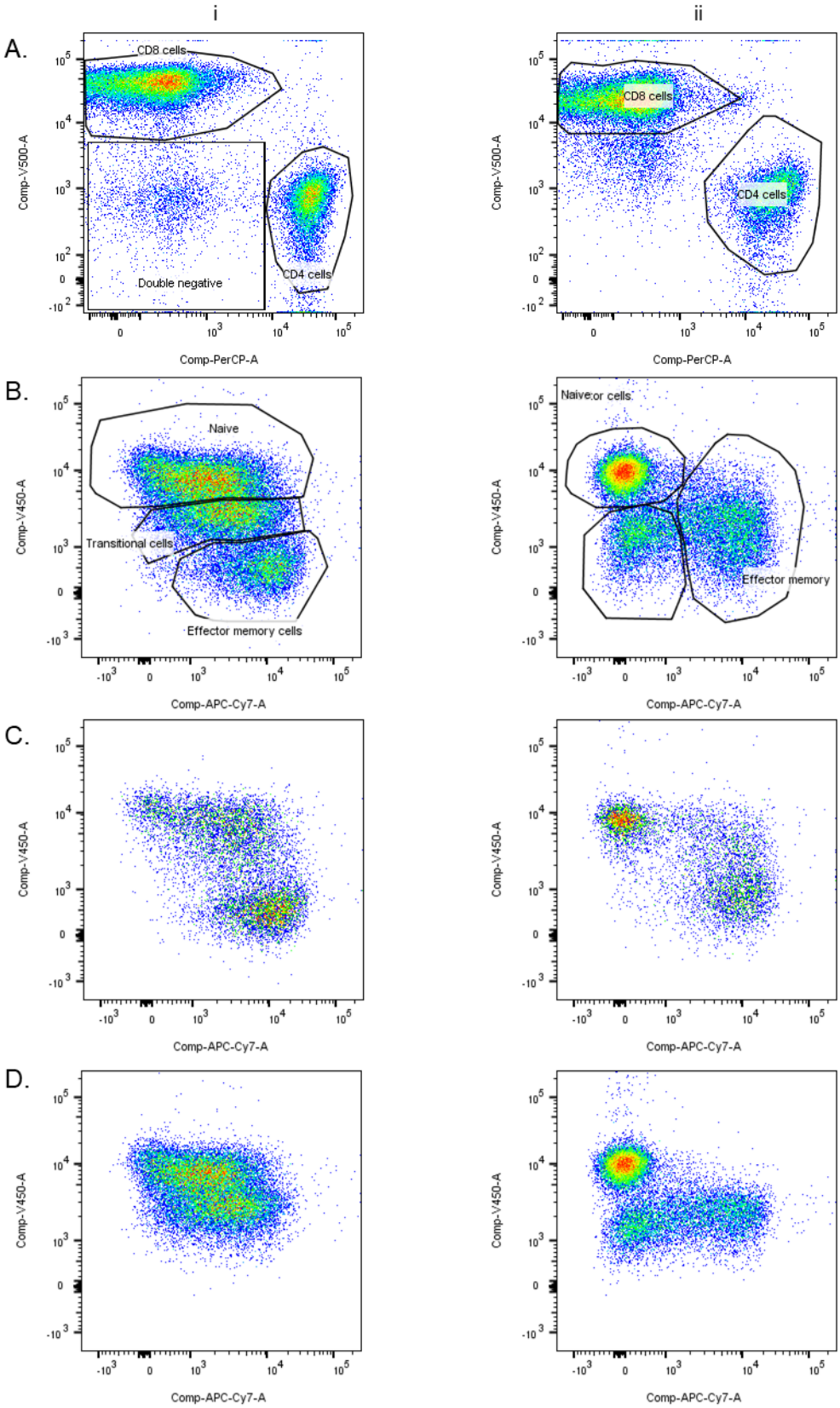


Figure 3.3: Flow cytometry plots showing the phenotypic balance of cell populations isolated by different methods and the impact they have on culture growth. (A) Shows the CD4+ and CD8+ profiles of cell population isolated by CD3 beads (i) or CD4 and CD8 isolation beads (ii). It can be seen that both isolation methods give rise to similar CD4+/CD8+ profiles. (B) shows the variation in maturity profile of the cells when observing them based on CD45RO (APC-Cy7) and CCR7 (V450), key markers in T cell lineage. (ii) appears to show relatively separate groups of Naïve (CCR7+, CD45RO-), effector (CCR7-, CD45RO-) and effector memory (CCR7-, CD45RO+) cells within the population. (i) meanwhile displays a broad naïve population that begins to appear central memory-like in marker profile (CCR7+, CD45RO+) and an effector memory population, with a transitional between the two that does not match traditional T cell marker profiles. (C-D) shows that this phenotypic distribution is not uniform across the CD4 (C) and CD8 (D) subpopulations.

It is not immediately clear why these expression levels were observed, it could indicate that this subpopulation is transitioning from Effector memory to Central Memory, or alternatively it could be showing that CD8+ Effector Memory cells have a higher level of CCR7 expression in culture than CD4+ Effector Memory subpopulations. In either scenario, the differences observed in CCR7 expression between the two subpopulations highlights that the single continuous cluster observed in the total event plot is not representative of both T-cell subpopulations. In comparison to the CD4/CD8 isolated cells, the distribution of events in the CD3 isolated cultures do not conform so easily to the standard phenotypic definitions. Analysis appears to show three main clusters of events, two of which overlap (Fig 3.3Bi) and one separate cluster. The level and range of CCR7 expression in the separate cluster identify it as low expression, while the range of CD45RO expression has high a predominantly high expression level with a small proportion of low expressing cells. This suggests that the independent cluster represents a predominantly Effector Memory populations with a small proportion of Effector phenotype cells also.

The two clusters which appear to somewhat overlap do not appear to sit within a single subpopulation definition. The cluster with the higher CCR7 expression has a high CCR7 expression level, and a wide range of CD45RO expression which covers both high and low expression. The minimum level of expression within this cluster is similar to the minimum level seen in the Naïve subpopulations of the CD4/CD8 isolated cultures. Meanwhile, the maximum level of expression is similar to the minimum level of the Effector Memory subpopulation. This cluster appears to contain Naïve cells that are beginning to transition to Central Memory cells, however more dynamic data would be required to validate this observation. The overlapping cluster with the lower CCR7 expression level showed a similar shape of expression to the first, shifted with different levels of expression. The minimum and maximum levels of CD45RO expression align relatively well with the middle level expression of the CD4/CD8 isolated Naïve and Effector Memory subpopulations respectively. Analysis of CCR7 expression in this cluster showed that it is relatively close to the threshold expression level for definition of homing or non-homing. Where the previous cluster appeared as if it could be a Naïve subpopulation extruded by transition into Central Memory subpopulation, this cluster is less tangibly linked to any one phenotypic profile, given that it contains events from all four maturity phenotype subpopulations.

The similar shape of the lower CCR7 expressing cluster and the Naïve-like subpopulation cluster could suggest that they are linked. It is suggested that the lower CCR7 expressing subpopulation expresses lower CCR7 and slightly higher CD45RO in response to a stimulus which has no, less or delayed effect on the higher CCR7 expressing population. It is not clear what this stimulus would be, or why the stimulus would have a different effect. If this were the case, these cells would be

considered a transitional cluster, however further investigation would be required to confirm these observations.

Comparison of the CD4+ and CD8+ subpopulations once again shows that there are differences between the two. The CD4+ subpopulation's densest cluster is the Effector Memory phenotype cells (Fig. 3.3Ci). The presentation of this cluster in the CD4+ subpopulation matches the presentation in the total cell plot extremely well, indicating little influence from the CD8+ subpopulation. The other cluster present in the CD4+ subpopulation resembles that of the Naïve subpopulation in the total cell plot, however the density of the cluster in this plot appears far lower than in that of the total event plot. In addition, the transitional cluster does not appear to be present in CD4+ subpopulation; events are present in the phenotypic space occupied by the transitional subpopulation, however these events do not appear to be sufficiently dense, nor congruent, that it would constitute a specific cluster. In comparison, the CD8+ subpopulation does not contain an Effector Memory cluster, correlating with the comments on the similarity in presentation of the cluster in the CD4+ subpopulation and the total cell plot previously. Present in the CD8+ subpopulation is the overlapping Naïve and transitional clusters, which represent nearly all events within the subpopulation, and correlate with the presentation of these clusters in the total event plot (Fig 3.3Di).

Considering the data presented, it is clear to see that there are substantial variations in the cultures isolated by CD3 and by CD4/CD8, both in population growth and phenotypic response. The viable cell counts of the CD3 targeted cultures show a substantial increase over time, while the CD4/CD8 targeted cultures did not show any substantial period of growth, but a gradual decline in viable cells over time. In addition, phenotypic analysis at 216 hours showed variation in maturity subpopulation presentation between isolation targets. The CD3 isolated T-cell data show a substantially higher level of growth, as well as subpopulations that show evidence of maturation. Comparatively, the CD4/CD8 isolated populations showed little evidence of subpopulation maturation and no overall growth during the culture period. These observations appear to correlate with the CD3+ targeted isolation inducing activation in the T-cell populations, counter to the advice provided.

In addition to differences between isolation targets, it is possible to see that the CD4+ and CD8+ subpopulations contain different maturity subpopulation distributions. There is some commonality in presentation between the isolation targets, with the CD4+ subpopulations both representing the Naïve and the Effector Memory subpopulations of their cultures. The CD8+ subpopulations also presented the Naïve populations, and comparison of the plots show some similarity between the presentation of the transitional population in the CD3 isolated population and the Effector/Effector Memory cluster in the CD4/CD8 isolated population. The different maturity profiles of the CD4+ and

CD8+ subpopulations highlight the differences between the Cytotoxic and Helper/Regulatory populations in the cultures. However, the variation in CD4/CD8 proportions are relatively conserved between the isolation targets, implying that the isolation methods affect the CD4+ and CD8+ populations similarly. It is further possible to see this when analysing the CD4+ and CD8+ subpopulation proportions in culture. The CD4/CD8 concurrently targeted cultures show a very similar level of variation from 72 hours to 216 hours to the CD3 isolated cultures. In this scenario it is the two-step isolation process which confers the negative effect, with the CD4+ cells, isolated second, appearing to have suffered with the extra isolation steps.

3.3.2 Comparison of different seeding populations and their effects on proliferation

CD4+ and CD8+ subpopulations were each isolated from separate PBMC aliquots. The resultant CD4+ and CD8+ populations were compared against the CD4+ depleted and CD8+ depleted populations as well as PBMCs which had not undergone T-cell isolation. Comparison of the viable cell counts over time shows separation at many points between the targeted isolation cultures and the depleted and PBMC cultures.

Analysis of the viable cell counts between 0 and 144 hours overall shows a separation between the depleted and PBMC cultures, and the directly targeted cultures early on. However, this variation is negated by the final timepoint. The CD4+ and CD8+ isolated cultures show a similar trend in performance over time. Initially, it is possible to see a slight drop in viable cell count from 0-24 hours, followed by a temporary increase at 48 hours and a subsequent drop to 72 hours, with these final values being lower than those observed at 24 hours (Fig 3.4A). From 72 hours to 144 the viable cell count remains comparable for the CD4 target isolated cells, whilst the CD8 target isolated cells showed a slight decrease over the time period.

The CD4 depleted and PBMC cultures also perform similarly to each other. Analysis from 24 hours shows that despite having a viable cell count $7x10^4$ cells/mL higher, the CD4 depleted and PBMC cultures both reduce in viable cell count to $2.7x10^5$ cells/mL at 48 hours. There is subsequently a substantial rise in the viable cell count of both cultures to 72 hours, however this is also matched by a substantial increase in the level of variability in the repeats, indicated by error bars. At 144 hours it is possible to see a substantial drop in viable cell count in these cultures, to a point below that of the CD4 targeted culture. The CD8 depleted culture shows a similar pattern of behaviour to the PBMC culture, however it is not to the same magnitude (Fig. 3.4). From 24 hours there is a slight decrease in viable cell count to 48 hours. This is followed by an increase to above the initial viable cell count at 72 hours, and a substantial drop in viable cell count to 144 hours as seen in the previously mentioned cultures.

A. B.

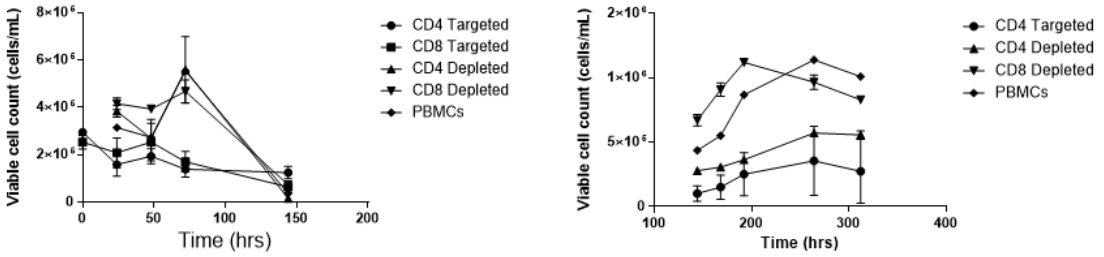


Figure 3.4: Analysis of viable cell counts from CD4 and CD8 targeted and depleted cultures show initial drop, but indicate growth in cultures after reseeding. (A) shows the viable cell count profile of CD4 target isolated, CD8 target isolated, CD4 depleted, CD8 depleted and PBMC cultures. CD4 and CD8 targeted cultures show a gradual trend in decline of viable cell count from 0 to 144 hours. CD4 depleted and PBMC cultures show an initial decrease, followed by sharp increase in viable cell count at 72 hours and a decline to 144 hours. The CD8 depleted culture performed similarly to the CD4 depleted culture, however the initial dip and subsequent rise were not as great. At 144 hours cultures were exchanged into fresh medium and reseeded. (B) The cultures that were reseeded showed an increase in viable cell count over time. CD4 targeted and CD4 depleted cultures showed similar growth from 144 hours to 264 hours before declining to 316 hours. The CD8 depleted culture showed substantial growth over 48 hours from seeding, followed by a gradual decline to 316 hours, whilst the PBMC culture showed more gradual increase in viable cell count to 264 hours before inflecting.

These data show that by 72 hours depleted and PBMC cultures showed evidence of strong growth. However, by 144 hours the viable cell counts are substantially reduced and comparable with the viable cell count of the CD8 targeted isolation. It is not entirely clear what induced the crash in viable cell count of the depleted and PBMC cultures, however it could plausibly be linked to a lack of medium exchange or supplementation over that time, especially with the elevated viable cell count relative to the targeted isolation cultures.

At 144 hours the cultures were reseeded due to low cell density. The target seeding density was $5x10^5$ cells/mL, however due to the insufficient concentration of CD8 target isolation the condition was discontinued. It is possible to see from viable cell counts at this timepoint that the target seeding density was not achieved in any cultures. Furthermore, it is possible to see a wide range of seeding densities at this timepoint (Fig. 3.4B). The only culture that was seeded above the target density was the CD8 depleted culture, while the other cultures were seeded below $5x10^5$ cells/mL. Analysis of the viable cell counts over time shows similar trends in the CD4 targeted and CD4 depleted cultures, contrary to what had been seen previously. Both of these cultures show a consistent increase in the viable cell count from 144 hours to 264 hours, followed by a reduction in viable cell count at 312 hours. The CD4 depleted culture shows a very slight decline in viable cell count from 264 to 312 hours, while the CD4 targeted culture shows a greater decrease. The higher loss of viable cells, as well as the lower cell count at 264 hours, indicates that the CD4 Targeted culture is responding more negatively at 312 hours than the CD4 depleted culture.

The PBMC and CD8 depleted cultures performed similarly for the first 48 hours after reseeding. They showed a consistent increase over this time period, both increasing by ~$4x10^5$ cell/mL. At 192

hours the CD8 depleted culture peaked at $1.1x10^6$ cells/mL and showed a consistent decrease in viable cell count to 312 hours where analysis showed it was $8.3x10^5$ cells/mL. Meanwhile, the PBMC culture showed continued increase in the viable cell count to a similar maximum level as the CD8 depleted culture at 264 hours. This was then followed by a reduction in viable cell count consistent with the rate seen in the CD8 depleted culture at 312 hours (Fig 3.4B). The similarity between the viable cell counts of the CD4 targeted and CD4 depleted cultures was unexpected considering the earlier growth of the cultures and the comparative performance of the CD8 Targeted and CD8 Depleted cultures.

The display of growth in the CD4 targeted culture after reseeding could indicate that the culture was suffering from some form of medium exhaustion, somewhat validated by the indication of decline in the culture at 264 hours (Fig. 3.4B). However, the viable cell count data from 0-144 hours does not appear to show a response to medium exhaustion. The lack of growth in the initial culture period could be linked to the starting cell concentrations being too low and having a negative effect on the cultures. However, the CD4 Depleted and CD4 Targeted cultures were reseeded lower or similar to the initial stage of the experiment and were able to demonstrate growth. Therefore, if the seeding density is an influential factor it appears that its influence is short lived. The combination of medium refreshment inducing growth and a lack of growth for the initial time period would suggest either that the populations need some time to recover from the extensive processing to get them into culture, and then needed replenishing of nutrients, or that there was some part of the isolation process that managed to negatively condition the medium to restrict growth initially, and replacing the medium removed this. In either case, or if both apply, it is possible to see that the reseeding of the cultures was of benefit and increased the growth to a level in line with a condition that had mostly substantially outgrown it. Comparison between the CD8 depleted and PBMC cultures appears to show that the two cultures achieved a similar rate of growth and decline, peaking at just over $1x10^6$ cells/mL. The main element that appears to stop these conditions overlapping each other almost exactly is the lower starting density of the PBMC culture. In addition, the performance of these two cultures in comparison to the CD4 depleted culture appears to imply that it is the CD4+ cells that are exhibiting the greater rate of growth in culture. This is also indicated in the CD4 targeted cultures, which were able to sustain a sufficiently high enough viable cell count to be able to reseed them compared to the CD8 targeted condition.

The primary observable difference between the culture period between 0 and 144 hours and between 144 and 312 hours is consistent level of growth seen in the latter that is not in the former. It is observable that over the first 144 hours all conditions tested produced a lower viable cell count than that which they had at 0 hours. However, on reseeding to a target concentration these

cultures, with the exception of the CD8 targeted population, which was below requirements, all showed some sustained level of growth. The CD4+ and CD8+ isolated cultures showed a similar pattern of growth in culture up until 144 hours, where the CD8+ subpopulation lost a sufficiently high concentration of viable cells that it was not possible to reseed them at an appropriate level. These data suggest that in culture independently, the CD4+ and CD8+ subpopulations will culture similarly.

3.3.2.1 Different subpopulations ratio Results

Culture samples were taken at semiregular intervals and analysed for viable cell counts. Analysis of viable cell counts over time shows a lack of growth across all cultures.

Initially, pattern of response could be combined into two groups. The CD4 Targeted, CD8 Targeted and CD4/CD8 cultures showed a consistent decline in the viable cell count from 0 to 89 hours of ~$2x10^4$ cells/mL. Meanwhile, the CD4/DD and CD8/DD cultures showed an initial drop of ~$1.5x10^4$ cells/mL to 16 hours, followed by relative stability in the CD8/DD culture and a slight increase in the CD4/DD culture (Fig. 3.5).

From this point all cultures performed relatively similarly, with a substantial drop of around $2.5x10^4$ cells/mL between 89 and 110 hours followed by a gradual decline in viable cell count in all cultures until 158 hours, sustained until 177 hours in the CD4/CD8, CD4/DD and CD8/DD cultures. There is a slight inflection in the CD4 and CD8 targeted cultures to 177 hours, however given the previous decline in cell count this is not necessarily indicative of a culture that would grow substantially.

The substantial decline in viable cell count at 89 hours coincided with a full medium exchange (FME), indicated by the two counts in quick succession. It is plausible that the FME is what caused the loss of viable cells, however it is not obvious by which mechanism this would have happened, especially as previous work has seen an increase in viable cell count after FME. The relative similarity of the counts before and after the exchange show that the cell loss did not occur within the process itself, however it is possible that these cultures were affected by the centrifugation step. FME was carried out previously on various T-cell populations which did not exhibit the same response, suggesting that if this was the cause, these cultures were particularly sensitive to this. Alternatively, this could be a seeding density issue. Due to restrictions with the donor material the cultures were seeded lower than was desired and then showed decline. At the point of FME, these cultures were potentially too sparse to sustain proliferation and subsequently underwent apoptosis.

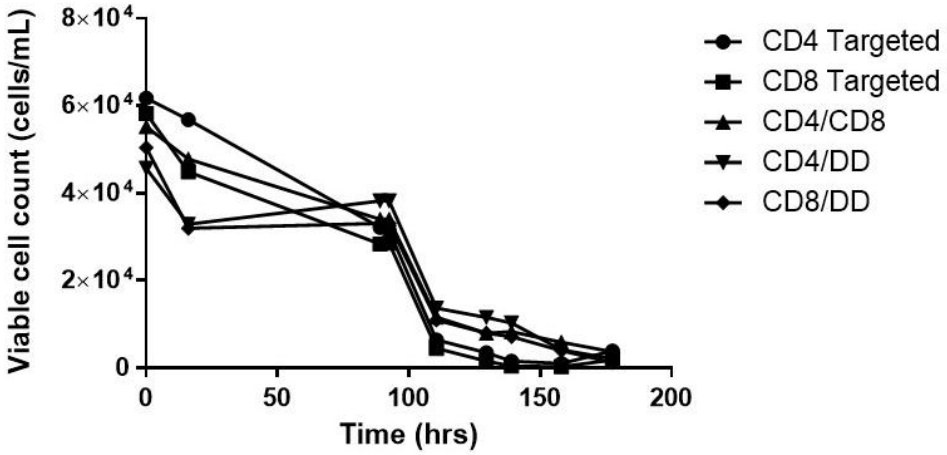


Figure 3.5. Analysis of viable cell counts in conditions with different subpopulation proportions over time showed little variation, with all showing overall loss of viable cell count through the experiment. The viable cell counts of the CD4 targeted, CD8 targeted and CD4/CD8 cultures dropped slowly until 72 hours and sharply afterwards, before remaining consistently low. Meanwhile, cocultures with double depleted (DD) cells supplemented in with CD4+ or CD8+ subpopulations show a substantial drop in viable cell count initially from 0 to 24 hours. This was followed by a slight increase in the CD4/DD coculture and relative stability in the CD8/DD coculture, before resuming decline in viable cell count in the same manner as was seen in the other cultures.

Overall, the range of subpopulation combinations tested does not show that there is any substantial difference caused by which populations are cultured together. This is in contrast to what was seen previously, where the depleted samples were shown to grow with more success than the isolation targeted cultures. This could potentially be explained by the relative differences in the seeding densities seen in the previous data, with the depleted cultures being seeded at a higher density than the targeted isolations, however it could be an example of variability in performance in culture depending on the donor.

3.3.2.2 Varied subpopulation ratio comparison

The previous data appeared to show the impact of a limiting seeding density, and the experiment prior to that appeared to show that at a higher seeding density CD4+ subpopulation grew better than CD8+ subpopulations. As such, an experiment was designed to analyse these elements in combination

3.3.2.2.1 Varied CD4/CD8 ratio Results

Cultures were sampled at semiregular intervals and assessed for viable cell counts. Analysis of the viable cell counts showed that the 1:3 ratio cultures had the highest viable cell counts over time and seeding at $5x10^4$ cells/mL produced substantially less growth in culture.

3.3.2.2.1.1 $5x10^5$ cells/mL seeded cultures

Viable cell counts at 0 hours show that although seeding with a target cell density of $5x10^5$ cells/mL, the cultures were measured to have been seeded with a viable cell count of ~$2x10^5$ cells/mL.

All cultures showed an increase in viable cell count from 0 to 89 hours, increasing by ~$5x10^5$ cells/mL on average, followed by a sharp decline after FME (Fig. 3.6A). The 3:1 and 1:1 cultures then show a very slight increase in viable cell count until 145 hours, before showing a consistent decline to 238 hours where they reach a negligible cell count which remains until the final count at 293 hours.

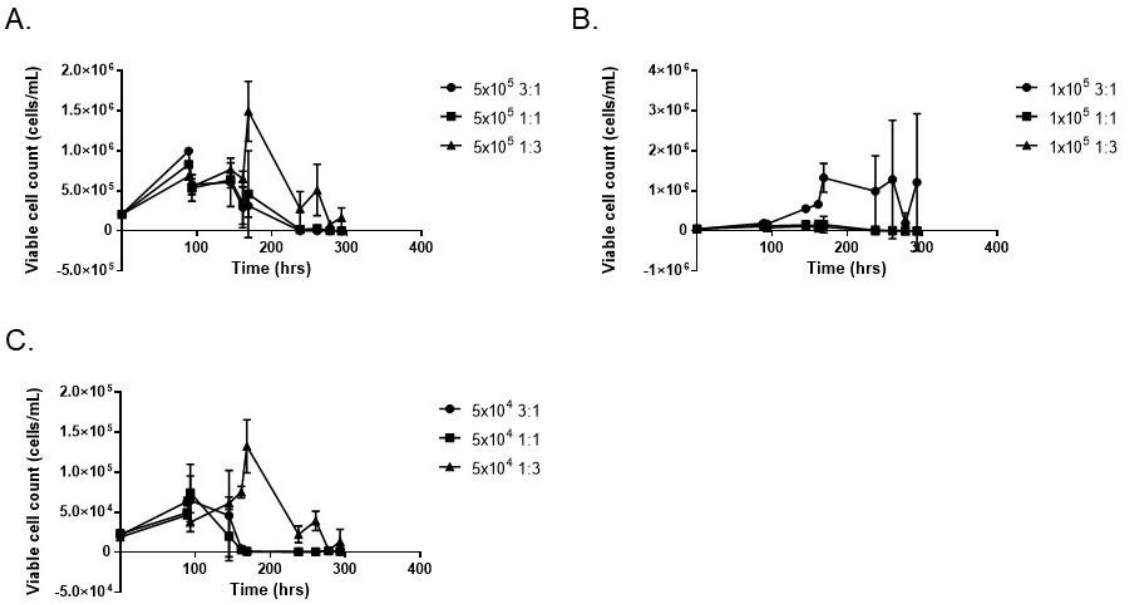


Figure 3.6. Viable cell counts over time of cultures with specific target seeing density and CD4:CD8 ratios. (A) Cultures with a target seeding density of $5x10^5$ cells/mL showed 3:1 ratio cultures grew best initially, however 1:3 cultures had the highest cell counts from 72 hours onwards. 1:1 ratio always performed between the two. (B) Cultures with a target seeding density of $1x10^5$ cells/mL show limited growth in the 1:1 and 1:3 ratio cells. There is high growth within the 3:1 cultures, however there is a high degree of variability. (C) Cultures with a target seeding density of $5x10^4$ cells/mL show 1:1 cultures with the greatest cell count at 72 hours, followed by 3:1 and 1:3. By 168 hours the 1:3 cultures have the highest cell count with the 3:1 and 1:1 cultures having reduced to limited viable cell count.

The 1:3 culture meanwhile shows an increase in viable cell count between 89 and 145 hours. There is a slight drop in viable cell count at 161 hours before a substantial increase at 168 hours. This increase is not sustained however, and there is a greater loss of viable cell count by 237 hours. This oscillating pattern somewhat repeats itself, with increases in the viable cell count observed, with decreasing magnitude, at 261 hours and 293 hours, and a decline observed at 278 hours.

The erratic behaviour of the 1:3 sample is not easily explained by behaviour in culture, as this level of expansion and crash has not previously been seen, nor does it appear biologically sustainable. Analysing the variation at these peaks suggests that repeats are consistently around this level, rather than spikes being caused by an individual repeat moving the average. This therefore suggests that there was underlying issue with the cell counting system, suggesting that cultures had higher viable cell counts than were present in reality.

3.3.2.2.1.2 $1x10^5$ cells/mL seeded cultures

The $1x10^5$ cells/mL seeded cultures also show a separation of conditions based on the subpopulation ratio.

Comparison of these cultures shows that, as seen in the $5x10^5$ cells/mL cultures, the 1:1 and 3:1 cultures perform similarly, whilst the 1:3 cultures show a different trend in viable cell counts.

The 1:1 and 3:1 cultures show a consistent level of growth from 0 to 145 hours, with a slight drop in viable cell count during FME at 89 hours. Subsequent to this there was a large decline in the viable cell count, reducing to the negligible levels seen previously by 238 hours and remaining there.

Meanwhile, the 1:3 culture showed a similar slight increase in viable cell concentration from 0 to 89 hours (Fig. 3.6B). From 89 to 161 hours the cultures continue to grow at an increased level, before sharply rising between 161 and 168 hours. At this point there is a much higher level of noise in the data, which increases as time goes on.

From 168 hours the viable cell count oscillates as seen in the cultures seeded at $5x10^5$ cells/mL. Peaks are observed at 260 and 293 hours, with troughs observed at 238 and 278 hours. Noticeably, the peaks correlated with a higher level of variation in the data, in contrast with the previous observations. This could indicate that one culture has a high degree of variability and is causing fluctuation in the average cell count, or that the method by which the viable cell count is being taken from one of the cultures is unreliable and is giving erratic results. As was mentioned before, this type of viable cell count is biologically illogical and unlikely, which leads to the conclusion that these samples, for reasons unknown, are not being accurately measured for viable cell count.

3.3.2.2.1.3 $5x10^4$ cells/mL seeded cultures

Cultures seeded at $5x10^4$ cells/mL showed a similar level of variability as the other conditions tested but given the lower seeding density they played out on a much smaller scale.

All cultures showed a similar increase in viable cell count over the first 89 hours. Subsequent to that the 1:1 and 3:1 cultures showed a substantial decline in viable cell count until 162 hours where the viable cell count remained consistently negligible (Fig. 3.6C).

The 1:3 culture shows an increase in viable cell count beyond 89 hours, continuing to grow until a peak at 168 hours. Subsequently the same oscillating pattern of increase and decrease is seen as was mentioned in analysis of the $1x10^5$ cells/mL and $5x10^5$ cells/mL cultures.

3.3.2.2.1.4 Comparison

The data analysed across all cultures appear to show that the 1:3 seeded cultures produced the highest cell counts, regardless of the seeding density.

Although the magnitude and executions vary, there is a relatively consistent pattern of behaviour across the conditions tested in this experiment. All cultures show an increase in the viable cell count from 0 to 89 hours, indicating that all seeding conditions are viable as they have demonstrated growth. After this point the 1:1 and 3:1 cultures ultimately show decline in viable cell count a minimal value, regardless of seeding density. In the cultures seeded at $5x10^5$ cells/mL and $1x10^5$ cells/mL this descent occurs after 145 hours, whilst in the $5x10^6$ cells/mL cultures this occurs from 89 hours onwards. Meanwhile, the 1:3 cultures showed an increase in viable cell count to 169 hours before showing an oscillating pattern of viable cell count increase and decrease of levels such that the viable cell count was overall decreasing until the final sampling point at 293 hours.

The conservation of the pattern across cultures of all seeding densities appears to indicate that the observations of the 1:3 cultures are not courtesy of anomalous T-cell cultures, nor of an individual anomalous analysis of viable cell count. Whether the viable cell counts obtained are truly representative of what is actually present in culture or not, some element of the 1:3 cultures has induced the observed response repeatedly.

This response observed in the 1:3 cultures implies that the more positive response has been induced either by the higher initial proportion of CD8+ cells, or the lower relative proportion of CD4+ cells. However, the substantially reduced responses observed in the 1:1 and 3:1 suggest that there is a threshold level that this subpopulation ratio must reach before this response could be observed. The increase in viable cell counts could have been induced by an increase in CD8+ T-cell subpopulation released cytokines, however the limited cytokine profile of these cells suggests that this is not the source. Alternatively, the cause could lie in the reduction in CD4+ subpopulation T-cells, this is particularly viable if these cultures had a particularly high level of Regulatory T-cells, which could suppress proliferation in the T-cell cultures and even induce apoptosis. Unfortunately, these cultures were not sampled for phenotypic data, and further work which monitored this facet of the T-cell cultures would be advised to further analyse the cause of the responses observed.

One of the common elements of the cultures was the decline in viable cell count over time, which would be considered a failing of the culture in all conditions except where cells were seeded at $1x10^5$ cells/mL in a 1:3 ratio. As sustained growth has been seen over time in previous cultures, it is surprising to see that nearly all conditions failed and brings into question as to why this was the case. Analysis of the culture conditions compared to other experiments that have shown growth does not appear to highlight any differentiating factor that may have induced the viable cell loss.

However, one element of variation that is present is the donor material. The variation of donor material and its influence on T-cell populations in culture is yet to be addressed substantially,

however there are obviously many factors such as the health state of the donor and their natural balance of T-cell subpopulations that could affect the trajectory of T-cells in culture. That is not to say that these data present in the way they do entirely because of the donor material variability, but that the performance of these cultures could plausibly be linked to it.

3.3.2.2.1.5 Conclusion

Analysis on the culture of CD4+ and CD8+ T-cell subpopulations appears to show that there are high levels of comparability between cultures, regardless of the combination of subpopulations which have been seeded.

Initial investigation into the performance of CD4 and CD8 targeted and depleted populations, as well as non-isolated PBMCs, showed lower viable cell counts in those conditions which were targeted for isolation compared to the PBMC cultures and those which were merely depleted. However, the continuation of these cultures appeared to show that the differences in the relative performances may well have been linked to the seeding density.

The effects of seeding density on T-cell growth were then further observed in investigation comparing the growth of CD4 and CD8 targeted subpopulations with 1:1 cultures of each combination of CD4, CD8 and DD subpopulations. The lack of growth in these conditions was linked to the density at which they were seeded, reinforcing the suggestion that the previous investigation showed reduced growth in targeted cultures due to lower seeding densities.

The relatively comparable performance of all conditions was contrary to what had been seen previously, with different subpopulations present inducing variable viable cell counts. However, this supplements the suggestion that seeding density is a cause for the variation in growth, as when all were seeded at the same density there was little variation.

The relative performance of the cultures seeded at $5x10^4$ cells/mL compared to those seeded at $5x10^5$ cells/mL appeared to identify that lowering the seeding density had a substantially negative impact on the culture of these T-cell subpopulations. Furthermore, as the relative ratios of CD4+ and CD8+ subpopulations had only been compared at a 1:1 ratio, there was a desire to manipulate this and identify any impacts that may result through an imbalance of CD4+ and CD8+ subpopulations.

In comparing the viable cell counts of cultures of varying density and CD4/C8 subpopulation balance it was possible to see that the 1:3 CD4:CD8 subpopulations grew in each of the seeding densities tested. The 3:1 and 1:1 ratio cultures showed a similar pattern of growth over time, regardless of seeding density, with a period of growth over the first 89 hours, before showing decline in viable cell counts without recovery.

The presence of growth in all the cultures was in contrast to what was seen previously, where the higher seeded cultures showed better growth, and cultures seeded at 5x104 cells/mL did not show growth. Considering the points differentiating factors between the cultures, these data appear to reinforce the idea that there is a large amount of variability in culture dependent on the donor material seeded.

The relatively high performance of the 1:3 cultures appears to show that the CD8+ subpopulations are having a preferentially positive effect on the cultures. The mechanism by which this effect occurs in culture is not fully understood. This is due in part to the lack of phenotypic data for these cultures, which would have helped to offer insight on the mechanisms and responses at play. It is not clear how the rise in viable cell count is reflected in the CD4+ and CD8+ subpopulations, nor in the maturity phenotype subpopulations. Information on these subgroups could highlight particularly proliferative behaviour, which would help to define the process by which these higher cell counts were achieved.

3.3.3 Assessing impact of supplementing CD3/CD28 Dynabeads into T-cell cultures on proliferation and phenotypic response

3.3.3.1 Delayed supplementation Results

Analysis of the viable cell counts shows culture performance pre-bead that is comparable to the behaviour seen previously. Meanwhile post-bead cultures appear to deviate depending on CD3/CD28 Dynabead supplementation, with their presence reducing the viable cell count.

3.3.3.1.1 Culture prior to CD3/CD28 Dynabead supplementation

The performance of both PBMC cultures show remarkably similar trajectories over time. They initially show a sharp decline in viable count, likely due to the unsustainable concentration of PBMCs. At 72 hours the cultures were diluted to ~$3x10^6$ cells/mL to bring the concentrations down and reduce the stress therefore placed on the cells. These cultures then saw a decline in viable cell count at 144 hours, potentially implying that the concentration was too high to sustain growth, before an FME was used to reduce the concentration of cells again to ~$1x10^6$ cells/mL (Fig 3.7 A).

After this point the cultures remained at a stable viable cell count, other than an attempt to increase the concentration with an FME at 264 hours. The concentration of viable cells dropped at 312 hours to the same level which had been seen previously, and was sustained to 336 hours, at which point the cells were harvested for the next stage of the experiment.

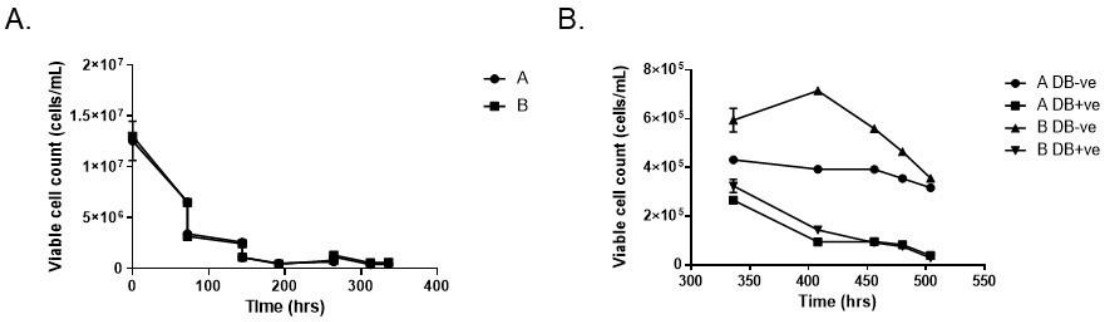


Figure 3.7. Cell counts over time of PBMCs cultured with and without Dynabeads. (A) shows the growth profile of PBMCs seeded at too high a density over an extended period. Cells appear to recover from overseeding and are able to show some growth when seeded at $3.5x10^5$ cells/mL, however they do not show growth when seeded at $1x10^7$ cells/mL. (B) Samples were taken at 336 hours of A and B and were reseeded both with and without CD3/CD28 Dynabeads. All cultures showed an ultimate decline, however those supplemented with the CD3/CD28 Dynabeads showed a swifter decline in the viable cell count. In addition, the sample from culture B not supplemented with CD3/CD28 Dynabeads showed a period of growth before losing viable cell count.

The viable cell counts show a substantial drop initially due to overseeding of the cultures. However, when the cultures reach a concentration that did not affect the viable cell count, the cultures remained relatively stable.

3.3.3.1.2 Culture post CD3/CD28 Dynabead supplementation

After 336 hours, the T-cell populations were reseeded to a target density of $5x10^5$ cells/mL and divide into cultures that would not be supplemented with CD3/CD28 Dynabeads.

The viable cell counts initially show that those cultures that were not supplemented with CD3/CD28 Dynabeads had a higher viable cell count than those which were. This trend continues as the viable cell counts are monitored over time (Fig. 3.7B). Those cultures that were not supplemented with CD3/CD28 Dynabeads show a comparable viable cell count at 456 hours before showing a declining rate of viable cell count. Meanwhile the cultures that were supplemented immediately show a ~$5x10^5$ cells/mL reduction in viable cell counts after 408 hours, which then continues to reduce but at a reduced rate until 504 hours.

These data clearly show that these cultures had a considerably negative response to being supplemented with CD3/CD28 Dynabeads. It is not clear why this would be the case when these beads are supposed to induce activation and promote proliferation.

3.3.3.2 DB supplementation at seeding Results

The PBMC cultures were semiregularly sampled to be tested for viable cell to monitor the progress of conditions. Phenotypic analysis was also performed at 192 hours.

3.3.3.2.1 Cell yield

Analysis of the viable cell counts shows that while the CD3/CD28 Dynabead supplemented cultures performed well, the cultures that were not supplemented at all did not grow and had a negligible viable cell count by 120 hours.

It is possible to see that both CD3/CD28 Dynabead supplemented cultures started at a similar cell density, while the unsupplemented culture started at just over half the starting density of the two (Fig. 3.8A). The CD3/CD28 Dynabead only and unsupplemented cultures decreased slightly over 48 hours, while the CD3/CD28 Dynabead and IL-2 supplemented culture showed a slightly higher decrease, being comparable to the level of viable cells in the unsupplemented culture.

From 48 to 120 hours the unsupplemented culture showed a decline to negligible viable cell count, and from which point the culture was no longer continued. Meanwhile, 48 hours marked the inflection point for the CD3/CD28 Dynabead supplemented cultures, with both showing a substantial increase in viable cell count at 120 hours, and then further still on to 192 hours. At 48 and 120 hours the culture supplemented with CD3/CD28 Dynabeads and IL-2 showed a slightly lower viable cell count, however the counts were not substantially different and by 192 hours the viable counts were once more comparable.

Overall, it is clear to see that the CD3/CD28 supplemented cultures perform better than the unsupplemented culture, as expected. It is also possible to see that the CD3/CD28 supplemented cultures perform similarly to each other, appearing to indicate that supplementation of IL-2 has a minimal effect on their culture. This is not only contrary to the anticipated result of IL-2 increasing the viable cell count substantially compared to its absence, but the IL-2 supplemented culture was also for most of the experiment marginally lower in viable cell count.

3.3.3.2.2 Phenotypic analysis

At 120 hours samples were taken from the CD3/CD28 Dynabead supplemented cultures and analysed for phenotypic profile. The phenotypic data was then analysed to identify the maturity subpopulation composition of the CD4+ and CD8+ subpopulations of each culture.

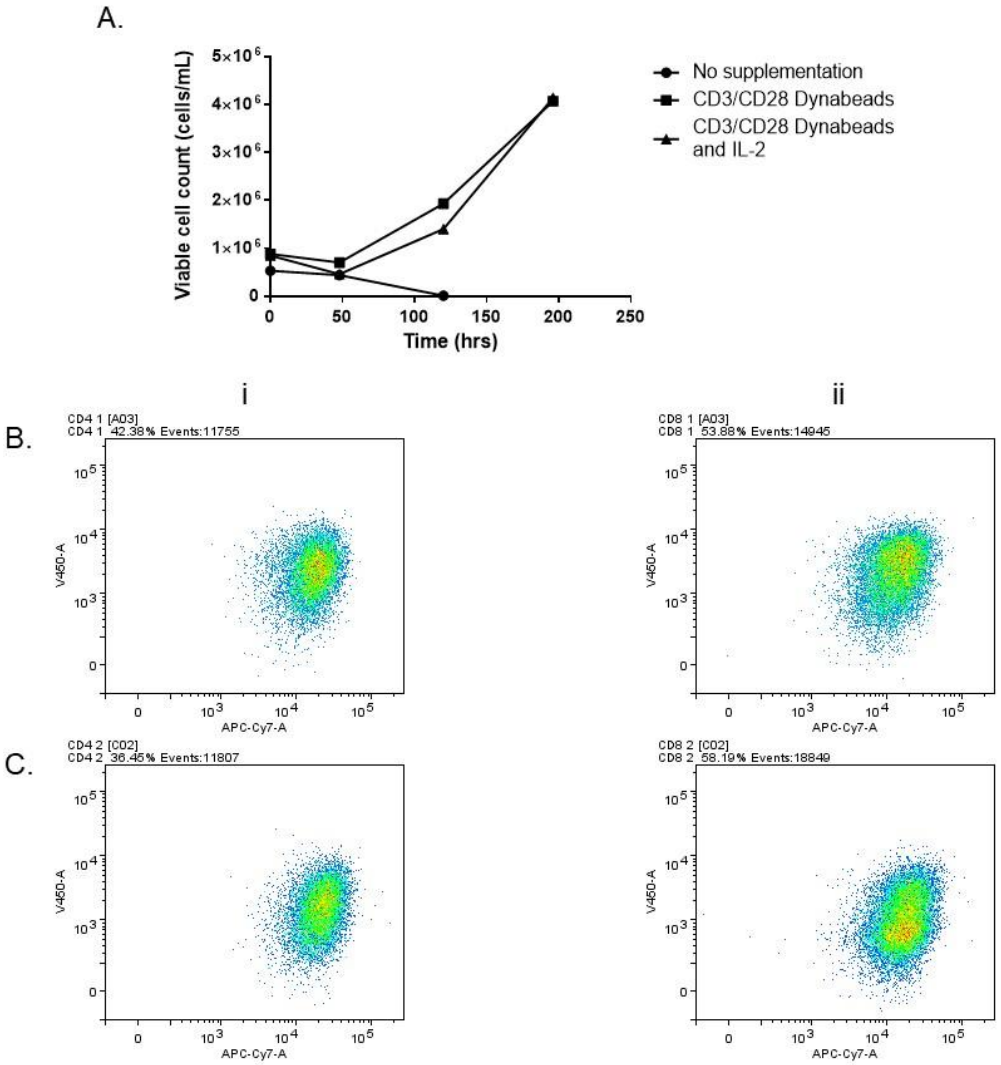


Figure 3.8. PBMC culture with CD3/CD28 Dynabeads shows the stimulating effect they have on cultures. (A) Graph shows that in the presence of CD3/CD28 Dynabeads cells will grow, whilst in the absence of any supplementation the viable cell count will decrease. The PBMC culture supplemented with CD3/CD28 Dynabeads and IL-2 has a comparable rate of growth to that supplemented with just CD3/CD28 Dynabeads. (B-C) Flow cytometry plots showing the phenotypic profile of the CD4+ (i) and CD8+ (ii) populations in the CD3/CD28 Dynabead (B) and the CD3/CD28 Dynabeads and IL-2 (C) supplemented cultures. All cells are expressing high levels of CD45RO (APC-CY7), indicating mature cell populations, and have mid to low expression of CCR7 (V450), indicating a predominantly, if not entirely, effector memory phenotype of the populations.

The data show that all the populations observed show a high degree of similarity to each other (Fig. 3.8B-C). All populations are high CD45RO expressing and show a range of CCR7 expression that indicates the presence of both CCR7+ and CCR7- events. The data appear to show that the presence of CD3/CD28 Dynabeads has induced maturation in the T-cell populations, nearly to a point of Effector Memory homogeneity. However, it is not possible to say without additional phenotypic data how the populations have changed over time.

There were no expectations with regards to the outputs of the phenotypic data from this investigation, however the lack of variation in the cultures between those supplemented with and without IL-2 appears to indicate that the addition of the growth factor has little effect on the phenotypic response, if any.

3.3.3.3 Comparison of DB, DB &2Iand IL2 Results

The data show that there are clear differences in the performance of PBMC populations in each different condition across both cell yield and phenotype.

3.3.3.3.1 Cell yield

Analysis of viable cell counts over time shows that all CD3/CD28 supplemented cultures underwent periods of growth, however of the non CD3/CD28 Dynabead supplemented cultures only the lower density seeded culture showed any signs of growth.

Comparing viable cell counts of cultures seeded at the lower density shows that the IL-2 supplemented culture spends a long time in lag phase before showing growth (Fig. 3.9A). The viable cell count initially drops between 0 and 24 hours, followed by a very gradual decline to 120 hours. However, after this point it is possible to see an incline in the viable cell count where the culture appears to show a substantial increase.

The culture supplemented with CD3/CD28 Dynabeads meanwhile shows increasing viable cell count for nearly the entire duration of the experiment. The viable cell count increases gradually for the first 72 hours, appearing to reach plateau at 96, however after this there is a period of substantial growth to 144 hours before a drop of roughly a third of the viable cell count by 168 hours. This behaviour of sudden drop off appears to be indicative of medium exhaustion, whereby the cells in culture has expended all resources they require for proliferation and sustenance, however further investigation would be necessary to confirm this.

Analysis of the culture seeded at the lower density rendered an unusual pattern of behaviour. The viable cell count initially drops, as was anticipated in these cultures with the loss of PBMCs not sustained by the culture conditions and was proceeded by growth. However, after showing substantial growth between 72 and 96 hours the trend reversed course and was followed by substantial loss of cells until the end of the experiment. Considering the performance of the CD3/CD28 Dynabead supplemented culture and both of the higher seeded density Dynabead supplemented cultures this appears to be abnormal behaviour for these cultures. The cause of this drop is not immediately obvious. The point at which the inflection is seen does not correlate with a medium exchange, likely eliminating that as a possible cause for the observation. As this response is

not replicated in any other Dynabead supplemented cultures in this experiment, nor in data observed in future experiments, this has been put down to being anomalous and of unknown cause.

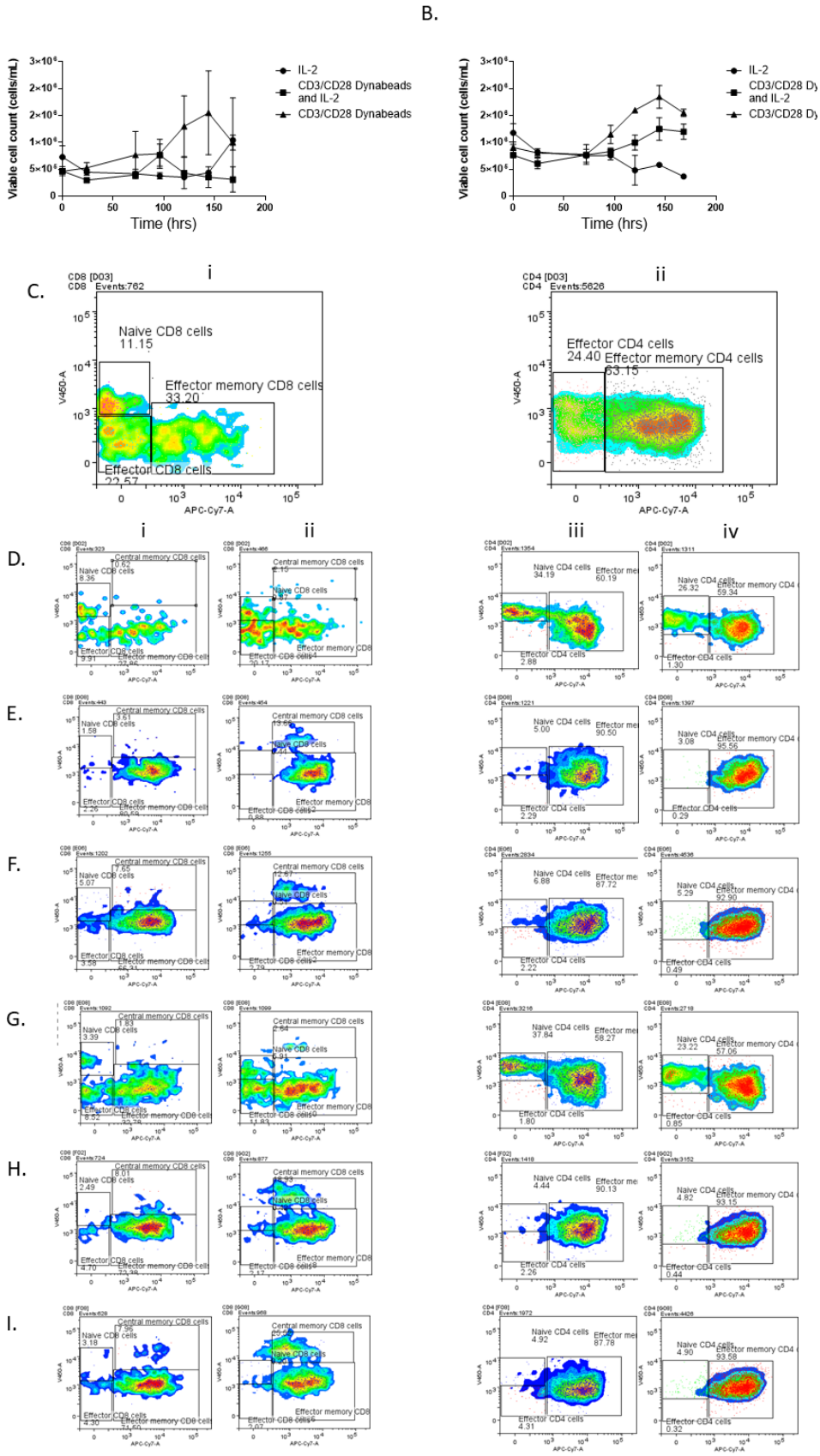


Figure 3.9. Viable cell count and phenotypic data of PBMCs with different stimulants supplemented into culture highlight the effects of CD3/CD28 Dynabeads. (A,B) Viable cell counts of IL-2, CD3/CD28 Dynabead, and IL-2 and CD3/CD28 Dynabead supplemented cultures seeded with a target density of (A) $1x10^6$ cells/mL and (B) $2x10^6$cells/mL. (C) phenotypic profile of CD8+ (i) and CD4+ (ii) cells after thawing from cryopreservation. Density plots (D-I) show the expression of CD45RO (APC-Cy7) and CCR7 (V450) in CD8+ populations (i,ii) and CD4+ populations (iii,iv) on day 3 (i,iii) and day 5 (ii,iv) of culture of cells cultured with IL-2 (D), IL-2 with CD3/CD28 Dynabeads(E), and CD3/CD28 Dynabeads (F) seeded at the lower density ($1x10^6$ cells/mL). (G-I) shows the same data for the same culture supplements and timepoint of cells seeded at the higher density ($2x10^6$ cells/mL).

It is still possible to see within the data that the conditions induce a preference for growth in cultures, with the cultures supplemented with only CD3/CD28 Dynabeads showing nearly constant growth, the culture supplemented with both the CD3/CD28 Dynabeads and IL-2 showing growth after an initial drop at 24 hours, and finally the culture supplemented with IL-2 only requiring 120 hours in culture before showing any signs of growth.

Comparison of the viable cell counts in those cultures seeded at the higher density shows similar variation, however the presentation is somewhat different to that observed in the lower density seeded cultures (Fig. 3.9B). The IL-2 only supplemented culture showed a more or less constant decline over time. The was a relatively gradual level of decline between 24 and 96 hours, whilst at all other points there was a greater decline, except between 120 and 144 hours where there was a small incline in the viable cell count. The slight rise in viable cell count appears to correlate with a medium exchange, which could be the cause of this growth, either through replenishment of nutrients or removal of inhibitory waste elements, but it was not sufficient to sustain a long term period of growth.

The culture supplemented with only CD3/CD28 Dynabeads showed a slight decline in viable cell count from 0 to 72 hours, followed by substantial growth. This growth period increased the viable cell count two fold over 48 hours, and the count continued to rise to 144 hours, before seeing a decline to 168. There are elements of this pattern that are similar to what was seen in the condition seeded at the lower density, with a substantial growth between 96 and 144 hours, followed by a decline in viable cell count. However, the high density seeded culture does not show the same initially high growth as the lower density seeded culture, potentially indicating some initial benefit to seeding the cultures at a lower density.

The culture supplemented with both CD3/CD28 Dynabeads and IL-2 also showed an initial drop in viable cell count. This was followed by a gradual increase in viable cell count until 144 hours before seeing a slight decrease at 168 hours. The level of growth seen in this culture was not as great as that in the CD3/CD28 Dynabead only supplemented culture, with viable cell count only increasing by 25% in the same time frame the Dynabead only culture was able to double.

Comparison between cultures seeded at the two densities show similarities in the CD3/CD28 Dynabead supplemented cultures, other than that of the lower density seeded Dynabead and IL-2 supplemented culture due to the aforementioned drop off (Fig. 3.9A-B). The comparable cultures show a lag period, followed by sustained growth and an inflection point at 144 hours where lower cell counts are seen at 168 hours. This inflection point was originally suggested to due to medium

exhaustion, however given the varying densities of these cultures and the similar point of inflection, there is a possibility that this could be a temporal inhibition caused by something yet unobserved.

The data presented show that which was previously assumed, that the lack of stimulation is inhibitory to substantial growth, with the Dynabead stimulated cultures generally showing a higher viable cell count over time than those that were not supplemented. However, these and previous data do also show that the absence of stimulation does not mean that growth in T-cell cultures would not be possible. These viable cell count data also show that cultures supplemented with only CD3/CD28 Dynabeads produce a higher cell yield than those supplemented with both CD3/CD28 Dynabeads and IL-2 which is contrary to what was predicted. Further investigation is required to identify the reason the supplementing with IL-2 causes a reduced level of growth in cultures, as this is not only a common factor of T-cell culture, but should also be an element that contributes to the proliferation of these cultures. If the supplementation of IL-2 is going to hinder the growth of Dynabeads stimulated cultures, this would be a significant change to T-cell culture practises.

3.3.3.3.2 Phenotypic analysis

Phenotypic analysis was performed on the experimental cultures at 0, 72 and 120 hours to analyse the progression of the phenotypic response to the experimental conditions.

Analysis of the phenotypic data at 0 hours shows that both CD4+ and CD8+ subpopulations have a very high Effector subpopulation proportion, with both displaying some level of Effector Memory phenotype cells also. Although the CD4+ and CD8+ subpopulations categorise their events similarly, it is possible to see variation in the way these express themselves between the two subpopulations.

The CD4+ subpopulation cells appear to present as one cluster when observing expression of CCR7 and CD45RO (Fig. 3.9Cii). The cluster expression encompasses nearly the full range of CCR7 expression that denote non 'homing' events, whilst the range of CD45RO expression shows that the populations ranges from no CD45RO expression to that of Effector Memory cells, indicating the presence of mature populations. The cluster demonstrates a consistent range of CCR7 expression across the whole range of CD45RO expression, and vice versa. However, the density of events within the cluster show that there is a high concentration of events with low CD45RO expression, and the densest area of events is around the border between Effector and Effector Memory subpopulations cells.

The CD8+ subpopulation shows a similar range of both CCR7 and CD45RO expression however the expression is not as consistent as the CD4+ subpopulation. The CD8+ subpopulation presents itself as a single cluster, however the level of CCR7 expression is not maintained, narrowing by reduction of the maximum level roughly halfway between the minimum level of CD45RO expression and the

level at which cells are deemed positive for CD45RO (Fig. 3.9Ci). Although below the level of CCR7 expression of homing populations, this presents itself as if a Naïve subpopulation extension of the Effector subpopulation. The CD8+ subpopulation also shows a high density area around the low CD45RO expression region of the cluster, however the densest area of this cluster is in the higher CCR7 expressing area, indicating that this subpopulation's phenotypic balance is more towards immature and potentially Naïve subpopulations.

Analysis of the phenotypic data taken from the other two time points shows a clear effect of supplementing CD3/CD28 Dynabeads into culture, as well as the progression of cultures phenotypically over time, but shows commonality between different subpopulations seeded at different densities.

Comparison of the CD8+ subpopulations of cultures supplemented with only IL-2 at both 72 and 120 hours seeded at both densities shows very little difference in total profile to the phenotypic data observed at 0 hours. It is possible to see the larger density within the data shifting to the more CD45RO positive events, however the profile of the data remains similar. This lack of loss of the most immature events, especially compared to the other data shows the effect of CD3/CD28 Dynabeads in inducing maturation in T-cell populations (Fig. 3.9Di,ii,Gi,ii).

Analysis of the CD4+ subpopulations shows a similarity between the phenotypic profile of the cultures seeded at the two densities, as well as between the profiles at 72 and 120 hours, however the profile does not exactly match that of the samples from 0 hours. At the higher levels of CD45RO expression the culture samples resemble the 0 hour phenotypic profile, maintaining not only the range of expression, but also the relative density of the cells in the region. In comparison, the later samples show a loss of the lower level of CCR7 expression in the lower CD45RO expressing region, with the point at which the full range of CCR7 expression returning appearing to coincide with where the denser cluster begins (Fig. 3.9Diii,iv,Giii,iv).

Analysis of the cultures supplemented with only CD3/CD28 Dynabeads shows a dynamic response that changes in the phenotypic profile from 0 hours to 72, as well as when measured at 120 hours. Comparison of the CD4+ subpopulation maturity profile shows that at 72 hours there is a substantial shift in the profile of the cells, increasing the minimum level of CD45RO expression and condensing all events towards the greatest cluster seen at 0 hours (Fig. 3.9Fiii,Iiii). Whilst at 72 hours there was still a trail of events as part of the previous CD45RO low cluster, by 120 hours the cells were almost entirely focused around the cluster observed from the start (Fig. 3.9Fiv,Iiv). This cluster, as was observed at 0 hours, straddles the threshold level of CD45RO expression considered the point at which T-cells show maturity, and thus contains both Effector and Effector Memory cells. These

changes in the phenotypic profile were observed both in the culture seeded at $1x10^6$ cells/mL and $2x10^6$ cells/mL implying that the seeding density did not impact this subpopulation phenotypically.

The CD8+ subpopulations also showed similarity between those populations seeded at $1x10^6$ cells/mL and $2x10^6$ cells/mL. The profile of the cultures at 72 hours showed a similar clustering around the Effector Memory subpopulation area as was seen in the CD4+ subpopulation, although with a higher proportion of cells sustaining the Effector phenotype profile, giving it the profile of a tailing population (Fig. 3.9 Fi,Ii). In the culture seeded at $2x10^7$ cells/mL it is possible to see the presentation at 72 hours of some events that are CD45RO+ and CCR7+ (Fig. Ii). indicating that they are Central Memory phenotype cells. By 120 hours the populations had further concentrated to the right, with there being even fewer events present in the lower CD45RO region identified than before in conditions seeded at either density (Fig. 3.9Fii,Iii). Furthermore, it is possible to see an increase in the Central memory population in the culture seeded at $2x10^6$ cells/mL and the appearance of one in the culture seeded at $1x10^6$ cells/mL.

The cultures supplemented with CD3/CD28 Dynabeads and IL-2 exhibited very similar behaviour to those supplemented with only CD3/CD28 Dynabeads. The CD4+ subpopulations showed a substantial concentration towards higher CD45RO expression at 72 hours (Fig. 3.9Eiii,Hiii), continued to 120 hours (Fig3.9Eiv,Hiv), as was seen in the Dynabead only cultures. In comparison to the CD3/CD28 only supplemented the CD4+ maturity profile appears to be slightly more concentrated to the highly CD45RO expressing region in both the high and low density seeded cultures and at both 72 and 120 hours.

The CD8+ subpopulation of the cultures supplemented with both CD3/CD28 Dynabeads and IL-2 also show a high level of comparability to the Dynabead only supplemented cultures. The population clusters around a high CD45RO, low CCR7 point at 72 hours, however there is no substantial evidence of a Central Memory subpopulation at this stage (Fig. 3.9Ei,Hi). By 120 hours it is possible to see that in both cultures seeded at $1x10^6$ cells/mL and at $2x10^6$ cells/mL the Central Memory subpopulation has appeared, however the size of the cluster appears to be reduced compared to that of the cultures supplemented with only CD3/CD28 Dynabeads at their respective seeding densities (Fig. 3.9Eii,Hii). These data appear to show that the combination of CD3/CD28 Dynabeads and IL-2 are inducing a greater level of activation in the cultures, increasing the minimum CD45RO expression level at a greater rate than when supplemented with only the CD3/CD28 Dynabeads. However, the presence of the IL-2 supplemented into culture is also having a slightly inhibitory effect on the emergence of the Central Memory subpopulation from the Effector Memory subpopulation. It is not known what is causing this, however there is the possibility that IL-2 is promoting the Effector Memory subpopulation or inhibiting the Central Memory subpopulation directly.

Alternatively, this effect could be an indirect result of the impact these conditions are having on cell growth, with more proliferative conditions promoting Central Memory subpopulations, and growth inhibitors therefore selecting for Effector Memory populations.

3.3.3.3.3 Conclusions

Investigation into the impact of supplementing CD3/CD28 Dynabeads into culture has identified a strong effect in increasing culture growth and promoting maturation in cultures.

The initial investigation into the supplementation of CD3/CD28 Dynabeads into experienced cultures showed no positive effect and suggested that their inclusion was actually having a negative effect on conditional growth. The lack of response was important for establishing the level of effect CD3/CD28 Dynabeads had when they were introduced as part of the input material.

The protocol for use of this product included supplementing the beads into culture at seeding, and therefore this was tested to identify what effects, if any, they would have when supplemented immediately. The effects were immediately obvious, with cultures supplemented with the CD3/CD28 Dynabeads showing far superior growth to unsupplemented cultures. Phenotypic data after 120 hours of culture showed that all populations had converged around a single cluster of high CD45RO and mid level CCR7 expression. This population therefore manifested itself as an Effector Memory/Central Memory cluster, however without any other knowledge of the phenotypic state it was not possible to ascertain the experimental effect.

Further investigation compared the PBCM samples supplemented with either IL-2 or CD3/CD28 Dynabeads or both. Viable cell count data showed that the cultures supplemented with only IL-2 did not perform as well as those supplemented with Dynabeads, as was expected, due to the lack of activation which stimulates proliferation. Meanwhile, of those cultures supplemented with CD3/CD28 Dynabeads, those also supplemented with IL-2 appeared to not grow as well as those that were cultured without it, which was not expected as the increased level of IL-2 in cultures supplemented with both was anticipated to increase the level of growth. There was no obvious reason for an increased level of IL-2 being inhibitory to growth in T-cell cultures, necessitating further research to understand the cause of that response.

Phenotypic data was collected at 0, 72 and 120 hours in order to monitor the phenotypic response of the T-cell populations and how the experimental variables affected the subpopulation profile over time. Analysis of the phenotypic data showed that the cultures supplemented with only IL-2 showed a very similar phenotypic profile across all timepoints, with the only substantive difference lying in the increase of the lower CCR7 expression level in the low CD45RO expressing events. This appeared to indicate that there was little in the way of phenotypic progression from the culture conditions.

The CD3/CD28 Dynabead supplemented cultures, however, had a much stronger response to the experimental conditions, showing a substantial shift of the majority of events to a mid/high level of CD45RO expression.

In the CD4+ subpopulation, the cultures remained predominantly Effector Memory subpopulation, with no events being identified as 'homing'. The concentration of these populations was seen, at each time point, to be slightly greater in the CD3/CD28 supplemented culture with IL-2, rather than independently. The CD8+ subpopulations also show the shift towards mature phenotype subpopulations, with this once more being more pronounced in cultures supplemented with both CD3/CD28 Dynabeads and IL-2, rather than just the beads independently. However, within the CD8+ subpopulations it is also possible to identify Central Memory phenotype cells. At 72 hours this population is only present in the culture seeded at $2x10^6$ cells/mL and supplemented with only CD3/CD28 Dynabeads. At 120 hours it is possible to see evidence of the subpopulation in all CD3/CD28 Dynabead supplemented cultures, however this is a larger and denser subpopulation in the Dynabead only supplemented cultures, and is more prominent in the cultures seeded at the higher density than the lower. This implies that although the CD3/CD28 Dynabeads are helping to mature the CD8+ subpopulation, the additional supplementation of IL-2 is inhibiting the promotion of the Central Memory subpopulations.

Overall, the effect of CD3/CD28 Dynabeads at seeding is substantial. There is clear evidence that they are promoting proliferation in culture, however this does come at the expense of any immature cell populations. Supplementation of the CD3/CD28 Dynabeads induces activation in the T-cell subpopulations, causing an upregulation in IL-2 production by the cells, and the expression of CD25, which increases the affinity with which it binds to IL-2. As such it was anticipated that supplementing IL-2 alongside CD3/CD28 Dynabeads would enhance the effect and increase proliferation further, however what was observed was the opposite, with lower cell yields from those cultures that were supplemented with the growth factor compared to those that weren't. Analysis of the phenotypic data showed the effects, maturation, of the CD3/CD28 supplementation, however there was little difference between them other than the slight increase in Central Memory presentation in the CD8+ subpopulations.

3.4 Conclusion

The experiments presented in this chapter were designed to test the impact of the input material on T-cell cultures, from how they were sourced to the balance of subpopulations and finally the level of stimulation afforded to the cultures. Overall, the data showed that there were some potential differences between the culture growth when the balance of subpopulations input was manipulated,

and that stimulation had a substantial effect both on the growth of cultures and their phenotypic profile.

The comparison of isolation targets highlighted the benefit to growth of selecting for CD3+ cells, compared to CD4 and CD8, whilst also highlighting a variation in phenotypic profile that suggested the proliferation may be linked to stimulation. This was further qualified when PBMC samples were cultured with CD3/CD28 Dynabeads, specifically designed to stimulate activation in T-cell populations. The similarity in the level of growth and the effect that stimulation had on maturity subpopulations over time strongly suggests that the CD3+ isolation was inducing activation in T-cells, contrary to what the stated effects of the microbeads would be.

With the lack of activation presented by the CD4/CD8 isolation, this process was used to isolate the CD4+ and CD8+ subpopulations individually for manipulation of subpopulation proportion in cultures. The investigation into this appeared to show that there were relatively little differences between the growth profile of CD4+ and CD8+ subpopulations, nor when they were cultured in equal combination with each other or non T-cell PBMCs. Investigation into supplementation of CD4+ and CD8+ subpopulations at different relative concentrations appeared to identify a 3:1 CD8:CD4 subpopulation ratio induced a greater level of growth in culture, however this would seem to require a greater level of investigation to validate.

The comparison between culture in the presence and absence of CD3/CD28 Dynabeads clearly highlights the strong effect they have on promoting proliferation in culture, as well as the maturing effect they induce phenotypically. However, current observation appears to show that beads need to be supplemented into culture at seeding to benefit in this way from their presence. Analysis of the effect of seeding PBMCs at different seeding densities showed that the variation was either minimal or inconsistent across those conditions tested. Meanwhile, comparison of CD3/CD28 activated PBMC cultures supplemented with and without IL-2 appear to show that IL-2 has some level of inhibitory effect of T-cell culture growth when supplemented into culture at seeding. The evidence also shows that although non IL-2 supplemented cultures appear to mature T-cell populations faster, increasing the average CD45RO expression level at an increased rate, the CD8+ subpopulations were also slightly slower to develop Central Memory subpopulations in certain cultures.

It became clear with the last sets of experimental data, that although culture of T-cell populations and growth in culture was possible without stimulation, carried out here by CD3/CD28 Dynabeads, it would not be a viable method of culture as the yield was just too low over time relative to what could be achieved in stimulated cultures. Moving forwards the cultures were to be supplemented

with CD3/CD28 Dynabeads, however further investigation could consider whether the concentration of Dynabeads supplemented into culture could be adjusted to stimulate growth, and promote the selection of Naïve and Central Memory subpopulations.

4 Investigating the effect on growth and phenotypic profile of T-cell cultures when delaying the supplementation of IL-2

4.1 Introduction

There are two main areas of interest for optimisation in T-cell culture for CAR T-cell therapies in the expansion stage prior to transduction. An expensive to produce and variable quality therapy, research is focussed on increasing yield from the input material and improving purity for target subpopulations[84,137,165]. Optimising and improving these elements can reduce time and cost of the manufacturing process, whilst improving the efficacy of the transplanted therapy doses. This could increase access to CAR T-cell therapies to more patients with less risk.

4.1.1 Effects of IL-2 and CD3/CD28 Dynabeads on proliferation

Previous data had shown that supplementing cultures with both CD3/CD28 Dynabeads and IL-2 had a less positive effect on proliferation when compared against cultures supplemented with just CD3/CD28 Dynabeads. The difference in phenotypic output between the two appeared to be relatively minimal despite the conditional variance demonstrably affecting the proliferation of the cultures, but nevertheless warranted further observation. The data observed in 3.3.3 suggested that exposure to IL-2 before sufficient stimulation and activation could result in a reduced response to the growth factor, which was complemented by colleague's anecdotal evidence. As a result, not only would T-cells in culture not respond to the supplemented IL-2, but also to the endogenous IL-2 produced by the cells themselves.

Delaying the introduction of IL-2 for a period of time after seeding would theoretically give time for the activation of cells within the cultures by CD3/CD28 Dynabeads before exposure to the growth factor. This could explain the difference in culture growth between the CD3/CD28 Dynabead only supplemented cultures and the CD3/CD28 Dynabead and IL-2 supplemented cultures in 3.3.3.3.1. These conditions would also more closely resemble the *in vivo*modality, with T-cells normally only being exposed to high concentrations of IL-2 after activation by exposure to their target antigen[39,40]. Hypothetically, introducing the IL-2 subsequent to stimulation would then aid in promoting proliferation, as opposed to being inhibitory, and the greater concentration of IL-2 would allow stimulation of growth beyond that of the Dynabead only cultures.

Furthermore, in order to validate the previous observation that cultures supplemented with CD3/CD28 Dynabeads will proliferate more than those also supplemented with IL-2 at the point of seeding cultures, it was decided that it would also be appropriate to perform T-cell cultures with no IL-2 supplemented.

4.1.2 Observed effects of IL-2 and CD3/CD28 Dynabeads on phenotypic profile

As mentioned, the previous evidence did not show a substantial difference in the phenotypic profile of samples cultured with CD3/CD28 Dynabeads or CD3/CD28 Dynabeads and IL-2, however there was a stark difference between these samples and the samples cultured with only IL-2. The IL-2 only supplemented cultures were shown to retain their phenotypic heterogeneity over time, relatively maintaining Naïve and Effector subpopulations. However, all conditions supplemented with CD3/CD28 Dynabeads showed a distinct progression, even at 72 hours, towards mature phenotypes. This was mainly presented as a loss of Naïve phenotype (CCR7+CD45RO-) cells and retention of only small populations that match an Effector phenotype (CCR7-CD45RO-) profile. In addition, the mature subpopulation that the cultures transitioned into was Effector Memory (CCR7-CD45RO+), rather than the Central Memory (CCR7+CD45RO+) phenotype subpopulation which is a target output population, although the data did appear to show the emergence of a Central Memory population as time progressed.

This information would be vital in the context of CAR T-cell therapy manufacture moving forwards, as inducing a superior proliferation in cultures could end up becoming a hinderance if an appropriate phenotypic profile is not achieved or maintained as an output of this section of the process. A greater variation from the desired population composition could result in a product that requires a greater amount of processing steps, and therefore time, at further downstream stages in the manufacturing process. It may also negatively impact the efficacy of the treatment after administration.

4.1.3 CD3/CD28 Dynabeads' activating properties

The previous evidence indicates strongly that despite IL-2's maturation properties[108], it is the CD3/CD28 Dynabeads that are inducing the maturation of the T-cell population. This was achieved using the manufacturers recommended concentration of CD3/CD28 Dynabeads, which has been calculated with proliferation in mind. It was hypothesised that reducing the Dynabead concentration could induce sufficient stimulation in cultures to allow a positive proliferative response to IL-2, but without progressing to the point of total maturity, allowing them to maintain some level of Naïve phenotype cells.

4.1.4 Impacts of seeding density on T-cell culture

Seeding density was another consideration for this experiment. Although IL-2 could be manually added to cultures in order to raise the concentration, it is naturally produced by T-cells, especially when stimulated for example with CD3/CD28 Dynabeads. Cultures seeded at a higher density would have a higher concentration of endogenous IL-2, however they would also have a higher

concentration of cells with which it would interact. The interaction between these elements would help inform culture processes moving forwards as there is a desire to reach maximum yield per input and seeding density may impact this.

Furthermore, when increasing the seeding cell density, it is important to consider the possibility of medium exhaustion. Reaching a point of exhaustion will decrease the proliferative capacity of the cultures and may influence their phenotypic profile as well.

4.1.5 Experimental setup

It was anticipated that thorough testing of these factors: IL-2 concentration, timing of IL-2 addition, Dynabead concentration and seeding density, would provide a comprehensive data output that would aid in substantially improving the understanding of the impact these variables have on culture through defining their effects on proliferation and promotion of phenotypic subpopulations. In order to sufficiently test these parameters, Design of Experiment (DoE) methodology was utilised for planning and then implementation to enable exhaustive investigation into not only the effects of these factors individually, but also in combination.

Cell counts were able to be performed with relative ease, therefore giving the ability to monitor growth in cultures. It was preferable to take cell counts at a high frequency to allow high resolution monitoring of culture proliferation, and as a result cell counts were taken daily.

Phenotypic analysis would continue to be performed using the existing 5 colour analytical panel. Similar to cell counting, there was a desire to obtain this information at a high frequency to increase the resolution of data and enable a more detailed assessment of experimental impacts.

Table 4.1. Range of experimental conditions tested to identify relationship between cell density, Dynabead concentration, IL-2 concentration and timing of IL-2 addition.

Condition No.	Cell Seed Density ($1x10^6$/mL)	Dynabead Concentration ($1x10^6$/mL)	IL-2 Concentration (UI/mL)	IL-2 Addition Delay (Hrs)	Replicates
1	1	0.2	0	0	2
2	1	0.2	0	96	2
3	1	0.2	200	0	2
4	1	0.2	200	96	2
5	1	1	0	0	2
6	1	1	0	96	2
7	1	1	200	0	2
8	1	1	200	96	2
9	1.5	0.6	100	48	3
10	2	0.2	0	0	2
11	2	0.2	0	96	2
12	2	0.2	200	0	2
13	2	0.2	200	96	2
14	2	1	0	0	2
15	2	1	0	96	2
16	2	1	200	0	2
17	2	1	200	96	2

4.1.6 Hypotheses

4.1.6.1 Cell yield hypotheses

The previous data regarding the proliferation of cells in the presence of IL-2 and CD3/CD28 Dynabeads, and the common assertion that IL-2 is positive for T-cell growth[160], appear to indicate that delaying the supplementation of IL-2 will have a positive effect on proliferation of T-cell cultures.

Similarly, the same data highlights the fact that stimulation of the CD3 and CD28 pathways, for example with CD3/CD28 Dynabeads, is essential for T-cell culture proliferation[105,129]. As the higher concentration of CD3/CD28 Dynabeads to be supplemented in this experiment is the same as the manufacturers recommended concentration, it would be predicted that the greatest level of proliferation would be achieved when supplementing with the higher concentration of CD3/CD28 Dynabeads.

Regarding seeding density, it would be expected that cultures seeded at the lower density of $1x10^6$ cells/mL would show a higher level of proliferation than those seeded at the higher concentration.

This is in part following on from the previous prediction; Dynabead concentration was calculated in a volume, rather than cell density dependent fashion and therefore cultures seeded at a lower density would have a greater bead per cell interaction. This in turn would increase the number of anti-CD3 and anti-CD28 proteins the cells would be exposed to and could increase the level of stimulation. This increase in relative concentration applies to the supplementation of IL-2 as well, as it is also calculated volume dependently. Finally, increasing the seeding density would increase the risk of medium exhaustion, which could reduce the ability of cultures seeded at the higher density to proliferate to the same degree as those seeded at the lower density.

4.1.6.2 Phenotypic data hypotheses

Phenotypic profile is somewhat linked to proliferation in the sense that those conditions that stimulate growth tend to show higher proportions of mature phenotype cells. This is particularly true of cultures supplemented with CD3/CD28 Dynabeads, with their interaction with T-cell inducing activation and upregulating the expression of maturity marker CD45RO[113,114]. Therefore, it can be said that all the prior hypotheses regarding proliferation would be the same of Effector Memory subpopulations. As such, these conditions will also reduce the concentration of the immature subpopulations of Naïve and Effector cells.

4.1.6.3 Chapter Objectives

To test the variables outlined above for effects on phenotypic profile and population growth using efficient DoE methodology.

To analyse the viable cell count data for effects of experimental variables.

To analyse phenotypic data using conventional manual flow cytometry gating.

To develop a novel method of phenotypic data analysis based on algorithmic cluster formation to optimise phenotypic resolution and compare to conventional analysis.

4.2 Materials and methods

4.2.1 Cell source material

The following work was completed using frozen PBMC aliquots from a single donor provided by Axol Bioscience as described in 2.1.1.2.

4.2.2 T-cell Culture methods

Cell cultures were set up and maintained in accordance with the processes set out in 2.1.2, 2.1.4 and according to the parameters outlined in Table 4.1.

4.2.3 Sampling of cultures for viable cell count and phenotypic analysis

Viable cells count and phenotypic analysis was carried out as described in 2.1.6.2 and 2.1.7 respectively. Cultures were sampled for viable cell count at approximately 24, 48, 72, 96, 120, 144 and 168 hours after seeding. Cultures were sampled for phenotypic analysis at approximately 48, 72, 96, 120, 144 and 168 hours after seeding.

4.2.4 Standard gating protocol

In order to isolate and quantify different subpopulations for further analysis, a conventional gating process was performed on the phenotypic data collected. Initially cell events were isolated from the total events based on forward scatter and side scatter (Fig. 4.3A) to remove cell debris and CD3/CD28 Dynabeads from the analysis.

Next, cells were isolated based on CD4 and CD8 expression. In the event that the total T-cell population was to be analysed, non T-cell events were gated out of the population, defined by their lack of CD4 or CD8 expression (Fig. 4.3B). Where specific CD4+ and CD8+ subpopulations were to be analysed independently, a quadrant gate was used to identify the CD4+CD8- and CD4-CD8+ subpopulations (Fig. 4.3C).

Once total T-cell or CD4+ and CD8+ subpopulations were defined, they were then analysed for maturity phenotype subpopulations. Subpopulations were plotted for CCR7 and CD45RO expression and quadrant gates were applied to identify Naïve (CCR7+CD45RO-), Effector (CCR7-CD45RO-), Effector Memory (CCR7-CD45RO+) and Central Memory (CCR7+CD45RO+) subpopulations.

4.3 Results

A series of T-cell cultures were conducted with variable cell density, Dynabead concentration, and timing and concentration of IL-2 supplementation. The variable levels were organised in a 4^2 DoE structure with an intermediate centrepoint in the design to identify the main effects on culture outputs and any interactions between variables. Analysis of the T-cell cultures was concerned with two specific culture outputs, viable cell count and expression of the phenotype defining markers CD4, CD8, CD45RA, CD45RO and CCR7.

Viable cell count analysis was carried out with the Vi-Cell automated cytometer. Phenotypic data were firstly analysed using conventional manual flow cytometry gating and analysis. Novel analysis based on the outputs of automated phenotype clustering was then performed to identify if the same conclusions could be extracted with a less subjective analysis, or if different or additional conclusions would be drawn.

4.3.1 Analysis of Conventional Manual Analysis Outputs with Design of Experiment

4.3.1.1 Growth of Tcell cultures in response to variable stimulation and seeding density

The viable cell count data over time (Fig. 4.1) show that all conditions on average show a period of growth. Conditions were separated based on CD3/CD28 Dynabead and IL-2 concentration for clarity of presentation. Centrepoint data were included on all plots to aid comparison between conditions. The general trend in the data showed a lag period in cell growth from 24-72 hours (0 hours data unavailable) followed by consistent growth until the last measurement at 168 hours. Conditions 11, 13, 5 and 8 showed no growth from 144-168 hours, whilst conditions 12 and 16 showed longer periods without growth from 96 and 120hr respectively. The average cell count at 48 hours for condition 12 appears to be erroneously high; the individual sample count producing this high average was considered anomalous and was not carried forward to further analysis.

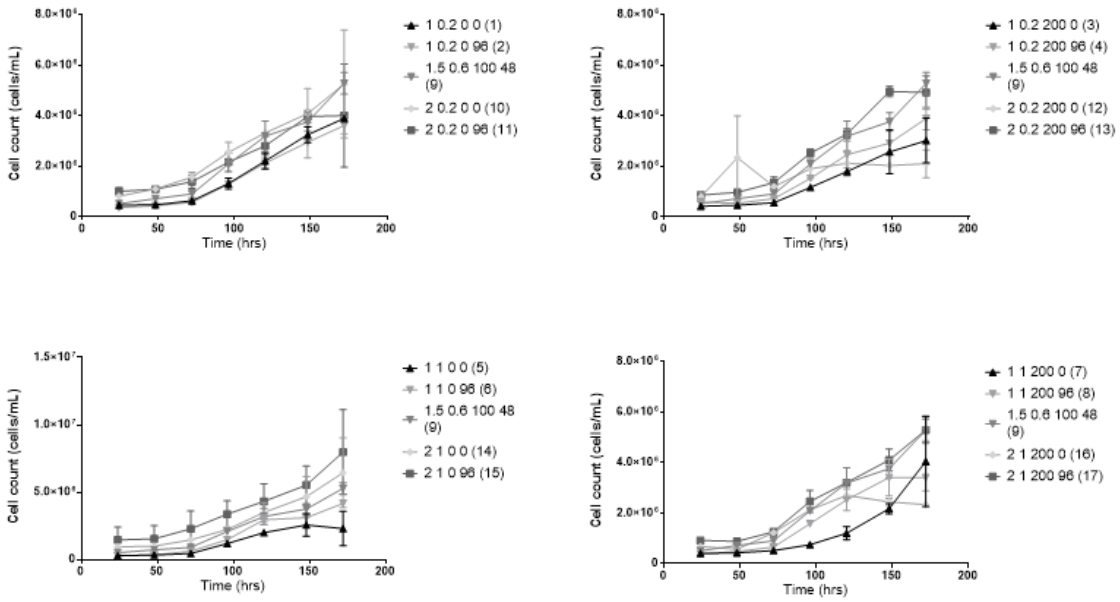


Figure 4.1. Average live cell counts from culture samples over time. PBMC cultures were sampled every 24 hours up to 168 hours and analysed for live cell count. Figure legend shows experimental conditions in order: seeding cells density (10^6 cells), CD3/CD8 concentration (beads/mL), IL-2 concentration (UI/mL), delay of IL-2 supplementation after seeding (hours), and the condition number in parentheses. All conditions experienced a period of growth, generally conserved until the end of the experimental period. Culture conditions had a significant impact ($P<0.005$) on cell counts over time.

The highest endpoint average viable cell count was induced by condition 15 (7.97×10^6 cells/mL), and the lowest was conditions 12 (2.1×10^6 cells/mL), more than 4 times lower. Where a single seeding density is used in an experimental setup, the end point viable cell count can be considered a measure of both the total yield and the yield per input unit. Within this experimental setup seeding density of the cultures was a variable, and therefore the previous assertion could not be made in this scenario. As one of the objectives of the experiment was to identify the effects that experimental variables had on yield per input, the end point viable cell count was neither a sufficient nor an

appropriate measure of conditions to answer the objective. As such the growth rate was calculated by taking the natural log of the value produced by the LOGEST function in excel. This calculated value considered the seeding density, as well as how the viable count changed over time, and could therefore be suitably used as a measure of the yield per input of the cultures. In addition, there is a level of approximation in the calculation that allows for a more representative analysis of the data. A temporary fluctuation in viable cell count would have limited influence on the calculated growth rate produced, whilst a sustained deviation would have a greater impact and would vary the growth rate in the direction of the deviation. This should give a more genuine representation of culture behaviours in analysis and allow for more accurate analysis of the impacts of experimental factors.

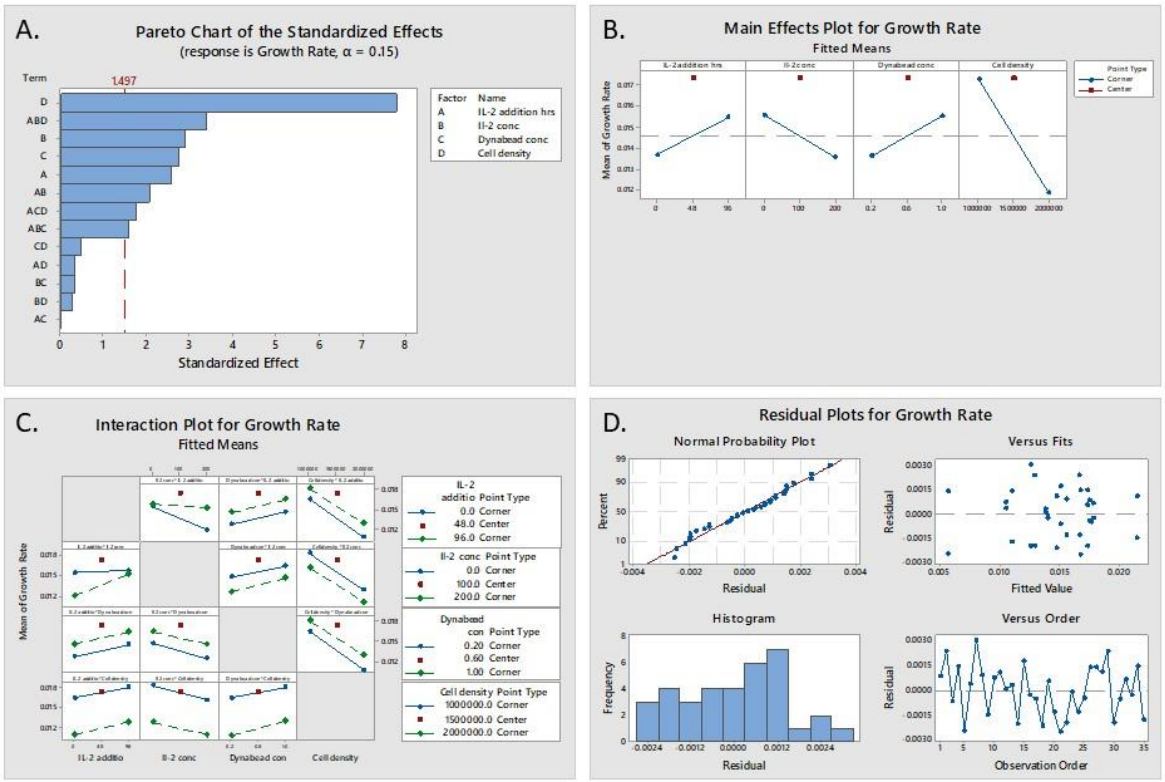


Figure 4.2. Seeding density has a substantially higher impact on growth rate than other culture variables. Calculated growth rates were analysed using Design of Experiment approach to identify how seeding density, CD3/CD28 Dynabead and IL-2 concentration and timing of IL-2 addition impacted them. Analysis of standardised effect showed that seeding density of cultures has twice impact than the next most significant factor, and that all tested variables had a significant effect on growth rate (a). Later IL-2 addition and a higher CD3/CD28 Dynabead concentration had a positive impact on growth rate whilst higher IL-2 concentration and seeding density had a negative effect. Midpoint data suggested a non linear relationship for all variables (b). The significant interacting factor effect showed adding IL-2 at seeding reduced growth, however supplementing at 96 hours removed that detriment (c). Comparison of residual data with the observation order implied a negative correlation between the order and observed growth rate, including a step change around sample 24 (d).

Analysis of growth rates with Design of Experiments showed that all experimental variables had a significant effect on the growth rate. Seeding cell density had over twice the impact of any other effect observed (Fig. 4.2A), where increasing the seeding density had a negative impact on growth rate. IL-2 concentration was the next most impactful variable, followed by Dynabead concentration

and the timing of IL-2 addition. Increasing IL-2 concentration had an overall negative impact on growth rate, increasing Dynabead concentration overall increased the culture growth rates, and delaying IL-2 addition had a similarly beneficial impact on growth rates (Fig. 4.2B). Centrepoint data on the main effects plot (Fig. 4.2B) indicates that all conditional variables have a non-linear effect on the growth rate of T-cell targeted PBMC cultures.

There were several combined variables that had a significant effect on growth rate (Fig. 4.2A). The only two factor combination that had significance was timing of IL-2 addition and IL-2 concentration. Analysis of the effects of this variable interaction showed that with no IL-2 addition there is little difference between the timing of 'addition' (Fig. 4.2C), which would be anticipated in cultures with no functional difference. In cultures where 200 UI/mL IL-2 was added there was a minimal drop in growth rate when the growth factor was supplemented at 96 hours, but a substantial drop in growth rate when it was added at seeding, implying in this scenario that there is a negative effect of adding IL-2 at seeding compared to delaying addition. Similar to the single variable analysis, the centrepoint data provided in this plot suggests a non-linear effect of the combined variables on growth rate; the centrepoint (addition of 100 UI/mL IL-2 at 48 hours) had a positive effect compared to the other combinations of these variables.

The normal probability plot produced from the model (Fig. 4.2D) show that the data is approximately normally distributed. Similarly, the histogram of residual against frequency does not imply a distribution of data that is drastically shifted from normal, although there is a slight shift towards the higher end before a sharp drop. There does appears to be a slight trend in the plot describing the residual against observation order. The experimental order was randomised specifically to avoid any conditional bias, and therefore any trends observed in response by order would suggest a systematic effect of the measurement process or other influence of experimental order. A trend downwards can be observed from observation order 1 to around 24 or 25. Following a step up these is then a second trend downwards until the end of the observation. Due to the size of the experiment, samples were added to the cytometry equipment in multiple plates, split unevenly, with24 samples on the first plate and 11 on the second, in a consistent order. The step change in the responses in this plot would plausibly correlate with the change in plates. Randomisation of the sample order means that the observed trend did not increase the chance of a false effect being identified, however it did reduce the statistical power to detect any effect.

4.3.1.2 Analysis of phenotypic response to variable stimulation and seeding density in culture

The phenotypic analysis also produced data indicating significant influences of the experimental variables. CD45RA, CD45RO and CCR7 are all indicative of certain traits of immaturity, maturity and likelihood of migrating back to the Secondary Lymphoid Organs in T-cell populations. As such, the

changing expression level of these proteins on T-cell surface can change the maturity subpopulation the cells would be sorted into based on phenotypic profile. Additionally, when monitored over time these changes could show a trend in the expression levels indicating that the cell, or population, will soon transition to another subpopulation phenotype. As a result, the variation both within and between samples has biological significance, especially as maturity subpopulation defines part of the target output. This will help to establish which experimental variables significantly effect target outputs and in what way.

4.3.1.2.1 Standard gating protocol

Only CD4 or CD8 will be expressed on T-cell surface as their expression is mutually exclusive. They define the cell as belonging to either the cytotoxic subpopulation (CD8+/CD4-) or the helper/regulatory subpopulation (CD4+/CD8-). Currently T-cells have not been shown to transition between these two subpopulations, and therefore variation in CD4 or CD8 expression does not have the same functional significance as variation in the other monitored markers. Dot plots comparing CD4 to CD8 expression show how it is often the case that CD4+ and CD8+ subpopulations can be easily identified (Fig. 4.3C) due to the relative levels of CD4:CD8 expression for T-cells, or the absence of expression in non T-cell PBMCs. T-cells were gated into maturity subpopulations from the total T-cell population and CD4+ and CD8+ subpopulations. Analysis of CD4 and CD8 expression is important to fully define the phenotype of a cell, however as the marker expression is binary, varying expression within a cluster would not have the same level of functional implication as with the maturity subpopulation markers.

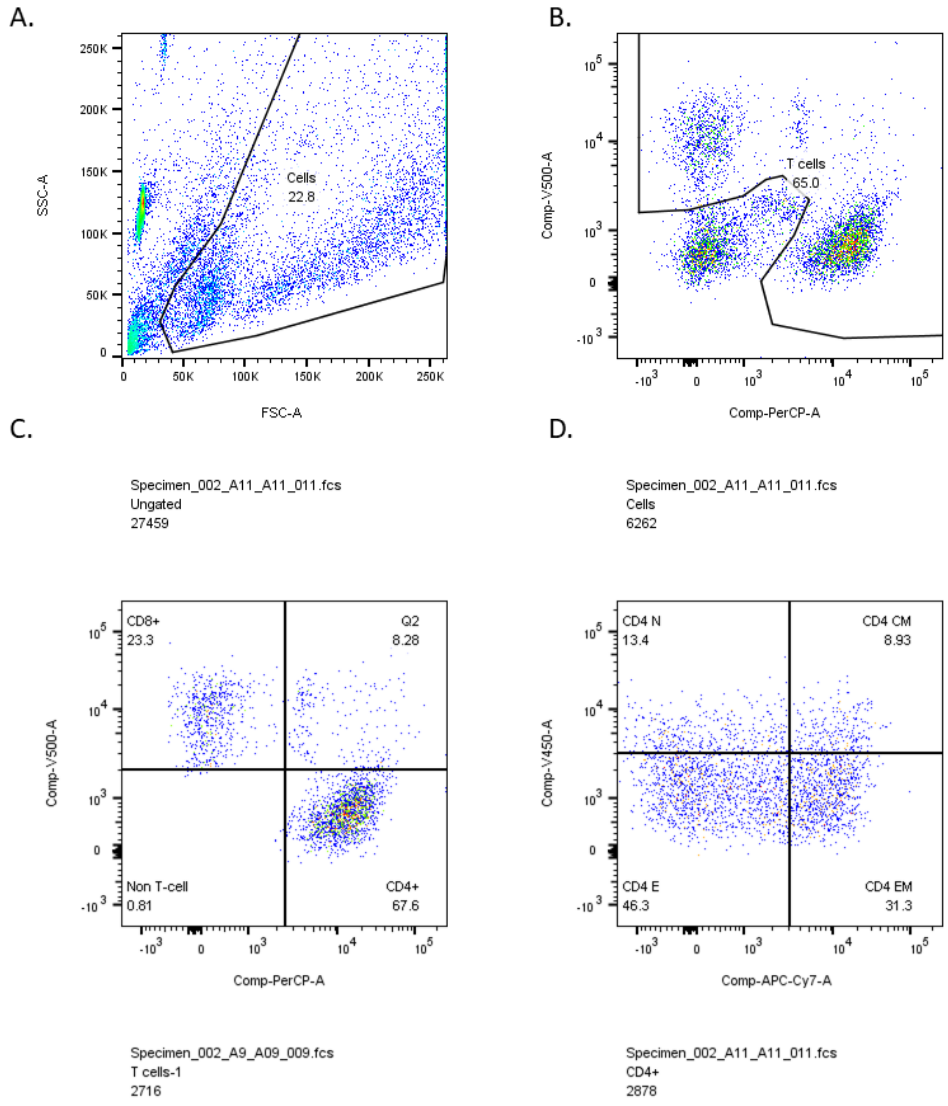


Figure 4.3. Demonstration of standard gating protocol currently in place. Samples were labelled to identify CD4, CD8, CCR7, CD45RA and CD45RO expression and assessed using flow cytometry. Data was analysed to identify the number and proportion of events that had expression profiles matching those of established subpopulations. Initially 'cell' events were isolated from debris and CD3/CD28 Dynabead populations using forward scatter and side scatter data (a). T-cells were then isolated from non T-cell PBMCs by CD4 (Per-CP) and CD8 (V500) expression (b). These populations could also be gated using quadrants where needed to further analyse specifically CD4+ or CD8+ populations (c). Using CD45RO (APC-Cy7) and CCR7 (V450) marker expression levels a quadrant gate was placed to identify Naïve (CD45RO-CCR7+), Effector (CD45RO-CCR7-), Effector Memory (CD45RO+CCR7-) and Central Memory (CD45RO+CCR7+) subpopulations (d).

4.3.1.2.2 Average subpopulation variation over time

At seeding, phenotypic analysis of the samples showed heterogenous populations, with a range of CD45RO and CCR7 expression that matched the phenotypic profile of all 4 maturity subpopulations

(N, E, EM, CM) (Fig. 4.4A and B). It is evident to see that over time there is a transition from the heterogenous population seen initially to predominantly Effector Memory phenotype (Fig. 4.4). This trend is also observable in the percentile proportion of T-cells, both overall and the subpopulations of CD4+ and CD8+ T-cells, that belong to each maturity subpopulation. Across all parentage (total T-cell, CD4+ and CD8+), the Naïve population is on average 11.8% at 48 hours, which then declines until 96 hours to an average of 1.2% and approximates that level until the final measurement. The Central Memory population in all parentage begins with a lower proportion than the Naïve subpopulation and rises slightly to an average of 14.2% at 72 hours, before reducing to a similar plateaued level beyond 96 hours as the Naïve population (Fig. 4.5).

The trend and values in proportion of Effector and Effector Memory populations of total T-cells and CD4+ T-cells match each other very closely. As CD4+ cells make up the larger proportion of total gated T-cells this is the likely cause of this correlation as the larger subpopulation will have a greater influence on what is observed in the total population. The CD8+ Effector and Effector Memory populations match the observed trend in the total gated T-cell and CD4+ Effector and Effector Memory subpopulations regarding the direction of change in the subpopulations, however the CD8+ subpopulations do not progress with the same similarity.

There is an observable drop from 48 hours in average Effector subpopulation proportion regardless of parentage. This drop in average subpopulation proportion continues at each timepoint until 144 hours, at which point the average subpopulation proportion increases. In the total T-cell and CD4+ Effector subpopulation, this rise in average subpopulation proportion is to a level approximating that seen at 72 hours, whilst in the CD8+ Effector subpopulation proportion this increase was to a level approximately halfway between that seen at 120 and 144 hours.

The Effector Memory populations show an inverse trend to the Effector subpopulations. The phenotypic definition of Effector and Effector Memory subpopulations have the same level of CCR7 expression, and only differ in their CD45RA and CD45RO expression levels. Where the immature Effector T-cell subpopulations have a high CD45RA and low CD45RO expression level, the mature Effector Memory phenotype cells have the opposite levels of expression for these two markers (CD45RA-/CD45RO+). The dominant observation of the cultures with regards to CCR7 and CD45RO expression is a shift from low to high CD45RO expression (Fig. 4.5). Therefore, it is probable that this inverse trend is a result of the increasing level of CD45RO expression over time giving the effect of shifting events from the Effector subpopulation gate to the Effector Memory subpopulation gate during phenotypic analysis.

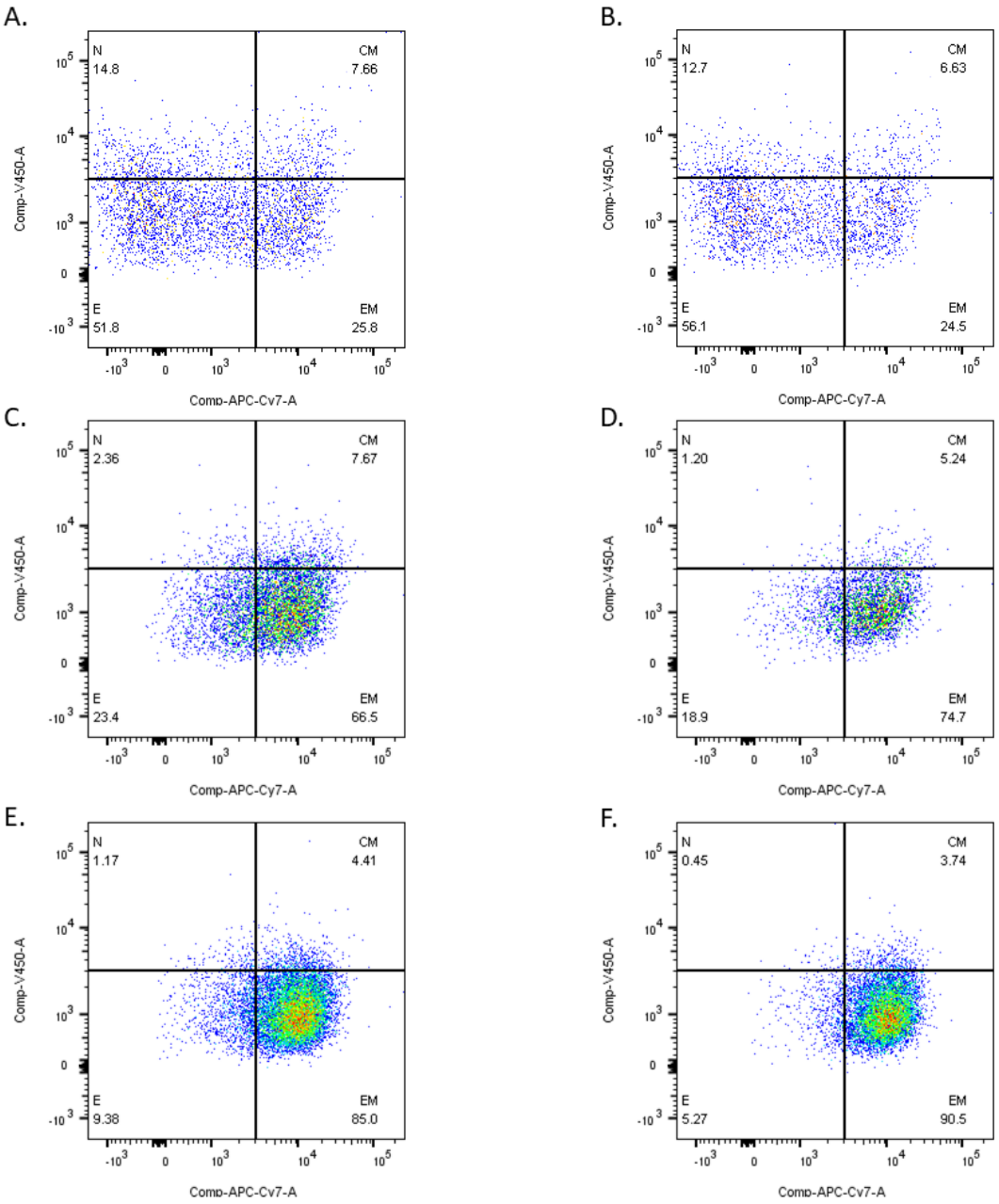


Figure 4.4. Phenotypic profile transition in culture from heterogenous to predominantly Effector Memory population over time. Marker expression data for CD45RO (APC-Cy7) and CCR7 (V450) from two midpoint conditions present the overall trend seen across all conditions. At day 2 (a,b) the population is still relatively distributed, showing at least 5% events present in all quadrants, with the greatest concentration lying in the Effector quadrant. At day 4 (c,d) there has been a substantial reduction in the expression of CCR7 as well as an increase in CD45RO expression. By day 6 (e,f) the cultures are over 80% Effector Memory and it is possible to the see that all events are clustered around a central point of high CD45RO, low CCR7 expression.

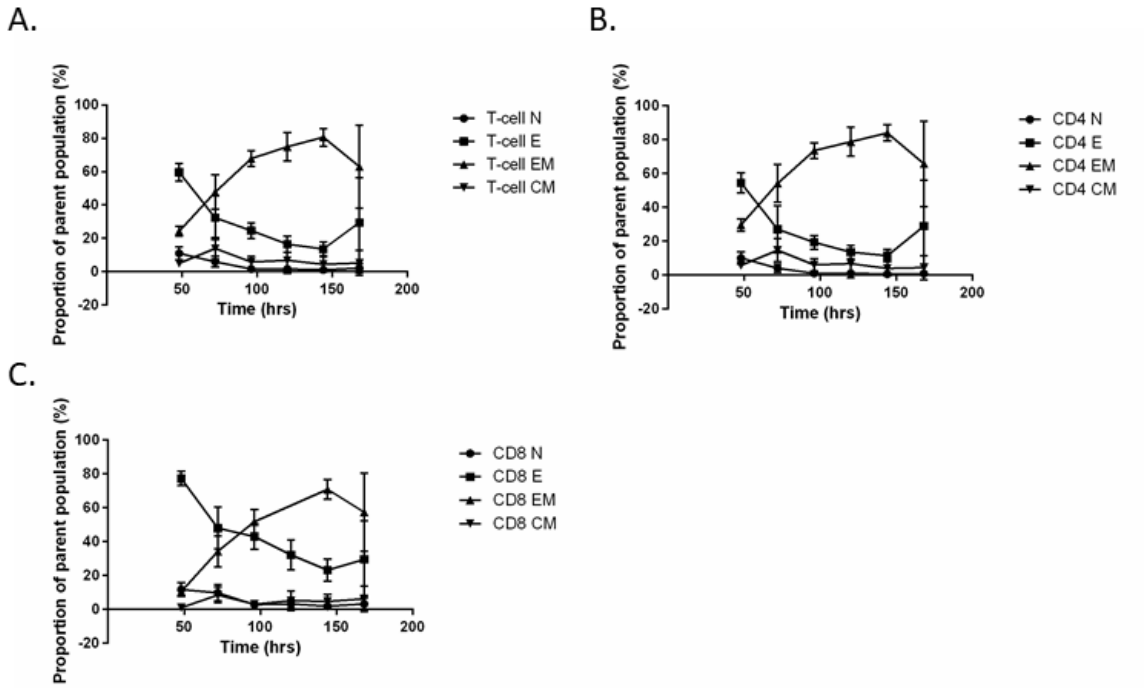


Figure 4.5. Tracking maturity subpopulation proportions over time shows a shift to predominantly Effector Memory phenotype populations. Percent proportion of each maturity subpopulation (Naïve, Effector, Effector Memory and Central Memory) was calculated for each sample for total gated T-cell (a), CD4+ T-cell (b) and CD8+ T-cell (c) parent populations. The average proportion was calculated for each time point and plotted over time. The error bars represent standard deviation of the dataset across all experimental conditions.

The Effector Memory subpopulations show an increase from 48 hours until 144 hours. As with the Effector subpopulations there was a change in opposition to the trend at 168 hours, with the Effector Memory subpopulation proportions reducing regardless of parent population.

The data in this experiment show that CD4+ and CD8+ populations respond the similarly to the stimuli provided. Both CD4+ and CD8+ T-cell parentage show immediate changes in Effector and Effector Memory populations however the CD8+ subpopulations were initially observed at both a higher and lower proportion respectively. Within the Effector subpopulation trend it is possible to see a sharper drop between 48-72 hours, followed by a less steep decline until 144 hours, whilst within the Effector Memory subpopulations it is possible to see a greater increase sustained from 8 hours until 96 hours, before the rate of increase appears to drop somewhat until 144 hours. As such, although the relative proportions differ, the trend in change of the Effector and Effector Memory subpopulation proportions within the CD4+ and CD8+ subpopulation are similar. The pattern of response in the Effector and Effector Memory subpopulations is consistent with what

would be expected from cultures in which subpopulations have different but consistent growth rates. The data therefore could suggest that the change in subpopulation proportion comes from a greater stimulation of the Effector Memory subpopulations than the Effector. However, knowledge of the activating properties of CD3/CD28 Dynabeads on T-cells[62,108] would suggest that any selective stimulation of Effector Memory subpopulations is complemented by the maturation of immature subpopulation cells, indicating that the effect seen is a combination of expansion of the Effector Memory cells and transition to the Effector Memory phenotype from Effector.

From the data it is possible to say that the CD8+ Effector and Effector Memory subpopulations are stimulated into change at the same point, implying that Effector and Effector Memory cells are affected similarly by the same stimulus regardless of their CD4+ or CD8+ phenotype. However, it is not possible to state conclusively, due to the two parent populations having different starting proportions, if they are affected at the same rate. The intended target subpopulation for this experiment are Naïve and Central Memory, regardless of parent population, and thus this observation is an informative addition rather than essential for analysis.

4.3.1.2.3 Selection of a suitable timepoint for statistical analysis

The nature of the phenotypic data available, marker expression data at multiple timepoints, meant it was necessary to select a timepoint from which data could be taken to statistically analyse impact of experimental variables on maturity subpopulations. The subpopulation proportion data appear to show that as the experiment progresses, the rate of change, specifically of Effector and Effector Memory subpopulations, is reducing up until 144 hours (Fig. 4.5). Analysing the data at an early timepoint would identify those conditions and variables that have a greater immediate influence on the subpopulation proportions. If the objective of the experiment was to identify these effects, this would be pursued; however, the experiment was designed to give a better description of the way the experimental variables impacted subpopulation proportion at the point of transduction. These data suggest that towards the end of this experiment the cultures reached a point of some equilibrium, with populations predominantly consisting of Effector Memory phenotype cells.

In order to effectively answer the objective of the experiment, and given the aforementioned nature of the data, analysis would be performed on data from the final timepoint. As previously mentioned, at 168 hours the change in proportions of Effector and Effector Memory subpopulations from 144 hours goes against the trend of the data, increasing for Effector subpopulations and decreasing for Effector Memory subpopulations. With this the standard deviation also increases substantially from what has been observed previously. These data could be indicative of genuine influences of the experimental conditions affecting the cultures and altering the proportion of Effector and Effector Memory cells present in the cultures. Fresh medium and IL-2 was

supplemented to all cultures after sampling for counting and phenotyping at 144 hours, which when taken in isolation could plausibly have induced this reaction in the cultures. However, the same process, supplementation of fresh medium and IL-2 also occurred at after sampling at 48 hours (only fresh medium in conditions where IL-2 was first added at 96hours) and 96hours, which did not see the same buck in the trend. With this evidence it would appear more likely that either an unobserved element affected the cultures between the samplings at 144 and 168 hours, or that an unobserved factor impacted the phenotyping process at 168 hours.

As the phenotypic data collected at 168 hours, the final timepoint, is now considered inappropriate, the data from the penultimate timepoint should be assessed for its suitability. The population proportion data at 144 hours show the lowest and highest Effector and Effector Memory percentage respectively in each parentage. In addition, the standard deviation is substantially lower than that observed at 168 hours and comparable to those deviations seen previously in the experiment. It would normally be more beneficial to see a higher standard deviation when wishing to identify differences between experimental conditions as it would be indicative of variation in the dataset. However, given the trend of standard deviation of the data sets over time compared with the deviation observed at 166 hours, a reduced standard deviation would be more indicative of the data at this time point being more in line with the general trend, rather than what was observed at the final time point. With this it appeared that the phenotypic data at 144 hours was the most suitable to determine the impact of experimental variables with regards to maintaining higher levels of Naïve and Central Memory phenotype cells in cultures (Fig. 4.5).

4.3.1.2.3.1 Statistical analysis of phenotypic data at 144 hours

Statistical analysis of subpopulation proportion data at 144 hours showed that only IL-2 concentration and Dynabead concentration were independently significant, however all factors had some significant impact when taking into account interactive effects. IL-2 concentration was the most significant factor, with a significantly negative effect on Effector subpopulation proportions of all parent populations when increasing from 0 to 200 UI/mL (Fig. 4.6). Increasing the Dynabead concentration from $0.2x10^6$ to $1x10^6$ significantly increased the proportion of Effector Memory cells in the CD8+ parent population, but not in the total T-cell or CD4+ parent population. No individual factor had a significant impact on Naïve or Central Memory subpopulation proportions within all parent populations.

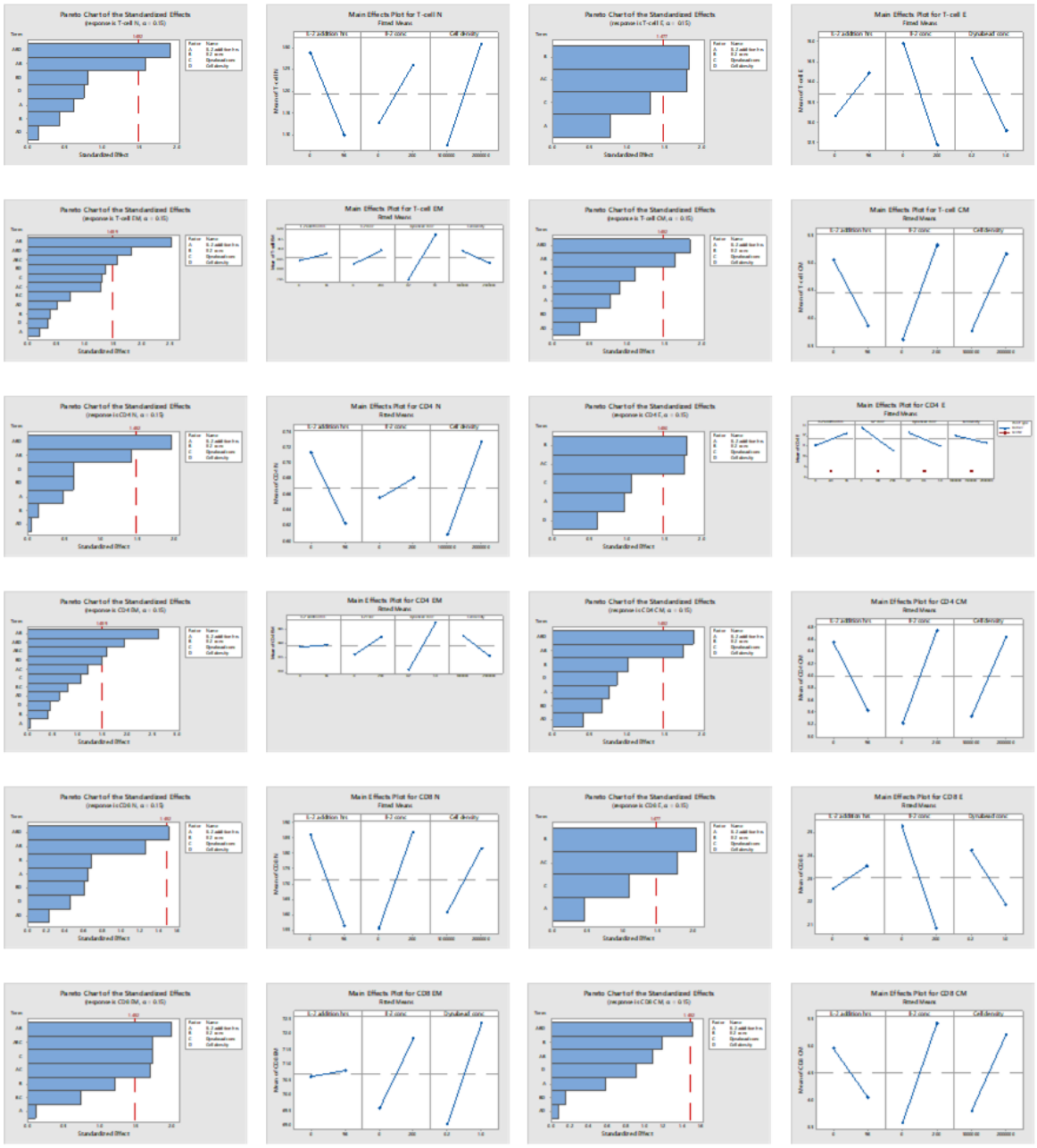


Figure 4.6. Design of Experiment analysis of data at 144 hours shows that IL-2 concentration negatively impacts Effector subpopulation selection. Maturity subpopulation proportion data from 3 parent populations (total T-cell, CD4+ T-cell and CD8+ T-cell) were obtained from analysis of samples taken at 144 hours. These data were then assessed to identify any significant effects of experimental conditions on subpopulation proportions. All subpopulations were significantly effected by at least one individual or combination of factors. No single factor had a significant effect on the proportion of Naïve (N) or Central Memory (CM) proportion at 144 hours from any parentage.

Whilst being the most significant factor independently, IL-2 concentration was also the most significant interacting factor, being an influential part of 19 of the 23 significant factor interactions across all subpopulations. The most common significant two factor interaction was IL-2 concentration and the timing of IL-2 supplementation (Fig. 4.6). Interaction plots show that in total T-cell Naïve and Central Memory and CD4+ Central Memory subpopulations, supplementing 200

UI/mL IL-2 at seeding has a positive effect on their proportions compared to adding at 96 hours. Conversely, the interaction plots show that addition 200 UI/mL IL-2 at seeding has a significantly negative effect on Effector Memory subpopulations of all parent populations compared to adding at 96 hours. These data potentially suggest a relationship between later IL-2 supplementation and a higher Effector Memory subpopulation proportion at a point when the culture reaches an equilibrium of subpopulation proportion at the cost of Naïve and Central Memory subpopulation cells. As the average proportions of these subpopulations across all samples do not reach equilibrium, this is more hypothetical speculation, however that does not preclude the statement from still being true at this timepoint. Meanwhile, the relative range of these variations, from 1.6-0.9% in the total T-cell Naïve, and 78-84% and 7.5-3.5% in Effector Memory and Central Memory respectively, suggests more that the transition is from Central Memory to Effector Memory.

The conclusions drawn so far describe the impact of supplementing 200 UI/mL of IL-2, the upper concentration of this variable within the experimental design, at 0 or 96 hours, however the validity of these conclusions is brought into question when observing the lower level of IL-2 added to cultures, certainly with respect to the Effector Memory subpopulation behaviour. Within this experiment, the lower level of IL-2 (0 UI/mL) was 'supplemented' at 0 and 96 hours as well. As the conditions allocated the lower level of IL-2 would have no IL-2 supplementation, regardless of their allocated supplementation time, it would stand to reason that there would be little to no variation between these two categories of conditions (0 UI/mL IL-2 at 0 hours and 0 UI/mL IL-2 at 96 hours). However, using the Effector Memory subpopulation interaction plots as a demonstration, for which the IL-2 concentration and supplementation time is a significantly interacting pair of factors under all parentage, it is quite clear that this is not the case. Some variation could assume to be observed due to the nature of cell culture, however it is possible to see in the Effector Memory interaction plots of all parent populations that the drop in average subpopulation proportion from 'supplementing' 0 UI/mL IL-2 at 0 hours to 96 hours is roughly equivalent to the rise in average subpopulation proportion from supplementing 200 UI/mL IL-2 from 0 hours to 96 hours. In the case of the total T-cell and CD4+ Effector Memory interaction plots, this effect manifests itself as the 0 hour 0 UI/mL IL-2 and 96 hour 200 UI/mL IL-2 and the 0 hour 200 UI/mL IL-2 and 96 hour 0 UI/mL IL-2 average Effector Memory proportions each approximating each other. Within the CD8+ Effector Memory interaction plot the average Effector Memory proportions of the 0 and 200 UI/mL conditions when supplemented at 0 hours are shown to be more comparable (71.4% and 69.9% respectively), however the same trend is still observed when comparing to the average Effector Memory proportion change in conditions supplemented at 96 hours, where the drop observed between the

early and late supplementation time for the 0 UI/mL supplemented cultures is the same as the rise seen in the 200 UI/mL supplemented cultures.

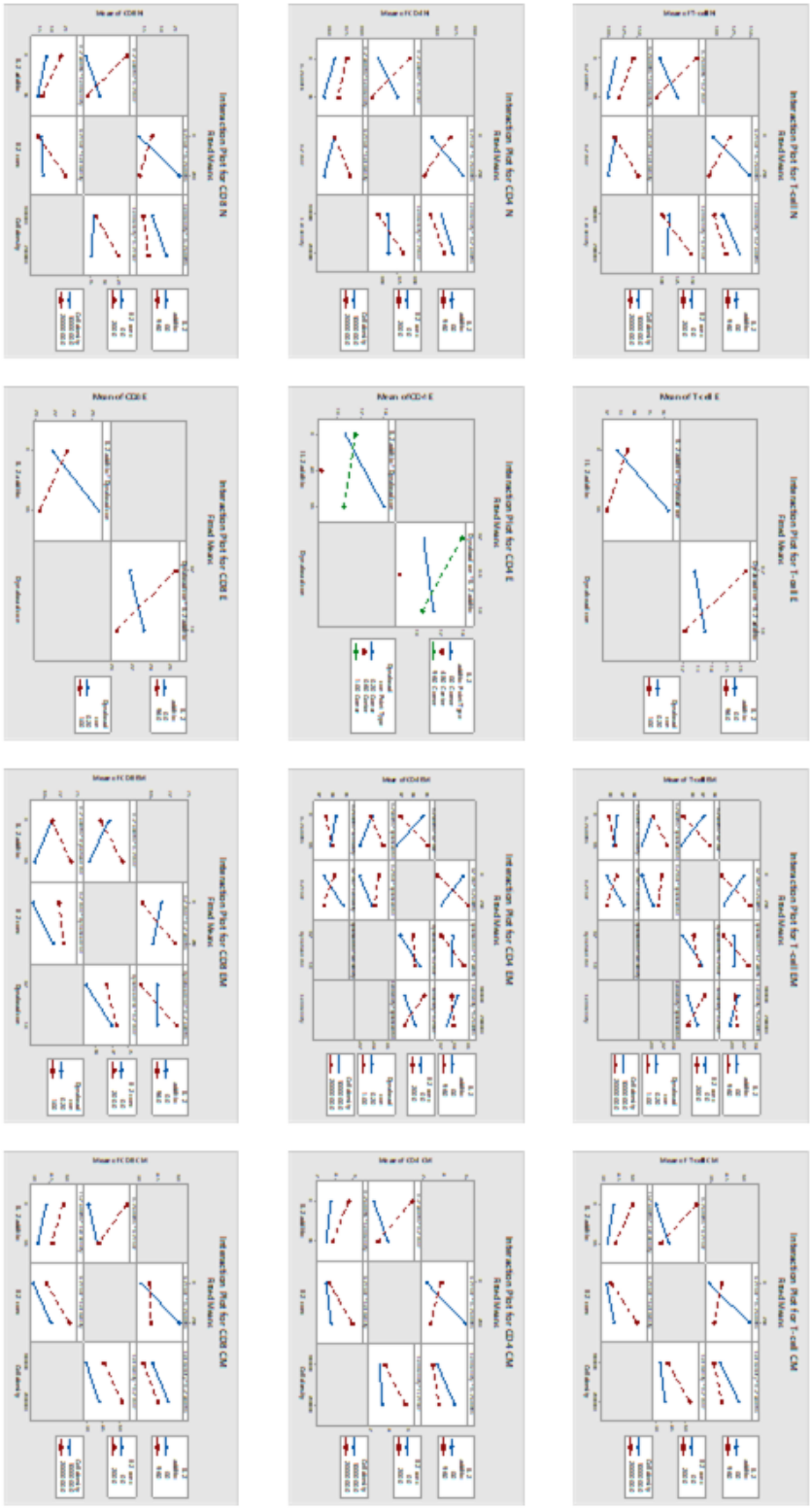


Figure 4.7. Analysis of interacting effects on T-cell maturity subpopulations at 144 hours shows that delayed supplementation of IL-2 had a significantly negative effect on total Naïve and Central Memory proportion, and a significantly positive effect on Effector Memory proportion. Maturity subpopulation proportion data of three different parent populations (Total T-cell, CD4+ T-cell and CD8+ T-cell) were obtained from samples taken at 144 hours and statistically analysed to identify combinatorial effects of experimental variables. Significance of effects observed is presented in Fig. 4.6.

The variation of the average subpopulation proportions of the 0 hour and 96 hour 0 UI/mL IL-2 supplemented cultures, in particular in the case of the Effector Memory subpopulation, identifies a

potential issue with some of the conclusions drawn so far from these data. The difference in average subpopulation proportion between 0 hour and 96 hour supplementation was, as previously described, as great in the 200 UI/mL IL-2 supplemented cultures as it was in the 0 UI/mL IL-2 supplemented cultures, even though the directions of variation were opposed (Fig. 4.7). If it were possible to observe the same level of variation in cultures that were different only by which supplementation time category they had been split into, which had no implementable difference, as there was in two sets of conditions that had genuinely implemented differences, it brings into question whether or not the observed outputs are a real effect from interactions of experimental factors or if they are a result of either coincidental noise or other experimental factors that were not observed.

The randomisation of the experimental order was used to reduce the chance of identifying false effects in the data that could be created by systematic bias. There were no differences in the way that the 0 and 96 hour supplementation samples within the 0 UI/mL IL-2 subgroup cultures were treated during the experiment. As randomisation could still generate patterns, the plate position of cultures were analysed to identify if this could elucidate any information which could be the cause of this variation. Identification of the distribution of samples within subgroups to the first and second row of culture plates was analysed, however across all plates the distribution was at most a 3:5 balance, suggesting that the variation was not caused by back and front row of plate positioning. Secondly the experimental layout was analysed to assess whether culturing within a specific plate would have an impact, however there is no discernible pattern that would correlate with the results that have been identified in the interaction plot. Cultures were also sampled in the randomised experimental order. Given the previously mentioned assessment that the different subgroups of conditions under scrutiny here had no discernible pattern, this reasonably precludes the phenotyping process from causing this effect in the data as it was performed in the same order.

It would appear that the immediately apparent avenues of investigation have been explored regarding the cause of the variation seen in the 0 UI/mL IL-2 conditions, and none of them look to provide an explanation as to why there was variation at the same level as seen in the 200 UI/mL conditions. Therefore, in this scenario it would be concluded that it was the inherent variability in the cultures that caused the difference in average Effector Memory subpopulation proportion between the cultures in which 0 UI/mL IL-2 was added at 0 hours, and those where the same concentration was added at 96 hours, and that this coincidentally correlated to present a significant effect. This also goes for the other subpopulations where this interaction was considered significant, total T-cell Naïve and Total T-cell and CD4+ Central Memory subpopulation proportion. Although in these interaction plots the level of variation of average subpopulation proportion between the

conditions supplemented with 0 UI/mL IL-2 at 0 and 96 hours were not as great as the 200 UI/mL supplemented cultures, the calculation of significance was based on their interaction. If there is noise in the data that is distorting the perception of significance of IL-2 concentration and supplementation timing interaction within one subpopulation, it cannot be accepted in other subpopulations. This is due to the fact that subpopulation presence was measured as a proportion, and as all events in the parent population would be gated into one of the four maturity subpopulations, what affects one subpopulation affects them all and therefore this effect can be considered to be present in all subpopulations.

The false statistical significance of the cultures supplemented with 0 UI/mL IL-2 brings into question the validity of those cultures supplemented with 200 UI/mL IL-2. The statistically significant response in cultures not supplemented with the growth factor at different timepoints could potentially just be a type I statistical error in just those cultures. However, it could also be indicative of there being too great a level of noise in the cultures compared to the genuine response in all cultures analysed in this dimension, which would therefore invalidate the cultures supplemented with 200 UI/mL IL-2. It is not possible to definitively say from the data presented which applies in this scenario, and further work would be needed to validate these responses.

The patterns in the data observed in the Effector Memory subpopulation IL-2 concentration vs supplementation timing interaction plot are not observed in any other significant interactions. The only other interactions identified as significant were IL-2 concentration and Dynabead concentration, and IL-2 concentration and cell density. When assessing the interaction plots that involve IL-2 concentration, the confounding variation would be averaged within each level of the variable. As such it would not invalidate the data, or the observed effects, but reduce the statistical power of the analysis. Meanwhile the same could be said of the interaction of the second two factors, only the averaging of the concerning data would occur within the two IL-2 concentration subgroups. Similarly, in the case of assessing individual factors, the noise in the data would be averaged out between the two levels, and the same statement would apply, that it does not invalidate the data, but does reduce the statistical power of the analysis.

The interaction of timing of IL-2 supplementation and Dynabead concentration had a significant effect on the proportion of Effector subpopulation cells from all parent populations, and the CD8+ Effector Memory subpopulation proportion. The effect that the interacting experimental variables had on the Effector subpopulation proportions was similar across all parent populations. The interaction plot shows that when IL-2 is added at 0 hours, increasing the Dynabead concentration from $0.2x10^6$ beads/mL to $1x10^6$ beads/mL has a relatively small positive effect on Effector subpopulation proportion of between 1-2 percentage points. Supplementing IL-2 at 96 hours has a

large positive effect of Effector subpopulation proportion in conditions supplemented with $0.2x10^6$ beads/mL compared to those where IL-2 is supplemented at 0 hours of at least 3% across all parent populations. Meanwhile, supplementing IL-2 at 96 hours in conditions that had been supplemented with $1x10^6$ beads/mL substantially reduced the average subpopulation proportion, bringing it below the average subpopulation proportion of conditions where IL-2 was supplemented at 0 hours, with either $0.2x10^6$ beads/mL or $1x10^6$ beads/mL regardless of parent population. The main effects and interaction plots of the CD4+ Effector subpopulation were also the only parent population/subpopulation combination that plotted the midpoint data. These data indicate a non-linearity in the relationship between timing of IL-2 supplementation and Dynabead concentration, and average Effector subpopulation proportion. The main effect in this interaction appears to be that when the supplementation of IL-2 is delayed in conditions with low Dynabead concentrations, Effector subpopulation proportions will be increased.

The transition to Effector Memory phenotype subpopulation from Effector is linked to maturation of the cells. Both IL-2 and CD3/CD28 Dynabeads drive this maturation process and it would appear that given the relative similarity in average subpopulation proportion between conditions that have the earlier IL-2 supplementation, higher Dynabead concentration, or both, that culturing with either of these variables at the level expected to induce greater maturation will do so. The main deviation from this is when both variables are at the level at which they would be less expected to induce maturation, $0.2x10^6$ beads/mL and supplementation of IL-2 at 96 hours. When these variables are present in culture a substantial increase in Effector subpopulation proportion is observed. This suggests that either of these two variables at a high level is able to induce a greater level of maturity in cultures, however using them in combination at these levels will not further increase the maturation of the culture. This could potentially be due T-cells responding to a threshold level of IL-2 early in their culture. CD3/CD28 Dynabeads as part of their mechanism of action induce endogenous production of IL-2 in T-cells, and therefore increasing the Dynabead concentration would plausibly increase the level of IL-2 production through increased strength of activation in T-cells and increased number of cells activated.

The evidence that these conditions induce higher Effector subpopulation proportion to the detriment of the Effector Memory subpopulation proportion is present in the CD8+ Effector Memory subpopulation proportion data. The interaction plot for the CD8+ Effector Memory subpopulation shows that there is very little difference in the average subpopulation proportion between conditions supplemented with $0.2x10^6$ beads/mL and $1x10^6$ beads/mL when IL-2 is supplemented at 0 hours. However, delaying the supplementation to 96 hours has a relatively negative impact on average subpopulation proportion in cultures supplemented with $0.2x10^6$ beads/mL and a relatively

positive impact on those supplemented with $1x10^6$ beads/mL. The variation between the 0 and 96 hour IL-2 supplementation was equal if in opposite direction. This evidence concurs with the previous statement that in cultures with later IL-2 supplementation and a lower Dynabead concentration, the Effector Memory subpopulation proportion is reduced. However, unlike the previous evidence, this also shows that supplementing IL-2 at 96 hours to cultures with a Dynabead concentration of $1x10^6$ beads/mL has a positive effect on Effector Memory subpopulation proportion over those where IL-2 was supplemented at 0 hours. It is not clear why a later supplementation of IL-2 in conditions supplemented with $1x10^6$ beads/mL would increase the Effector Memory subpopulation proportion. However, when supplementation occurred at 96 hours the T-cells would have had a longer exposure to CD3/CD28 Dynabeads, and therefore greater level of activation, before exposure to a larger concentration of IL-2. Therefore, this evidence suggests that T-cell cultures can respond differently to IL-2 supplementation depending on activation level in agreement with the hypothesis of this experiment, if not observed in the same output.

Analysis of the residual data from the models produced in statistical assessment for the Naïve, Effector Memory and Central Memory subpopulations of all parent populations showed that they approximated a normal distribution and identified no systematic trends in the residuals when observing by order (data not shown). The same analysis of Effector subpopulation residuals identified a normal distribution as with the other subpopulation analyses. However, analysis of the residuals against the order of observation identified a positive trend as the observational order progressed (Fig. 4.8). The positive correlation is maintained from initial observation through to the final one, and unlike the residual data from the cell yield assessment (Fig. 4.8) there does not appear to be a substantial variation between the 24^{th} and 25^{th} observation despite there being a plate change at this point in the phenotyping process also. There does appear to be a negative shift at this point for the total T-cell and CD4+ Effector subpopulation plots, however this shift moves the residuals to around the level of that seen around 5-6 observations previous, as opposed to that seen in cell yield which returned the residuals to that seen around the beginning of the observations. Furthermore, the randomised order of the experimental conditions will have reduced the chance of false effects being identified. There were previous issues with observed noise in the system, however that was observed most extensively in the Effector Memory subpopulation analyses, which did not show this trend in observational bias. As a result, the shifts observed do not appear to have substantially impacted any conclusions of the analytical outputs, but may have reduced the statistical power of analyses to identify significant effects.

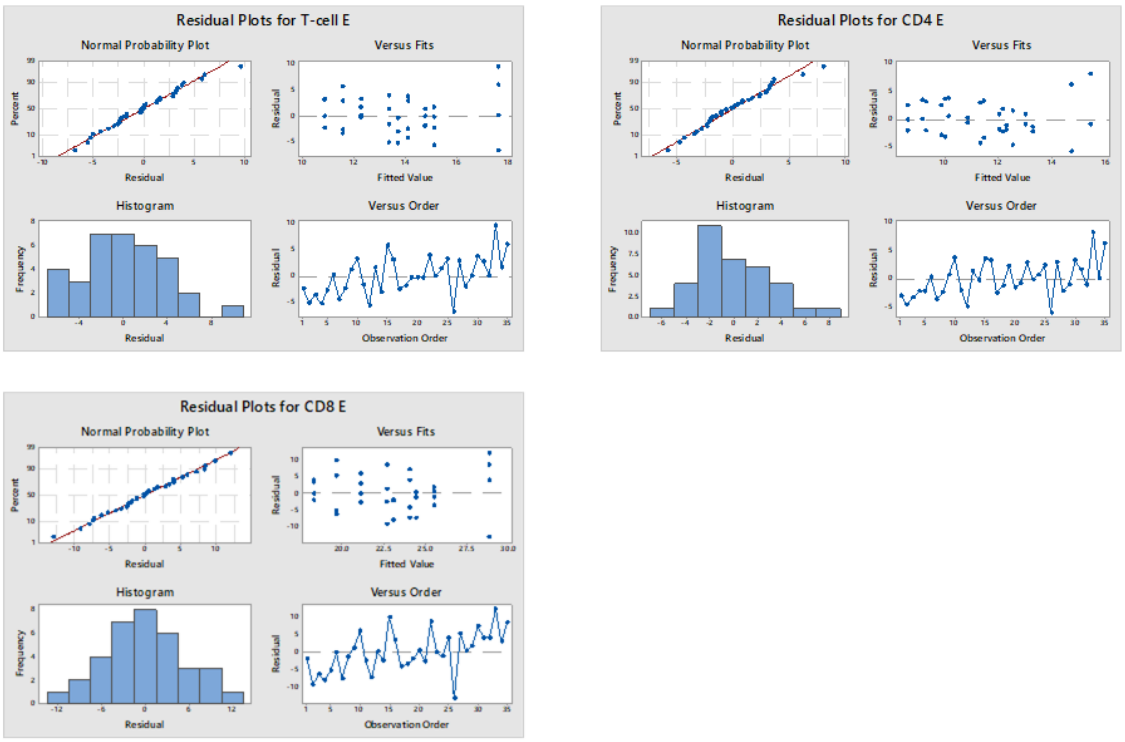


Figure 4.8. Residual plots from analysis of Effector subpopulation proportions at 144 hours show positive correlation between residual and observation order. Statistical analysis of maturity subpopulation proportions of samples taken at 144 hours identified significant experimental variables. Residual data was then assessed to identify if there were any irregularities in the data. Assessment of the residual Versus Order plots shows a positive correlation between the residual and the observation order. This trend is not observed in the residual Versus Order plots for Naïve, Effector Memory or Central Memory subpopulation analysis (data not shown). Due to randomised order of analysis this should not result in false effects being identified, however it will have reduced the statistical power of the analysis of these data.

4.3.1.3 Discussion

The aim of this experiment was to identify if T-cell cultures being exposed to IL-2 before sufficient activation were responding less, or not at all, to IL-2 supplemented and endogenously produced in culture. It was suggested that this was having a negative impact on the growth of T-cell cultures. However, it was speculated that this effect could also be observed in, or could even be caused by, the maturity subpopulations (Naïve, Effector, Effector Memory and Central Memory) proportions in cultures.

In order to assess this hypothesis, the timing of IL-2 supplementation was varied between 0 and 96 hours. To further test if supplementation of IL-2 was having an effect on cultures stimulated with CD3/CD28 Dynabeads, the concentration of IL-2 supplemented was varied between 0 UI/mL and 200UI/mL. CD3/CD28 Dynabead concentration was supplemented between $0.2x10^6$ beads/mL and $1x10^6$ beads/mL to identify if varying the concentration of CD3/CD28 Dynabeads had an effect on the level of maturation or growth. To test if the seeding density in static culture would influence

growth rate or subpopulation proportions, cultures were seeded between $1x10^6$ cells/mL and $2x10^6$ cells/mL

With T-cells expressing either CD4 or CD8, indicative of their function within the body, it was proposed that the maturity subpopulation proportion of CD4+ and CD8+ subpopulations may have varied from each other. Therefore, maturity subpopulations were analysed using CD4+ and CD8+ gated parent populations as well as the total T-cell gated population.

4.3.1.3.1 Response of culture growth rates to experimental variables

All cultures showed a period of growth in culture which correlated with an increase in T-cell event count, which indicated that all cultures were growing T-cells. This was a positive reflection of the input material and culture protocol, regardless of experimental conditions. Growth rates were calculated using viable cell counts taken every 24 hours in order to assess conditions based on their output per input, as opposed to greatest outright yield.

Statistical analysis of the growth rates showed that all experimental variables were independently significantly affecting growth. Meanwhile, the midpoint data indicated that all of these relationships were non-linear, and offered the greatest growth rates when comparing conditions by concentration and timing of IL-2 supplementation, as well as CD3/CD28 Dynabead concentration.

Increasing the CD3/CD28 Dynabead concentration had a significantly positive effect on growth rate, indicating that a greater level of stimulation could induce greater growth. Meanwhile, cell concentration was the experimental variable with the most significant effect on growth rate with the highest seeding density negatively affecting growth rate. The reduced average growth rate for conditions with higher seeding density could have been due to some level of exhaustion of the medium with greater requirements than that of the lower seeding density conditions. Alternatively, it could also have been a reduced level of activation as CD3/CD28 Dynabead concentration was calculated relative to the volume rather than the cell concentration, and increasing the CD3/CD28 Dynabead concentration had been shown to have a significantly positive effect on growth rate. Contributing to the lower average growth rate of the higher density seeded cultures is the substantial drop off in growth of certain conditions from around 96 hours. Although it is possible that this was caused by exhaustion of medium, these conditions were similar in that they were both supplemented with 200 UI/mL IL-2 at 0 hours, and this same trend was not seen in other conditions that were supplemented with 0 UI/mL IL-2 or those where supplementation of 200 UI/mL IL-2 was delayed until 96 hours. This seemingly suggests that these conditions negatively impacted growth in the higher density seeded cultures.

Supplementing IL-2 at 96 hours was shown to have a significantly positive effect on the growth rate of T-cell cultures, correlating with the hypothesis. However, increasing IL-2 concentration from 0 UI/mL to 200 UI/mL has a significantly negative effect on growth rate, contrary to the expectation that increasing IL-2 concentration would have a positive effect on growth rate. The significant interaction of IL-2 concentration and timing of supplementation identifies that conditions where 200 UI/mL IL-2 was supplemented at 96 hours had an average growth rate akin to that where 0 UI/mL IL-2 was supplemented, and that the average growth rate of conditions supplemented with 200 UI/mL IL-2 at 0 hours was substantially lower.

These data validate the hypothesis that there is a negative impact of supplementing IL-2 at seeding on growth rate. However, these cultures show that delaying the supplementation by 96 hours did not improve proliferation beyond those not supplemented with additional IL-2, and that supplementing IL-2 at seeding had a negative effect. Increasing the Dynabead concentration was also shown to have a positive effect on growth rate, indicating that variation in the level of stimulation could vary the growth rate output of the cultures. The non-linear relationship suggested by the midpoint data in all these scenarios suggests that somewhere between the maximum and minimum tested concentration of IL-2, timing of IL-2 addition and Dynabead concentration would be a condition that generated the greatest growth.

4.3.1.3.2 Low level of significant effects on phenotypic profile indicates limited phenotypic effect

Statistical analysis of the maturity subpopulation (Naïve, Effector, Effector Memory and Central Memory) proportions at 144 hours showed very few significant effects over the 12 sets of data (4 maturity subpopulations from 3 different parent populations).

The only independently significant variables were IL-2 concentration and Dynabead concentration. Increasing the concentration of IL-2 supplemented into cultures had a significantly negative effect on Effector subpopulation proportions from all parent populations, meanwhile increasing the CD3/CD28 Dynabead concentration had a significantly positive effect on CD8+ Effector Memory subpopulation proportion.

The negative effect of increasing IL-2 concentration on Effector subpopulation proportions is likely due to IL-2 inducing maturation faster in T-cells and causing transition from Effector to Effector Memory phenotype subpopulations. Similarly, the positive effect that increasing the Dynabead concentration had on the CD8+ Effector Memory subpopulation proportions is likely down to a greater level of stimulation inducing faster maturation and therefore faster transition from Naïve and Effector subpopulation phenotype to Effector Memory subpopulation phenotype.

The interaction between concentration of IL-2 supplemented to culture and the timing of supplementation was also identified as having a significant effect on the total T-cell Naïve, Effector Memory and Central Memory, CD4+ Effector Memory and Central Memory, and the CD8+ Effector Memory subpopulation proportions. However, analysis of the data showing this identified the same variation in average subpopulation proportion between the conditions 'supplemented' with 0 UI/mL IL-2 at 0 and 96 hours as was between the conditions supplemented with 200 UI/mL IL-2 at 0 and 96 hours. Potential causes of the variation were investigated, predominantly focussed around plate positioning due to the randomised experimental order but yielded no obvious answer. As a result, it was concluded that the variation seen in the 0 IL-2 supplemented cultures was the result of natural variation in the cultures that happened to present as a meaningful difference despite no differences in the way that the cultures were treated. Because the level of variation of average subpopulation proportion observed in the 0 UI/mL IL-2 supplemented cultures was the same as that in the 200 UI/mL IL-2 supplemented cultures when comparing timing of supplementation, it was further concluded that these interactions should be discounted as their validity was called into question. However, this did not invalidate other observations of individual variables or 2 factor interactions.

The interaction between the timing of IL-2 supplementation and Dynabead concentration was significant in the Effector subpopulation proportions from all parent populations, as well at the CD8+ Effector Memory subpopulation proportion. In Effector subpopulations, varying the Dynabead concentration made little difference when supplementing IL-2 at 0 hours. However, supplementing IL-2 at 96 hours had a slightly negative effect on the average Effector subpopulation proportion relative to the 0 hour supplementation proportions in those conditions supplemented with $1x10^6$ beads/mL. Conversely a substantially positive effect was seen in those conditions supplemented with $0.2x10^6$ beads/mL. Meanwhile, supplementing IL-2 at 0 hours negated any effect of CD3/CD28 Dynabead concentration on the CD8+ Effector Memory subpopulation proportion, but in conditions where IL-2 was supplemented at 96 hours the inverse of the Effector subpopulations was seen, where a higher CD3/CD28 Dynabead concentration positively affected Effector Memory proportion while a lower concentration negatively affected it.

IL-2 and CD3/CD28 Dynabeads both induce maturation in T-cells through activation and activated cells in culture will transition to a memory phenotype. Therefore, it is logical that conditions with low Dynabead concentration and delayed IL-2 supplementation have a higher Effector subpopulation proportion and lower Effector Memory subpopulation proportion. The relatively large drop in Effector subpopulation proportions observed where IL-2 is supplemented earlier or Dynabead concentration is higher (or both), paired with the significant decrease observed with

higher IL-2 concentration shows that this subpopulation is sensitive to conditions which promote maturation.

The interaction between the concentration of IL-2 supplemented into culture and the seeding cell density was significant in the CD4+ Effector Memory subpopulation. The data show that when no IL-2 is supplemented into culture, increasing the seeding density slightly increases the CD4+ Effector Memory subpopulation proportion. However, with additionally supplemented IL-2, it is the reduced seeding density that has a substantially positive effect on subpopulation proportion. Meanwhile, the higher seeding density reduces the average subpopulation proportion observed to the same level as the non-supplemented, lower cell density conditions. Potentially this was a result of increased cell density, and induction of greater proliferation through increased IL-2 concentration, exhausting the medium of an unmonitored factor required for transition to Effector Memory subpopulation phenotype. However, further investigation would be required to identify the nature and cause of this relationship.

4.3.1.3.3 Phenotypic Analysis Process

The analysis of phenotypic data from individual timepoints is a common method of assessing the effects of experimental variables on subpopulation proportions. Analysis of phenotypic data from two different timepoints can be compared to identify how the shift in subpopulation proportion relates to experimental conditions. With each increase of the temporal resolution the definition of the culture system that is being analysed is improved.

The aim of this experiment was to identify what effects the experimental conditions would have on T-cell subpopulations, with specific interest in the Naïve and Central memory subpopulation proportions. Therefore, in this scenario it was not necessary to monitor subpopulation proportions over time, and the selection of data from samples at 144 hours was previously justified.

A further limitation of this style of analysis is that the user must either select to evaluate subpopulations based on the number of events identified within their gate, i.e. the size of the subpopulation, or based on the number of events identified within their gate compared to the total events within the parent population, i.e. the proportion of the subpopulation. There are advantages and disadvantages to each process. If the user evaluates subpopulations based on their size then analysis could identify conditions that would produce a high yield of the specific subpopulation, with no consideration for the purity of the population that would be produced. Similarly, if the user evaluates subpopulations based on their proportion, analysis would be able to identify conditions that would induce a higher concentration, or more pure population of the specific subpopulation,

but without cross referencing with relevant cell counts would have no understanding of the cell yield these conditions would produce.

In addition, the analysis relies on the accuracy, and when analysing multiple timepoints consistency, of user defined gates applied to data during assessment. Furthermore, due to the multiple levels of gating that occurred in this experiment, consistent with other work in the field, a relatively small variation in gate placement early on could have a large impact downstream. This also highlights the issue of how suitable the gates used to identify T-cell subpopulations are. One theoretical example of the inadequacy of this method would be where the number of events in a subpopulation gate of two different samples could be similar or identical, however density plots of the populations would show them to be substantially different given the variation in marker expression that the gate size allows. The current process would not allow for effective or efficient classification of these events as different and distinct. Analysis of density plots produced by phenotypic data in this experiment indicate instances of these occurrences and suggest that the current analytical method does not have a phenotypic resolution sufficiently high to effectively assess experimental cultures.

4.3.1.4 Conclusions

This experiment hypothesised that supplementation of IL-2 into cultures immediately after seeding was having at least no positive effect on growth and possibly a negative effect on the growth rate. It was speculated that this was due to T-cells not responding to IL-2 in the same way, or at all, when exposed before activation via stimulation such as CD3/CD28 Dynabeads.

Although the mechanics and pathways of how this effect comes to pass have not been defined as part of this experiment, the evidence shows that delaying the supplementation of IL-2 does positively affect the growth rate of T-cell cultures. Furthermore, it suggests that delaying the supplementation of IL-2 for 96 hours has no positive effect on growth relative to cultures where IL-2 was not supplemented, and that supplementing IL-2 into cultures immediately after seeding will have a negative effect on growth rate.

Increasing Dynabead concentration had a significantly positive effect on growth rate, indicating a correlation between level of activation and proliferation. Increasing the cell density had a negative effect on growth rate, however given the observed effect of Dynabead concentration, and the fact that the concentration of beads supplemented was calculated based on well volume rather than bead to cell ratio it is plausible that this reduction was observed courtesy of the cells' reduced exposure to stimuli and ergo a reduced level of activation.

It was also speculated that the deferred supplementation of IL-2 may also have significant effects on maturity subpopulation (Naïve, Effector, Effector Memory and Central Memory) proportions in

culture. Although all effects are of interest to better define the T-cell culture system, any effects on the CAR T-cell manufacturing targets of Naïve and Central Memory were of particular interest.

Analysis at the selected timepoint of 144 hours showed some anticipated trends such as increasing IL-2 concentration significantly reducing Effector subpopulation proportions and Increasing Dynabead concentration significantly increasing some Effector Memory subpopulations. Analysis also showed that delaying supplementation of IL-2 in cultures with lower Dynabead concentrations also had a significantly positive effect on Effector subpopulations at 144 hours, while those same conditions had a negative effect on some Effector Memory subpopulation proportions.

Some variable interactions identified as significant had to be discounted due to variation between conditions 'supplemented' with 0 UI/mL IL-2 at 0 and 96 hours being as great as those where 200 UI/mL IL-2 was supplemented at those same intervals. Given that the cultures supplemented with 0 IL-2 at different timepoints were functionally identical, it suggested that the natural variation in the cultures was producing a false effect, and that the same level of variation identified between conditions that were functionally different could potentially not be considered a genuine effect courtesy of this. The chance that the significant response seen in cultures supplemented with 0 UI/mL at different timepoints was a type I statistical error, rather than being indicative of noise across all cultures, means that further work is required for a definitive answer.

The general lack of significant effects on subpopulation proportions, especially when compared to the growth rate analysis, appears to show that at 144 hours there was not a substantial variation in subpopulation proportions between different conditions. This is particularly true of the target subpopulations, Naïve and Central Memory, which were only significantly affected by the interaction of timing and concentration of IL-2 supplementation and seeding density and the interaction of timing and concentration of IL-2 supplementation. As a three factor interaction, the relationship between the concentration and timing of IL-2 supplementation could not be visualised in 2 dimensions for analysis, and the interaction between concentration and timing of IL-2 addition had to be discounted due to the previously mentioned issue.

The limited significant effects observed in the phenotypic analysis could also have been caused by the inadequacy of the analytical process they underwent. Development of a novel analytical process that did not rely on user defined gates, dynamically evaluated phenotypic data across multiple timepoints and was able to optimise the phenotypic resolution to the dataset would give more reliable analytical outputs. Furthermore, it could potentially identify effects that could not be observed using the standard protocol .

4.3.2 Development of optimal phenotypic resolution and definition analysis

Phenotypic analysis of a series of T-cell cultures using common analytical methods in the field highlighted the inadequacy of the common practises used to define phenotypic subpopulations in T-cell cultures. Current gating strategies do not account for the level of variability in marker expression seen within populations, and user defined gates are subject to bias based on numerous factors. Furthermore, the analytical process is temporally static, not acknowledging the dynamic nature of T-cell subpopulation behaviour in culture over time. It is possible to take data from multiple timepoints to analyse and then compare results, but this process is still individually applied to each timepoint, and it is the comparison data between these timepoints which adds the dynamic element.

The analytical process fails to effectively define variations between cultures and doesn't take into account the dynamic nature of marker expression over time, and as such it is not sufficiently fit for purpose. These issues also reduce the power of statistical analysis on this data, as a less resolute definition of a culture system can only result in less resolute conclusions being drawn regarding the effect of experimental variables and could potentially overlook important relationships in culture.

A novel analytical method was developed to analyse phenotypic data from T-cell cultures using the observations above as a basis from which to improve the process. This process was then applied to the previously analysed data to identify if there were any differences in the main conclusions and if there were any additional observations.

4.3.2.1 Spanning-tree Progression Analysis of Density-normalised Events (SPADE)

SPADE is an algorithm based analytical tool for processing flow cytometry data. This tool uses an algorithm and user-defined parameters to assess phenotypic data for nodes of commonality and will individually associate all events to a node based on similarity of phenotypic profile.

4.3.2.1.1 SPADE process

The SPADE process involves 4 main steps to analyse flow cytometry data. Density dependent downsampling removes outlier events and those in high density clusters to ensure that smaller phenotypic groups are proportionally increased for the clustering step. Iterative clustering is then carried out to concentrate the remaining events to as many points as is outlined in the analysis parameters. Once the nodes are selected a minimum spanning-tree is calculated to link clusters according to phenotypic similarity. Finally, all previously removed events are upsampled into the SPADE tree by association with the node which their phenotypic profile most similarly resembles.

The clustering process is performed on all samples within the analysis as a multidimensional data cloud. In order to effectively define how populations vary over time, data from all timepoints was

analysed together. As a result, this process is able to identify similarities and differences between samples across time and condition. However, there are some initial preparation tasks that must be completed before the analysis can be performed.

4.3.2.1.1.1 Preparation of data and parameters for SPADE analysis

4.3.2.1.1.1.1 File upload

SPADE is a web hosted analytical tool provided by Cytobank. As it is not device based, the first step in SPADE analysis is to upload FCS files to Cytobank's external servers via their web portal.

4.3.2.1.1.1.2 File naming

The flow cytometer that was used to acquire the phenotypic data would give the same filename to samples that were run from the same wells at different timepoints. This raised issues when identifying which samples were to be associated with which timepoints subsequent to analysis. It was possible to go through the folders and manually change the filenames to easily identify them post analysis, however this was laborious and time intensive as well as increasing the risk of human error. Therefore, a program was written in python code and compiled as a windows executable. The program would append a user defined timepoint to all files within a folder, allowing for faster and more reliable labelling of files for easy identification.

4.3.2.1.1.1.3 Gating requirements and strategy

Having uploaded samples to external servers, the next step is to apply gates to identify populations for processing.

One of the main issues with the traditional phenotypic assessment process is that the gating process is not sufficiently nuanced enough to identify differences between cultures, however it is important to note that gating in this context is not the same as in standard phenotypic analysis. Gating was previously implemented to identify which subpopulation cells belonged to. In this scenario the application of gates is to identify the groups of events to be analysed rather than to define the subpopulations. This includes removing unnecessary or irrelevant events that would confuse, obfuscate or unnecessarily increase the optimal node number of the analysis.

In line with this goal, two gates were applied to data prior to analysis. This gating process fell in line with the commonly used practice seen previously, as the first steps in that protocol are also to identify T-cell populations from total collected data. Polygonal gates were used to ensure that the gates were most effective at capturing all relevant events, whilst eliminating the majority of those that were not. As the aim of the gating process different to that of the common protocol, they were applied in a fashion such that in the event of uncertainty on the part of the user, populations or

events were included rather than not so as to reduce user bias on the data prior to analysis with SPADE.

The initial step was to identify events that matched the correct size profile on a plot of forward scatter area (FSA) against side scatter area (SSA). Having gated the 'Cell' population, this subpopulation was then plotted on a graph of CD4 expression against CD8 expression. A gate was then applied to identify events that would be classified as T-cells, those which expressed high levels of CD4 or CD8, and isolate out non T-cell events that would be present. The latter gate was particularly important at earlier timepoints due to the higher concentration of non T-cell PBMCs still in culture.

Initial investigation into gating prior to SPADE analysis tested both no gating and gating only 'Cell' events. However, the resulting SPADE diagrams and outputs produced were saturated by the level of non T-cell activity, making it an ineffective dataset for T-cell analysis, particularly in the ungated analyses. As the objective was to give greater sensitivity to T-cell subpopulation analysis, it was considered appropriate to gate for T-cell events and run SPADE analysis on this population.

All gates were applied globally; sample specific gating was possible, however creating bespoke gates for all samples would have introduced user bias and variability and as such was deemed unnecessary and inappropriate. At both gating levels the gate was applied to the first sample presented and checked against all other samples. Where target populations were observed outside of gates, borders were moved to ensure inclusion and this automatically updated all other samples.

4.3.2.1.1.1.4 SPADE analysis parameters

After identifying the populations for analysis, the final step before the SPADE assessment could be performed was to define the parameters under which the analysis would be run. The parameters for definition were the Target Number of Nodes (TNN), Downsampled Events Target, Population and Clustering Channels.

Population refers to the population that will be analysed and would be the gated T-cell population. Clustering Channels refers to the flow cytometry channels, synonymous for this purpose with markers, by which data should be analysed and then clustered in the assessment. A 5 marker panel was used to phenotypically assess populations and define which T-cell subpopulations, if any, they would be classified under. It was these 5 markers (CD4, CD8, CCR7, CD45RA and CD45RO) that were selected as clustering channels. As a result, all analysis of data is in 5 dimensions.

Downsampling in SPADE, to be discussed further in 4.3.2.1, is the process by which events are removed in a density dependent manner to give a more equal value to rare dense populations that may usually be overlooked in standard analysis. Downsampling is carried out in the context of events

in the population selected for analysis, rather than the total populations. SPADE analysis requires the user to define the level of downsampling that will occur, and this can be evaluated in two ways. The Downsampling Events Target can either be set as a percentage of total events or as an absolute number, and is applied to each sample individually. A fixed volume was run for analysis at each timepoint, rather than a fixed number of events, meaning that the number of events over time, and between samples, varied based on the total cell count at that time. As it was anticipated that different subpopulations would have different growth rates it would have been unsuitable to downsample based on an absolute value as this may have unfairly favoured certain clusters, even when executed in a density dependent manner. Meanwhile, downsampling on a proportional basis would ensure an appropriate representation of the phenotypic clusters present. On creation of a new SPADE analysis via the Cytobank website the default setting was to downsample to 10%, and this was left unchanged. The value indicates the percentage of total events that will remain after downsampling, rather than the proportion of events that will be removed. For example, with 10% downsampling of a sample with 1000 events, 900 would be removed and 100 would remain.

The final parameter to define is one of the key benefits of SPADE analysis as a tool to improve over the standard analytical process, the TNN. During the clustering stage of SPADE analysis this value defines the number of nodes to which the population is reduced to represent the full phenotypic landscape the whole population occupies. Manipulation of this parameter is what provided the ability to vary the phenotypic resolution with which the analysis was carried out, or in other terms vary the number of subpopulations the total population was divided into. As such this value was varied in due course of the analytical process in a manner which will be described later.

4.3.2.1.1.2 Downsampling stage

In SPADE analysis, events are removed in a density dependent manner to preserve rare populations that may normally be overlooked by many clustering algorithms. This ensures that in the next step of the SPADE process the smaller, more rare subpopulations will still be represented in the diagram output.

The process is applied on a sample by sample basis. A 5 dimensional neighbourhood is calculated so that it is the smallest it can possibly be whilst ensuring that all events also have at least one other event in their neighbourhood. Once the neighbourhood is defined, the local density can then be calculated and treated as follows:

If the local density is below the algorithm calculated outlier density the event is removed

If the local density is above the algorithm calculated outlier density but below the target density the event is kept

If the local density is above the target density, then the likelihood that the event will be retained is target density divided by the local density

Once the number of events remaining matches the downsampling target the process progresses to the next step.

4.3.2.1.1.3 Clustering stage

Clustering is performed globally, rather than at a sample level, by analysing all downsampled events together. This is to ensure that the node definitions are conserved between samples for consistency and to aid comparison. At the beginning of the clustering process, each event remaining after downsampling is considered its own point. In the first round of clustering a random event is selected and grouped with the event nearest to it. Another random event is then selected and grouped with its nearest event. If the nearest event in a point's neighbourhood has already been grouped, that point is skipped. After this has been carried out so that all events have been either paired or removed the process repeats itself, this time using the average of the groups as their datapoints. This process of iterative clustering continues until the target node number is reached.

4.3.2.1.1.4 Spanning-tree construction

In the next step, SPADE links the nodes of most similarity using Boruvka's algorithm. This process is not of particular importance to the novel analytical process developed as each node is individually assessed for marker expression regardless of spanning-tree position.

4.3.2.1.1.5 Upsampling stage

Once the spanning-tree has been constructed, any events removed through the process so far are upsampled back into the diagram.

Every sample is compared against the downsampled data used for clustering to identify its 5 dimensionally closest neighbour. The event is then assigned to the node from which the closest neighbour ultimately came.

4.3.2.1.1.6 Results and outputs of SPADE analysis

There are two main outputs of the SPADE analysis; a comprehensive dataset for each node of each sample covering a range of categories and a SPADE tree (Fig 4.9).

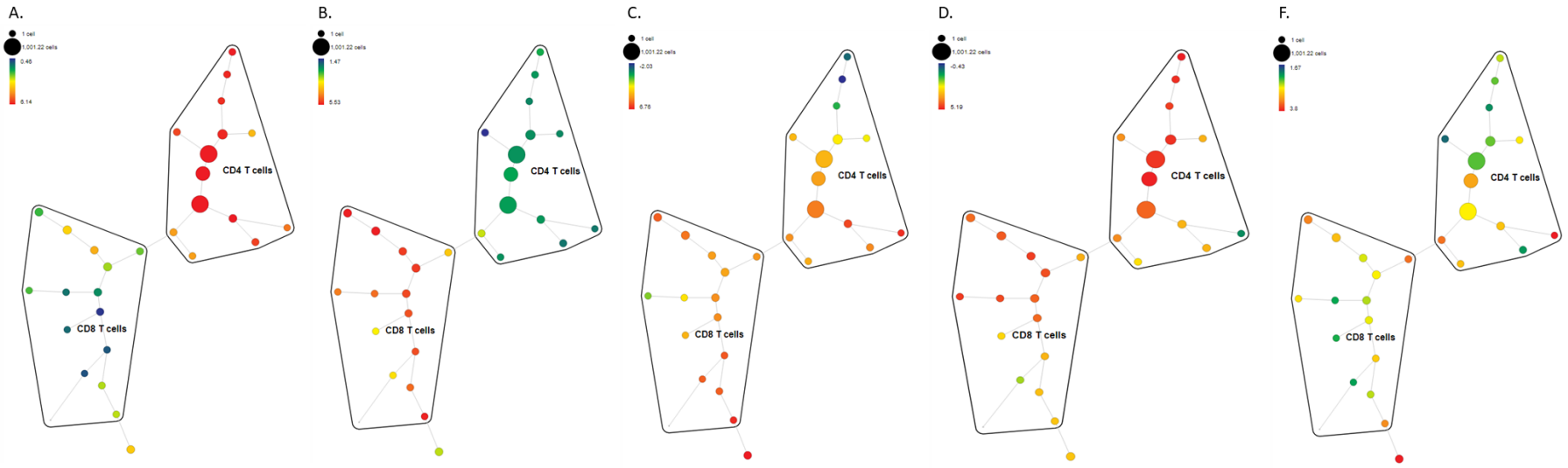


Figure 4.9. Representative SPADE diagrams heatmapped for marker expression from a 30 node analysis show the more nuanced approach the process has to subpopulation definition. Phenotypic data obtained via flow cytometry was processed using the SPADE analysis utility available from Cytobank. The nodes in resultant diagrams are coloured to represent the expression of CD4 (A), CD8 (B), CD45RA (C), CD45RO (D), and CCR7 (E) according to the individual scale with blue/green representing low expression and red representing high expression. The size of the node indicates the number of events that have been attributed to the node according to the attached scale. There is a relatively clear division between CD4+ and CD8+ cell containing nodes, having been separated by the analytical process. Candidate nodes can begin to be identified as potential nodes of interest, however further validation is required to confirm this.

The shape of the tree and links between nodes is the same across all samples, however via the online portal it is possible to view how this tree changes depending on sample and metric being viewed. A separate SPADE diagram exists for every sample, and each node represents a different subpopulation generated through the clustering and upsampling steps. Within a SPADE diagram the size of the node represents the event count within the node, whilst the colour corresponds to a heat mapping key for a marker currently being visualised. This key goes from blue, representing the lowest expression, through green, yellow and orange to red, which represents the highest expression.

SPADE diagrams are a good method for user friendly presentation of the data, and can assist in certain levels of subpopulation characterisation, such as facilitating simple identification of CD4+ and CD8+ nodes.

While the SPADE diagrams provide a digestible overview of the data, the comprehensive dataset allows for a more in depth analysis. These data are essential for the effective statistical analysis of data which is to come.

4.3.2.1.2 Comparison to traditional gating

The process outlined here highlights how SPADE is a beneficial tool in identifying T-cell subpopulations and is more considerate of observable population clustering than the traditional gating method. The algorithmic classification of the subpopulations reduces the user role to outlining the population to be analysed and defining values for the parameters within which the process will run. As a result, this reduces the chance of user bias being introduced into the process due to the inherent variability of user gating. Furthermore, the algorithmic clustering and upsampling processes used to generate and populate nodes does not restrict subpopulations to rigid gates and can instead generate populations that have strong intrinsic similarities despite appearing initially diverse.

The ability to define the TNN also allows flexibility in the resolution of the phenotypic analysis, one of the main flaws highlighted with the traditional method. This ability to increase the resolution gives the opportunity to define populations that would otherwise be assimilated into broad gates in traditional gating, as well as those that may be missed or mistaken as part of larger event clouds in subjective visual user analysis.

There is a trade off in balance when selecting the TNN of a SPADE analysis. A low TNN will likely cluster phenotypically different subpopulations together, potentially rendering the analysis either only as good as, or worse the traditional method. Increasing the TNN will increase the separation of these subpopulations as more phenotypically distinct clusters can be defined, however eventually

the number of clusters formed exceeds the number of subpopulations different in practical significance for any particular purpose. At this point the clustering process will begin to produce nodes, or populations, that are minimally distinct from others. The objective of this new analysis process is to increase the resolution of the analysis in line with the number of distinct populations, and subdividing it beyond this level will lead to repeat conclusions of similar populations, and will also increase noise in the system as minimal differences dictate node association, introducing large variation in the number of events in a node.

4.3.2.2 Effect to Noise ratio analysis

In order to optimise the TNN that should be used to analyse a dataset in SPADE, a process was required that could assess an analysis of a specific TNN for its suitability and compare to others. The Effect to Noise ratio (E2N) analysis process was developed to compare these analyses and identify an optimal TNN for analysis of the dataset.

4.3.2.2.1 Premise of E2N calculation

Any data set produced by varying experimental parameters with repeat conditions will have a level of variation induced by two elements, the inherent noise of the dataset, and variation caused by different combinations of experimental factors. Taking the standard deviation of the total dataset would give the total variation in the dataset, combining the variation induced by both the noise and the experimental conditions. Calculating the standard deviation between replicates of the same condition would negate the experimental condition effect and give the level of variation caused by noise. Calculating this value for every set of repeated conditions and taking the mean would give the approximate level of noise across the dataset. Comparing the standard deviation of the total dataset to the average standard deviation of the replicates would give a ratio of experimental effect to noise in the system. This value would be expected to change with changing node resolution as increasing population discrimination with increasing nodes will lead to increasingly coherent behaviour of cells within a node in response to experimental variables.

4.3.2.2.2 Selection of node measurement

SPADE calculates many node specific values for each sample in the analysis that could be used to compare this level of variation. These outputs are predominantly concerning the clustering channels, describing the average marker expression level of all events associated with a node. However, these values only assess one dimension of the node's phenotype. For example, when comparing CD4 or CD8, there would be a large variation between those that were cytotoxic and those that are helper/regulatory, but within those groups there would be little variation. This would almost completely negate the differences that would be observed in other channels which help to define the maturity subpopulations.

The output that was ultimately selected for this comparison was the number of events that are associated with a node. Association of an event with a node is dependent on similarity of expression profile, and as such this metric considers all channels that were used for clustering. Variation in the node count between samples taken at the same timepoint would be indicative of different numbers of cells in the represented subpopulation at that timepoint, meanwhile variation between node counts of samples taken from the same condition over time would be indicative of subpopulation variation over time.

4.3.2.2.3 Effect to Noise ratio implementation

As demonstrated previously, the phenotypic profile of cultures varied over time. Phenotypic data from any single timepoint, even across all cultures, could represent a different phenotypic space to that of another, and would result in a different distribution of SPADE defined clusters. Therefore, SPADE analysis was carried out on all experimental data to ensure that the nodes generated were representative of the full spectrum of phenotypic activity across the course of the experiment. In addition, analysis of the full dataset would allow tracking of subpopulations over time to identify experimental impact on proportion and transition. The phenotypic variability of the cultures over time and the increase in event count over time due to increasing viable cell counts indicate that calculating the Effect to Noise ratios across the full timeframe of the experiment would not involve comparing data sampled from cultures in a like for like state. Comparison of conditional repeats over time would increase the level of variation in node subpopulations that substantially changed through the experiment. Although the standard deviation of the total dataset would also increase, it is likely that these would not be proportional, producing an E2N that was less effectively representative of the SPADE analysis.

Calculating E2N ratios for each node within each timepoint will allow for more appropriate comparison of data between conditional replicates as well as with the total variation within a node. This method would also allow tracking of E2N over time and improve the understanding of which timepoints or periods measured within this experiment show greatest experimental effect.

As such, the E2N was calculated for each node, at each timepoint, using the following calculation:

$$\frac{\textit{Standard deviation of the counts within a node for all samples at timepoint } x}{\textit{Mean of the standard deviations of conditional replicate counts at timepoint } x}$$

With this calculation, theoretically the lowest E2N that could be calculated would be 1, as this would represent that all the variation in the node at a timepoint is due to the noise in the system, rather than any experimental effect. However, due to the use of duplicate replicates in the experiment the STDEV.S function in excel was used for calculation of the standard deviation. This took the provided data as representative of a sample from the total available responses, rather than defining it as the

complete set. Testing E2N calculation process with random values showed that the lowest value of E2N produced was consistently closer to 1.25, likely due to the approximation of the standard deviation value from the sample provided. Courtesy of this, the threshold for experimental effect in subpopulations will be at 1.25 rather than 1. In datasets where experimental variables are having effects on the phenotypic expression of cells, the calculated E2N ratios can then be used to compare SPADE analyses with different TNN for optimisation.

4.3.2.2.4 Running SPADE analyses with varying target number of nodes

Having developed a method to assess SPADE analyses, the next step was to generate the data by running SPADE analyses with different TNNs. The traditional method of culture analysis generated 8 subpopulations, the 4 maturity subpopulations (Naïve, Effector, Effector Memory and Central Memory) of the two T-cell classifications (CD4+ and CD8+). This process was in part deemed inappropriate due to the insufficient number of subpopulations that were generated, and therefore it appeared reasonable not to begin with a TNN lower than this.

For simplicity, the lowest TNN tested was chosen to be 10. Further SPADE analyses were then run, increasing this value by 5 each time until a TNN of 30 was tested, before a 6th and final SPADE analysis was run at a TNN of 40. The limit was set here, and further analysis with a higher TNN could have been completed were it deemed necessary.

4.3.2.2.5 Comparison of SPADE analyses

After generation of the SPADE analysis data for different TNNs the data needed to be compared to define the optimal node number for the dataset.

4.3.2.2.5.1 Selection of point of greatest experimental effect

Over time the phenotypic impact of experimental conditions varies, identified in the variation of E2N over time. In order to streamline the optimal TNN selection process and avoid having to compare all 980 generated E2Ns with each other, it was decided to compare data from a single timepoint rather than all timepoints. For the optimisation to be most effective it made the most sense to compare E2Ns from the point at which there was the greatest experimental effect. At this point there would be the greatest difference between different conditions and therefore likely the greatest number of nodes would be required to describe the full dataset.

The data were assessed to identify the point of greatest experimental effect from which the optimal node number could be identified. Box plots were produced for each SPADE analysis, with each box representing a different timepoint. Each boxplot graph was analysed to identify which point or points showed the greatest variation (Fig. 4.10). These conclusions were then compared with each

other to identify any commonality between analyses of different TNN. Unfortunately, loss of data from the samples taken at 24 hours has resulted in their omission from the subsequent analysis.

Analysis of the data in this fashion shows a common trend relatively conserved between the analyses. It is possible to see a general decrease in E2N from 48 to 96 hours, a step change is seen between 96 and 120 hours, before seeing the same decrease from 120 to 168 hours as was seen between 48 and 96 hours.

The two points of greatest variation observed over this experiment are at 48 and 120. In comparing these two across all analyses it is possible to see that the mean at 120 hours approximates or is greater than the mean at 48 hours. Furthermore, the upper quartile is always higher than that of the 48 hour results. As a result, 120 hours was selected at the point of greatest experimental effect moving forwards.

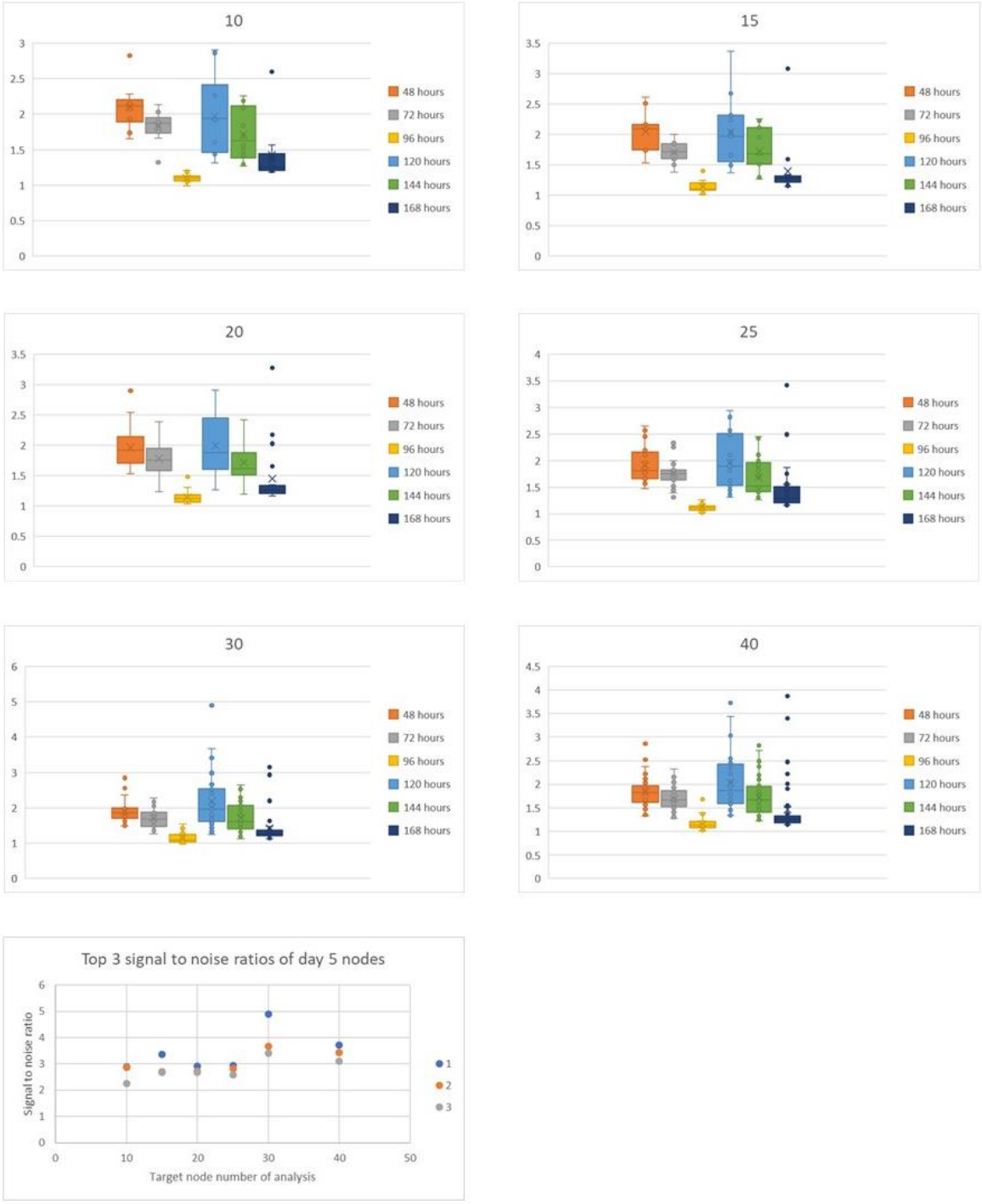


Figure 4.10. Grouping signal to noise ratios by timepoint identifies day 5 as the point of greatest variation, from which 30 nodes can be identified as the optimal target node number for this dataset. Data from analysis at each phenotypic resolution were grouped by timepoint and displayed in boxplot format. Plots showed a conserved trend of overall decreasing signal ratios from day 2 to day 4, followed by a positive shift to day 5, and then a similar decline until day 7. Day 5 was identified as the day of greatest variation and the top 3 signal to noise ratios from each resolution of analysis at day 5 were plotted. There was an increase in ratio from 10 to 15 nodes, which when plateaued until 25 nodes. The ratio then increased again at 30 nodes and stayed similar for the 40 node assessment.

4.3.2.2.5.2 Selection of optimal Target Number of Nodes

After selecting the point of greatest experimental effect, the SPADE analyses of different TNN were compared to identify which produced the greatest E2N.

It was evident from analysis of the E2Ns produced that although there were some nodes that fluctuated with the experimental effects, there were similarly some that remained low for the duration of the experimental period. The nodes that produced consistently low E2Ns were unhelpful in establishing the optimal TNN. This is in part because they are less variable than the higher E2N values, which makes it challenging to differentiate any pattern that may be observed in the data. However, it is predominantly because the lower E2N indicates that they do not represent the nodes, and therefore subpopulations, affected most by the experimental conditions. Those nodes are therefore not indicative of the phenotypic region for which the optimisation is being performed. This does not preclude the lower E2N nodes from being included in further analysis, as will be discussed, only that when attempting to establish the optimal TNN for cultures, they are not a suitable contributor.

Taking this into account, it was decided that the top 3 E2N values would be compared against each other from each SPADE analysis. This avoided any potential issues with the single highest value being an outlier. It also overcame the issue of plotting so many values that the consistently low E2N values did not become part of the optimisation process in the lower TNN analyses. The anticipated trend in these data was an increase in E2N as the TNN increased and separated populations based on more nuanced but practically significant differences. This would then reach an apex where the E2N would begin to decline as the events were separated based on relatively minimal and arbitrary differences, increasing the noise in the system without substantially increasing the standard deviation of the total node dataset. With analyses performed at a sufficiently low and high TNN, with regular intervals, it was predicted that plotting the top 3 E2N from each analysis would approximate a bell curve as the TNN increased closer to the optimal value and then further away.

The general trend observed in the data plotted was an increase in the top 3 E2N values from TNN of 10 up to 30, before a slight decline. There was an initial increase from TNN of 10 to 15 where both the top and third highest E2N increased by a similar value of roughly 0.5, whilst the second highest value changed from being similar to the highest value to approximating the third highest value between the two analyses. Between the 15 and 20 TNN analyses the second and third highest values remained at a similar level, whilst the highest value reduces to about the level of that seen in the 10 TNN analysis, whilst the top values in the 25 TNN analysis are similar to that of the 20 TNN. The 30 TNN analysis shows the highest E2Ns for each of the top positions with the second and third highest values being between ~3.3 and 3.7, and the highest value being ~5. There is a drop in all

three highest E2N values in the 40 TNN analysis, however these values are the second highest of each of their respective positions seen, and therefore do not drop to the level seen in analyses with a TNN lower than 30.

It is not clear why there is a decrease in the top 3 E2Ns of the 20 and 25 TNN analyses relative to the 15 and 30 TNN analyses. These are all analyses of the same data, rather than analyses of separate datasets, and therefore this variation is down to the TNN that is being implemented on these data rather than any culture variation. As this variation is being caused by the subpopulations that are being formed, one explanation of the reduction could be to do with the phenotypic space in which the additional cluster points are being formed. It is plausible considering the observable changes that are happening in the data around 120 hours that several cluster points will be within a similar area of the phenotypic space when the TNN is increased. As previously stated, part of the purpose of increasing the TNN of analyses is to ensure that genuinely different subpopulations are not combined into other groups, obfuscating any varying effects that cultural conditions are having on them. At the lower (10, 15) TNN SPADE analyses the lower E2N compared to other analyses suggest that this is the case, while the highest observed E2Ns in the 30 TNN analysis would suggest that this is, of those tested, the optimal TNN for separating subpopulations.

Where there are subpopulations identified in the 30 TNN analysis which are grouped together in the lower TNN analyses, it serves logic that there could be a TNN which would partially separate these groups. These 'in between' definitions would likely sit, as a phenotypic identity, somewhere between those of the subpopulations identified in the 30 TNN SPADE analysis. In this situation it would be plausible that events of a genuine subpopulation between these new definitions would be unevenly, and potentially inconsistently, distributed to surrounding nodes, leading to increased noise between repeats. With the increase in variation between conditional repeats, the bottom half of the E2N calculation becomes larger, without proportionally increasing the top half, leading to a decreased E2N ratio. This would appear to apply in the case of the 20 and 25 TNN analyses and would explain the previously unexpected drop as TNN increases.

As the 30 TNN analysis has the greatest E2N values when comparing the first, second and third highest values from each of the analyses, it is reasonable to say that this is the optimal TNN of those compared and will be used moving forwards.

4.3.2.3 Subpopulation selection rate analysis

With the optimal TNN selected, this gave a dataset that was optimally algorithmically divided into subpopulations based on a full representation of the phenotypic landscape across the duration of

the experiment for all conditions. The next process was to analyse how these newly defined subpopulations interacted, if at all, with the experimental variables in this experiment.

4.3.2.3.1 Selection of nodes of interest

The previous phenotypic analysis process resulted in 12 groups of data, that of the 4 maturity phenotypes within CD4+, CD8+ and total T-cell parent populations. The new analytical process generated 30 different groups, each with their own profile. Within CAR T-cell therapy manufacture the Naïve and Central Memory are the desired subpopulations, and therefore one of the objectives of the experiment was to identify how the experimental conditions affected the growth and purity of these subpopulations in culture. With these subpopulations the main targets of the phenotypic analysis, it was decided that rather than analyse all nodes against the experimental variables, only those that contained events that would fit into either of the two target subpopulations would be assessed.

The Cytobank website can display SPADE trees of all the analysed data, heat mapped to whichever marker the user desires. Given the biologically mutually exclusive nature of CD4/CD8 expression and the relatively minimal overlap between CD4+ and CD8+ populations it is possible to identify the 'sides' of the SPADE trees that pertain to either CD4+ or CD8+ populations. It is also possible to begin to identify the maturity subpopulations that nodes would be categorised into based on the 'heat' of nodes for CCR7, CD45RA and CD45RO expression. Considering the objectives of this novel analytical technique are to reduce the level of user bias in analysis and generate a process with a greater founding in logic, it was prudent to pursue further methods of analysis beyond user based identification of subpopulation classification based on colour gradient of nodes.

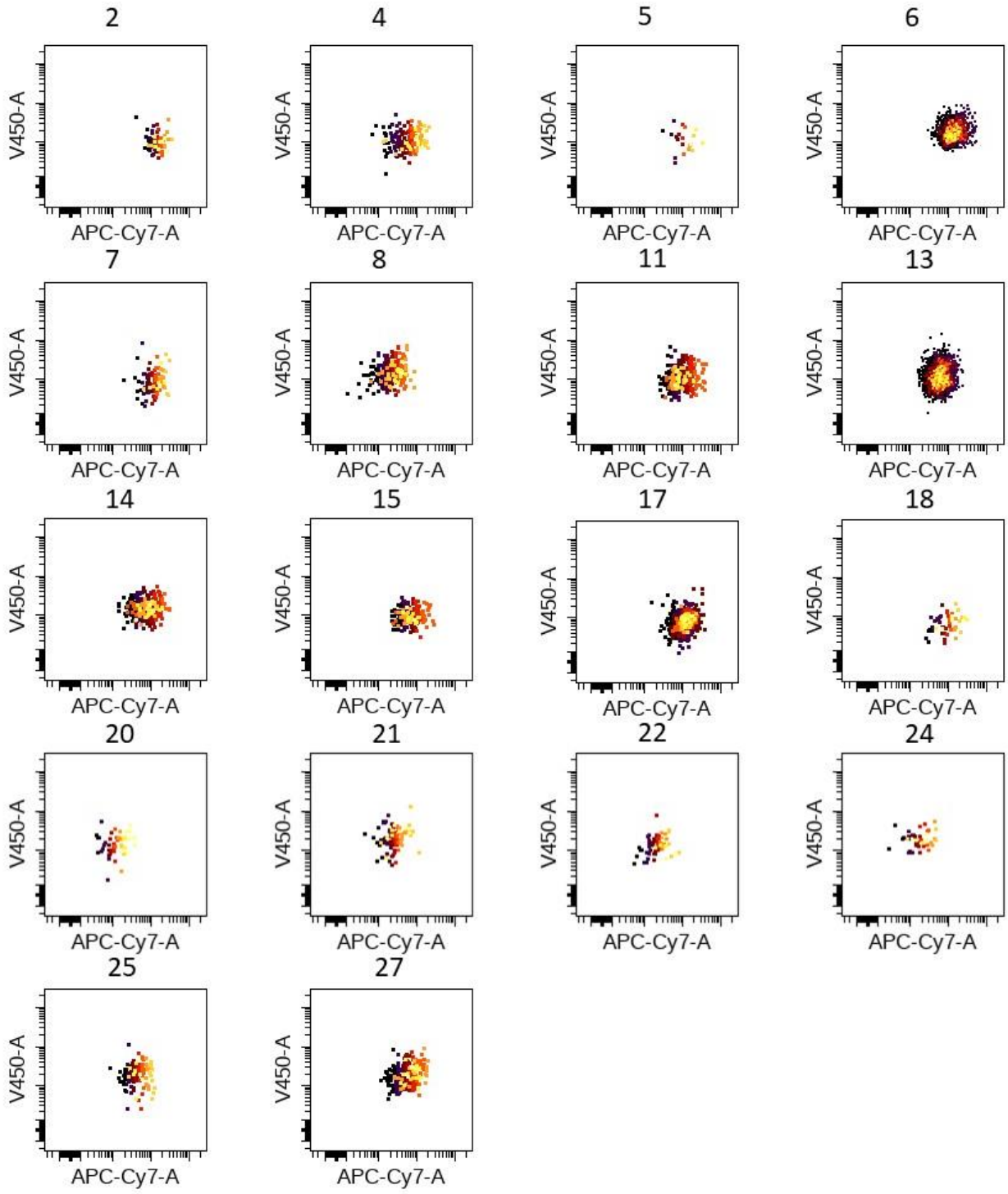


Figure 4.11. Plots of CCR7 (V450-A) and CD45RO (APC-Cy7-A) expression of events within nodes identifies nodes of interest for further assessment. Nodes from the SPADE analysis of optimal resolution for the dataset were assessed to identify those that contained events falling into the definition of Naïve (CCR7+/CD45RO-) and Central Memory (CCR7+/CD45RO+). All node event plots presented were identified as having sufficiently high CCR7 expression to qualify as containing events of the specified subpopulations.

The online interface of Cytobank allows the user to select a node, or nodes, of a SPADE tree and will display the events allocated to the selection from a specific sample as a dot plot. The user can define the axes that the data is presented on which allows the visualisation of event data attributed to nodes on the CCR7/CD45RO axes that were utilised for analysis in the original analytical technique (Fig. 4.11). Using these plots it would be possible to identify those nodes which presented events fitting the phenotypic profile of Naïve (CCR7+/CD45RO-) or Central Memory (CCR7+/CD45RO+), the Nodes of Interest (NoI). The analytical process generated 245 SPADE diagrams, and it was therefore not feasible to analyse every node on every diagram for their CCR7/CD45RO distribution, requiring a streamlined but representative method of identifying these NoI.

The experiment was set up using a full factorial 2^4 design with midpoint. This involved testing all permutations of the high and low set values of the 4 experimental values, as well as the midpoint conditions which were cultured at the average level of all variables. Midpoint conditions are used to highlight any non-linearity in the relationship between culture behaviour and the experimental variables. The intermediate nature of these conditions means that they were the closest that the experiments came to an average response to the experimental conditions, and as such identifying the NoI using these conditions appeared to be a suitable method for global analysis of the dataset. The requirement of picking a timepoint at which to analyse the nodes was deferred back to the previous selection of point of greatest experimental effect, as this would most likely be the point at which data were distributed through the greatest number of nodes, making it easier to identify events of interest. Furthermore, the greater density of cells at 120 hours would also make identification of events of interest easier.

Implementing this method identified 18 NoI, described in Table. 2, selected for the presence of events meeting the threshold level of CCR7 expression. The density plots for these nodes of this sample visibly vary in density, indicative of different subpopulation sizes, and vary in expression of CCR7 and CD45RO. The range of expression of CD45RO over the NoI shows populations that are immature, mature, and those that lie across the threshold used to define between the two. Meanwhile, CCR7 expression shows that all populations observed within the selected NoI have events either side of the threshold for 'homing' cells. This indicates that although there are populations identified within this experiment that are more or less 'homing' there are no populations identified by SPADE's analytical process that are explicitly Naïve or Central Memory, only those that have a greater or lesser expression of attributes associated with those populations.

Table 4.2. Description of phenotypic presentation in Nodes of Interest, as identified in Fig 4.11

Node number	CD4/ CD8	Maturity phenotype	Additional notes
2	4	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression range spans –ve and +ve.
4	8	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression range spans –ve and +ve.
5	8	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression range spans –ve and +ve.
6	4	Central Memory	High CD45RO expression. CCR7 expression borders but does not present as –ve.
7	4	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression range spans –ve and +ve.
8	4	Naïve/Effector/ Effector Memory/ Central Memory	Expression of CD45RO and CCR7 spans both –ve and +ve. –ve expression of CD45RO is limited.
11	8	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression range spans –ve and +ve.
13	4	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression range spans –ve and +ve.
14	8	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression is predominantly positive.
15	8	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression range spans –ve and +ve.
17	4	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression is predominantly negative.
18	8	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression is predominantly negative.
20	4	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO and CCR7 are both expressed equally as +ve and –ve. The range of CCR7 expression is greater than that of CD45RO expression.
21	8	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression range spans –ve and +ve.
22	8	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression predominantly +ve. CCR7 expression equally spans +ve and –ve.
24	8	Naïve/Central Memory	CD45RO expression predominantly +ve. CCR7expression borders but does not present as –ve
25	4	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression range spans –ve and +ve.
27	8	Effector Memory/ Central Memory	CD45RO expression borders but does not present as –ve. CCR7 expression predominantly +ve.

It is possible to see where some subpopulations from different nodes would overlap, were they presented on the same CCR7/CD45RO plot. The 5 dimensional analysis of these data mean that it is not possible to fully define the differences between the populations seen in different nodes using only the CCR7/CD45RO plots, requiring further analysis using the other markers used for clustering. Two nodes from a midpoint sample at 120 hours may produce extremely similar density plots on a CCR7/CD45RO scale, but these populations could vary in CD4/CD8 expression, separating them by their cytotoxic/helper function, and similarly could have a different CD45RA expression level, indicating the level of transition between immature and mature states. Considering this, it is worth mentioning that where node subpopulations occupy a similar space, that does not mean that they are occupying the same 5 dimensional phenotypic space and should still be considered truly separate subpopulations.

4.3.2.3.2 Subpopulation analysis

With the NoI identified, the next step was to ascribe a value to each sample within the node that would give the ability to assess what impact cultural conditions were having on cells that would fall into that distinct subpopulation. As mentioned in the traditional analysis method, part of the decision is between using the absolute number of events attributed to that node for each sample, or the proportion of the sample represented in that node relative to the total sample.

4.3.2.3.2.1 Total event count vs proportional analysis

Although the sampling method of analysing a fixed volume sample from conditions at each timepoint would allow the tracking of subpopulations through event count, it does not consider other populations within the samples and is therefore suboptimal for several reasons. Firstly, this method only acknowledges the count of the sample within this node, and in not recognise other subpopulations it is not possible to identify the purity of the sample with regards to this subpopulation. If the objectives of the experiment included generating the greatest number of cells from a particular subpopulation then this would not be an issue, however as the target is purity in culture rather than quantity produced per input, the relative numbers of cells from other subpopulations is relevant.

Secondly, the reason for phenotypic analysis of cultures at multiple timepoints is due to the change in phenotypic profile of cells over time. With a greater number of subpopulations that overlap in multiple dimensions there is also a greater opportunity for transition between subpopulations over time. T-cells are unable to transition between CD4+ and CD8+ state, but variable expression of CCR7, CD45RA and CD45RO could all induce transition from one subpopulation to another. Comparatively, only analysing the number of events from a sample assigned to a node could lead to ambiguity in results. It would fail to highlight whether the cause for an increase or decrease in the

growth rate of a subpopulation was the result of a global change in growth rate, or selection for or against the specific subpopulation. Meanwhile, a proportional approach to analysis may not fully explain any variation observed but would give a greater definition of the population relative to the total growth of the culture, and therefore allow for easier assessment of experimental variables' effect on target populations.

4.3.2.3.2.2 Subpopulation proportion calculation

The subpopulation proportion needed to be calculated as it was not automatically done so as part of the SPADE analysis. Traditional analysis would compare the number of events in the node to the total number of events for the sample at that timepoint. However, this process means that in the event that there is one node that carries a substantial proportion of the total events to the point where other subpopulation counts are relatively negligible, all proportions will essentially be taken as relative to this large proportion. To balance this somewhat, the proportion was calculated by comparing the number of events for a sample within a node to the number of events for the sample in all other nodes. This analysis was performed on all NoI, for all samples, for nearly all timepoints. A lack of data from samples run at 168 hours meant that it was not possible to run this analysis for data from the samples tested at that timepoint and it therefore had to be omitted from this analysis.

4.3.2.3.2.3 Subpopulation proportion tracking

With the comparison between the NoI and the remaining nodes produced as a relative proportion, it was then possible to plot this value against time. Observing the data in this way made it possible to identify how the subpopulation proportions changed over time.

Plots of subpopulation proportions over time showed a high degree of variation in subpopulation behaviour between nodes. However, plotting the average subpopulation proportion within a node across all samples at each timepoint showed a relatively conserved pattern in the data (Fig. 4.12). 17 of the 18 NoI showed an increase in the average subpopulation proportion from 48 hours to 72 hours, followed by a decline to a similar level at 96 hours. Furthermore, in 16 of those 17 NoI, the average subpopulation from 96 hours to 144 hours remained relatively stable. The changing level of variation seen in the last three timepoints shows that the different NoI were affected differently by experimental conditions, it is none the less noteworthy that there was such a conserved pattern across all NoI when averaging across samples.

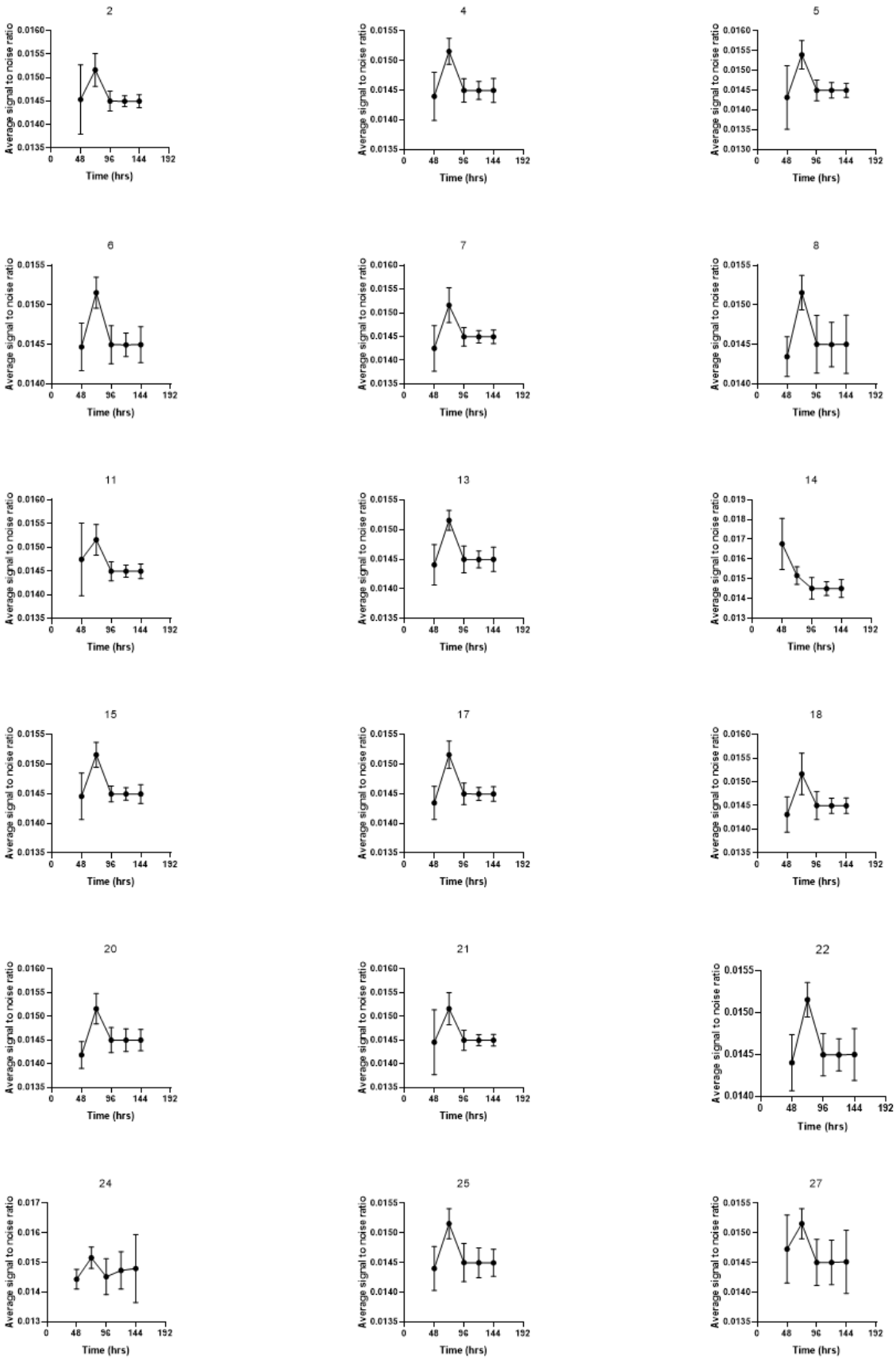


Figure 4.12. The trend in average subpopulation proportion for all samples over time remains relatively well conserved across all Nodes of Interest. Proportions were calculated for each sample by comparing the number of events from a sample within a node to the sample's events in all other nodes. The mean of this value across all samples was plotted over time, and standard error of the mean (SEM) error bars added to indicate variation.

Node 24, a relatively central CD8+ cluster, with events that fall into all 4 traditional maturity phenotype categories showed a shallow incline in average subpopulation proportion after 96 hours, compared to the relative stability of the 16 comparable nodes. Meanwhile, node 14, a CD8+ Central Memory subpopulation, showed a decline in the average subpopulation proportion from 48 hours to 96 hours, after which the average subpopulation proportion remained at a similar level until 144 hours, akin to the pattern seen in the majority of other node analyses. Considering the method for calculating the subpopulation proportion, the pattern of a spike in subpopulation proportion at 72 hours is indicative of a drop in the subpopulation proportion of a large population, distributed amongst the other subpopulations at this timepoint. The reasons for this temporary redistribution is not clear; the change does correlate with the first supplementation of medium in the experiment, and therefore there may be some link between them, however this same response is not seen at 120 hours, another supplementation timepoint, and it also is not apparent how these would relate.

4.3.2.3.2.4 Subpopulation selection rate

Calculation of subpopulation proportions across time identified that the pattern of change over time changed depending on sample being observed. This implication of condition relative effects on subpopulations required further processing of the data to identify the nature of the relationships. In order to get an approximation of the behaviour of the subpopulation proportions over time, so that they could be compared against the experimental conditions, the gradient of a linear estimate of the data was calculated. This was done using the LINEST function in excel, with the subpopulation proportion and time acting as the Y and X data respectively.

The calculated gradient was indicative of the change in subpopulation proportion over time. A negative gradient would indicate that the subpopulation was not being selected for in the culture conditions, whilst a positive gradient would imply a positive selection for that subpopulation in the culture conditions. A gradient of 0 would either imply that the absolute number of cells in the node was increasing at the same rate as the total population, whether entirely due to growth of cells in that subpopulation, or in combination with transition in and out of the subpopulation from/to others. As the gradient represents the rate at which the subpopulation proportion changes, if at all, this value can also be considered the selection rate for the subpopulation as a proportion of the total culture.

4.3.2.4 Minitab analysis of output data

Data were statistically analysed in Minitab to identify effects, both independently and in combination, on the selection rates generated from SPADE analysis data.

4.3.2.4.1 Analysis of experimental effect on calculated selection rates

Analysis of the selection rates shows that all the experimental variables had some significant effect across the subpopulations from the NoI. Furthermore, it shows that all subpopulations were significantly affected by at least one independent factor except node 20, a CD4+ subpopulation that shows Naïve and Effect phenotype cells (Fig. 4.13).

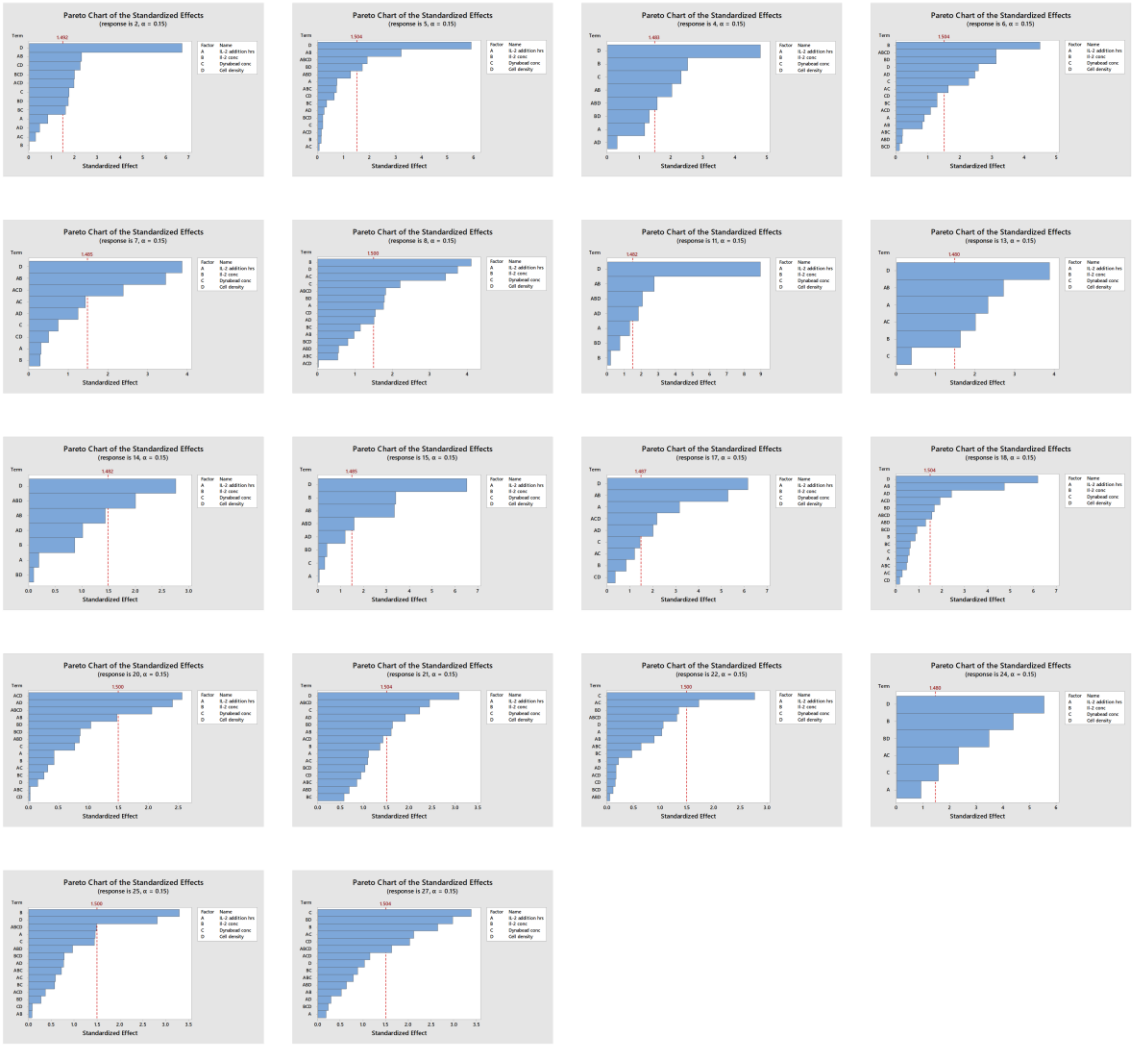


Figure 4.13. Statistical analysis of Nodes of Interest shows that cell density significantly impacts selection in the majority cases. Calculated subpopulation selection rates were analysed using Design of Experiment framework for significance of experimental variables. All subpopulations were significantly effected by experimental conditions in combination, and all but one subpopulation (node 20) were significantly impacted by at least one individual experimental factor. Effects of the significant variables are presented in Fig 4.14

Analysis of factor significance shows that cell density was the most common individual factor of significance, having a significant impact on 15 of the 18 analysed subpopulations (Fig 4.13).

Increasing the cell density from $1x10^6$ cells/mL to $2x10^6$ cells/mL generally had a negative effect on the subpopulation proportion, with negative correlation identified in 13 of the 15 subpopulations significantly affected by seeding density (Fig 4.14). IL-2 concentration and Dynabead concentration each significantly affected 8 of the 18 targeted subpopulations, 5 of which were affected by both factors. Increasing the Dynabead concentration had a predominantly negative effect on subpopulation proportion, with 6 of the 8 effected nodes negatively affected. Meanwhile, increasing the IL-2 concentration mostly had a positive effect on those nodes that were significantly affected, with 5 of the 8 effected nodes positively affected (Fig 4.14).

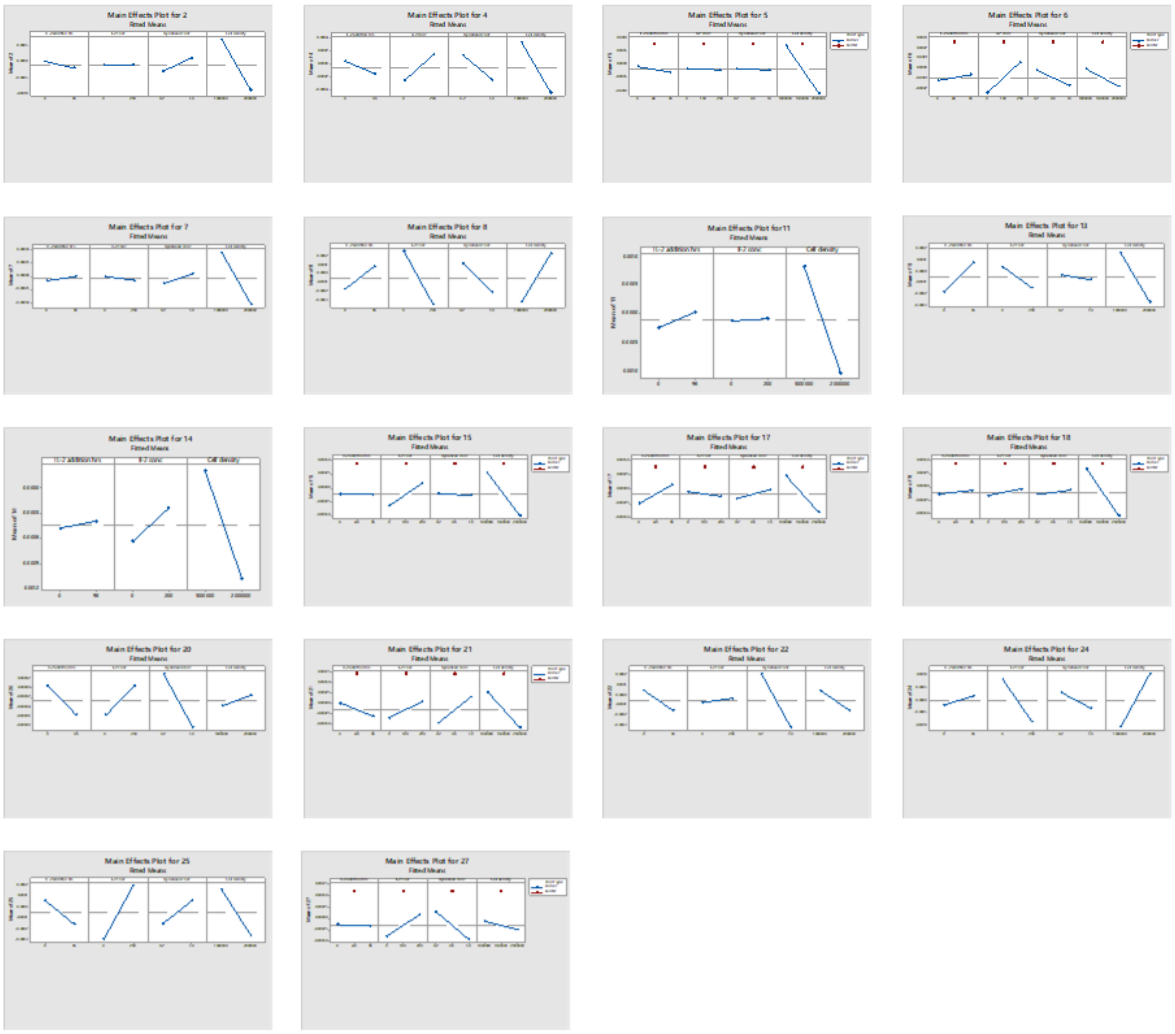


Figure 4.14. Analysis of significant variables shows that cell density impacts selection negatively in the majority cases. Significant variable, identified in Fig 4.13 were analysed to identify their impact on subpopulation selection rate.

No pattern is immediately obvious in the data other than that the nodes that have been selected for further analysis are those which show events with sufficiently high CCR7 expression to classify as part of the 'homing' maturity phenotypes (Naïve and Central Memory), although this is not a ubiquitous effect. A potential reason for this is that the effected subpopulations are more effected

by transition of cells between phenotypic definitions, as the experimental conditions tested were predicted to have an impact on this behaviour. In addition, there was no observable pattern relative to CD4/CD8 expression, but CD45RA expression was not investigated here and this may better inform any understanding of the reason that some subpopulations were affected and others were not.

The least impactful experimental factor within the nodes analysed was the timing of IL-2 addition, which only effected 3 of the 18 analysed nodes. Where a significant factor, delaying the addition of IL-2 always had a positive effect on subpopulation proportion. All nodes with a significant response to timing of IL-2 addition were also significantly affected by at least one of the other experimental factors. The significant response to other factors was in line with conditions that would not be conducive to stimulation of proliferation and maturation, negative to IL-2 and Dynabead concentration increases and positive to increasing the seeding density of cultures. As these other factors correlate with less maturing conditions, it suggests that delaying the addition of IL-2 until 96 hours could also be considered a condition that does not induce the same level of maturation as adding at 0 hours.

Of all the nodes which presented all 4 maturity phenotype (Naïve, Effector, Effector Memory and Central Memory) events, only one had a significantly negative correlation with increasing the cell seeding density. The two conditions which were positively affected by increasing seeding density were nodes 8 and 24. These two subpopulations were also significantly negatively affected by increasing the IL-2 and Dynabead concentrations from 0 UI/mL to 200 UI/mL and $0.2x10^6$ beads/mL to $1x10^6$ beads/mL respectively. Both factors are commonly associated with increasing proliferation and maturation in T-cell cultures, whilst a higher seeding density in a proliferating culture would be assumed to deplete nutrients and exhaust medium faster than that of cultures with a lower seeding density. Therefore, it would appear that these subpopulations benefit from conditions that do not promote growth or maturity in culture and are more restrictive.

The lack of stimulation and nutrition could potentially restrict the maturation of certain cell populations, causing the generation of a stunted population unable to reach full maturation. Alternatively, rather than generating a new subpopulation, these restrictive conditions could be reducing transition from these subpopulations onto other existing subpopulations. As mentioned, nodes 20, 21 and 22, which represent events of all 4 maturity subpopulations, do not have a significantly positive response to increases in cell density. Node 22 is significantly negatively affected by increasing Dynabead concentration, whilst node 20 is the only subpopulation analysed at this stage that does not have a significant response to any individual factor.

Node 21 has a significantly positive response to an increase in Dynabead concentration and significantly negative response to increasing the cell density, which is counter to the trends seen in the other like nodes. As mentioned, increasing the Dynabead concentration would aid in stimulating the maturation of cultures, and reducing the seeding density would reduce the chance of medium exhaustion, allowing greater proliferation and allowing maturation to continue in T-cell cultures. The positive response to these conditions could be indicative of this subpopulation being a genuine endpoint phenotype for certain populations, which are stimulated or enabled to proliferate better in cultures with higher Dynabead concentration and lower seeding densities. However, these data could also show a subpopulation that is a key transitional state for cells maturing to other subpopulation phenotypes, and in conditions that stimulate greater maturation and proliferation it would be logical to observe a greater proportion of cells going through this transition to reach a more mature state.

Looking at combinatorial responses, interaction between the concentration of IL-2 and the timing of addition was the most commonly significant pair of factors. However, of those nodes that were significantly affected by this interaction, only nodes 13 and 17 did not display the same patterns as was seen in the traditionally gated and analysed data, whereby there was a similar level of variation identified in the response of cultures supplemented with 0 UI/mL IL-2 at 0 and 96 hours as there was in the cultures supplemented with 200 UI/mL IL-2 at those two timepoints. The previous analysis concluded that the observation invalidated any conclusions drawn from this interaction but did not invalidate any observations of either the factors independently or the interactions of these factors with other experimental variables due to their interdependent nature. It is nonetheless worth note that these data were analysing the change in subpopulation proportion over time, as opposed to subpopulation data at a specific point, indicating that this inherent variation was present throughout the duration of the cultures and not just at the timepoint previously examined. Furthermore, it was hoped that in increasing the phenotypic resolution that the data causing this issue would be separated into subpopulations that had cohesive behaviour, eliminating this effect, however the data shows that this has not been the case.

The next most commonly significant interaction was between the concentration of IL-2 supplemented into cultures and the seeding cell density. The majority of the subpopulations effected by this interaction saw the lower seeding density, regardless of the level of IL-2 supplemented have a more positive effect than any of the conditions that were conducted at the higher seeding density. The only nodes where effects between the two seeding densities crossed was node 6 and node 27.

In node 6, at a higher IL-2 concentration the responses were still separated by the observed trend, with the lower seeding density having a positive effect compared to the higher seeding density. However, at the lower IL-2 concentration, the difference between the two seeding densities appeared to be negligible, with the reduction in IL-2 seeming to negate any impacts that the seeding density would have on this subpopulation. At a lower IL-2 concentration the effect on the subpopulation proportion is negative, regardless of the seeding density, whilst at higher IL-2 concentration a slightly less negative effect on subpopulation proportion is observed at the higher seeding density, and a substantially positive, both relative and actual, effect on subpopulation proportion is observed in the lower seeding density. This potentially suggests that this subpopulation requires a higher concentration of IL-2 to increase its subpopulation proportion, but at a higher seeding density, this subpopulation is affected more by medium exhaustion.

Node 27 shows a similar, but not identical pattern of response where the difference between the response at 0 UI/mL and the 200 UI/mL IL-2 conditions seeded at $2x10^6$ cells/mL was this time negligible. Meanwhile, the response difference between high and low IL-2 was the same as previously mentioned in the cultures seeded at $1x10^6$ cells/mL, with subpopulation proportion being positively affected by increasing the IL-2 concentration. However, in this node the negative effect on subpopulation proportion of low IL-2 in the lower seeding density cultures was greater than that seen in either group of the higher seeding density cultures.

There were two nodes significantly affected by the interaction between IL-2 concentration and cell density where subpopulation proportion was positively affected by seeding at $1x10^6$ cells/mL compared to $1x10^6$ cells/mL, nodes 8 and 24. In node 8, the level of selection for the subpopulation was reduced when increasing the IL-2 concentration from 0 UI/mL to 200 UI/mL at either seeding density. However, whilst at a seeding density of $1x10^6$ cells/mL the average selection rate moves from negative when supplemented with 0 UI/mL IL-2 to a more negative rate when 200 UI/mL is supplemented, at $2x10^6$ cells/mL the average selection rate decreases from substantially positive to slightly negative as the IL-2 concentration increases.

In node 24, increasing the IL-2 concentration similarly reduces the average selection rate for the subpopulation from relatively very positive to slightly negative in the cultures seeded at $2x10^6$ cells/mL. However, in this node, regardless of the IL-2 concentration supplemented, the average selection rate of cultures seeded at the lower density is always lower than that of those seeded at the higher density. Seeding at the lower density therefore always had an average selection rate that was negative.

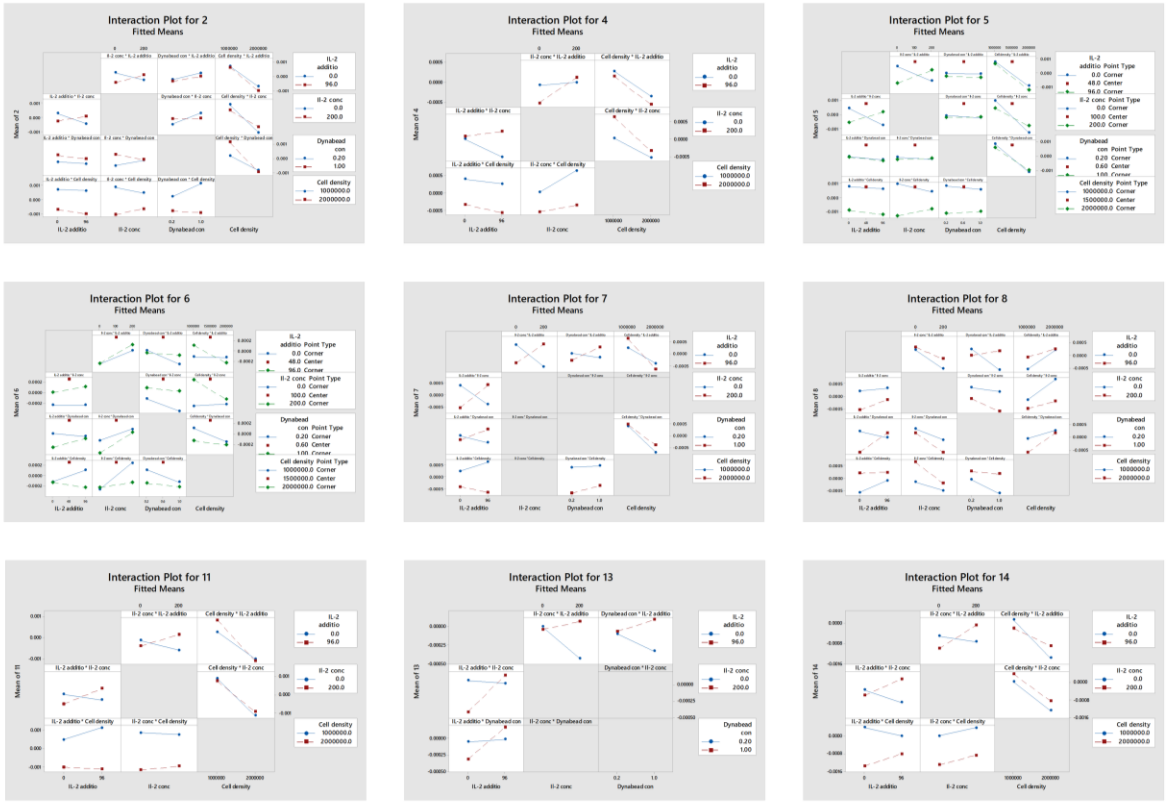


Figure 4.15. Graphs showing the effects of conditional variable interactions that had a significant impact on subpopulation selection rates. Interactions plots were generated by analysis through Design of Experiment framework; significance of these combinations are outlined in Fig. 4.13. Only data from nodes 5, 6, 15, 17, 18, 23 and 27 generated an effect model that included a midpoint.

Nodes 8 and 24 were similarly the only nodes where the selection rate was significantly increased in reaction to increasing the cell density. This further supports the conclusion that these subpopulations are further stimulated and selected for in conditions that limit proliferation and maturation of the cell populations. However, this also shows that there are subpopulations that using the standard 2-dimensional analysis method may have been grouped together, in reference to the subpopulations that represent the same CCR7/CD45RO phenotypic space, which do not have similar responses to experimental conditions. The benefits of the new analytical method are further shown by the algorithmic selection of event clusters that fall into multiple standard phenotypic classifications, indicating that a more phenotypically resolute analysis of phenotypic data will garner results which better inform the interactions in the culture system.

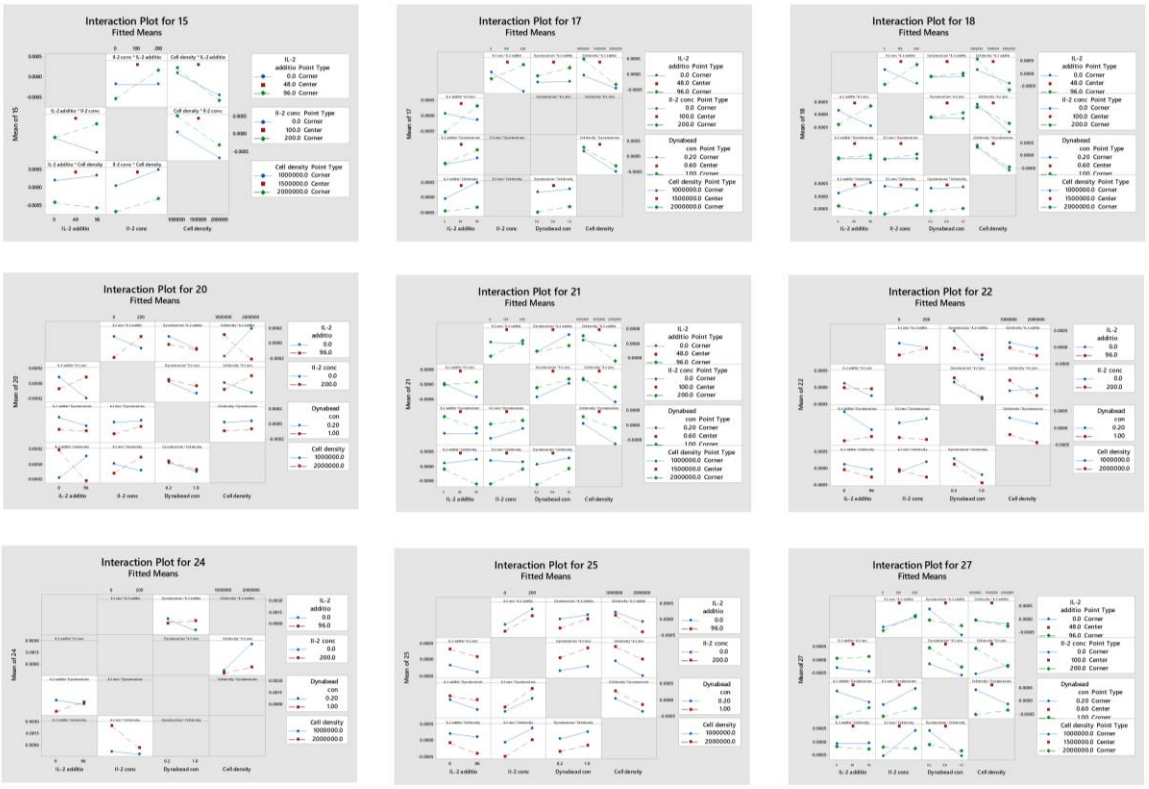


Figure 4.15 (continued). Graphs showing the effects of conditional variable interactions that had a significant impact on subpopulation selection rates. Interactions plots were generated by analysis through Design of Experiment framework; significance of these combinations are outlined in Fig. 4.13. Only data from nodes 5, 6, 15, 17, 18, 23 and 27 generated an effect model that included a midpoint.

The interaction between the timing of IL-2 addition and cell density also had a significant effect on several subpopulations. Four of these subpopulations, nodes 11, 17, 18 and 21, showed a similar type of response to the experimental factors (fig. 4.15). All four subpopulations showed that the average selection rate was negatively affected when cultures were seeded at $2x10^6$ cells/mL, with the average rate being negative regardless of the timing of IL-2 addition. Nodes 11, 17 and 18 show a similar rate of increase in the average selection in cultures seeded at $1x10^6$ cells/mL when the supplementation of IL-2 is delayed from 0 to 96 hours. In the $2x10^6$ cells/mL cultures, nodes 11 and 18 show a negative effect on selection rate when IL-2 supplementation is at 96 hours, whilst in node 17 this is shown to have a slightly positive effect on the average selection rate. Average subpopulation selection rate in node 21 of cultures seeded at $1x10^6$ cells/mL increased at a lower rate than that observed in nodes 11, 17 and 18 between subgroups where IL-2 was supplemented at 0 and 96 hours, but still observed the positive trend. Node 21 also showed a greater negative change in cultures seeded at $2x10^6$ cells/mL between those supplemented with IL-2 at 0 and 96 hours.

In node 6 the data show that there was a negligible reduction in the average subpopulation selection rate for cultures seeded at $1x10^6$ cells/mL and $2x10^6$ cells/mL when IL-2 is supplemented at 0 hours, as well as that supplementing at 0 hours always negatively selects for that subpopulation. Supplementing IL-2 at 96 hours had a negative effect on average selection rate compared to 0 hours when seeding at $2x10^6$ cells/mL, however it had a relatively positive effect when seeding at $1x10^6$ cells/mL, as well as producing a genuinely positive selection rate. Node 8, which has so far been shown to benefit from those conditions that are less likely to induce proliferation or maturation in cultures, shows the opposite trend to that which has been observed so far, with average selection rate of cultures seeded at the higher density being more positive than those seeded at the lower density. There appears to be little impact on delaying the supplementation of IL-2 from 0 hours to 98 hours to cultures seeded at $2x10^6$ cells/mL, however there is a small increase in the average selection rate when supplementing later. At the lower seeding density, delaying the supplementation of IL-2 has a substantial positive effect on the average selection rate, however the selection rate still remains negative.

Node 20 is the only subpopulation where there is a substantial difference in the effect of IL-2 supplementation timing between the two seeding densities tested. At the lower seeding density, the average selection rate when supplementing IL-2 at 0 hours is negative but delaying the addition of IL-2 to 96 hours has a substantially positive effect on the average selection rate, producing a positive selection rate. Meanwhile, in conditions seeded at the higher density of $2x10^6$ cells/mL the selection rate is positive when IL-2 is supplemented at 0 hours. Delaying the supplementation to 96 hours has a substantially negative effect, and induces a negative average selection rate, similar to the average rate of the $1x10^6$ cells/mL conditions supplemented at 0 hours.

Nodes 6, 8 and 24 were all similarly significantly affected by the interaction of the timing of IL-2 supplementation and the concentration of CD3/CD28 Dynabeads that was supplemented into culture. In these subpopulations, changing the concentration of CD3/CD28 Dynabeads supplemented has little effect on those cultures where IL-2 was supplemented at 96 hours, reducing slightly in node 6 and increasing a small amount in nodes 8 and 24. When supplementing IL-2 at 0 hours, conditions which were supplemented with $0.2x10^6$ beads/mL show a similar selection rate to those of the cultures supplemented at 96 hours. However, supplementing with $1x10^6$ beads/mL has a substantially negative effect on the average selection rate. Node 13 shows a similar change in the average selection rate in conditions supplemented with IL-2 at 0 hours, however when supplemented at 96 hours there is a more easily observable increase in the average selection rate between those cultures supplemented with $0.2x10^6$ beads/mL and $1x10^6$ beads/mL.

Nodes 22 and 27 were also identified as having similar significant responses to the interaction of Dynabead concentration and timing of IL-2 supplementation as each other. In both nodes, the supplementation of $1x10^6$ beads/mL CD3/CD28 Dynabeads had a negative effect on average selection rate relative to those which were supplemented with $0.2x10^6$ beads/mL, but also that delaying the supplementation of IL-2 to 96 hours reduces this negative effect. The opposite effect is seen in the cultures supplemented with $1x10^6$ bead/mL CD3/CD28 Dynabeads, with supplementing IL-2 at 96 hours having a negative effect on selection rate, while supplementing at 0 hours has a substantially positive effect on average selection rate.

4.3.2.4.2 Discussion

Analysis of the average subpopulation proportion over time for the subpopulations carried forward to the further analysis showed surprising similarities. The majority of subpopulations showed the same trend in the average subpopulation proportion, whilst the range of data at each point was more indicative of the variation between subpopulations. Unlike comparing the proportions of different subpopulations within a condition, the subpopulation proportions of different experimental conditions do not affect each other and there is not mathematical reason why they are likely to appear so similar. Therefore, it would appear that this is a genuine similarity between these subpopulations' behaviour on average. Apart from the spike in subpopulation proportion at 72 hours, the data showed that on average the subpopulation proportion remained relatively similar throughout the duration of the culture. However, as previously mentioned, the high degree of variation between the range of data at any one point between subpopulations indicates that overall there were substantial differences in the way these subpopulations behaved over time. This data also does not highlight the variation in how individual cultures behave within these ranges over time, which varied substantially.

Analysis of the selection rates generated show that the most significantly impactful experimental factor on the selection rates for the subpopulations assessed was the seeding density. The impact of increasing the seeding density from $1x10^6$ cells/mL to $2x10^6$ cells/mL on the subpopulations analysed, whether in isolation or in combination with other factors, was predominantly negative. Not only did the higher seeding density having a negative effect on the average selection rates compared to the lower seeding density, but also mostly induced a genuinely negative selection rate, implying that these subpopulations were not proportionally performing as well as others in the cultures when the seeding density was higher.

Where the CD3/CD8 Dynabead concentration had a significant effect, increasing the concentration had a negative effect on the average selection rate on the subpopulations more often than where the effect was positive. Meanwhile, increasing the IL-2 concentration had the opposite effect by

having a positive effect on more of the subpopulation selection rates than negative in those where IL-2 concentration was a significant factor. Three of the observed subpopulations were significantly affected by the timing of IL-2 addition, with the supplementation of IL-2 at 96 hours having a positive effect compared to supplementing at 0 hours in all three.

With all three of these factors there was no immediately obvious trend in the data to define a relationship between those experimental variables and the subpopulation selection rates. Both IL-2 and Dynabead concentration effect a mixture of CD4+ and CD8+ subpopulations, as well as a number of subpopulations that span the CCR7/CD45RO range seen across all analysed nodes. The effect of manipulating the supplementation timing for IL-2 only significantly affects subpopulations that were CD4+, however the phenotypic profiles of the events within these nodes still show limited congruity, if any, and a spread of events over the phenotypic space results in no clear relationships being defined.

Analysis of the selection rate data also didn't yield any obvious trends in which subpopulations would be significantly affected by interacting factors, and mostly didn't provide evidence there was a trend in how they would be affected.

Changing the perspective and viewing the interactions of factors with subpopulations it is possible to see that 2 nodes stand out from those assessed. Nodes 8 and 24 each represent a subpopulation of cells which have a range of CCR7 and CD45RO expression which results in cells falling into all 4 maturity subpopulation phenotypic categories. Furthermore, these nodes represent a CD4+ and CD8+ subpopulation respectively, suggesting that these nodes represent the same subpopulation of cells in either the helper/regulatory or cytotoxic subgroups. These nodes respond positively to all the conditions that would be anticipated to reduce the level of proliferation and maturation of cells in cultures, such as increasing the seeding density and decreasing the concentration of IL-2 and CD3/CD28 Dynabeads in culture.

Nodes 8 and 24 were the only nodes of all those analysed at this stage which showed a positive response to increasing the seeding density. It is not clear if these subpopulations benefit from these conditions due to a failure of cells to progress in the maturation process and transition to a different subpopulation, or if these limiting factors cause the creation of a new end point subpopulation that is not as mature. In either instance, this is evidence that this novel approach to phenotypic data analysis can identify subpopulations and trends in data that were not previously seen.

4.3.2.4.3 Conclusions

It was originally hypothesised that the supplementation of $1x10^6$ beads/mL CD3/CD28 Dynabeads at seeding and 200 UI/mL IL-2 after 96 hours into cultures seeded at $1x10^6$ cells/mL would prove

beneficial for Effector Memory phenotype subpopulations and be detrimental to the immature, Naïve and Effector, subpopulations. One of the limitations of using the novel analytical method to produce subpopulations is that it is not easy to compare the hypotheses to subpopulations generated as they span the borders of phenotypic classification the hypotheses were addressing. However, in the subpopulations carried forwards into further analysis the trends in data show that, where significant, supplementation of 200 UI/mL IL-2 at 96 hours with $1x10^6$ beads/mL CD3/CD28 Dynabeads into cultures seeded at $2x10^6$ cells/mL had negative impacts on selection for these nodes.

There was a high number of subpopulations that included events that fall into the Effector Memory phenotype profile, which could be the cause of so many subpopulations responding negatively to a higher seeding density and delayed supplementation of IL-2. However, there were several subpopulations that did not get selected, including predominantly Effector Memory subpopulations, and therefore this response is still somewhat unexpected.

One of the main conclusions from this analysis is that the novel analytical process was able to identify a subpopulation within both CD4+ and CD8+ subpopulations that had a similar phenotypic profile, but different responses to the same experimental conditions. This shows that the process has the capacity to identify subpopulations and draw conclusions at a higher phenotypic resolution that would have been assimilated into other groups and lost at a lower one.

4.3.2.5 Comparison with standard gating technique

This novel analytical method for phenotypic data was developed in response to observation of the shortcomings of the traditional method. The standard method was effective at analysing the proportion of events that fell into phenotypic categories, however their rigid definition resulted in analysis that did not consider the nuances of protein expression for the specific dataset being analysed. It also made no attempt to identify subpopulations within the cultures, beyond the 4 maturity phenotypes of CD4+ and CD8+ subpopulations, instead assuming the behaviour of all cells in the culture would have a similar response to the same stimulus.

The process developed addressed some of these issues. Using a platform that utilised algorithmic generation of subpopulations avoided rigid threshold expression levels to define subpopulations. User definition of the number of subpopulations generated allowed for optimisation of this against the data to identify the number of genuinely different subpopulations. Furthermore, analysis of data from all conditions and timepoints simultaneously allowed for the generation of subpopulations that represented the full phenotypic space occupied across the duration of the whole experiment. Data and outputs from both analysis techniques were compared to assess any different observations or conclusions that may have been drawn.

4.3.2.5.1 Subpopulations identified using SPADE analysis

The phenotypic profile of subpopulations produced in the 30 node SPADE analysis vary across time. However, it is still possible to see in the snapshot of data presented in Fig. 4.11 that the subpopulations produced do not fully fit within the parameters of the standard analysis protocol. In all nodes carried forward to further analysis within this culture at 120 hours, the spread of CCR7 and CD45RO expression results in clusters that span at least two maturity phenotype definitions, and some span all four.

That a non-biased, algorithmic approach to cluster definition would produce clusters of cells that span the typical subpopulation definitions shows the inadequacy of the standard analytical method. Subpopulations identified show a range of expression profiles which, by the process that the optimal TNN was identified, are affected differently by experimental conditions. The traditional phenotypic analysis would not have given this detailed an output of the distinct subpopulation makeup of the cultures.

A greater number of markers was used to generate the subpopulations represented by each node. However, as they were the markers that constituted the same panel used for standard analysis, it is possible to determine which traditional subpopulations the novel populations would have been placed into, and in what proportions where the population would not entirely fit into one boundary. With this, although the analysis produces novel populations, it is still possible to relate these back to the standard, biologically defined populations currently used, and therefore compare the conclusions obtained from using this method back to previous research.

The data clearly show that the novel analytical technique is superior to the traditional technique in identifying genuinely different subpopulations in these T-cell cultures. However, this was not the only comparison to be drawn between the two, as there is also the question of any differences or similarities in the conclusions of experimental effects.

4.3.2.5.2 Comparison of experimental conclusions between conventional and novel phenotypic analysis

One thing that is true of the statistical analysis of the subpopulation data obtained via the traditional method is that it did not identify a large number of significant impacts. For example, the only independently significant factors were IL-2 concentration and Dynabead concentration, with the latter only significantly affecting one of the subpopulations. The issues with the data presented of the interaction between IL-2 concentration and timing of supplementation (Fig. 4.7), leading to them being invalidated, also reduced the number of significant factors in the dataset.

Meanwhile, analysis of the data produced from the novel analytical technique showed a large number of significant factors, with each factor individually having a significant effect on at least one subpopulation. Having a larger number of significant factors does not immediately make the novel analytical technique better, especially given that theoretically this could be due to subdivision of populations already identified as being significantly impacted. However, the number of subpopulations significantly affected implies that this process of identifying subpopulations has enabled the identification of significant effects, where before they were obfuscated by poor definition of subpopulations. Furthermore, the increase in significantly affected subpopulations as a proportion of the total subpopulations, along with the increase in the number of significant factors, independently and in combination, appears to show that in this scenario the novel analytical technique has been able to better identify what effects the experimental conditions are having on different subpopulations of cells within the cultures.

The predominant output from individual factor analysis from the standard analytical technique was that increasing the IL-2 concentration had a significantly negative effect on Effector subpopulation proportion. In the data obtained from the novel analytical technique, those subpopulations observed to have the greatest proportion of cells as Effector phenotype significantly affected by IL-2 concentration also had a negative response to an increase in the concentration. However, there were also several other subpopulations that had a significantly positive response to increasing the IL-2 concentration from 0 UI/mL to 200 UI/mL.

The analysis of the interaction between the timing of IL-2 supplementation and Dynabead concentration between the two analytical techniques also highlighted differences in outputs. Analysis of data traditionally obtained showed that in Effector subpopulations the proportion was negatively affected by either supplementation of IL-2 at 0 hours or increasing the Dynabead concentration to $1x10^6$ beads/mL, or both. Effector subpopulation proportions were only positively affected by delaying the supplementation of IL-2 and decreasing the concentration of CD3/CD28 Dynabeads supplemented. Meanwhile, the analysis of the data obtained via the novel technique showed no subpopulations significantly affected by this combination of variables that had the same type of response. Of the three subpopulations where the largest proportion of cells would be categorised as Effector phenotype, two of them only showed a deviation of response when IL-2 was supplemented at 0 hours along with the higher concentration of CD3/CD28 Dynabeads, while the other showed a relatively positive response to the lower concentration of CD3/CD28 Dynabeads and IL-2 supplemented at 0 hours. It could be argued that the two subpopulations that were negatively affected by supplementing IL-2 at 0 hours and supplementing CD3/CD28 Dynabeads at $1x10^6$ beads/mL had a similar response to that seen in the standard protocol analysis as these conditions

would be considered to induce proliferation and maturation in T-cell cultures. However, not all subpopulations identified as mostly Effector phenotype in the novel analysis were significantly affected by this combination of factors. Furthermore, the standard analysis showed that a negative response could be induced by setting either of these factors at the higher level, rather than requiring them both. The difference in response here shows how the novel technique has benefits over the traditional technique. Identifying subpopulations that are mostly Effector phenotype, but recognising that the subpopulation these cells belong to also incorporates cells that fall into different phenotypic categories and that not all subpopulations that may appear similar, or fit into a similar phenotypic classification will react identically to the same stimuli.

These were the two main points of comparison between the two analyses due to the limited number of significant factors in the traditional analysis. However, as has been mentioned in discussing the experimental conclusions, the new technique has identified several more significant effects through the data. The method of defining subpopulations based on algorithmic representation of the full phenotypic space, before assigning events to these based on a multidimensional analysis, would appear to provide the opportunity for more effective analysis of cohesive populations, as opposed to rigidly defining groups by whether or not a threshold level of marker expression is met.

In addition to this, there were populations identified that responded differently to expected given the trend observed in the data and predicted effects of experimental variables. Nodes 8 and 24 each represent a cluster of cells that covers all 4 maturity subpopulations of CD4+ and CD8+ subgroups respectively. These subpopulations saw an increase in selection rate in conditions that were expected to negatively affect maturation of cultures, whilst other subpopulations that occupied the same CCR7/CD45RO phenotypic space did not see the same response. As previously mentioned, it is not possible to say here if the subpopulations saw a proportional increase due to a lack of maturation beyond this subpopulation, or if it was a completely different endpoint for the cells given the culture environment. Regardless, this analytical process has identified subpopulations with alternate responses that would have been combined into other subpopulations with their responses overlooked in the traditional analytical method.

4.3.2.5.3 Limitations of the novel analytical technique

Whilst the novel analytical approach is a substantial improvement on the old technique, it is still not a perfect system. Oftentimes when it comes to phenotypic analysis there is no perfect way; the new analytical process developed seeks to address the issues of the previous method and negate some of the issues commonly associated with measuring marker expression, but still has its limitations, some of which will be highlighted below.

4.3.2.5.3.1 Use of relatively inverse markers potentially increases weighting of maturity in clustering

One of the major benefits of using SPADE analysis for clustering cells for phenotypic analysis is that it is able to analyse all marker channels simultaneously, giving a multidimensional understanding of the datacloud and highlighting areas of similarity and separation that may otherwise be missed in standard 2D phenotypic analysis. However, one limitation of the process applied in this scenario is the use of CD45RA and CD45RO as markers in analysis. These markers represent naivety and maturity in T-cell cultures and have generally been observed as having a negative correlation when displayed against each other. Although there is a justification for using it in these analyses, the level of CD45RA expression in subpopulations with increasing CD45RO expression could be indicative of different maturation pathways and similar subpopulations that will respond differently to the same stimuli, it also potentially lends bias to the clustering step of the SPADE analysis. As clustering performed by linking events to their nearest neighbour in multidimensional space, having twice as much input to make that calculation is likely to skew the clustering somewhat in favour of maturity being the separating factor. The relevance of CD45RA as a marker beyond just being the inverse of CD45RO means that this marker will not be removed from analysis moving forwards, but nonetheless it could influence outputs moving forwards.

4.3.2.5.3.2 Variable subpopulation generation complicates comparison between experiments

The novel process developed generates subpopulations by analysing a multidimensional cloud and finding the points of greatest commonality to generate phenotypic definitions that events can be associated to. Although this results in a selection of subpopulations that are more representative of what is actually seen in the cultures, it does have the side effect of producing subpopulations that do not often fit into only one of the standard definitions of T-cell phenotypes. This was ultimately the point, to develop a method that took into account the nuances of T-cell subpopulation behaviour, which may not fit into the boxes described by meeting threshold levels of CCR7 and CD45RO expression, but this does cause some issue when trying to interpret data relative to previous experiments. It raises the question of whether subpopulations should be defined based on all maturity subpopulations they represent, that which they predominantly represent, or whether they should be judged based on the Median Fluorescence Intensity of the cluster. Such elements need further investigation and clarification moving forwards if this novel analytical process is to be expanded into the wider scientific community, or results will be to niche and specific for them to be easily comparable across experiments.

4.3.2.5.3.3 Developed subpopulation tracking process may not have universal suitability

Calculating the proportion of cells that belonged to a subpopulation within a culture was the beginning of the process for being able to understand the effect of experimental conditions on said

subpopulations. This was then taken further by analysing how this subpopulation changed over time using a linear estimate equation in Microsoft Excel. This calculated a straight line that best fit the data and output the gradient of the line, which was then taken as the selection rate of the subpopulation. The issue arises with the fact that not all the subpopulation proportion data progresses over time in a linear fashion. Many subpopulations did have a linear progression, while some changed in an exponential manner. Meanwhile, other subpopulations fluctuated in such a manner that they appeared to show that they were transitional populations. After analysing the data produced it was believed that the linear estimate was still a sufficient representation of the change in subpopulation proportion over time, however it is still recognised that this is not a perfect solution to defining the selection rate for subpopulations.

4.3.2.5.3.4 Current process protocol is time consuming

One of the benefits of the original phenotyping method over the novel method produced is the relatively quick process of obtaining results. Currently, the novel process requires multiple SPADE analyses to be performed, analysed for both the point of greatest experimental effect and optimal TNN, before selecting the NoI so that they can be further analysed for subpopulation selection rates. While it is still the case that this phenotypic analysis is an improvement over the traditional method, giving a more resolute picture of the effects of different subpopulations within the culture systems, it is still appreciated that as it stands this is a labour intensive process. Steps have been taken to simplify some elements of the analytical process, but for this method to be adopted widespread in the scientific field it requires some streamlining.

5 Investigating the effects of concentrated incubation with variable CD3/CD28 Dynabead concentrations prior to seeding on T-cell cultures

5.1 Introduction

One of the main components of T-cell culture, and therefore CAR T-cell therapy manufacture, is the stimulation of the activation pathways to induce proliferation in T-cell populations. The main method of isolation utilised in this research is CD3/CD28 Dynabeads. This product works by stimulation of the primary (CD3) and secondary (CD28) activation proteins that are stimulated during the typical activation process *in vivo*. Stimulation of the activation pathway is an important part of T-cell culture as it is the primary driver of proliferation in culture. The presence of stimulants has a significantly positive effect on growth, which can be seen both in literature and in previous work carried out as part of this research.

The effects of activation on proliferation in T-cell cultures is the reason that stimulation became a key element of CAR T-cell manufacture. However, this process also has an effect on the phenotypic profile of cultures. Activation *in vivo* occurs in response to exposure to the T-cell's target antigen. This process induces proliferation to induce a sufficient response in the T-cells, and maturation in the cells as they are 'experienced' in order to mount a quicker and more effective response on a repeat encounter. The maturation is manifested as an increase in CD45RO expression and downregulation of CD45RA expression. The implications of this for T-cell culture *in vitro* are that in the presence of stimulants cultures will see a phenotypic shift from low to high CD45RO expression, moving cells from Naïve and Effector phenotype to Effector Memory and Central Memory phenotype profiles[166]. Depending on the nature of the transition, this could have positive or negative effects on the total number of cells that fall into the target subpopulations of the initial expansion period in CAR T-cell manufacture, Naïve and Central Memory, with stimulation always having a negative effect on Naïve subpopulations, but variable effect on Central Memory subpopulations. As described in 4.3.1.2.2, evidence appears to suggest that stimulation with CD3/CD28 Dynabeads will increase the concentration of Effector Memory cells in the cultures to the detriment of Central Memory subpopulations. However, the data analysing the impact of CD3/CD28 Dynabeads on culture in 3.3.3.3.1 appear to show that longer culture with CD3/CD28 Dynabeads shows an increase in the level of Central Memory subpopulations.

Supplementation of stimulants also introduces another element to these cultures. Depending on the stimulant that is used in culture, it will usually involve the addition of non-human or non-

biological elements into culture. Although there are CAR-T therapy products which have gone through FDA approval that use CD3/CD28 Dynabeads as a stimulant[167,168], removal of the magnetic beads can result in loss of product[167] and there is also increasing pressure to move away from non-biological and animal-based products. The latter is evident in the large amount of research into manufacture of T-cell culture media which do not use animal products, so called xeno-free or serum-free media[145–147], however this element of T-cell culture is outside the scope of this research. With the benefits of stimulation on T-cell population growth evident, it is not practical to remove this from the culture process completely. However, reducing the level of stimulants used would reduce the reliance of T-cell culture on non-human products, and may also have additional effects on proliferation and phenotypic profile of the cultures.

If it were possible to reduce the level of stimulant used, whilst maintaining the level of activation of cultures, this would not only reduce the reliance of T-cell culture on non-human product but would also have substantially reduce the cost of this consumable in therapy manufacture. It would be preferable in reducing the level of stimulant supplemented to induce the same level of proliferation in cell cultures without the same impact on maturation of T-cell subpopulations. Were this possible, it would decrease the reliance on the stimulant whilst also increasing the concentration of Naïve and Central Memory subpopulations in T-cell cultures and retaining the growth for which the stimulant is added. In any case, investigation into the impact on cultures of different levels of CD3/CD28 Dynabead supplementation would improve the definition of the relationship between the stimulant and culture response.

The previous chapter investigated the impact on proliferation and phenotypic profile by a number of culture variables, including the concentration of CD3/CD28 Dynabeads supplemented into culture. Analysis of data identified that increasing the CD3/CD28 Dynabead concentration significantly increased the growth rate of T-cell cultures, however there was also a suggestion that this relationship was not linear. In addition, analysis also showed that there was no significant interaction between the CD3/CD28 Dynabead concentration and seeding density. The data appeared to show that increasing the CD3/CD28 Dynabead concentration elicited the same increase in average growth rate in cultures seeded at $1x10^6$ cells/mL or $2x10^6$ cells/mL. If response to the CD3/CD28 Dynabead concentration was not impacted by the seeding density of the culture, it implies that the absolute concentration of CD3/CD28 Dynabeads is the more important factor in the response observed, rather than the bead/cell concentration.

The previous investigation also suggested that supplementation of IL-2 was detrimental to T-cell cultures if they were not given sufficient time with CD3/CD28 Dynabeads to activate. New evidence suggested that T-cell cultures only needed to be incubated with CD3/CD28 Dynabeads for 30

minutes to induce the effects of activation, substantially less than the 96 hours given previously. It was suggested that it would only be necessary to incubate T-cell cultures with the necessary concentration of CD3/CD28 Dynabeads for that initial activation period to see a response, after which point the cultures would not have the same requirement for CD3/CD28 Dynabead stimulation. It was not a practical suggestion to remove C3/CD28 Dynabeads after this time as the interaction between the beads and cells which induces activation would result in loss of cells from the culture when beads were removed. In addition, it would also put the cells through unnecessary additional manipulations, which increases stress on cultures and removes them from optimal culturing conditions. As such, removal of CD3/CD28 Dynabeads post-activation would not reduce the initial requirement of beads, and with arguments not to remove beads post-activation it would also not reduce the concentration in the final product.

Although removing CD3/CD28 Dynabeads after activation did not appear viable, activation of cultures with a reduced initial number of beads appears feasible due to the previous evidence that concentration was of greater importance than bead to cell ratio. It was theorised that incubating cells at a higher concentration, with Dynabeads at the manufacturers recommended concentration, for a period of time prior to seeding into culture plastic would convey sufficient activation. If possible, this process would enable activation of the T-cell populations, whilst using a fraction of the CD3/CD28 Dynabeads that would be used otherwise. On dilution of the cultures into culture, cell concentration would be at a standard level, whilst CD3/CD28 Dynabead concentration would be at a reduced level without having to extract any beads manually.

There was some consideration for the impact of the concentrated incubation on cell populations to ensure that this process would not negatively impact them. The supplied protocol for the CD3, CD4 and CD8 MicroBead isolation used in chapter 3 specified incubation of cells at $1x10^8$ cells/mL for 10 minutes, whilst initial investigation into this process considered storing of cells at a maximum of $1x10^7$ cells/mL for a minimum of 30 minutes. The processes would differ somewhat in that the new process would be performed in standard medium rather than isolation buffer and would rely on the cells being metabolically active, as opposed to labelling which only relied on binding to externally expressed markers. However, it was believed that this was sufficient proof that the cells would survive the concentrated incubation period.

After an initial investigation indicated that there was limited negative impact, and some potential benefit to concentrated incubation, a larger set of variables was tested which attempted to establish the boundaries of this relationship. Further investigation incorporated surface protein analysis to identify the impacts of concentrated incubation prior to seeding on the balance of subpopulations in

culture. Specific conditions were selected for repeat testing to assess if observations appeared to be repeatable.

5.1.1 Chapter objectives

Validate that the concentrated incubation process will produce viable cultures by monitoring viable cell counts of test conditions in an initial investigation.

Analyse the viable cell counts of test conditions for effects of experimental variables.

Use the novel analytical method developed in 4.3.2 to assess phenotypic data and produce algorithmically defined subpopulations.

Analyse the impact of experimental conditions on subpopulations containing events that matched Naïve (CCR7+CD45RO-) and Central Memory (CCR7+CD45RO+) phenotypic profiles.

Select sample conditions to repeat and compare for validation of observed effects.

5.1.2 Hypotheses

5.1.2.1 Hypothesis on the impact of concentrated incubation on the growth rate of T-cell cultures

Data analysed in 4.3.1 appeared to show that the relative concentration of CD3/CD28 Dynabeads in culture had a greater influence in the promotion of proliferation in T-cell cultures than the bead:cell ratio. In addition, commercially available isolation productions specified protocols that stored T-cell populations at concentrations as high as $1x10^8$ cells/mL, and still producing viable cell cultures. These data became the basis on which the following experiments investigating concentrated incubation were planned. It was predicted that incubating cell suspensions in a reduced volume with the same relative concentration of CD3/CD28 Dynabeads as the standard culture protocol would result in the same level of proliferation as seen in those cultures.

5.1.2.2 Hypothesis on the impact of concentrated incubation on the phenotypic profile of T cultures

Analysis of phenotypic data in chapter 4 show limited examples of significant interaction between seeding density and CD3/CD28 Dynabead concentration, similar to what was observed in the proliferation analysis. As a result, it was predicted that phenotypic response would resemble that of the control conditions. However, it was also speculated that the lack of a sustained standard level of CD3/CD28 Dynabeads in culture, due to the dilution of seeding, could have a positive effect on the Naïve and Central Memory target populations.

5.1.2.3 Predicted relative performance of validation conditions

The balance of T-cell subpopulations can be highly variable between donors[169,170], as has been observed in the experimentation seen so far. This variability between source materials has the

potential to impact and influence the outputs of the experiment both phenotypically and proliferatively. As such it was anticipated that there would be differences in the response between the initial response and the secondary validation. The nature of this change was unknown as it was dependent on the donor material, however it was still believed that despite a different level of response, the cultures would have the same type of response to the experimental variables. For example, if material from donor A showed a positive response in growth rate to increased incubation period, it would be anticipated that this would also be observed in material from donor B, just to a different degree.

5.2 Methods and materials

5.2.1 T-cell source material

The following work was completed using frozen PBCM aliquots from Axol Bioscience as described in 2.1.1.2. The initial investigation was performed using cells from a single donor. Further work was all completed using aliquots of cells from another single donor.

5.2.2 T-cell culture methods

Cell cultures were set up and maintained in accordance with the processes set out in 2.2 and 2.4 except culture seeding in concentrated incubation conditions as described in 5.2.3 and specific parameters listed in 5.2.4, 5.2.5 and 5.2.6.

5.2.3 Methods of concentrated incubation prior to seeding in culture

Two different methods of concentrated incubation were used during experimentation. In the initial investigation, cells were prepared from thaw to a concentration of $1.5x10^7$ cells/mL. For each condition, the appropriate absolute number of cells was transferred for the relevant incubation concentration when diluted to 300 µL. The samples were then supplemented with the required absolute number of prepared CD3/CD28 Dynabeads for the required concentration. The appropriate volume of standard medium was then added to each tube to bring the total volume to 300 µL. Tubes were then placed into an incubator at 37°C and stored for their test period. On removal cultures were transferred to a 6 well plate and supplemented with 2.7 mL standard medium and 200 UI/mL IL-2. Control conditions were seeded as described in 2.4.5 and placed into the same 37°C incubator. These cultures were supplemented with 200 UI/mL IL-2 at 50 minutes, in line with the intermediate condition.

In the initial investigation the absolute cell number was changed to vary the cell/mL concentration during incubation. In the subsequent experimentation the absolute number of cells was maintained across conditions and the suspension volume was changed to vary incubation concentration. This enabled a consistent seeding density across all cultures, avoiding perceived issues with varying this

factor discussed in 5.1.2. Seeding density was set at $1x10^6$ cells/mL, with a culture volume of 3mL this meant that the absolute number of cells per test condition was $3x10^6$. Cells were prepared from thaw to a concentration of $6x10^7$ cells/mL and 50 µL transferred to each tube. CD3/CD28 Dynabeads were prepared as described in 2.4.4 other than the final resuspension, which was in half the initial volume of beads taken for preparation. This was to accommodate the smaller working volume for supplementation. Cultures were then supplemented with an appropriate volume of CD3/CD28 Dynabeads for the condition. Standard medium was then added to all experimental conditions to bring to a total volume appropriate for the condition. These were 100 µL for those incubated at $3x10^7$ cells/mL, 150 µL for those incubated at $2x10^7$ cells/mL and 300 µL for those incubated at $1x10^7$ cells/mL (Fig. 5.2).

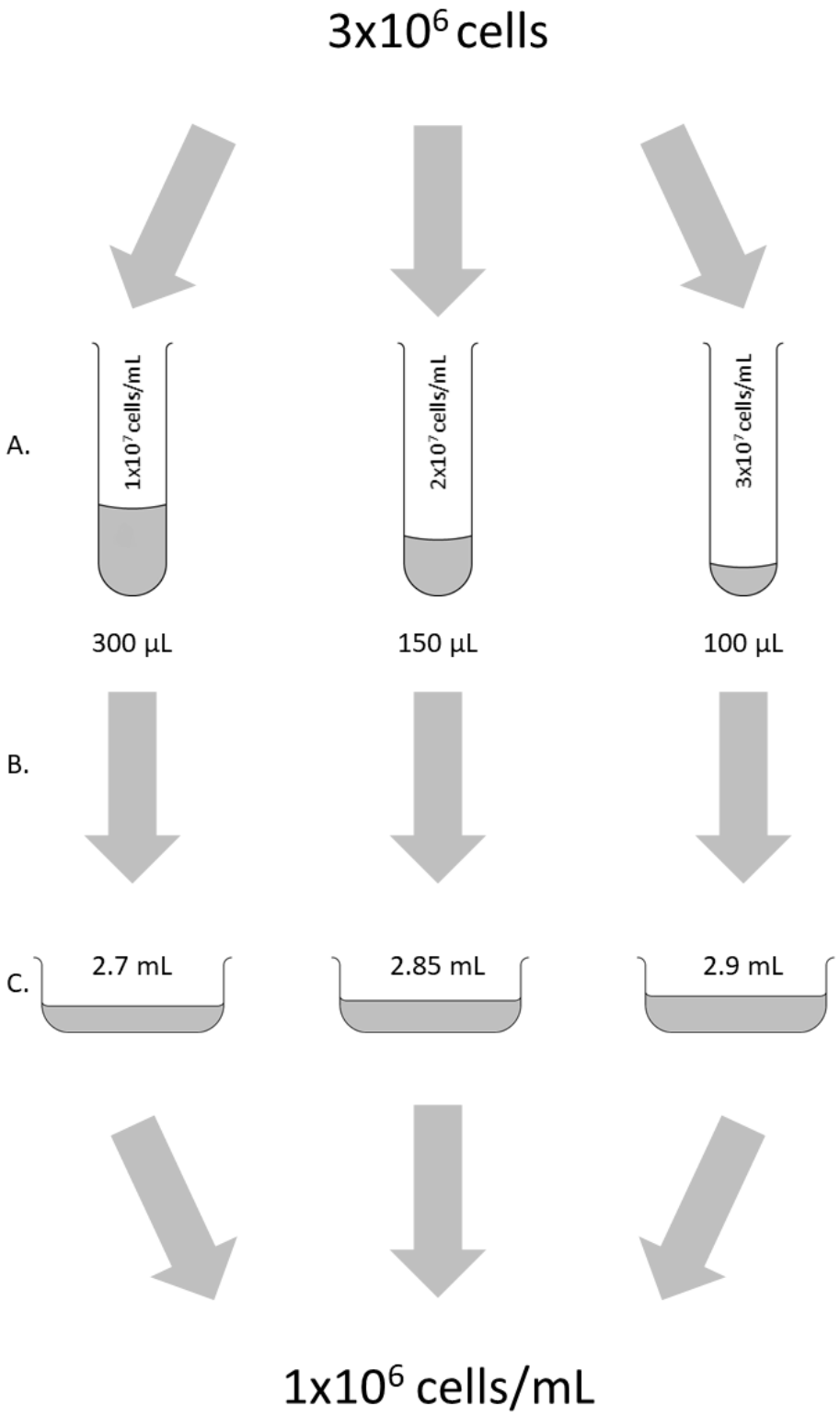


Figure 5.1. Process of concentrated incubation prior to seeding into culture. $3x10^6$ cells were transferred to Eppendorf tubes and supplemented with CD3/CD28 Dynabeads and standard culture medium to bring sample to required cell/mL concentration (A). Samples were then incubated for their designated duration (B). Samples were then transferred into culture wells with standard medium for a total volume of 3 mL (C). Final seeding concentration of all conditions was $1x10^6$ cells/mL

Non-incubated conditions were prepared and placed into incubation alongside the concentrated incubation experimental conditions. After their designated incubation period, samples were removed from incubation and transferred to a 6 well plate and supplemented with standard medium to make up to 3 mL. Once the final samples had be transferred to 6 well plates, all cultures were supplemented with 200 UI/mL IL-2 and returned to incubator. This process was also used when validating the results of the expanded concentrated incubation outputs.

5.2.4 Sampling of cultures for viable cell counts and phenotypic analysis

Viable cells count and phenotypic analysis was carried out as described in 2.6.2 and 2.7 respectively. Timing of culture sampling for analysis is detailed on an experimental basis in 5.2.4, 5.2.5 and 5.2.6. Further phenotypic analysis was performed on marker expression data using the novel analytical technique outlined in 4.2.1-3.

5.2.5 Specific methods and materials used in initial investigation into effect of concentrated incubation prior to seeding with variable cell and CD3/CD28 Dynabead concentration

Concentrated incubation was anticipated to put cells in conditions that may be more stressful than standard culture conditions. As such, efforts were taken to reduce further impact after transferring into culture. Common seeding density of T-cells into culture varied between 1-2x10^6 cells/mL[132], and as such it was decided to include conditions that would be at this density after dilution into culture. At a concentration factor of 10, and a seeding density of 1x10^6 cells/mL, this would result in an incubation concentration of 1x10^7 cells/mL. Conditions incubated at a concentration of 2x10^6 cells/mL were tested so as to include concentrated samples that were within the limits of standard seeding density of T-cells. Between them, these two incubation concentrations would be within the standard range of T-cell seeding densities at concentrated incubation and at seeding after incubation.

Regarding incubation duration, it was posited that the length of time the cultures were incubated at a high concentration with the CD3/CD28 Dynabeads could impact either the level of T-cell activation or the number of cells the beads were able to interact with. This could then impact either the level of proliferation in the culture or the phenotypic response. In addition, it was understood that given sufficient incubation period there would be a point at which medium exhaustion would occur, negatively impacting the cells. 30 minutes was speculated as the minimum period of time necessary to stimulate growth and was therefore the minimum period of incubation included. It was not clear when medium exhaustion would occur, and therefore during initial investigation the maximum duration tested was 70 minutes.

A midpoint condition for incubation period and concentration was included to increase the analytical power. Maintaining the same absolute concentration during the incubation period as was standard in culture, taking into account the 10-fold concentration, meant that incubated samples were supplemented with $1x10^6$ beads/mL. A standard protocol control condition was included, along with an incubation control which used midpoint incubation parameters with the standard concentration of Dynabeads added. Experimental parameters of conditions tested are described in Table 5.1. Each condition was tested in duplicate, and asterisks indicate a condition that did not undergo concentrated incubation. Cultures were sampled for viable cell count analysis at 0, 14, 36, 58 and 114 hours.

Table 5.1. Experimental conditions examined in proof of concept experiment.

Condition no.	Incubation Concentration (10^7 cells/mL)	Incubation Period (minutes)	Dynabead Concentration (10^6 beads/mL)
1	0.2	30	0.1
2	0.2	70	0.1
3	0.6	50	0.1
4	1	30	0.1
5	1	70	0.1
6	0.6	50	1
7	*	*	1

5.2.6 Specific methods and materials used in subsequent expanded analysis of effects of concentrated incubation prior to seeding with variable cell and CD3/CD28 Dynabead concentration

The results of the initial investigation informed the parameters of subsequent investigation into concentrated incubation. The experimental conditions that were tested are listed in Table 5.2, conditions with an asterisk did not undergo concentrated incubation. Each condition was performed in duplicate. Cultures were sampled for viable cell count and phenotypic analysis at 0, 18, 37, 62, 66, 86, 111, 115, 138 and 158 hours.

Table 5.2. Experimental conditions examined. Starred conditions were seeded straight into culture wells and did not undergo any concentrated incubation.

Condition no.	Incubation concentration (10^7 cells/mL)	Incubation period (minutes)	Dynabead concentration (10^6 beads/mL)
1	1	30	0.1
2	1	60	0.1
3	1	60	0.2
4	1	60	1
5	1	90	0.1
6	1	120	0.1
7	1	120	0.2
8	1	120	1
9	1	180	0.1
10*	-	-	0.1
11*	-	-	0.2
12*	-	-	1
13	2	60	0.1
14	2	90	0.1
15	2	120	0.1
16	3	30	0.1
17	3	60	0.1
18	3	60	0.2
19	3	60	1
20	3	90	0.1
21	3	120	0.1
22	3	120	0.2
23	3	120	1
24	3	180	0.1

5.2.7 Specific methods and materials used in experimentation to validate the expanded analysis of the impact of concentrated incubation prior to seeding with variable cell and CD3/CD28 Dynabead concentration

Validation conditions, 8, 12, 19 and 21, outlined in Table 5.2, were selected as described in 5.1.3. These conditions were run in triplicate and sampled at 0, 20, 43, 46, 67, 88, 96, 113, 137, 145 and 159 hours for viable cell count and phenotypic analysis.

5.3 Results

Analysis of T-cell growth data in 4.3.1.1 suggested that the concentration of CD3/CD28 Dynabeads supplemented into culture had a greater impact on the proliferation of a culture than the bead:cell ratio. A series of PBMC samples were incubated at a concentrated level with varying quantities of CD3/CD28 Dynabeads to assess if it were possible to reduce the process's reliance on stimulant particles whilst maintaining proliferation. Analysis was also performed on the phenotypic profile of these cultures to help define the impact of the concentrated incubation process on the expression of phenotypic markers.

5.3.1 Initial investigation into the impacts of concentrated incubation with variable CD3/CD28 Dynabead concentration on T-cell culture growth

5.3.1.1 Impact of concentrated incubation on the final cell yield of T cultures

Observation of viable cell counts shows that all cultures showed a higher final cell count than was seeded, indicating that they all experienced some period of growth in culture. Comparison of the conditions (Fig 5.1A), especially at 115 hours, shows a clear separation between the different experimental conditions. The conditions incubated at the highest concentration consistently had the highest viable cell concentrations, regardless of the period of incubation. Meanwhile, the viable cell counts of the condition incubated at $5x10^6$ cells/mL were not substantially lower than the yield of the conditions incubated at $1x10^7$ cells/mL at the final measurement.

The conditions incubated at the lower concentration of $2x10^6$ cells/mL, however, had a substantially lower yield than the other experimental conditions observed at 115 hours. The conditions incubated at the lower concentration failed to proliferate sufficiently to produce a concentration greater than $1x10^6$ cells/mL across the experiment, while the other test conditions all had a final viable cell count above $1.75x10^6$ cells/mL.

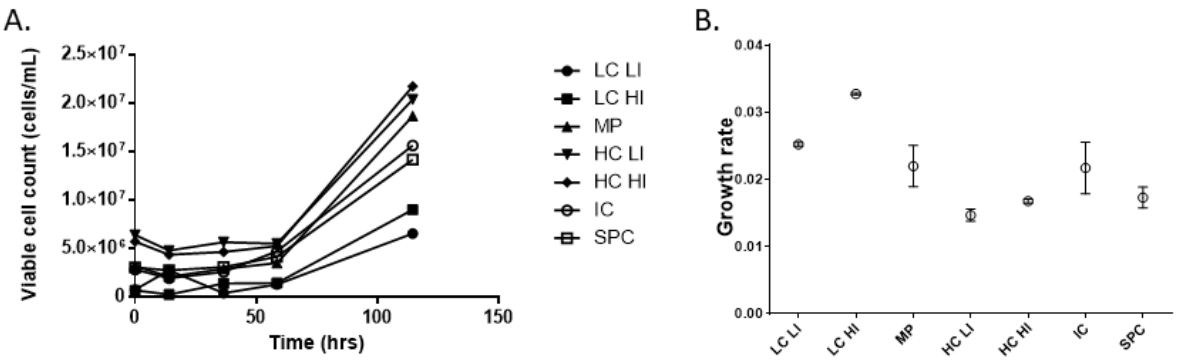


Figure 5.2. Analysis of viable cell counts over time shows comparable performance between test and control conditions. PBMC cultures were incubated at varying concentrations for a different periods with 10 fold reduced concentration of CD3/CD28 Dynabeads to the standard concentration. The conditions were sampled at regular intervals and tested for viable cell count (a). An estimated growth rate was then calculated from 24 hours to identify differences in proliferation. Data shown as mean with SEM error bars (b).

Meanwhile, the control conditions appeared to perform similarly to each other, with the Incubation Control (IC) having a slightly higher viable cell concentration at the final count than the Standard Protocol Control (SPC). The control samples both ended the experiment at ~1.5x10^6 cells/mL, slightly lower than both the mid-level condition and the high incubation concentration conditions.

These data suggest not only that the concentrated incubation is not having a negative effect on the T-cells, but that these conditions could also outperform the control conditions. Furthermore, the final yield seen in these cultures appears to show that the T-cells are being sufficiently activated despite the reduced concentration of CD3/CD28 Dynabeads being supplemented into culture. The intermediate condition, seeded at the same density as the control conditions, showed a greater yield from the same start material than either the IC or the SPC. This suggested that the reduced concentration of CD3/CD28 Dynabeads may have a comparable effect to the recommended concentration on final yield, and that the concentrated incubation was also promoting that.

5.3.1.2 Effects of concentrated incubation on growth rate of T cultures

The process of controlling cells for incubation in this experiment varied the quantity of cells in 300 µL medium, then diluted to 3mL in culture plastic. This method resulted in cultures being seeded at different concentrations. Although total cell yield is an important measure in understanding what effects the experimental conditions are having on culture performance, if the starting concentration is not considered these values can become misleading. To account for them, growth rates were calculated from the viable cell counts over time and compared to identify which conditions proved the most proliferative.

Comparing growth rates of the different conditions immediately showed a different picture to comparing the final viable cell counts (Fig 5.1B). Whilst the conditions incubated at higher concentrations produced the highest cell yield, they also produced the lowest growth rates. The next highest growth rate, of the experimental conditions, was once again the mid-level condition, while low concentration incubation conditions had the highest growth rates. Between the two low concentration incubated conditions, the culture incubated for 70 minutes had a higher growth rate than those only incubated for 30. This effect was also observed in the cultures incubated at the high concentration, however the difference in growth rate was not of the same magnitude. Comparison with the control condition growth rates shows that SPC has a similar growth rate to that of the high concentration, long incubation period condition, however the range of values produced by the SPC is greater. Meanwhile, the IC produced both a growth rate and range similar to that of the mid-level condition.

These data somewhat flip the conclusions from the cell yields regarding the test conditions. They show that the best growing conditions were those seeded at $2x10^5$ cells/mL rather than $1x10^6$ cells/mL, although they still both suggest that longer incubation period is of benefit. However, the relative performance of the control conditions showed that test conditions still outgrew them and also appeared to benefit from this process.

5.3.1.3 Conclusions on initial investigation into the concentrated incubation protocol

The preliminary investigation was carried out to identify the effect of the concentrated incubation process on T-cell growth in culture and ensure that the process would produce viable T-cell cultures to avoid wasting time and materials. The outputs of the experiment suggested that the concentrated incubation protocol was worthy of further investigation. Calculated growth rates showed that the test conditions which grew worst still grew in at a rate similar to that of the SPC, indicating that neither the concentrating of the cells prior to seeding, nor the reduced concentration of CD3/CD28 Dynabeads was substantially hampering growth of the T-cell populations.

This evidence alone would have been sufficient to warrant further investigation into concentrated incubation, however there is also evidence to suggest that the concentrated incubation improves the growth rate compared to the SPC. Observation of the response in the incubation control showed an increase in growth rate in the mid-point condition and IC by a similar amount regardless of the concentration of CD3/CD28 Dynabeads supplemented.

Further experimentation needed to address the methodological issue of seeding cultures at different concentrations. The relationship between seeding density and proliferative capacity is not fully understood and removing this element from experimentation would help to reduce confounding

factors in post hoc analysis. In addition, it would be prudent in further examination of these parameters to test further concentrations of CD3/CD28 Dynabeads to try and improve the definition of the relationship between the level of CD3/CD28 Dynabead reduction and growth. Finally, further experimentation would include phenotypic analysis using the novel analytical technique previously developed to attempt to identify any phenotypic relationship with experimental variables.

5.3.2 Increasing the limits of concentrated incubation variables tested to further test the relationship with growth and compare to phenotypic response

5.3.2.1 Analysis of how concentrated incubation with varying levels of CD3/CD28 Dynabeads changes T-cell population proliferation

Analysis of viable cell count over time (Fig 5.3) shows that on average nearly all conditions showed some level of growth. These plots also show the inflection point of the cultures, where the growth of viable cells begins to outweigh the rate at which cells are dying, at 37 hours. This effect is a common and anticipated part of T-cell culture, with the majority of cell death coming as a result of non T-cell PBMCs that are not sustained by the culture conditions dying[126,133,140]. Growth rates were calculated using the viable cell count data from the inflection point, 37 hours, to compare proliferation in culture. The average growth rate for all cultures was positive apart from the condition incubated for 90 minutes a $1x10^7$ cells/mL and supplemented with $1x10^6$ bead/mL CD3/CD28 Dynabeads.

Comparison of calculated growth rates with the Incubation Concentration (Fig. 5.4a) shows a general negative trend in growth rate as the incubation concentration increased. 3 of the top 4 average growth rates calculated came from conditions that did not undergo any concentrated incubation. The average growth rates then correlated somewhat negatively from the non-incubated conditions as the incubation concentration increases. However, although the average growth rates can be seen to decrease as the concentration increased, the range of growth rates observed also increased. This increase in range is great enough that the highest growth rate observed in conditions incubated at $3x10^7$ cells/mL is greater than any of those incubated at $1x10^7$ cells/mL, and greater than the non-incubated condition supplemented with $1x10^6$ beads/mL CD3/CD28 Dynabeads. One condition, incubated at $1x10^7$ cells/mL for 90 minutes and supplemented with $1x10^5$ beads/mL CD3/CD28 Dynabeads, showed a negative calculated growth rate. Considering this performance of this culture in the context of all other conditions, this response can be considered anomalous. It is also not clear what caused this anomaly to occur as the culture was treated in the same manner as all others.

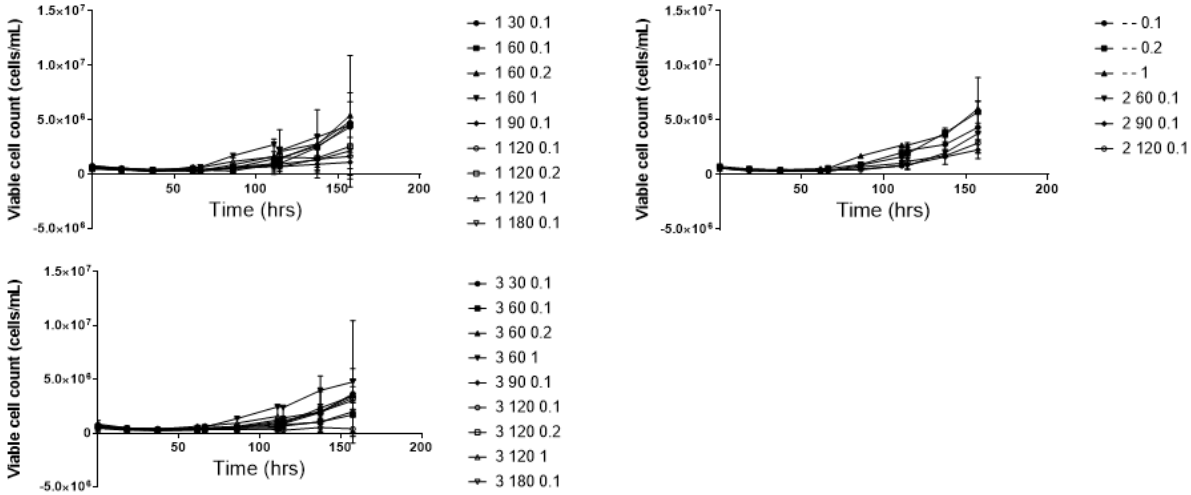


Figure 5.3. Analysis of viable cell counts over time shows that longer incubation periods are more likely to negatively effect final cell yield. Cultures were sampled regularly and tested for viable cell count to monitor proliferation in cultures over time. Data are separated based on cell concentration during incubation for improved clarity and presented as mean viable cell count with SEM error bars.

Statistical analysis shows that, in omission of anomalous results, there is a significant negative correlation between increasing Incubation Concentration and growth rate ($P<0.05$). Although experimental differences mean they are not directly comparable, these data show a similar response to what was observed in the initial investigation. This appeared to show that that conditions incubated at a lower concentration produced a greater growth rate. Conversely, the conditions that did not undergo concentrated incubation produced the greatest growth rates, compared to the initial experiment, where incubated conditions outgrew non-incubated conditions seeded at the same density.

Comparison of growth rates with Incubation Period shows a similar relationship as that with Incubation Concentration, in that there appeared to be a negative trend in the calculated average growth rates when Incubation Period was increased (Fig 5.4b). The trend can be most clearly seen comparing the conditions incubated for 30, 90 and 180 minutes due to the limited number of conditions incubated for these periods. This trend was still visible in those conditions incubated for 60 and 120 minutes, however due to the greater range of conditions tested at these incubation periods the increase in range of growth rates produced which masks this somewhat.

As was observed also when comparing Incubation Concentration, the range of growth rates increased as the duration of incubation is increased. It is possible to see a greater range of growth rates produced in conditions incubated for 120 minutes compared to 60. These data suggest that a

longer Incubation Period will increase the separation between the greatest and lowest growth rates induced by other experimental variables.

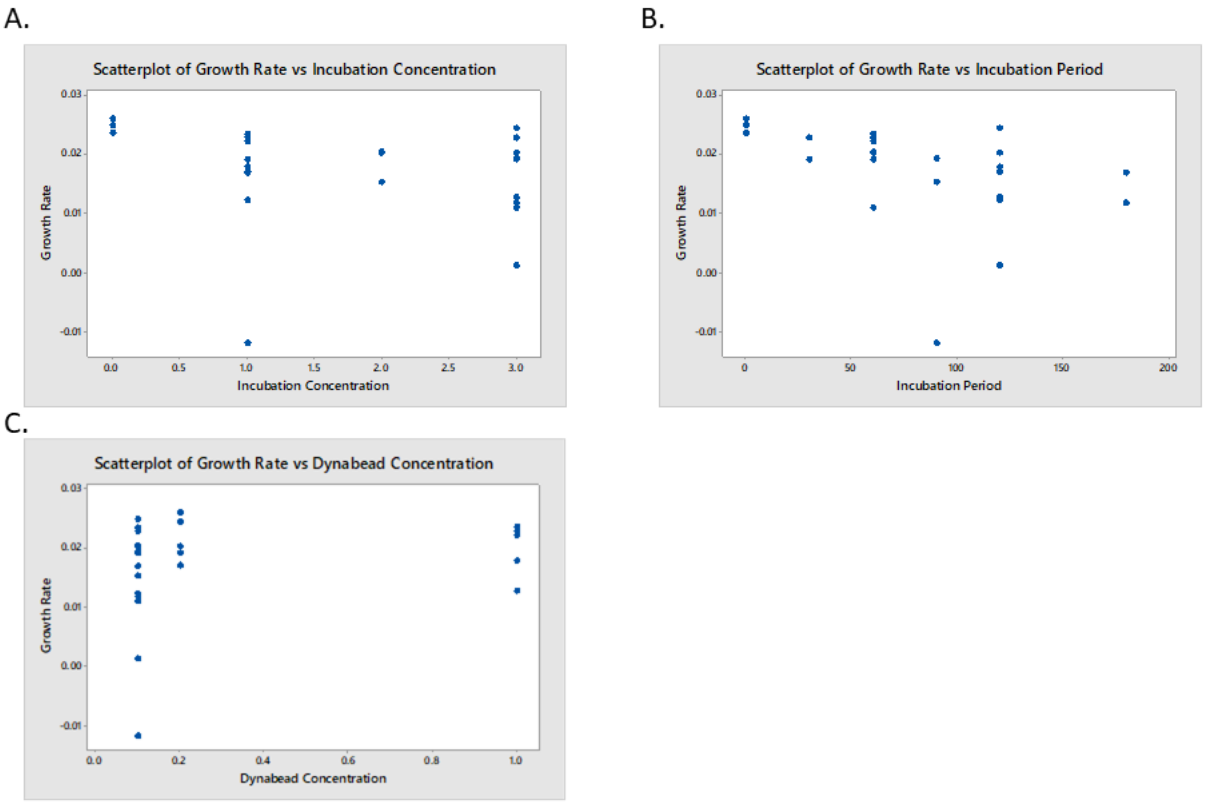


Figure 5.4. Comparison of variables against growth rates shows that increasing incubation concentration and incubation period have a significantly negative effect. Growth rates were calculated from viable cell counts between 37 and 157.5 hours. Growth rates were then compared against Incubation Concentration (a), Incubation Period (b) and Dynabead Concentration (c). Statistical analysis showed a significant negative corelation when comparing growth rates to Incubation Concentration ($P<0.05$) and Incubation Period ($P<0.005$).

However, the conditions incubated for 30, 90 and 180 minutes did not see the same increase in range as incubation period increased. The conditions incubated for these periods of time were incubated at $1x10^7$ and $3x10^7$ cells/mL, with $2x10^7$ cells/mL also incubated for 90 minutes. Observing the difference between the growth rates of the cultures at 30 and 180 minutes shows that incubating at $1x10^7$ cells/mL had a positive effect relative to incubating at $3x10^7$ cells/mL. Meanwhile, the anomalous data from the conditions incubated for 90 minutes at $1x10^7$ cells/mL does not allow for the same comparison at this point, but the difference between the cultures incubated at $3x10^7$ cells/mL and $2x10^7$ cells/mL is still noticeably of a similar magnitude. All conditions incubated for either 30, 90 or 180 minutes were supplemented with $1x10^5$ beads/mL CD3/CD28 Dynabeads. The data show that an increasingly separating dataset was seen between incubation periods of 60 and 120 minutes, where multiple CD3/CD28 Dynabead concentrations were

compared, as opposed to incubation periods of 30, 90 and 180 minutes which tested one. This implies that the effect of CD3/CD28 Dynabead concentration was magnified by increasing the incubation period, however increasing the incubation period with a consistent concentration displayed a more linear relationship.

Further investigation into the nature of the variation in growth rates produced by cultures incubated for 60 and 120 minutes showed that cultures incubated at $3x10^7$ cells/mL with $1x10^5$ beads/mL had the lowest growth rates. As a result, the trend of growth rates for this condition fluctuates in a pattern without a clear cause. The growth rate decreases when increasing the incubation period from 30 to 60 minutes, this growth rate is then seen to increase when this incubation period is further increased to 90 minutes to a level higher than that originally seen when incubated for 30 minutes. Beyond this the growth rate drops again when incubated for 120 minutes to a level that is only just positive, before increasing substantially when incubated for 180 minutes, increasing the growth rate to a level similar to that seen when incubated for 60 minutes.

While it is still possible to see general trends in the data, such as those cultures supplemented with $1x10^6$ bead/mL CD3/CD28 Dynabeads at 60 minutes having slightly higher growth rates than those supplemented with $2x10^5$ beads/mL, it is also possible to see confounding data. Following from the previous examples, this can be seen where growth rates for cultures incubated at $1x10^7$ cells/mL for 120 minutes were slightly higher when supplemented with $1x10^6$ beads/mL compared to $2x10^5$ cells/mL. However, when incubated at $3x10^7$ cells/mL the growth rate is substantially higher than either condition incubated at $1x10^7$ cells/mL when supplemented with $2x10^5$ beads/mL, and substantially lower than them when supplemented with $1x10^6$ beads/mL.

It is unclear within these conditions if there are other influential factors that have not been recorded, or if it is merely noise within the data caused by the inherent variability of the T-cell culture system. The data suggest that there is a negative correlation between the increasing of incubation period and the growth rate achieved. Statistical analysis of the correlation shows that there is a significantly negative correlation ($P<0.005$). However, the further assessment of these data brings this conclusion into question, with the somewhat erratic behaviour cultures with common incubation and CD3/CD28 Dynabead concentrations being the source of this uncertainty.

The overall negative trend observed when increasing the Incubation Period of cultures contradicts what has been seen previously, where an extended Incubation Period had a positive effect on growth rates. Considering the fluctuations seen in these data within experimental variable groups, further investigation needs to be carried out to establish if there are any consistent trends that can be observed in T-cell culture when increasing Incubation Period in this protocol.

Comparison of growth rates against Dynabead Concentration does not show the same level of trend as has been seen when compared against the other experimental variables. It is possible to see an increase in the average growth rate of all conditions when increasing from $1x10^5$ beads/mL to $2x10^5$ beads/mL. In addition, the range of growth rates reduces with this increase in CD3/CD28 Dynabead concentration, with the highest growth rate produced showing a slight improvement at $2x10^5$ beads/mL compared to $1x10^5$ beads/mL, but the lowest growth rate produced improving substantially. The average growth rate then decreases when the CD3/CD28 Dynabead concentration is increased to $1x10^6$ beads/mL, however the range of growth rates produced remained similar (Fig 5.4c).

For each supplemented concentration, those cultures that did not undergo concentrated incubation produced the highest growth rates, however beyond this there were few observable patterns in the data. The conditions incubated at $3x10^7$ cells/mL performed better than those incubated at $1x10^7$ cells/mL when supplemented with $2x10^5$ beads/mL. However, when supplemented with $1x10^6$ beads/mL those conditions incubated at $3x10^7$ cells/mL produced both the highest and the lowest growth rates. It is possible to see that conditions incubated for 30 minutes produced the highest growth rates of the incubated conditions at this level of CD3/CD28 Dynabead supplementation.

The growth rates of the cultures supplemented with $2x10^5$ and some cultures supplemented with $1x10^5$ beads/mL CD3/CD28 Dynabeads confirm one element of the hypothesis and repeat what was observed in initial experimentation. These data show that the supplementation of T-cell cultures with a reduced concentration of CD3/CD28 Dynabeads, even when supplementing directly into culture without concentrated incubation, does not have a reductive effect on growth rate. Reducing the concentration of CD3/CD28 Dynabeads 5 or 10 fold would substantially reduce the quantity of beads required to generate the quantity of cells required for T-cell therapies. These data would have to be reconciled with any phenotypic effects the reduction in CD3/CD28 Dynabeads were having, but remains an important conclusion in the effects of these experimental variables on growth rates.

5.3.2.2 Analysis of phenotypic response to concentrated incubation with variable CD3/CD28 Dynabead concentrations

Phenotypic analysis was performed using the novel analytical process outlined in 4.3.2. SPADE analyses were run with a TNN of 10, 15, 20, 25 30 and 40, and E2N ratios were calculated from the data produced as described in 4.3.2.2. These data were then analysed for the point of greatest experimental effect.

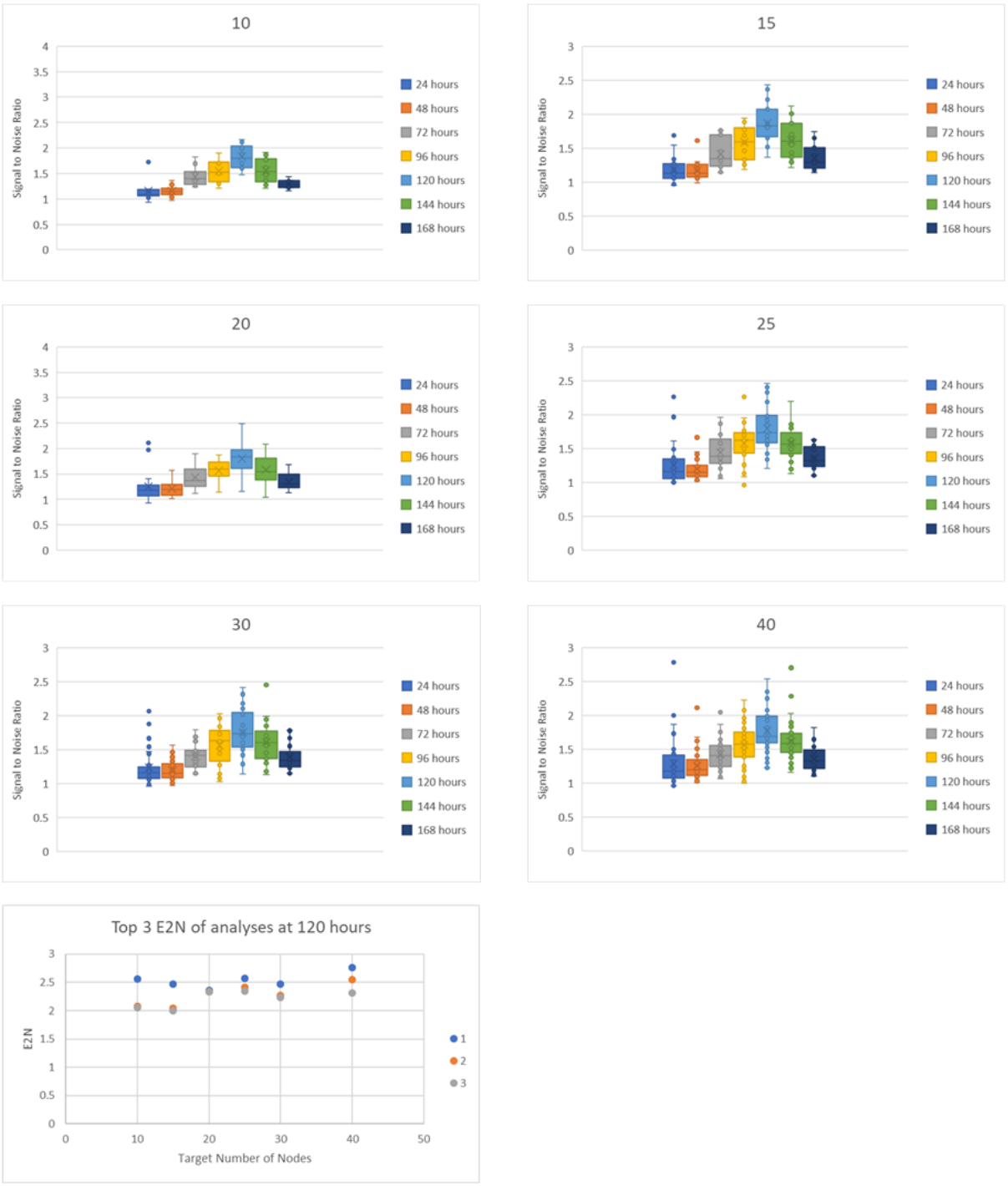


Figure 5.5. Analysis shows the point of greatest experimental effect is 120 hours, and the optimal Target Number of Nodes (TNN) is 20. Box plots were produced for each SPADE analysis of different TNN run, with data separated by day of collection to identify the point of greatest E2N values, indicative of the greatest experimental impact. This was identified as day 5 (120 hours). The top 3 E2N from each analysis at this timepoint was then plotted to identify the optimal TNN for this analysis.

It is possible to see across all analyses that there is a common trend in the data. Each analysis displays an increase in E2N over time from Day 2 until Day 5, followed by a sharp decline until Day 7. This trend of increase and subsequent decrease can be seen in the upper and lower quartiles and

the median E2N values of the box plots. In the 10, 15, 20 and 30 TNN analyses the E2N ratios for Day 1 are at a similar level to Day 2, whilst in the 25 and 40 TNN analyses Day 1 has a higher upper quartile of E2N ratios with a similar lower quartile value (Fig 5.5). The pattern of these data and the repetition of this trend through the whole of the data clearly show that the point of greatest experimental effect was at Day 5, which was then carried forward for further analysis.

The top 3 E2N from the SPADE analyses of different TNN at Day 5 were then plotted for comparison. Analysis of the top E2N ratios from each analysis shows a gradual decline as the TNN increases from 10 to 20. There was a sharp increase when the TNN was 25, followed by a decrease at a similar rate to that seen previously when the TNN was increased to 30. Meanwhile, the analysis with the highest TNN, 40, also showed the highest E2N ratio.

Assessment of the 2nd and 3rd highest E2N ratios show a similar trend across the analyses. There is a decrease in the E2N ratio from 10 to 15 TNN, followed by an increase to 20 TNN. The 2nd highest E2N value increases slightly at 25 TNN, whilst the 3rd highest stays comparable to the value it had at 20 TNN. The 2nd and 3rd greatest E2N values at 30 TNN, however they did not reduce to below the level seen in the 10 TNN analysis. This was followed by an increase as the TNN was raised to 40 (Fig 5.5) in both the 2nd and 3rd greatest values. With the top and 2nd highest E2N values of the 40 TNN analysis being the greatest of their respective groups, the 40 TNN analysis was a consideration as the optimal TNN. However, the 25 TNN analysis showed the greatest 3rd highest E2N, and also showed the second greatest top and 2nd highest E2N values. Ultimately 25 TNN was selected as the optimal resolution and that analysis was used moving forwards.

At the selected optimal TNN, each node of the 25 node analysis was then analysed for presence of events that met the threshold CCR7 expression level to indicate the presence of Naïve or Central Memory cells. This analysis showed that these NoI were nodes 1, 3, 9, 11, 13, 14, 16 and 19. Assessment of the SPADE diagrams showed that nodes 1, 3, 11 and 13 represented CD8+ subpopulations, while nodes 9, 14, 16 and 19 represented CD4+ subpopulations (Fig 5.6, Table 5.3).

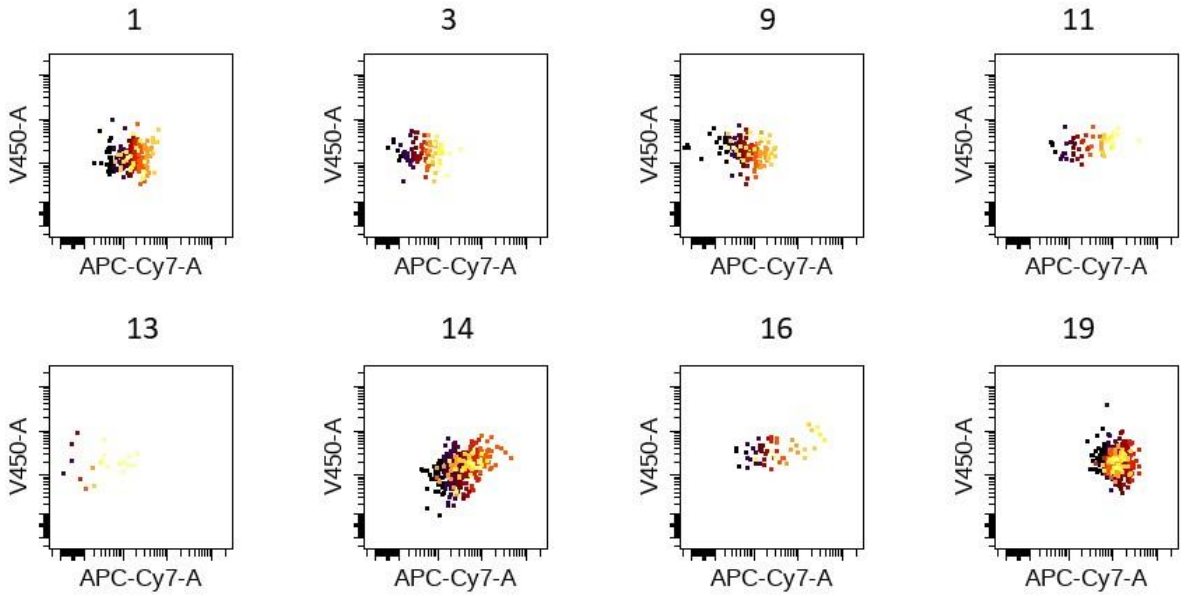


Figure 5.6. Plots of CCR7 (V450-A) and CD45RO (APC-Cy7-A) expression of events within nodes identifies nodes of interest for further assessment. Nodes from the SPADE analysis of optimal resolution (Target Number of Nodes 20) for the dataset were assessed to identify those that contained events falling into the definition of Naïve (CCR7+/CD45RO-) and Central Memory (CCR7+/CD45RO+). All node event plots presented were identified as having sufficiently high CCR7 expression to qualify as containing events of the specified subpopulations.

All nodes appear to cover a similar range of CCR7 (V450-A) expression except nodes 11 and 16. Nodes 11 and 16 each cover a smaller range of CCR7 expression, however the ranges they cover are similar. The expression levels within nodes 11 and 16 suggest that they are predominantly or entirely 'homing' subpopulations, while the other nodes also show low levels of CCR7 expression, indicating the presence of 'non-homing' T-cell subpopulations.

Comparison of CD45RO (APC-Cy7-A) expression shows a wide range of expressions across the NoI. Nodes 3, 9 and 13 show low levels of CD45RO expression, indicating that these nodes are comprised of Naïve and Effector subpopulation T-cells. Meanwhile node 19 shows a high level of CD45RO expression, indicating the presence of Central Memory and Effector Memory subpopulation T-cells. Nodes 1, 11, 14 and 16 show the presence of both CD45RO high and low expressing event. The range of CCR7 and CD45RO expression observed in nodes 1 and 14 show that they contain cells from all 4 maturity subpopulation (Naïve, Effector, Effector Memory and Central Memory), while nodes 11 and 16 can be seen to contain events from both Naïve and Central Memory subpopulations. It is also observable that although node 9 has low CD45RO expressing events and node 14 has some high

CD45RO expressing events, the predominant in the data is that the CD8+ nodes (1, 3, 11 and 13) have lower CD45RO expression levels than the CD4+ nodes.

Table 5.3. Description of phenotypic presentation in Nodes of Interest, as identified in Fig 5.6

Node	CD4/ CD8	Maturity Phenotype	Additional notes
1	CD8	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression is predominantly +ve. CCR7 expression spans both +ve and –ve.
3	CD8	Naïve/Effector	CD45RO expression is –ve, and borders but does not present as +ve. CCR7 expression spans both +ve and –ve.
9	CD4	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression spans both +ve and –ve. CCR7 expression is predominantly +ve.
11	CD8	Naïve/Central Memory	CD45RO expression is predominantly +ve. CCR7 expression is +ve and borders but does not present as –ve.
13	CD8	Naïve/Effector/ Central Memory	CCR7 expression is predominantly +ve. CD45RO expression in the CCR7+ve region is predominantly –ve, but also presents a +ve populations. CD45RO expression in the CCR7–ve region is entirely –ve.
14	CD4	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression is substantially +ve. CCR7 expression spans +ve and –ve. CD45RO expression is higher in the CCR7 +ve region.
16	CD4	Naïve/Central Memory	CD45RO expression is predominantly positive. At maximum, CD45RO expression is considered high. CCR7 expression is +ve, and borders but does not present as –ve.
19	CD4	Effector Memory/ Central Memory	High CD45RO expression. CCR7 expression spans +ve and –ve.

Subpopulation relative selection rates were calculated as discussed in 4.3.2.3 for each of the nodes of interest. The relative selection rates of these subpopulations were then compared against experimental variables to identify any consistent effects that can be observed. When comparing relative selection rates of the selected NoI against the Incubation Concentrations tested, the most common effect observed was a decrease in the relative selection rate as the Incubation Concentration was increased (Fig 5.7). The data from every node showed relative selection rates either side of 0, identifying that the range of experimental variable was able to induce both positive and negative selection rates in the NoI populations. Statistical analysis of correlation also shows that

there is a significant negative correlation between the relative selection rates and the Incubation Concentration in nodes 1, 3, 9, 14 and 19 ($P<0.05$).

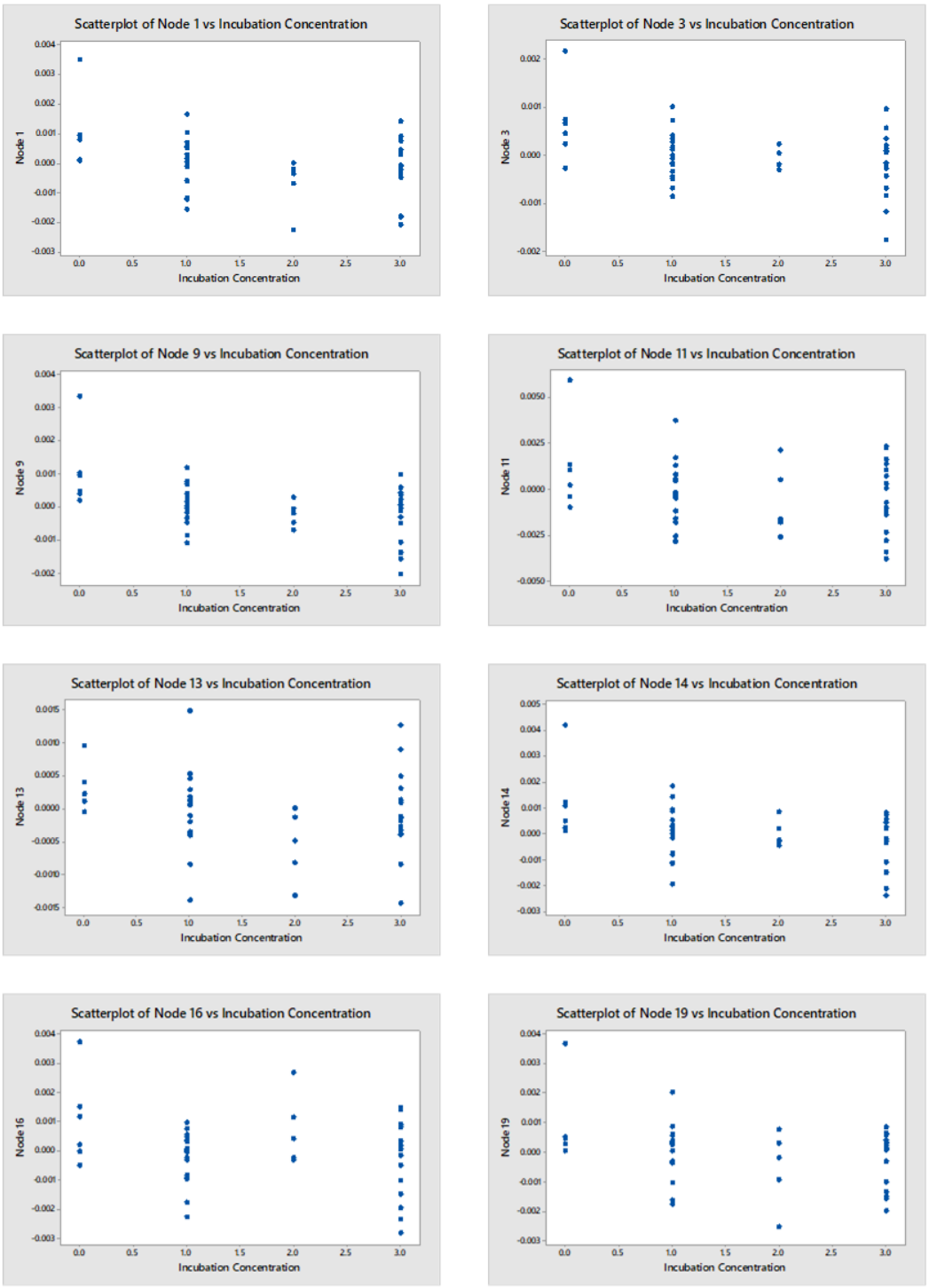
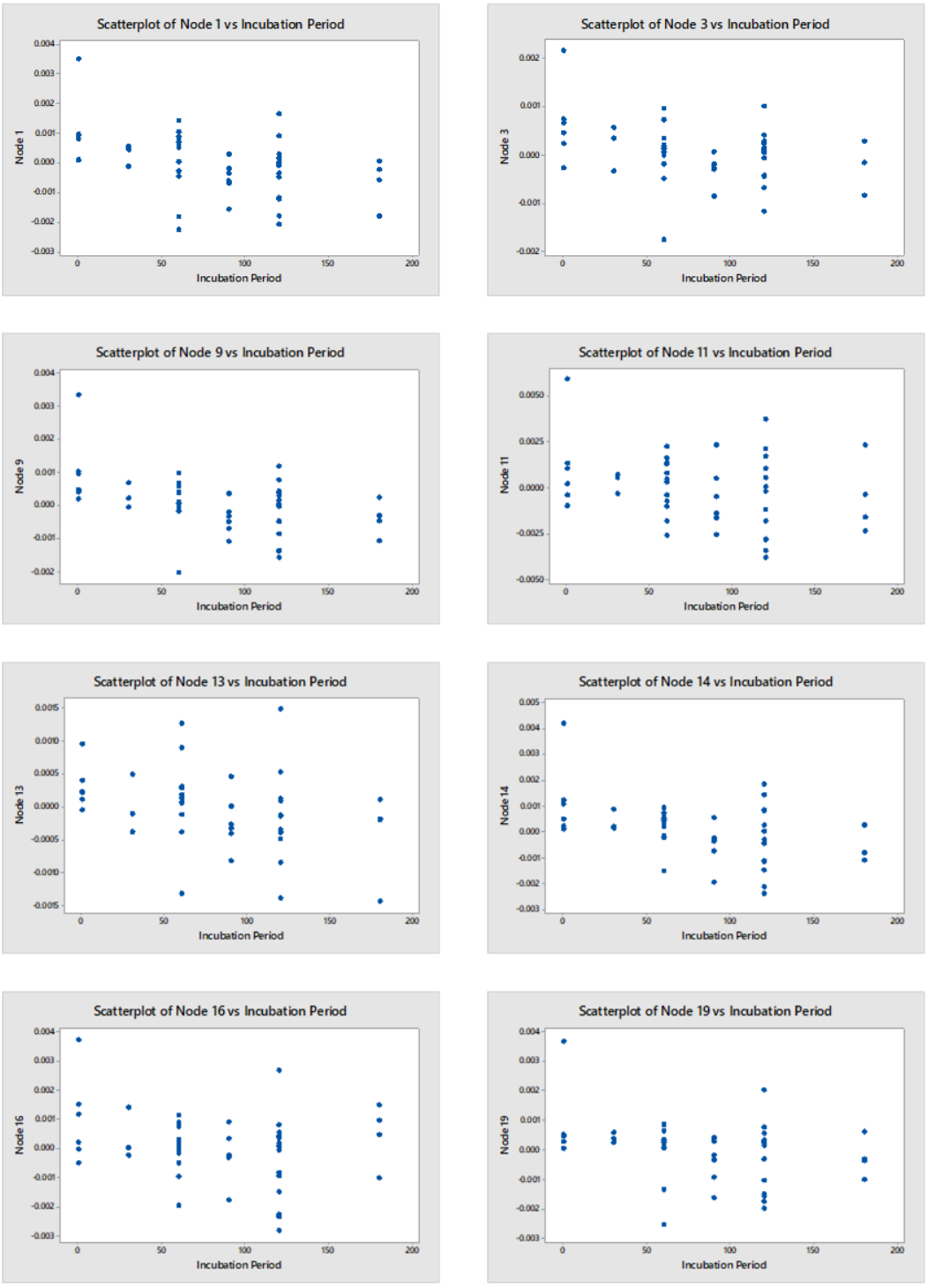


Figure 5.7. Comparison of node E2N against conditions shows that where effects are observed, increasing the Incubation Concentration and Incubation Period predominantly has negative effects on selection for these selected populations, whilst increasing CD3/CD28 Dynabead Concentration is has a positive effect. E2N data were generated for Nodes of Interest and compared against culture conditions. Analysis for significance of correlation showed that nodes 1, 3, 9, 14 and 16 had a significantly negative correlation (all $P<0.05$) with an increase in either the Incubation Concentration or Incubation Period. Node 13 had a significantly negative correlation with increasing Incubation Period. Nodes 1, 9, 11 and 13 also showed a significantly positive correlation (all $P<0.01$) with increasing Dynabead concentration.



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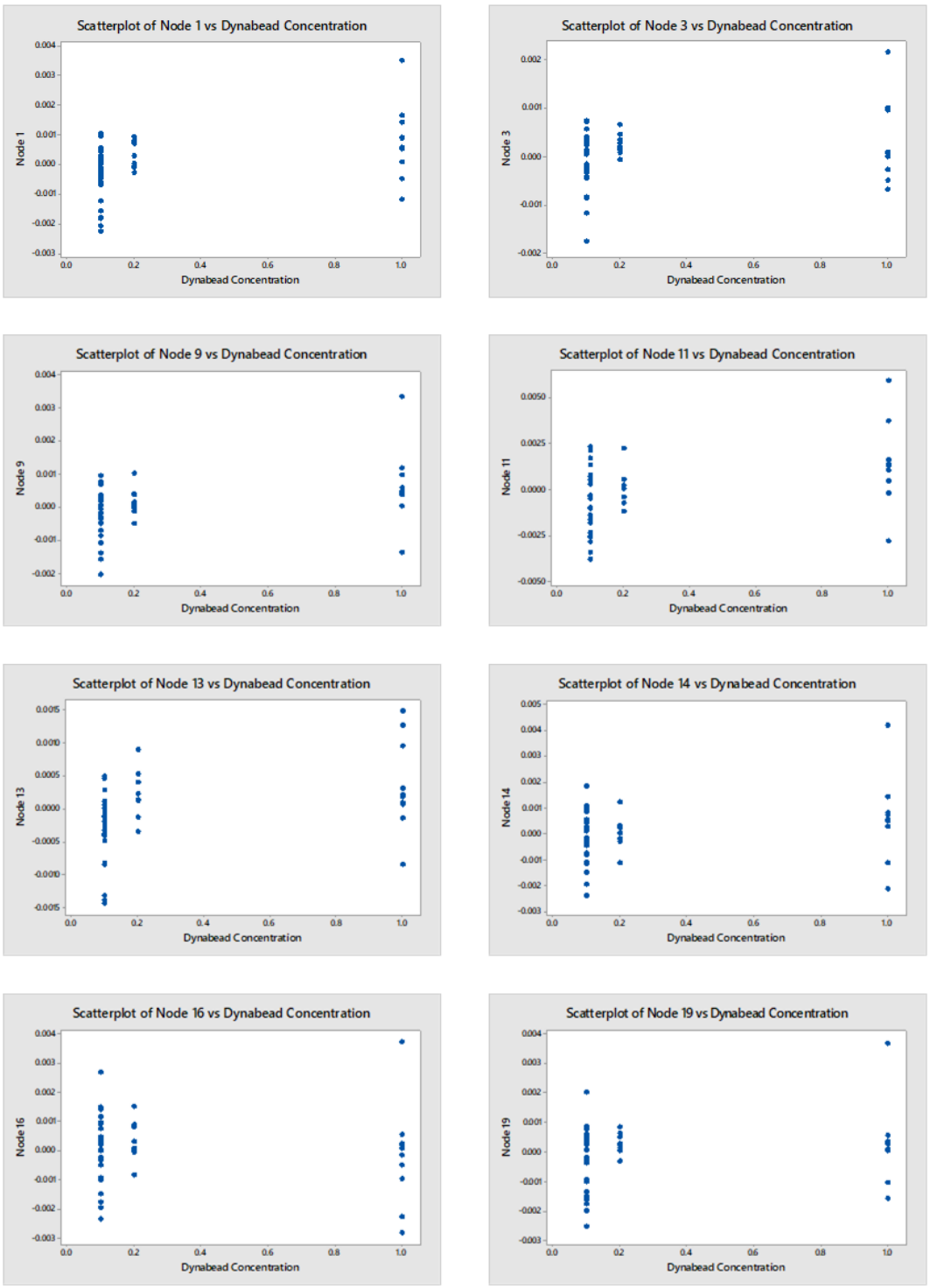

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When looking at the highest and lowest relative selection rates at $1x10^7$ cells/mL and $3X10^7$ cells/mL, it is possible to see a decline as concentration increased in nodes 1, 3, 9, 11, 13, 14 and 19. In nodes 3, 9, 11 and 14, the relative selection rates when incubated at $2x10^7$ cells/mL were between the range of the lowest selection rate at $1x10^7$ cells/mL and the highest relative selection rate at $3X10^7$ cells/mL suggesting a relatively linear relationship between incubation concentration and relative selection rate. The relative selection rates for conditions incubated at $2x10^7$ cells/mL in node 13 also fall in this range, however given that the highest relative selection rate appears to be roughly at the median value for both $1x10^7$ cells/mL and $3X10^7$ cells/mL this does not necessarily represent a linear relationship, dependent on the distribution of similar conditions within those data.

The data for $2X10^7$ cells/mL at in node 13 is similar to the presentation of data from the same incubation concentration in nodes 1 and 19, however here the lowest relative selection rate is below the level of either of the other two incubation concentrations and the highest relative selection rate is at the level of the median of the $1X10^7$ cells/mL incubation concentration in node one and the highest relative selection rate of $3X10^7$ sales mill in node 19 .

In node 16 there is a drop in both the highest and lowest relative selection rate from non-incubated conditions to those incubated at $1X10^7$ cells/mL However, when increasing the incubation concentration to $2x10^7$ cells/mL the relative selection rates increase to levels similar to that seen in non-incubated conditions. When the incubation concentration is increased further to $3x10^7$ cells/mL the relative selection rates trend negatively again but with an increase in the range of responses. The lowest relative selection rate observed when incubating at $3x10^7$ cells/mL was lower than that observed at $1x10^7$ cells/mL, but the highest relative selection rate was also higher than that observed at $1X10^7$ cells/mL.

In nearly all nodes the overall highest relative selection rate was produced in a condition that did not undergo concentrated incubation. However, although not anomalous, this result is often proportionally higher than the relative selection rates for other conditions that did not undergo concentrated incubation. The other relative selection rates for non-incubated conditions do not appear substantially higher than those produced by conditions that underwent concentrated incubation, and higher relative selection rates were achieved in conditions incubated at $1x10^7$ cells/mL in nodes 1, 3, 9, 11, 13, 14 and 19.

These data appear to show that the conditions that underwent concentrated incubation are not being negatively selected compared to those conditions that did not undergo concentrated incubation. Furthermore, the data show that in six of the 8 nodes of interest over half of the relative selection rates are positive. This suggests that increasing the incubation concentration is likely to

reduce the level of subpopulation selection but does not necessarily indicate that negative selection will be the result. Those nodes that do have a greater number of negative selection rates, nodes 11 and 13, only show that 51% of selection rates are negative, indicating that it is still not a substantial proportion that are negatively selecting for the subpopulation.

Furthermore, given the range of CD45RO expression across the nodes of interest and the level of positive selection rates observed within these data across all nodes, there does not appear to be a preference for positive or negative selection based on CD45RO expression. This appears to indicate that both immature and mature cell populations are being affected equally. However, comparison of the percentage of positive selection rates against whether the nodes represent cytotoxic or helper T-cells shows that the nodes representing CD8 positive populations have lower percentages of positive relative selection rates than those which are CD4 positive. This appears to indicate that there was a more negative effect on cytotoxic T cell populations than there is on CD4 populations overall. With a predominantly negative correlation between relative selection rate and Incubation Concentration, the implication was that as Incubation Concentration increased, CD8+ populations are being more negatively affected than CD4+ populations. In conclusion, in order to get the highest relative selection rate for cultures containing homing maturity subpopulation events, the data suggest that $1X10^7$ cells/mL would be the preferred concentration, although further investigation would be needed to be complete to verify this.

Statistical analysis of correlation showed a significantly negative correlation between relative selection rates and increasing incubation period in nodes 1, 3, 9, 13, 14 and 19 ($P<0.05$). This was of note, as the only two nodes that did not have a significant relationship with incubation period were those in which the events were predominantly or entirely CCR7+. It was also possible to see that in general the range of relative selection rates generated in conditions incubated for 60 and 120 minutes was greater than that of conditions incubated for the other periods tested in the experiment.

Further analysis shows that, as with the comparison with incubation concentration, there was little to no trend generated within these data when comparing against the other experimental variables in this experiment. Nodes 11, 13, 14 and 16 showed an increase in the range of relative selection rates in conditions incubated for 120 minutes compared to those incubated for 60 minutes. Furthermore, both highest relative selection rates were higher and the lowest selection rates are lower in conditions with the longer incubation period.

In nodes 1, 3, 9, 13, 14, 16 and 19 it is possible to see a reduction in the highest and lowest relative selection rates when increasing the incubation period from 30 to 90 minutes. However, in nodes 1,

9, 13, 14, 16 and 19 the reduction in the lowest relative selection rate was greater than the reduction in the highest relative selection rate, showing a broadening in range of relative selection rates at 90 minutes in these nodes. In nodes 1, 3, 9 and 19 the level and range of relative selection rates observed in conditions incubated for 90 minutes was similar to conditions incubated for 180 minutes. Meanwhile, in node 13 there was a sustained reduction in the highest and lowest relative selection rates from conditions incubated for 90 minutes to conditions incubated at 180. In node 14 there was a similar level of reduction seen in the highest relative selection rate from conditions incubated for 90 minutes to conditions incubated for 180 minutes as there was from conditions incubated for 30 minutes to conditions incubated for 90 minutes.

The trend of node 16 was contrary to what had been generally observed and showed an increase in both the highest and lowest relative selection rates in equal measure when increasing the incubation period from 90 minutes to 180 minutes. Node 11 showed a relatively high increase in highest relative selection rate and drop in lowest relative selection rate from when increasing the Incubation Period from 30 to 90 minutes, with this range and level being sustained when increasing the incubation period to 180 minutes.

It is not clear why, as was also seen in the comparison of conditions against growth rates, the conditions that were incubated for 30, 90 and 180 minutes are so consistent compared to the conditions incubated for 60 and 120 minutes at the same concentration and supplemented with the same concentration of CD3/CD28 Dynabeads. The relative consistency between the former conditions allows identification of nodes where there was little variation in the range and level of relative selection rates between cultures incubated for 90 and 180 minutes. In those nodes, 1, 3, 9, 11 and 19, the data suggested these sub populations reached their maximum impact from concentrated incubation at 90 minutes. It is noteworthy once again that nodes 1, 3 and 11 were representative of CD8+ subpopulations, indicating that CD8+ subpopulations are potentially less sensitive to the extended incubation period than the CD4+ subpopulations. In conclusion, it is possible to say that as a general trend, as incubation period increases relative selection rate decreases. However, there does not appear to be a substantial decrease in the relative selection rates when incubation period is increased from 120 minutes to 180 minutes.

Comparison of relative selection rates with supplemented CD3/CD28 Dynabead concentration identified a relatively similar trend across all nodes. This trend consisted of a broad range of relative selection rates in conditions supplemented with $1X10^5$ beads/mL followed by a reduction in the range of relative selection rates produced when this concentration was increased to $2X10^5$ beads/mL. This range was then subsequently increased once more when the concentration of CD3/CD28 Dynabeads supplemented into culture was increased to $1X10^6$ beads/mL.

In all nodes the range of relative selection rates produced when supplemented with $1X10^6$ beads/mL was at least great as, if not larger than, when supplemented with $1X10^5$ beads/mL. In nodes 3, 9, 16 and 19 this was predominantly down to one larger relative selection rate that increased this range, however in other nodes there was a relatively consistent spread of data. In nodes 1, 3, 9 and 11 the highest relative selection rate when supplemented with $2X10^5$ beads/mL was relatively similar to that when supplemented with $1x10^5$ beads/mL. Therefore, the reduction in range of relative selection rates when the CD3/CD28 Dynabead concentration was increased to $2x10^5$ beads/mL was due to a substantial increase in the lowest relative selection rate. In nodes 14, 16 and 19 there is a slight reduction in the highest relative selection rate when increasing CD3/CD28 Dynabead concentration from $1x10^5$ beads/mL to $2x10^5$ beads/mL. However, there is also a substantial increase in the lowest relative selection rate, indicating that these subpopulations are responding in a similar way to those mentioned previously. Node 13 showed an increase in the highest relative selection rate from $1x10^5$ beads/mL to $2x10^5$ beads/mL, also showing a substantial increase in the lowest relative selection rate.

In nodes 1, 3, 9, 11, 14 and 19, after omitting the highest relative selection rate observed in conditions supplemented with $1x10^6$ beads/mL, the data showed little difference between the top selection rate and when comparing to those supplemented with $2x10^5$ beads/mL. In node 16, omitting the highest relative selection rate in conditions supplemented with $1x10^6$ beads/mL resulted in a reduction in relative selection rate when compared with lower CD3/CD28 Dynabead concentrations. These data appeared to show, as with the previous experiment, that reducing the concentration of CD3/CD28 Dynabeads supplemented into culture does not substantially affect performance of cultures. This observation was previously validated against growth rate data and is being transferred to the performance on different phenotypic subpopulations. However, statistical analysis of correlation shows that in nodes 1, 9, 11 and 13 there is a significant positive correlation between relative selection rate and increasing CD3/CD28 Dynabead concentration ($P<0.05$).

The data also show that when supplemented with $2x10^5$ beads/mL or $1x10^6$ beads/mL, it was almost always a condition incubated for 120 minutes that produced the lowest relative selection rate, complementing the previous observation that increasing the incubation period has a negative effect of relative selection rate. In conclusion, although CD3/CD28 Dynabeads appeared to be having some effect on relative selection rate, the commonality in relative selection rates that existed between conditions supplemented with different concentrations of CD3/CD28 Dynabeads suggested that variation in relative selection rate is being effected predominantly by incubation period and incubation concentration rather than the concentration of bead supplementation.

The results observed also help to demonstrate some of the benefits of the novel analytical method. Fig 5.6 shows that nodes 1, 3, 9 and 13 represent similar populations when measured against CCR7 and CD45RO. In traditional phenotypic analysis, these populations would have been split based on their expression of these markers, and these subsections combined with other populations that had substantially different expression profiles. For example, the slightly CD45RO positive events would have been analysed the same as the highly CD45RO positive node 19. Fig 5.7 shows a general conservation of trend between nodes 1, 2, 9, 13 and 19, with the increase of both Incubation Concentration and Incubation Period correlating with a negative trend of the selection rate. In addition, the range of selection rates in these nodes includes both positive and negative values, indicating that depending on the conditions tested, the selection could be either positive or negative. However, closer analysis of these data shows that in nodes 3 and 9 the results are slightly more concentrated to positive selection (52.1% positive), closer to the 62.5% positive selection rates observed in node 19. Meanwhile, nodes 1 and 13 show slightly more negative selection rates, being only 47.9% and 45.8% positive respectively.

5.3.2.3 Conclusions of the expanded analysis of the effects of concentrated incubation on T cell populations in culture

PBMC cultures were tested to identify if concentrated incubation prior to seeding into culture plastic would allow reduction in the concentration of CD3/CD28 Dynabeads being used, whilst retaining the same level of proliferation and as standard protocol conditions, and not impacting phenotypic selection of CCR7+ populations negatively. A large screening experiment was set up to enable a broad assessment of incubation concentrations, incubation periods and CD3/CD28 Dynabead concentrations. Having performed this experiment and analysed the data produced it has been possible to identify the effects of these variables.

Of the three variables tested, Incubation Concentration and Incubation Period were the experimental factors which had an effect on growth rate of cultures. The evidence presented shows that there is a significantly negative correlation between growth rate and an increasing Incubation Concentration. This observation is also true of the relationship between the growth rate of cultures and the period of the concentrated incubation, with a significantly negative correlation between growth rate and increasing Incubation Period. Meanwhile, it was possible to see that the growth rates, when supplemented with a reduced concentration of CD3/CD28 Dynabeads, were comparable to that of the conditions supplemented with $1x10^6$ beads/mL.

Comparing these results with the data from the previous experiment concludes similarities when looking at the effects of incubation concentration and CD3/CD28 Dynabead concentration. With regards to Incubation Concentration, it was identified that the conditions incubated at a lower

concentration performed better when measuring growth rate than those which had been seeded at a higher density. It is understood that the method by which the previous experiment was executed doesn't necessarily mean that these results are directly comparable, however this perceived relationship still warrants further analysis for validation.

With regards to the supplementation of CD3/CD28 Dynabeads, the previous experiment concluded that reducing the concentration of beads did not negatively affect growth rate compared to conditions that were cultured with the suggested concentration. The data obtained in this experiment concludes the same response in growth rate, suggesting that a reduced quantity of CD3/CD28 Dynabeads could be used in culture and would still generate the same level of response as a seen at a higher concentration. This effect was also seen in cultures that did not undergo concentrated incubation suggesting that the concentrated incubation was not necessarily a required element of the culture protocol to elicit the same level of growth in the presence of a reduced quantity of CD3/CD28 Dynabeads.

The observation that growth rate reduced with increase of incubation period was counter to that which had been seen previously, where conditions incubated for a longer period of time saw improved growth rate. The upper level of the incubation periods tested previously was lower than executed here, and although there was some similarity between conditions incubated for similar periods to that in the previous experiment, the data better fit the general downward trend that was observed.

Phenotypic data was analysed using the novel analytical tool that had been developed as discussed in the previous chapter. Using this tool to analyse data showed that 120 hours was the point of maximum experimental effect and that there were similarities between the 25 TNN and 40 TNN SPADE analysis when trying to identify the optimal resolution for phenotypic analysis of this dataset, however ultimately it was concluded that 25 TNN would be the optimal number of nodes for this analysis and was carried forwards.

Analysis of the optimal TNN SPADE analysis data identified 8 nodes of interest. These nodes consisted of four CD4+ nodes and four CD8+ nodes. These nodes were all selected for the presence of CCR7+ events, showing that they presented either Naïve or Central Memory subpopulation phenotype events or both. Although these nodes also covered a range of CD45RO expression it was noticeable that the CD8+ nodes showed overall a lower level of CD45RO expression than the CD4+ nodes.

Calculated relative selection rates for the populations represented by the nodes of interest identified relationships with both the Incubation Concentration and Incubation Period. There were variations

in response to these factors between nodes, however the general effect was that as Incubation Concentration increased the relative selection rate decreased. It was also possible to see a similar response in comparing relative selection rates against Incubation Period, in that as the Incubation Period was increased, the relative selection rate for these selected nodes decreased.

Overall, the similarity between the effects seen in the relative selection rates and the growth rates appears to suggest that with a lower growth rate comes a relatively lower relative selection rate for subpopulations which have a higher CCR7 expression level. The conditions which have produced lower growth rates and relative selection rates are those which would more likely put cells into more harsh conditions, with both higher Incubation Concentrations and increasing Incubation Periods increasing the chance of medium exhaustion. Having observed the effect that these have had on growth rates it is therefore plausible that some populations represented by the nodes of interest are more sensitive to these adverse culture conditions than T-cells of other subpopulation phenotypes, which would cause the relative selection rates of these subpopulations to behave in a similar manner to the growth rates. It is not reasonable to assume that at all points the behaviour of these subpopulations would be linked to growth rate. However, given the broad similarity in response of the relative selection rates and the growth rate, these data suggest that performance of these subpopulations and growth are linked, rather than the phenotypic performance of the cultures and level of proliferation being separately influenceable outputs.

Comparison of the relative selection rates to the concentration of CD3/CD28 Dynabeads supplemented into culture shows that there is variation induced by changing the concentration added. However, further analysis of the data appears to show that the variation within the conditions supplemented with the same Dynabead concentration was predominantly due to the other experimental variables, rather than the Dynabead concentration itself. Furthermore, relative similarities in the relative selection rates observed at different Dynabead concentrations suggest that the concentration of beads is not having a substantive effect on the relative selection rate of the subpopulations represented in the nodes of interest.

In addition, it was possible to identify subpopulations produced as part of the novel analytical technique which have more or less positive selection rates across all conditions. Being able to identify this with a more nuanced approach may help to identify populations that are more susceptible to negative selection, and subsequently determine which conditions have a greater impact on positive or negative selection rates and prioritise their importance in culturing target populations.

The hypothesis established at the beginning of this experiment predicted that the cultures incubated at $1x10^7$ cell/mL and incubated for 30 or 60 minutes would have the same level of proliferation as those conditions that did not undergo concentrate incubation, regardless of the concentration of CD3/CD28 Dynabeads supplemented into the culture. In execution this was not the case, however the growth rates produced by the conditions incubated at $1x10^7$ cells/mL for 30 and 60 minutes were not substantially different from those that did not undergo concentrated incubation.

5.3.2.4 Identification of conditions for use in validation

Naïve and Central Memory subpopulations have been the main focus for optimisation of T-cell culture due to their desirability in the CAR T-cell manufacturing process[67,68,70,171]. The expanded investigation into the impact of concentrated incubation prior to seeding in 5.3.2 identified that, generally, as Incubation Concentration and Incubation Period increased, the relative selection rates in Nodes of Interest decreased. Therefore, any conditions which went against this trend were of interest for further investigation.

Further analysis of the NOI relative selection rates identified that certain conditions retained a higher relative selection rate across all of the subpopulations analysed. Specifically, those conditions incubated at $3x10^7$ cells/mL for 60 minutes and $1x10^7$ cells/mL for 120 minutes with $1x10^6$ beads/mL CD3/CD28 Dynabeads showed the highest relative selection rates within their Incubation Period subgroups in nodes 1, 3, 9 and 13, and were represented relatively highly in nodes 11 and 14. To perform some level of validation of these observations, these conditions would be retested and analysed to identify if they would be able to repeatably induce relative selection rates for similar phenotypic subpopulations.

To use as a positive control, these conditions would also be compared against cultures that did not undergo concentrated incubation and were also supplemented with $1x10^6$ beads/mL Dynabeads. Not only was this condition consistently shown to produce high relative selection rates, it is also a common culture process within T-cell industry and research, providing a good benchmark for comparison with the wider field. In addition, it was decided to select a negative control condition that had shown poor relative selection rates for cultures across all NoI. Cultures incubated for 120 minutes at $3x10^7$ cell/mL and supplemented with $1x10^5$ beads/mL CD3/CD28 Dynabeads had the lowest relative selection rate in node 14, and the lowest relative selection rate of those incubated for 120 minutes in nodes 1, 3, 9, 11 and 19, as shown in 5.3.2.2.

These conditions were selected for testing to validate the observations from previous experiments. The data would be used to validate the observations of growth rate in cultures, however there was also a particular interest in the outputs of the phenotypic analysis. The impact of the experimental

variables on phenotypic profile is of interest for application to the wider field of T-cell culture and CAR T-cell therapy manufacture, however in this scenario it was also of interest as the novel analytical technique used in the phenotypic analysis had not been used to validate phenotypic responses yet. Disparity between the phenotypic conclusions would not inherently be a comment on the accuracy of the analytical process, but could highlight issues or developments moving forwards.

5.3.3 Repeat testing of specified experimental conditions to validate observed effects of concentrated incubation on T-cell proliferation and phenotypic response

5.3.3.1 Assessing the influence of concentrated incubation on T-cell population growth rates

Analysis of viable cell counts over time shows that all cultures tested showed periods of growth. All cultures showed a lag phase from 0 to 46 hours, and from 46 hours it is possible to see a substantial increase in the viable cell count until 159 hours. From 0 to 46 hours there is a drop in viable cell count observable in certain conditions. This drop is consistent with previous observations and is likely due to the death of non T-cells in the PBMC cultures that are not being sustained by the T-cell supporting conditions. The increase in viable cell count between 46 and 159 hours is not consistent, with there being a substantial dip in viable cell count observable at 96 hours, and a similar reduction in the condition incubated for 60 minutes at $3x10^7$ cells/mL at 145 hours (Fig 5.8).

The drop in viable cell count observed at 96 hours correlates with the conditions undergoing a medium exchange. The volume of cultures was measured before and after to consider any dilution that may have occurred, and therefore wasn't the cause of the reduction. It is unclear what the source of the reduction in viable cell count was, however the subsequent drop seen in a single condition at 145 hours is also at a point at which the cultures will have undergone a medium exchange. This level of correlation would likely suggest that the cause of the reduction in viable cell count is linked to the process of exchanging the medium. Within this process it is not clear what is causing this effect, especially as this has not been observed in other viable cell count data in the previous cultures, however it could be plausibly linked to the centrifugation the cells undergo to separate them from the medium in which they are suspended. The lack of observation of this effect in previous cultures could also suggest that the susceptibility to this process could be donor related.

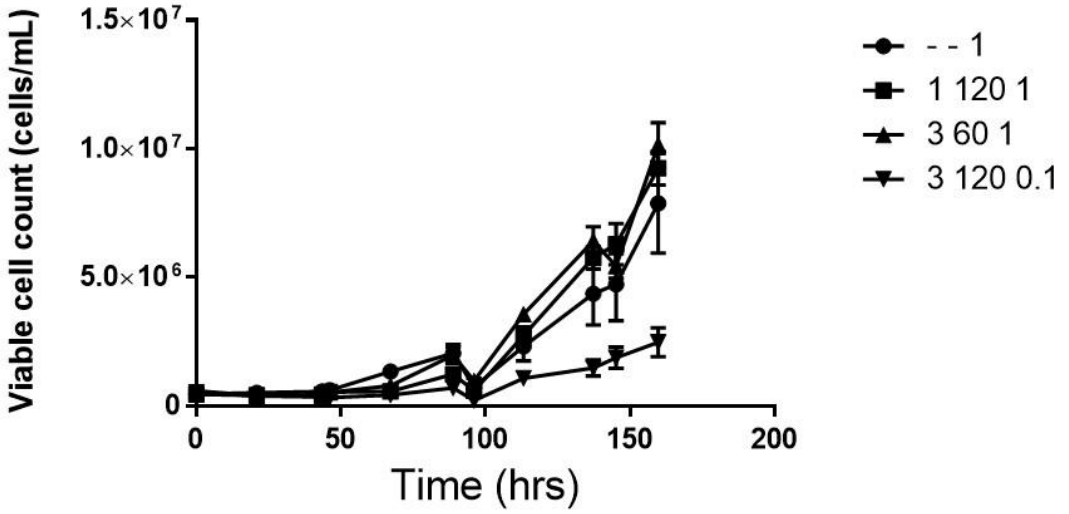


Figure 5.8. Analysis of viable cell counts over time show growth in all cultures. Cultures were sampled regularly and tested for viable cell count to monitor proliferation in cultures over time. All cultures showed growth over time. All cultures also showed a dip in viable cell count at 96 hours, before increasing further. Cultures where cells were incubated at $3x10^7$ cells/mL for 60 minutes and supplemented with $1x10^6$ beads/mL also observed a similar dip at 145 hours.

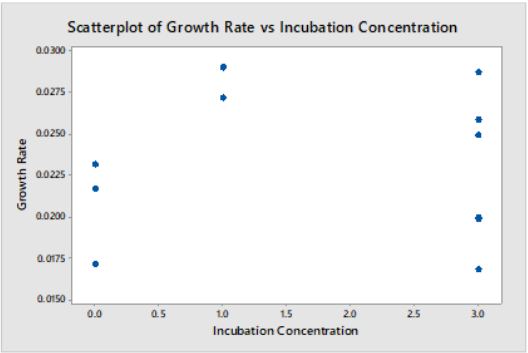
Growth rates were subsequently calculated from the viable cell counts and analysed to assess experimental impact. The growth rates were all calculated based on viable cell counts from 46 hours onwards as this was the inflection point of the cultures. All calculated growth rates were positive, showing that all conditions induced growth in the T-cell populations (Fig 5.9).

The growth rates show that when supplementing with $1x10^6$ beads/mL CD3/CD28 Dynabeads, concentrated incubation at any concentration induced a higher growth rate. Growth rates were highest on average when incubated at $1x10^7$ cells/mL for 120 minutes, although the rates of cultures incubated at $3x10^7$ cells/mL for 60 minutes were not substantially lower. The lowest average growth rate was observed in the cultures incubated for 120 minutes at $3x10^7$ cells/mL and supplemented with $1x10^5$ beads/mL CD3/CD28 Dynabeads.

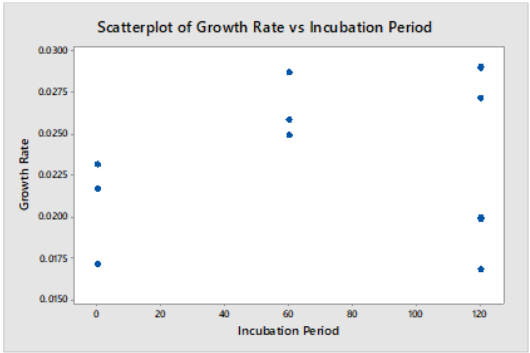
Comparison of the conditions that were not incubated and supplemented with $1x10^6$ bead/mL and those incubated for 120 minutes at $3x10^7$ cell/mL, supplemented with $1x10^5$ beads/mL, showed that the former has a higher average growth rate. However, the average growth rates for these two conditions are not substantially different, and the lowest growth rates within each subgroup are sufficiently similar that more data is needed to identify if they are truly separate. Statistical analysis of growth rates from cultures supplemented with $1x10^6$ beads/mL CD3/CD28 Dynabeads showed a

significant positive correlation between increasing growth period and growth rate (P<0.01). The data also show that concentrated incubation generated higher growth rates than those that did not undergo that process, although this relationship did not have a significant correlation.

A.



B.



C.

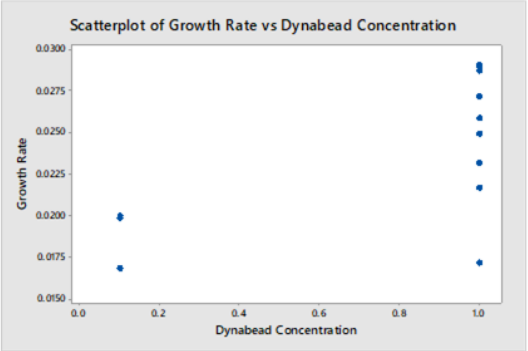


Figure 5.9. Comparison of growth rates against experimental conditions shows that decreasing CD3/CD28 Dynabead concentration has a significantly negative effect. Calculated growth rates were compared against Incubation Concentration (a), Concentration Incubation (b), and Dynabead Concentration (c). Negative control groups, which did not undergo concentrated incubation were represented by 0 on Incubation Concentration and 0 Incubation Period scales. Statistical analysis showed a significant positive correlation (P<0.05) between Dynabead Concentration and growth rate.

With the conclusions that were being drawn it is easy to see that the data were not going to be particularly comparable to the previous test of the conditions. This is predominantly due to the relatively high growth rates of the concentrated incubation conditions supplemented with the higher concentration of CD3/CD28 Dynabeads. However, the relatively similar performance of the incubated concentration conditions supplemented with the reduced concentration of CD3/CD28 Dynabeads also highlights the variation observed in the validation conditions compared to the initial observations. Although analysis of growth data is of great importance to ensure that any phenotypic benefits are not coming at the expense of growth, especially as the previous data appeared to link the subpopulation performance to the growth rate elicited by the experimental conditions, the main aim of this experiment was to identify if there is commonality in the phenotypic response.

5.3.3.2 Comparison of growth rate response to experimental conditions

Initial comparisons of growth rate data showed some deviations from what was seen in the previous experiment. The negative control, incubated for 120 minutes at $3x10^7$ cells/mL with $1x10^5$ beads/mL CD3/CD28 Dynabeads, produced growth rates that were on average lower than the positive control, non-incubated and supplemented with $1x10^6$ beads/mL (Fig 5.8), which was in line with what was observed previously. However, the previous experiment concluded that these conditions should induce substantially different growth rates, with the negative control proliferating significantly less than the positive control (Fig 5.4). The data from the previous experiment show that there is a clear and substantial distinction between the positive and negative controls, however the validation conditions show an overlap of the growth rates from these two conditions. This immediately highlighted that the outputs of this experiment are likely to differ from before.

Despite this it is also possible to see from the viable cell count data that the viable cell yield in the negative control conditions were roughly 3 fold lower at the final cell count. This observation does not change the fact that the positive and negative control had similar growth rates in culture, showing that they are not as different as was previously observed. However, it could highlight that cultures undergoing the incubated concentration protocol suffer a greater loss of initial material after plating into culture plastic than those that do not undergo the concentrated incubation process.

Meanwhile, the experimental conditions tested showed a substantially higher growth rate than those observed in the positive control. In the previous experiment, the standard protocol conditions generated the highest growth rates, and therefore the performance of the experimental conditions is counter to what was seen before. This level of performance is not unprecedented though, as the first investigation into this process showed that conditions undergoing concentrated incubation would perform better than those which underwent the standard protocol as described in 5.3.1.

Therefore, the data appears to validate the response observed from one set of T-cell cultures, but not another. These variations in response could potentially be due to the size of the expanded investigation experiment. The purely practical requirements of cultures to be out of the incubators for greater periods of time every 24 hours due to the number of conditions being sampled could have negatively affected cultures that underwent concentrated incubation. This could potentially have been that after the high stress environment of their incubation period they were more susceptible to further disruption. Alternatively, these data could highlight the inherent variability of T-cell cultures, showing that donor variability could be a considerable issue moving forwards in creating a universal 'best practice' protocol. The likelihood is that it was a combination of factors that may have contributed to the effects seen in the results.

5.3.3.3 Assessing changes in phenotypic response based on concentrated incubation with variable CD3/CD28 Dynabead concentrations

Samples were taken as discussed in 5.2.7 and analysed for expression of marker proteins and analysed using the novel analytical method outlined in 4.3.2. The data was processed via the SPADE analysis platform and E2N ratios were calculated for each node of the analyses run using the sample data generated. The resulting data was separated by TNN and timepoint collected for analysis of the point of greatest experimental effect. Analysis of the E2N data shows a consistent increase in upper quartile and median values from 24 to 72 hours across all analyses, with the increase in upper quartile value to 96 hours observable across analyses with TNN from 10 to 30. In all analyses there is a substantial drop in the E2N values at 120 hours, followed by an increase of varying magnitude across the analyses to 144 hours, and then a substantial reduction again to 168 hours (Fig 5.10). With consistently higher upper quartile and maximum E2N values across analyses with TNN 10-30, 96 hours was selected as the point of greatest experimental effect within this dataset. The top 3 E2N values from each analysis at 96 hours were then compared to identify the optimal Target Number of Nodes for the dataset.

Analysis of the 3rd highest values shows a relatively consistent increase from TNN 10 to TNN 25 where the values begin to plateau. Meanwhile, the second highest values show a relative plateau from 10 TNN to 20 TNN. From 20 TNN there appears to be a relatively consistent increase in E2N value to TNN 25 and on to TNN 40, however TNN 30 shows a drop in second highest E2N value to the same level as the third highest value. Analysis of the top values from the analyses shows an initial decrease when increasing TNN from 10 to 15, followed by a sharp increase to TNN 25. As with the second highest data, the TNN 30 top value drops, substantially in this case, again to the same level as the third highest E2N value from the analysis. The top value increases from 30 TNN to 40 TNN, however this value is still lower than that of the 25 TNN analysis (Fig 5.10). With the greatest top E2N values, second greatest second highest value and third highest value at the beginning of the plateau seen in the subgroup, the selected Target Number of Nodes for this dataset was selected to be 25.

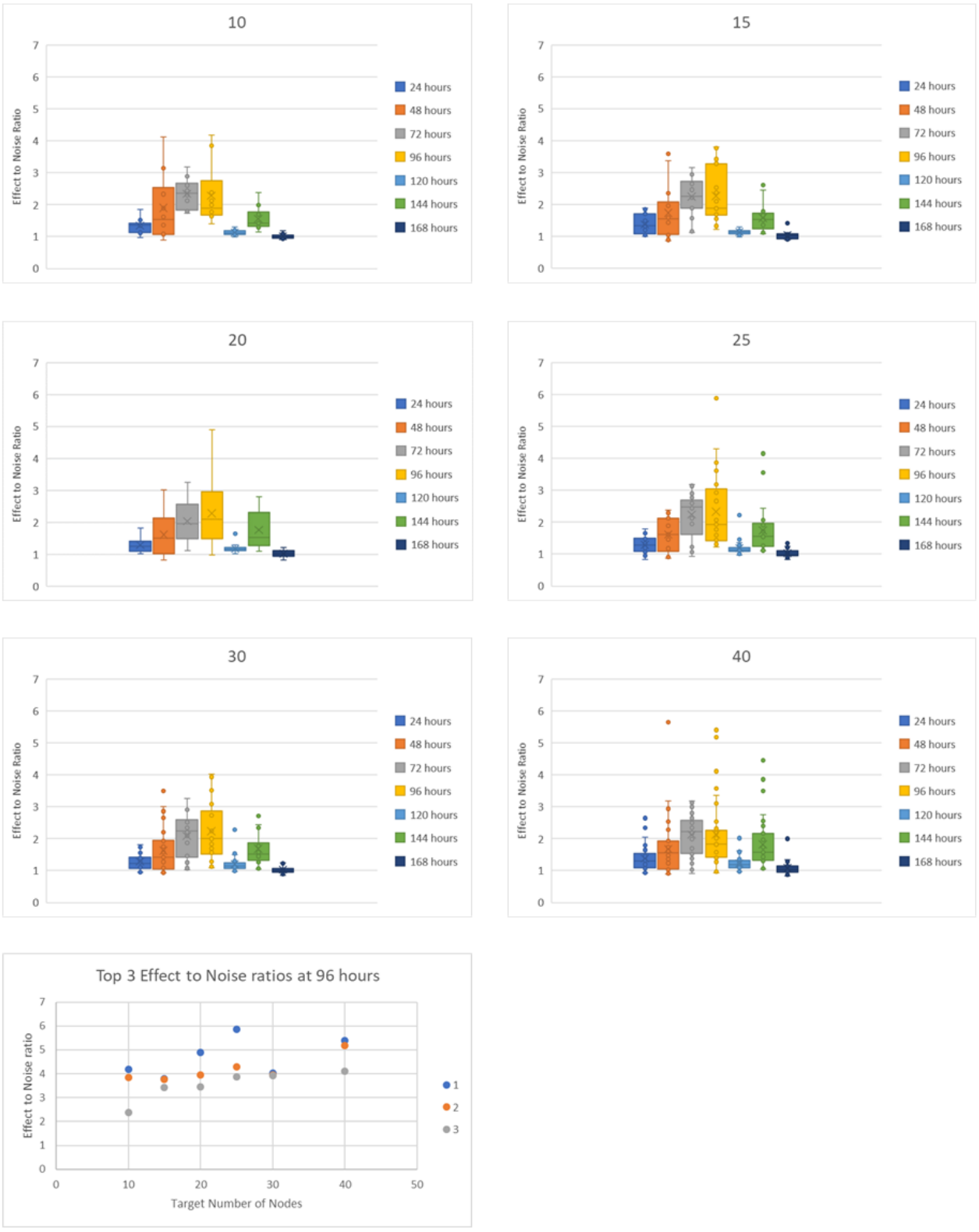


Figure 5.10. Analysis of Effect to Noise ratios (E2N) identifies 96 hours as the point of greatest experimental effect and 25 as the optimal Target Number of Nodes (TNN). Box plots were produced for each SPADE analysis of different TNN run, with data separated by day of collection to identify the point of greatest E2N values, indicative of the greatest experimental impact. This was identified as day 4 (96 hours). The top 3 E2N from each analysis at this timepoint was then plotted to identify the optimal TNN for this analysis.

Each node of the 25 TNN analysis was analysed to identify nodes which contained CCR7+ events. Through this process nodes 4, 8 9, 10, 14, 16, 17, 18 and 19. Nodes 4, 16, 18 and 19 represent CD8+ populations, whilst the remaining nodes all represent CD4+ populations (Fig 5.11, Table 4.4). Due to the parameters of selection all nodes contain some level of CCR7+ events, however unlike previously there are no nodes that appear to be predominantly or entirely CCR7+ events. A considerable range of CD45RO expression is covered across all NoI, and certainly covers both mature (Effector Memory and Central Memory) and immature (Naïve and Effector) subpopulations phenotypes.

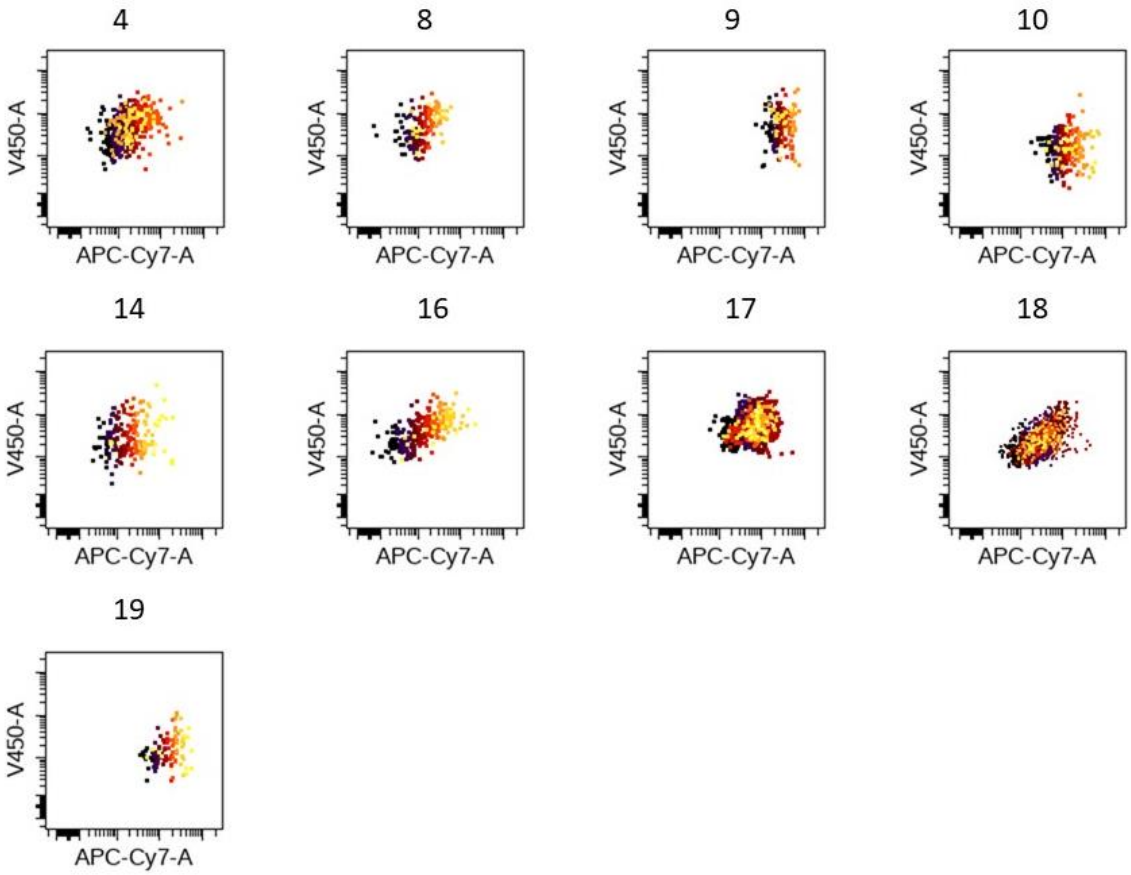


Figure 5.11. Plots of CCR7 (V450-A) and CD45RO (APC-Cy7-A) expression of events within nodes identifies nodes of interest for further assessment. Nodes from the SPADE analysis of optimal resolution for the dataset (Target Number of Nodes 25) were assessed to identify those that contained events falling into the definition of Naïve (CCR7+/CD45RO-) and Central Memory (CCR7+/CD45RO+). All node event plots presented were identified as having sufficiently high CCR7 expression to qualify as containing events of the specified subpopulations.

Nodes 4 and 14 show similar levels of CCR7 and CD45RO expression, both presenting events that fit in to all 4 maturity phenotype subpopulation definitions. Node 8 has a similar overall profile, but has fewer CCR7-CD45RO+, or Effector Memory subpopulation events. Nodes 16 and 18 have similar profiles to each other, with a distribution of events that shows a positive correlation between CCR7

and CD45RO expression. The CCR7- events in node 16 are predominantly Effector phenotype events, with the CCR7+ spanning the delineation between Naïve and Central Memory phenotype. Meanwhile, the events displayed in node 18, although with a similar profile to node 16, are shifted more positively on the CD45RO axis, meaning that the events are predominantly Effector or Central Memory, although there is some overlap at the centre into Naïve and Effector Memory phenotype definition.

Table 5.4. Description of phenotypic presentation in Nodes of Interest, as identified in Fig 5.11

Node	CD4/ CD8	Maturity Phenotype	Additional notes
4	CD8	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression is predominantly +ve. CCR7 expression is also predominantly +ve, with expression ranging from slightly –ve to highly +ve
8	CD4	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression spans –ve and +ve. CD45RO expression is predominantly +ve, with expression ranging from slightly –ve to highly +ve.
9	CD4	Effector Memory/ Central Memory	CD45RO expression is highly +ve. CCR7 expression is predominantly +ve, spanning from slightly –ve to highly +ve.
10	CD4	Effector Memory/ Central Memory	CD45RO expression is highly +ve. CCR7 expression spans –ve and +ve
14	CD4	Naïve/Effector/ Effector Memory/ Central Memory	CD45RO expression is predominantly +ve. CCR7 expression is predominantly +ve. Expression of both markers ranges from slightly –ve to highly +ve.
16	CD8	Naïve/Effector/ Central Memory	CD45RO expression spans from –ve to highly +ve. CCR7 expression spans from slightly –ve to highly +ve. CCR7 expression is only –ve where CD45RO expression is –ve, and there is a positive correlation between CD45RO expression and CCR7 expression.
17	CD4	Central Memory	Both CD45RO and CCR7 expression are +ve, and border but does not present as –ve.
18	CD8	Naïve/Effector/ Effector Memory/ Central Memory	Both CD45RO and CCR7 expression spans from –ve to highly +ve. There is a positive correlation between CD45RO and CCR7 expression.
19	CD8	Effector Memory/ Central Memory	CD45RO expression is highly +ve. CCR7 expression spans –ve and +ve.

Node 17 shows a population that is predominantly Central Memory phenotype, however there is a proportion of events that would still be defined as Naïve, Effector and Effector Memory. Nodes 9, 10 and 19 have high CD45RO expression in all events, to the extent that there are very few to no

immature phenotype events in these nodes. Nodes 10 and 19 are very similar, although in node 19 as the CCR7 expression increases, the lower limit of CD45RO expression increases, while the full range of CD45RO expression appears to be maintained in node 10. Node 9 has no immature events and has events that fall into both the Central Memory and Effector Memory phenotype definitions; it would appear that a greater proportion of the events are Central Memory than Effector Memory.

The relative selection rate of these subpopulations within samples was then analysed and compared against experimental variables to identify the experimental impact on subpopulation selection.

Comparison of relative selection rates for subpopulations represented in the NoI show the limiting effect of not incubating at a concentrated level in this experiment.

Consistently across all nodes it is possible to see that there is an increase in both the lowest and highest relative selection rates when comparing the non-incubated conditions with those incubated for 120 minutes at $1x10^7$ cells/mL. Furthermore, it is possible to see that in nodes 4, 8, 17, 18 and 19 the non-incubated cultures do not produce a positive relative selection rate at all, a fact not observed in any of the other conditions across all nodes (Fig 5.12). Analysis of the relative selection rates induced by incubating at $1x10^7$ cells/mL for 120 minutes shows a consistent pattern across all NoI, apart from node 9, that two of the repeats perform similarly and the third will perform substantially worse. It is not clear why one culture would perform generally worse that the other cultures when they have been subject to the same conditions.

Analysis of conditions incubated at $3x10^7$ cells/mL shows that increasing the Incubation Period and decreasing the concentration of CD3/CD28 Dynabeads supplemented did not have a substantial effect on the relative selection rate within these nodes. Within all NoI other than node 14 there was some level of overlap between the conditions incubated for 60 minutes and supplemented with $1x10^6$ beads/mL and those incubated for 120 minutes and supplemented with $1x10^5$ beads/mL. This evidence is in stark contrast to the growth data where the negative control condition performs substantially worse than the other conditions incubated at $3x10^7$ cells/mL.

In nodes 8, 10, 16 and 17 it is possible to see a reduction in both the highest and lowest relative selection rate observed when increasing the Incubation Concentration from $1x10^7$ cells/mL to $3x10^7$ cells/mL. Nodes 4, 9, 14, 18 and 19 show that the highest relative selection rate for these subpopulations were incubated at $3x10^7$ cells/mL.

These data suggest that on average incubated concentration is beneficial for the relative selection rates of the subpopulations defined by the NoI. However, the variability of relative selection rates observed in those conditions that underwent concentrated incubation does not appear to sufficiently show that there is a consistently positive effect. Statistical analysis did not identify any

significant correlations between the relative selection rates and increasing Incubation Concentration. Furthermore, where similar effects are observed with increase to the Incubation Concentration, there does not appear to be any observable connecting factor that would like the subpopulations in question, suggesting that these are also coincidentally similar.

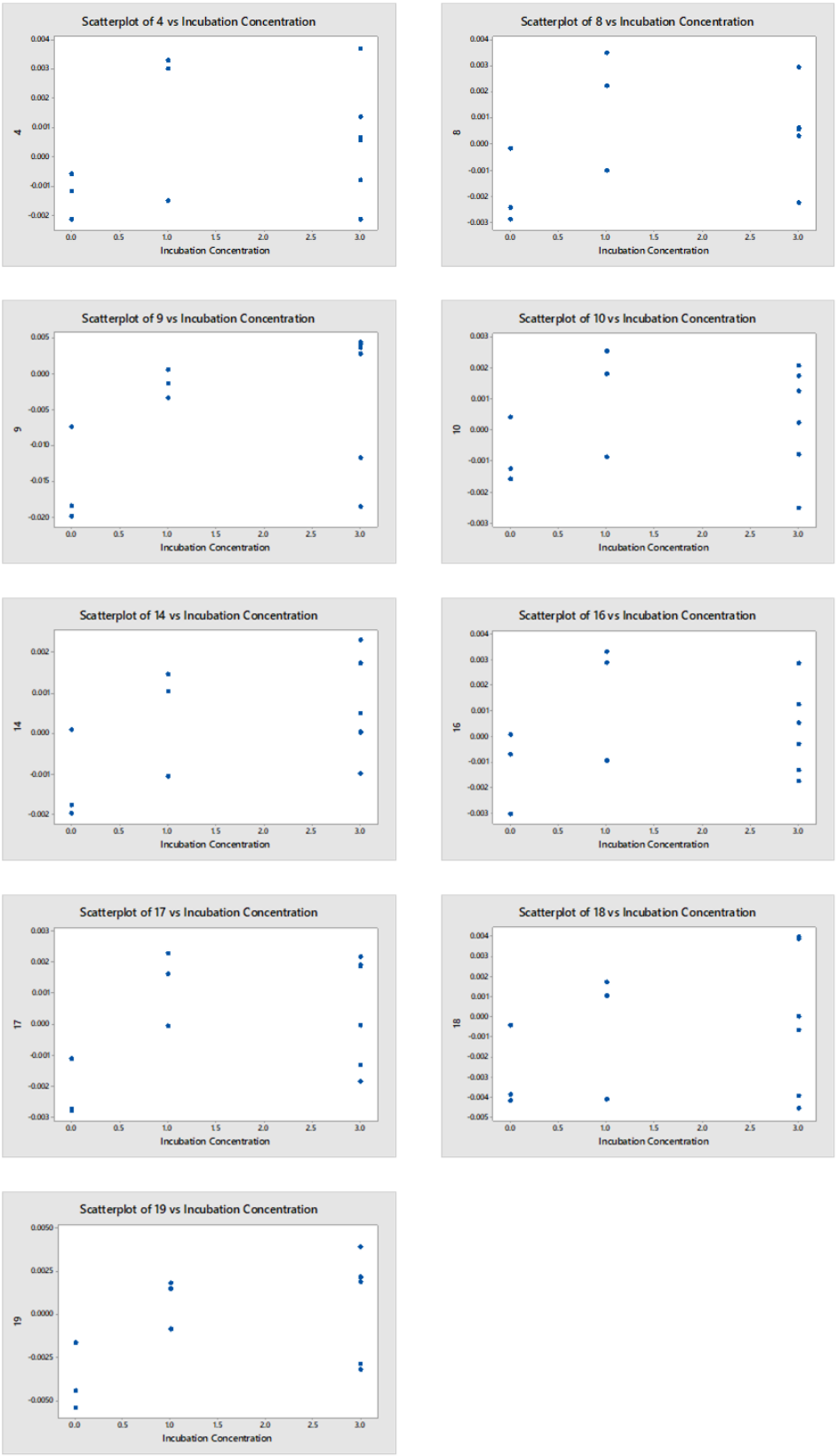
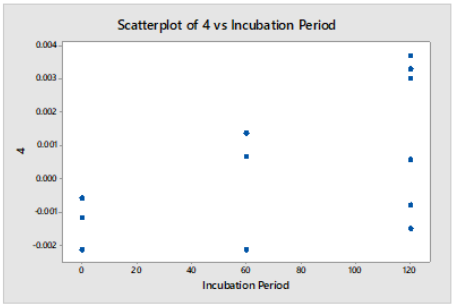
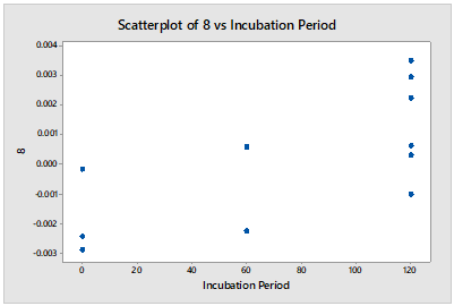
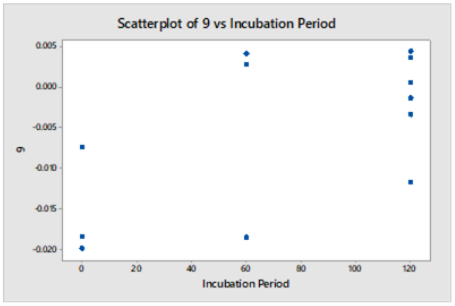
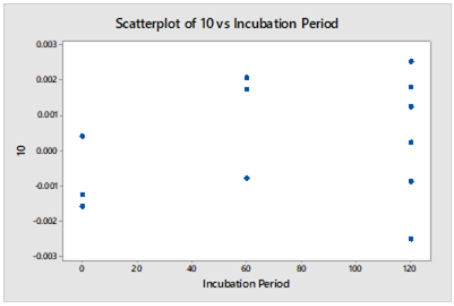


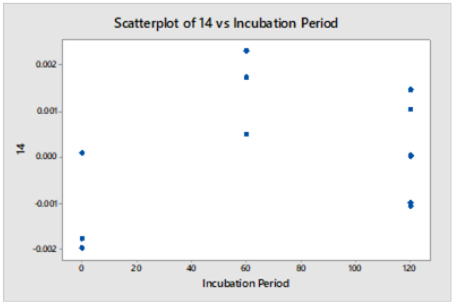
Figure 5.12. Analysis of subpopulation selection rates from Nodes of Interest (NoI) shows a trend of positive correlation between both Incubation Concentration and Incubation Period, and subpopulation selection rate. Calculated subpopulation selection rates of NoI were calculated and compared against experimental variables. Statistical analysis showed a significant positive correlation between Incubation Period and subpopulation selection rate in nodes 8, 9 and 19 ($P<0.05$).

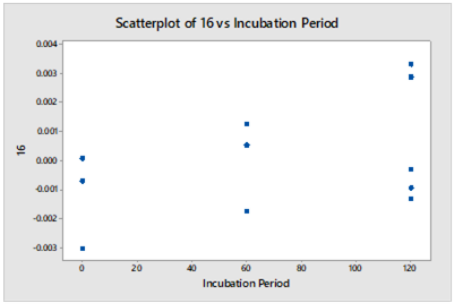


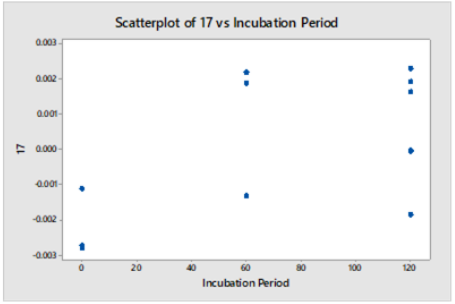


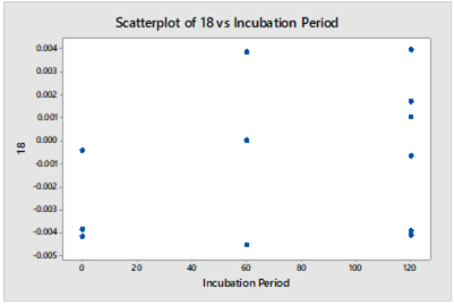












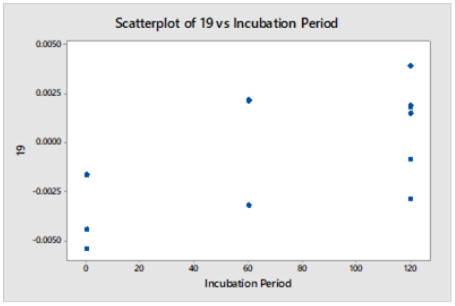


Figure 5.12'. Analysis of subpopulation selection rates from Nodes of Interest (NoI) shows a trend of positive correlation between both Incubation Concentration and Incubation Period, and subpopulation selection rate. Calculated subpopulation selection rates of NoI were calculated and compared against experimental variables. Statistical analysis showed a significant positive correlation between Incubation Period and subpopulation selection rate in nodes 8, 9 and 19 ($P<0.05$).

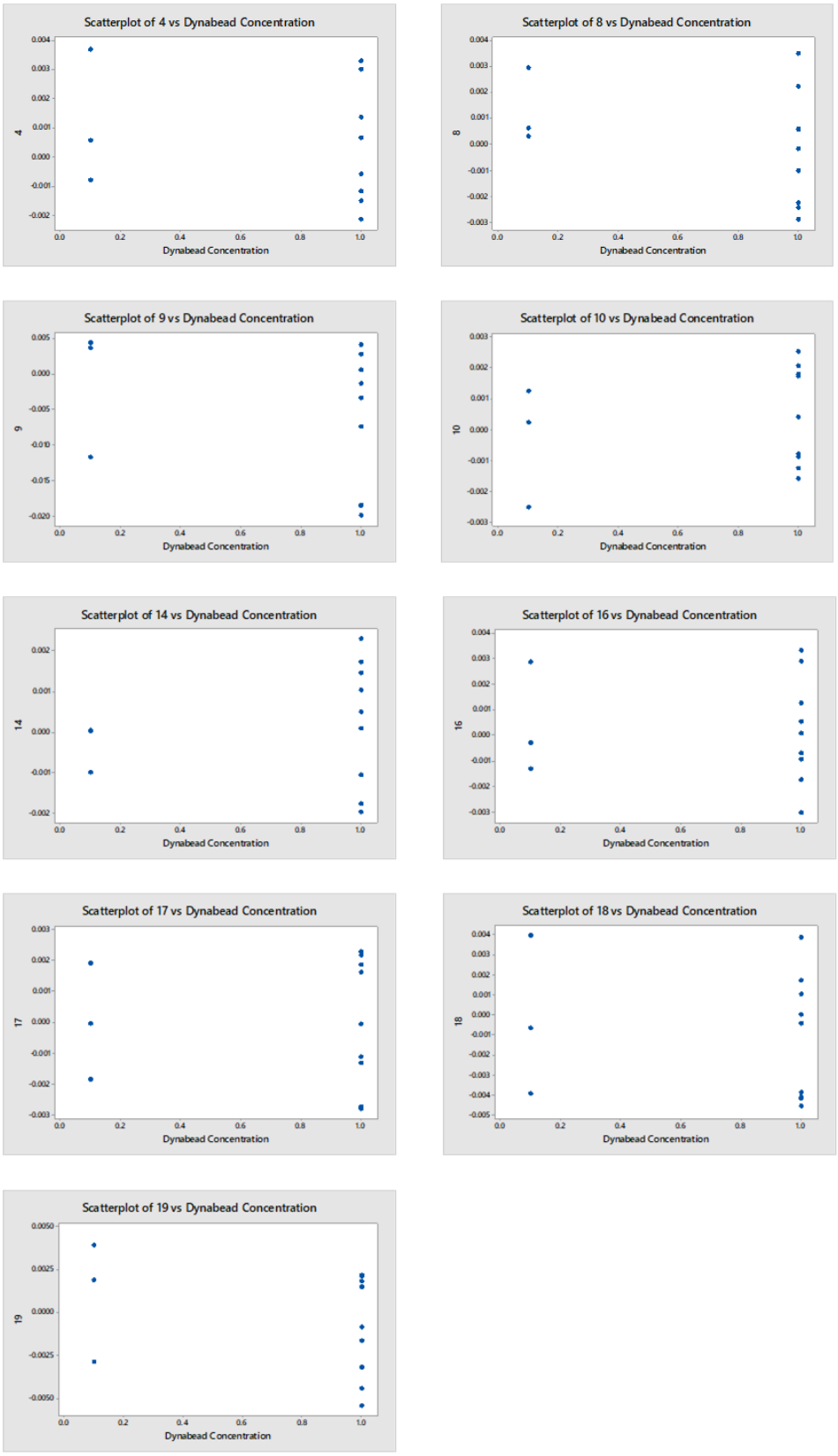


Figure 5.12. Analysis of subpopulation selection rates from Nodes of Interest (NoI) shows a trend of positive correlation between both Incubation Concentration and Incubation Period, and subpopulation selection rate. Calculated subpopulation selection rates of NoI were calculated and compared against experimental variables. Statistical analysis showed a significant positive correlation between Incubation Period and subpopulation selection rate in nodes 8, 9 and 19 ($P<0.05$).

It is possible to analyse the interaction between Incubation Period and the relative selection rate for NoI subpopulations. However, given the limited number of conditions tested within this experiment, a large number of observations will be similar or identical to those outlined in the previous section.

It is possible to see an increase in the lowest and highest relative selection rates produced between the non-incubated cultures and those incubated for 60 minutes in all nodes except nodes 4 and 18. Within these nodes it is possible to observe an increase in the highest relation selection rate when cultures undergo concentrated incubation, however there is also a slight reduction in the lowest relative selection rate. Statistical analysis shows a significant positive correlation between relative selection rate and increasing incubation period in nodes 8, 9 and 19. Comparison of relative selection rates against the concentration of CD3/CD28 Dynabeads showed varying response across subpopulations, however the statistical analysis of correlation did not highlight any significant relationships.

Node 4, 9, 18 and 19 show a decrease in the highest and lowest relative selection rate when increasing the CD3/CD28 Dynabead concentration, indicating that in these subpopulations a reduction in the CD3/CD28 Dynabead concentration is having a positive effect. Both nodes 9 and 19 represent subpopulations with relatively high CD45RO expression, containing a negligible quantity of immature events. CD3/CD28 Dynabeads promote proliferation through activation of T-cell and promote maturation and it was therefore somewhat surprising that reduction in the Dynabead concentration would induce a greater selection for these subpopulations. One theory for this scenario is that there are other high CD45RO expressing populations that do not show CCR7+ events, namely Effector Memory populations, that have reduced relative selection rate with a decrease in CD3/CD28 Dynabead concentration, increasing the relative selection rate of these subpopulations. There does not appear to be a consistent pattern of response across all subpopulations, however the data do highlight lack of negative effect on subpopulation selection rate when maintaining the recommended bead:cell ratio.

5.3.3.4 Main conclusions of the comparison between previous and current responses of T populations in culture

The primary purpose of testing the conditions implemented in this experiment was to validate the outputs and conclusions of the previous experiment. The main conclusions from this experiment were that growth rate of T-cell cultures were significantly affected by CD3/CD28 Dynabead concentration, and that concentrated incubation was positive for cultures supplemented with the same concentration of CD3/CD28 Dynabeads compared to the standard protocol. Phenotypically, all subpopulations had reduced selection rates if they had not undergone concentrated incubation, with varying responses to the experimental conditions based on subpopulation. Comparison with

previous data does not suggest validation of the conclusions and instead asks for more data to build a stronger definition of the interaction between these experimental parameters and phenotypic response.

Analysis of the growth rates of cultures in this experiment identified that when supplementing with $1x10^6$ beads/mL CD3/CD28 Dynabeads, a concentrated incubation had a positive effect relative to the non-incubated conditions. Furthermore, the cultures incubated at $3x10^7$ cells/mL for 120 minutes, supplemented with $1x10^5$ beads/mL did not grow at rates substantially worse than the non-incubated concentration. Overall these data show that the concentrated incubation had a positive effect on the growth rate and appeared to show that reducing the CD3/CD28 Dynabead concentration will not significantly inhibit growth relative to non-incubated conditions. The outperformance of the experimental conditions compared to the positive control, and the relative comparability of the positive and negative control conditions runs contrary to what was observed in the previous experiment. However, the higher growth rates of the experimental conditions do correlate with what was seen in the original proof of concept experiment, with concentrated incubation exhibiting improved growth over non-incubated controls.

Analysis of phenotypic data shows some patterns of response, including that the non-incubated conditions did not produce as high relative selection rates as those that had undergone concentrated incubation, similar to that observed in analysis of growth rates. Some nodes appeared to show a positive trend with either increasing Incubation Concentration or Incubation Period. However, there was a general lack of significant correlation, with only three of the nodes showing a significant relationship with Incubation Period. The data also show a varying response to reducing the CD3/CD28 Dynabead concentration, however they do appear to suggest that the concentration does not have a significant or substantial effect on the relative selection rates compared to those cultures supplemented with $1x10^6$ beads/mL. Furthermore, these perceived trends in the data did not appear to correlate with any particular observation of the cultures' growth rates or phenotypic profile. This suggests that the conditions are reacting in response to other experimental variables that have not been, or cannot be, monitored. Comparison of the variation within conditions to the variation between conditions speaks to the variability of T-cell cultures. Further investigation would be required to identify if there are elements of T-cell culture that could be affecting these outputs that it would be possible to control, or if there are any indications within the source material that could predict that a sample would be more or less variable.

Comparison of the general trends in phenotypic response to the experimental conditions in this experiment, as well as comparable subpopulation behaviours with the data of the previous experiment, show little correlation. It is possible to see some correlation between the relative

response in the positive control and experimental conditions of the growth rates and the phenotypic response, which was noted in the previous experiment. However, the relative response of the negative control against the two outputs contradicts this conclusion. Ultimately, further investigation is needed with these experimental variables to identify what relationships exist between them and impacts of growth rates and phenotypic response.

5.3.3.5 Comparing Nodes of Interest with similar profiles for effects of experimental conditions

Analysis of the relative selection rates of NoI in both the previous and current experiment showed broad trends that could be ascribed to most of the nodal responses, however responses between nodes of the same experiment did show more subtle differences in the way they responded. Therefore, although it would be possible to compare these broad assessments, it was more appropriate to try and identify comparable subpopulations that had been identified in both experiments and compare their responses. Using the CD4+/CD8+ nature of the node, and the level of CCR7 and CD45RO expression of the events, nodes were compared between experiments to identify comparable populations. Analysis of these criteria showed some populations that could be compared between the experiments to draw conclusions on variability or similarity in the effects experimental variables had on subpopulation selection.

When discussing comparisons between NoI, unless otherwise stated the first node mentioned refers to the previous experiment, with the second node mentioned referring to the current experiment. Furthermore, all relative comparisons in response regarding data from the previous experiment do so in the context of the conditions examined in this experiment rather than the full spectrum of conditions tested.

5.3.3.5.1 Node 1 and Node 4

Nodes 1 and 4 were noted for their similar CCR7/CD45RO profile. These nodes both presented subpopulations that contained events that fell into all four maturity phenotype expression profiles (Fig 5.6, 5.11). Node 4, from the validation experiment, does show a higher maximum CCR7 expression level, however all other limits of marker expression are similar and thus the subpopulations were considered comparable.

Comparing the relative selection rates shows that whilst in node 1 the positive control had the highest average relative selection rate for this subpopulation, in node 4 it gave the lowest. Analysis of the test conditions, 8 and 19 (Table 5.2), in the validation experiment showed that they performed relatively similarly initially (Fig 5.7). However, in the validation experiment this was not repeated as within node 4 the conditions incubated at $3x10^7$ cells/mL for 60 minutes supplemented with $1x10^6$ beads/mL showed lower relative selection rates compared to the other test conditions

(Fig 5.12). In addition, in node 1 the relative selection rate of the negative control was substantially lower when compared against the test conditions. Meanwhile in node 4, the negative control produced a higher individual and overall average relative selection rate that the conditions incubated at $3x10^7$ cells/mL for 60 minutes supplemented with $1x10^6$ beads/mL. Overall, the data in these nodes do not validate each other.

5.3.3.5.2 Node 9 and Node 8

Nodes 9 and 8 both represent populations that were lower in CD45RO expression. As was noted in the comparison between node 1 and node 4, node 8 of the validation experiment shows a slightly higher maximum CCR7 expression level, however these nodes both show the presence of Naïve and Effector subpopulations and are otherwise similar (Fig 5.6, 5.11).

Comparison of the relative selection rates shows that although the experimental conditions perform similarly to each other in node 9, the experimental conditions incubated at $3x10^7$ cells/mL has a lower relative selection rate compared to those incubated at $1x10^7$ cells/mL. Similar to the previous comparison, the relative selection rates of the positive control were greater than the experimental conditions in node 9 (Fig 5.7), whilst in node 8 they were lower than the experimental conditions incubated at $1x10^7$ cells/mL (Fig 5.12). Furthermore, in node 9 the selection rates of the positive control were all positive, whilst in node 8 they were all negative. The negative control's relative selection rates in node 9 were substantially lower than those of the experimental conditions, however in node 8 they had a higher average relative selection rate than the experimental conditions incubated at $3x10^7$ cells/mL and not substantially lower than those of the experimental condition incubated at $1x10^7$ cells/mL. Overall, the data do not show correlation in response between the two nodes.

5.3.3.5.3 Node 11 and Node 18

Nodes 11 and 18 both represent Effector and Central Memory phenotype cells. Node 18 of the validation experiment has a greater density of events than node 11 of the screening experiment. However, both nodes display events with a positive correlation between CCR7 and CD45RO expression, and presentation of events in node 11 suggest that increased density of events in this plot would look similar to what is presented in node 18 (Fig 5.6, 5.11).

Analysis of the relative selection rates shows that in node 11 the selection rates of the positive control are greatest. The experimental conditions show comparable relative selection rates that are slightly lower than that of the positive control, and the negative control selects wholly negatively for this subpopulation (Fig 5.7). Meanwhile, in node 18 the positive control has the lowest average relative selection rate of all the conditions tested. The experimental condition incubated at $1x10^7$

cells/mL showed an increase in the upper level of relative selection rates generated, however the lower level matches that of the positive control (Fig 5.12). The two conditions incubated at $3x10^7$ cells/mL show very comparable performance, with the upper limit of relative selection rates produced showing a continued increase from the previous incubation concentration. However, the lower limit remains comparable to both the previous incubation concentration and the positive control. The comparison of these data overall shows a lack of similarity and therefore they do not validate each other, however there are some elements of similarity seen between the conditions incubated at $3x10^7$ cells/mL. Further investigation would be required to establish if these similarities were genuine or coincidental.

5.3.3.5.4 Node 19 and Node 17

Nodes 19 and 17 both represent high CD45RO expressing populations. Analysis of the phenotypic distribution shows that these nodes represent Central Memory and Effector Memory phenotype events, although node 19 has a slightly lower shifted CCR7 expression level compared to node 17 (Fig 5.6, 5.11).

Assessment of relative selection rates shows multiple differences between the experiments. In node 19 the relative selection rates of the positive control condition show superior selection relative to the other conditions, with a relatively high, positive average relative selection rate (Fig 5.7). Conversely, in node 17 the positive control has the lowest average relative selection rate, and fails to produce a single positive relative selection rate across all replicates (Fig 5.12). The experimental conditions in node 19 have similar relative selection rates. The experimental conditions in this node select slightly less positively for these subpopulations than the positive control, while in node 17 the experimental conditions both have average relative selection rates higher than the positive control. Furthermore, the experimental cultures incubated at $3x10^7$ cells/mL show a reduction in the level of selection compared to those incubated at $1x10^7$ cells/mL. The negative control in node 19 did not positively select for the subpopulation at all and has a value substantially lower than both the experimental conditions and the positive control. In comparison, in node 17 the negative control has a slightly higher average relative selection rate than the experimental condition incubated at $3x10^7$ cells/mL and shows a similar range of responses. In addition, this average relative selection rate is positive, indicating that this condition is beneficial for the subpopulation. These data do not validate each other, and rather show that further investigation is required to better define the relationship between the experimental conditions tested in these experiments and phenotypic response.

5.4 Discussion

The purpose of this series of experiments was to test the theory that a concentrated incubation of PBMC samples prior to seeding into culture would be able to achieve similar growth rates in cultures with a reduced concentration of CD3/CD28 Dynabeads. If this proved correct it would allow reduction in the quantity of Dynabeads required in culture and limiting one of the greater costs of T-cell culture overall, as well as reduce the level of non-biological material supplemented into culture.

Initial experimentation appeared to show that the theoretical concept was sound with concentrated incubation improving the growth of the cultures tested. Furthermore, the data appeared to show that reducing the concentration of CD3/CD28 Dynabeads had a positive effect on the growth rate compared to concentrated incubation with the recommended bead to cell ratio. Evidence also appeared to suggest that increasing the incubation concentration had a negative effect on growth rate, whilst increasing the period of incubation was positive.

With the plausibility of this premise established practically, a further experiment was designed, extending both the incubation densities and periods, as well as introducing an additional level of CD3/CD28 Dynabead concentration. This experiment was designed to further expand the experimental parameters as well as test the relationships between the incubation concentration, period and Dynabead concentration and growth. In addition to this, the experiment was to monitor the phenotypic profile of cultures over time and attempt to identify relationships between the experimental parameters and phenotypic response.

Data showed that increasing either the incubation concentration or period prior to culture had a negative impact on the growth rate. The negative effects of increasing the incubation concentration were observed in the initial investigation, however the negative impact of increasing incubation period was not. In the initial investigation, increasing the incubation period was shown to improve growth in cultures, and comparison between test conditions and non-incubated controls showed that the test process appeared to be beneficial to cultures. In comparison, the trend in the data of the expanded investigation appeared to show that any incubation period would have a negative effect on the growth of the cultures. This was an early indication of the variable response to the concentrated incubation process. Increasing the concentration of CD3/CD28 Dynabeads supplemented appeared to have a positive effect on growth rate, however the relative similarity of cultures supplemented with $2x10^5$ beads/mL and $1x10^6$ beads/mL compared to the breadth of responses in cultures supplemented with $1x10^5$ beads/mL suggested there was not a linear relationship.

Similarly, analysis of phenotypic data calculated using the novel analytical technique developed previously showed that where present, the trends showed a negative correlation between increasing incubation concentration or period or reducing Dynabead concentration and the relative selection rates for the subpopulations of interest. It was not clear from only this experiment if the response in growth rate and selection rate of the NoI was linked or coincidental. Links between the two could be caused by the conditions which induce greater proliferation in cells causing a phenotypic change in T-cell populations so that they transition into another maturity subpopulation definition. Alternatively, the similarity in response could be due to the proliferative conditions selectively inducing growth in the non-target subpopulations. Understanding the biological mechanisms in T-cells, it is likely that any link between the two would be a balance of both causes mentioned.

Having extensively tested these parameters there was a desire to validate the outputs observed. The follow up experiment was executed with a positive and negative control and two experimental conditions. The condition that did not undergo concentrated incubation was selected as the positive control due to the performance of these conditions in the previous experiment, as well as being within the range of standard parameters in the field. The negative control was selected to be a condition incubated at $3x10^7$ cells/mL for 120 minutes and supplemented with $1x10^5$ beads/mL Dynabeads. This condition was selected for its poor growth rate and relative selection rates for target subpopulations. The experimental conditions were selected based on their ability to show high responses in both growth rate and relative selection rate compared to other conditions. As such, the two conditions selected would both be supplemented with $1x10^6$ beads/mL and incubated for 60 minutes at $3x10^7$ cells/mL and 120 minutes at $1x10^7$ cells/mL.

Initial analysis of these conditions showed that the growth rate was negatively affected relative to the experimental conditions in the negative control, however this was also seen in the positive control. The level of reduction was greater in the negative control, however the growth rates of the control conditions remained comparable, indicating that the longer incubation, higher concentration for the duration and lower CD3/CD28 Dynabead concentration supplemented were not having a substantially negative impact. Nevertheless, when observing the cell yield at the final timepoint it is possible to see that the negative control condition has a substantially lower viable cell count. With the growth rates that were compared being calculated using viable cell counts from 37 hours onwards, this appears to imply that the concentrated incubation does not negatively impact the proliferative potential of a culture, but may impact the level of viable cell loss in the initial stage of culture.

Phenotypic data was once again analysed using the novel analytical technique previously developed. Comparison was made of the overall trends in the responses, as well as between nodes that had

been identified as having similar phenotypic profiles. Comparison of the general trends showed little similarity to the previous experiment, with the positive control, as with growth, showing reduced relative selection rates. Responses varied depending on the node being assessed, however overall the responses to each of the experimental conditions were similar. The negative control also exhibited varying responses depending on the node, however more relevantly, the negative control often showed similar responses to both experimental conditions. Comparison of responses of subpopulations to similar subpopulations in the previous experiment also rendered the same verdict, that these data do not validate the previous conclusions. Overall these data indicate that concentrated incubation is of benefit to T-cell cultures, and that reducing CD3/CD28 Dynabead concentrations may cause a reduced final yield but will not reduce rate of proliferation relative to standard protocol and could improve the selection rate of targeted subpopulations.

The data collected from the validation experiment went little distance to validate the conclusions of the screening experiment. However, the improved response of the conditions that underwent concentrated incubation compared to those that did not and the relative indifference to reducing the level of CD3/CD28 Dynabead supplementation do correlate with what was seen in the proof of concept experiment.

It is not clear why there was such a variation in relative culture performance between the different experiments, however it could potentially be due to the size of the experiments. The screening experiment had a considerably larger number of samples, and the sheer practicality of sampling that many cultures could have resulted in samples remaining longer out of incubation or in other non-preferable conditions. In these situations, cultures which had already endured unfavourable conditions may be more responsive, or less tolerant, of similar conditions arising again. This could explain the difference in response between the proof of concept/validation experiment and the screening experiment.

Alternatively, these data could speak to the variability of donor material in T-cell culture. The multitude of factors that can affect the state at which material is input into culture, including the health of the donor, both acutely and chronically, their age, gender and ethnicity. Furthermore, this could manifest itself in two ways. Potentially, the input state directly defines the way in which the culture will behave, and the samples are merely behaving within that potential area of response depending on the condition. Alternatively, certain samples could be more or less responsive to the experimental parameters being tested specifically. It could be that there are elements or factors of the material that could be quantified which would elucidate how samples would respond when placed in certain conditions.

One of the objectives of this work was to validate the analysis technique in tandem with validation of the screening experiment outputs. The conclusions of the validation experiment, outlined above, clearly demonstrate that there is some disconnect between the phenotypic response identified in the screening and validation experiments to the same inputs, however this does not mean that the analytical technique is invalidated too. Rather, the ability of the process to identify differences between the experiments speaks to its merit and shows that the process is able to discern differences between cultures.

Ultimately further work is required to identify the source of this variation and to further define how PBMC cultures respond to an increased concentration incubation period with a reduced CD3/CD28 Dynabead concentration.

6 Thesis conclusions and further work

6.1 Conclusions on the impact of input material on T-cell culture

Hypothesis 1: Isolation targeting either CD3 or CD4 and CD8 would result in similar cultures both phenotypically and proliferatively.

Hypothesis 2: Culture of CD4+ and CD8+ subpopulations independently would produce different outputs in culture to each other and to when cultured in combination.

Hypothesis 3: Supplementation of CD3/CD28 Dynabeads will stimulate greater proliferation and maturation of subpopulation, which would be enhanced by the additional supplementation of IL-2.

The surge in interest in CAR T-cell therapies has increased the body of work analysing many aspects of manufacture, from transduction rates and efficiency to efficacy of treatments based on the therapeutic material. However, there was limited evidence of work on the impact of input material on the performance of cultures from a proliferative and phenotypic perspective. Factors of the input material considered were the isolation method of T-cells in non-CD3/CD28 stimulated cultures, the balance of CD4+, CD8+ and non T-cell PBMCs, and the presence of CD3/CD28 Dynabeads. It was proposed that by improving the understanding of the relationship between input material and culture outputs it may be possible to better predict the performance of cultures at seeding. In addition, it was suggested that this information may highlight ways to manipulate the input material to certain specifications for directed culture.

The initial work into target proteins for isolation of T-cells showed apparent benefits of CD3 targeted isolation in proliferation compared to CD4/CD8. There were also substantial differences in the presentation of maturity phenotype subpopulations in the CD3 targeted cultures to those targeted by CD4/8. The increase in proliferation and the nature of the variation between the phenotypic profiles of the two targets appeared to show that the CD3 isolation method was inducing activation in the T-cell populations.

Further work looking into the balance of CD4+, CD8+ and non T-cell PBMC populations highlighted the variability of donor material with variable proliferation observed which could not wholly be attributed to experimental set up. The evidence generally appeared to show that proliferation was greatest in cultures that underwent a reduced level of processing, suggesting that reducing the time and steps from start of processing to culture would be beneficial. Cultures that underwent similar levels of processing generally showed limited variation when altering the subpopulation composition. The greatest effect seen was increased proliferation in cultures seeded with a CD8/CD4 subpopulation ration of 3:1, however the results showed a high level of noise that

warranted further experimentation to validate these observations. As no phenotypic analysis was performed on these populations it is not possible to say what the impact of this was on subpopulation balance.

Testing the supplementation of CD3/CD28 Dynabeads as part of the input material showed that they had a positive impact on proliferation. The effect of supplementing the activating beads was also seen in the phenotypic analysis of the cultures, showing higher levels of mature subpopulations as well as a greater transition to those populations over time. The evidence also appeared to suggest that the supplementation of IL-2 with CD3/CD28 Dynabeads was slightly limiting with regards to proliferation and subpopulation transition. It was suggested that this may be due to the T-cell developing an anergy if exposed to too high a level of IL-2 prior to activation, however further work was required to validate this.

Overall, the data show that processing has a greater impact on non-CD3/CD28 stimulated cultures than the balance of populations, identifying the culture profiles of similarly processed conditions to be relatively comparable. In addition, the supplementation of CD3/CD28 Dynabeads appears to have a substantial impact on the proliferation and phenotypic response of T-cells in culture. The combined response of T-cell cultures to CD3/C28 Dynabeads and manually supplemented IL-2 also requires further investigation to validate the observed effects.

6.2 Conclusions on the effects on delaying supplementation of IL-2 into T-cell cultures

Hypothesis: Delaying supplementation of IL-2 into culture will have a positive effect on proliferation and maturation of subpopulations

Observations in the previous chapter showed that supplementation of additional IL-2 with CD3/CD28 Dynabeads at seeding reduced proliferation and inhibited the transition of cells to Central Memory phenotype. It was proposed that this inhibition was brought about by a level of anergy incurred by the T-cells to IL-2 on exposure prior to sufficient activation. Therefore, it was predicted that delaying the supplementation of IL-2 would allow time for sufficient activation that supplementation would have a positive effect. In addition, it was predicted that supplementing the CD3/CD28 Dynabeads at the highest bead:cell ratio would introduce the highest level of activation and therefore see the greatest positive impact on proliferation and maturation.

Analysis of the conditions tested showed that delaying the supplementation of IL-2 and higher concentrations on CD3/CD28 Dynabeads had significantly positive effects on growth rate, whilst supplementation of IL-2 and increasing the seeding density had significantly negative effects. Investigation of the significant relationship between concentration and timing of IL-2

supplementation showed that supplementing IL-2 at 96 hours produced the same response as those culture not supplemented with IL-2, whilst supplementing with IL-2 at 0 hours had a negative effect. This appears to validate the observation that supplementation of IL-2 at seeding inhibits proliferation, however it does not support the hypothesis that supplementation at the later timepoint will increase proliferation relative to non-supplemented cultures.

Traditional phenotypic analysis of the data showed minimal significant impacts of the data, with the predominant effect being the negative impact of supplementing IL-2 on Effector phenotype subpopulations. Assessment of the phenotypic method highlighted that the traditional method of analysis did not take into account the nuances of phenotypic profiles that may be presented and did not take a dynamic approach to subpopulation assessment. In addition, it was noted that user bias could easily influence the outputs of this analysis and bias conclusions. It was concluded that an analytical method that considered these elements, as well as eliminating as much of the user input as possible, could provide a greater insight into the influences of these experimental variables on phenotypic response.

Nodes of interest were identified which presented events that conformed to the Naïve and Central Memory phenotypic profiles. Analysis of these nodes showed that where responses were significant, they were generally in line with the observed effects on growth rate. The main difference was that the seeding density appeared to have a predominantly negative effect on the subpopulation selection rates. This appeared to suggest that proliferation of cultures was linked to the phenotypic response. However, it was not clear if this was because the conditions which stimulated proliferation would also induce maturation of T-cell cultures, or if they selectively induced proliferation in mature subpopulations.

Overall, the data confirmed the hypothesis that supplementation of IL-2 at seeding had a negative impact on T-cell cultures, whilst rejecting the hypothesis that delayed IL-2 supplementation would increase proliferation. Using the novel analytical method, the data also highlighted the link between maturation of T-cell subpopulations and proliferation.

6.3 Conclusions on the concentrated incubation of cells prior to seeding and how that impacts the subsequent culture of the material

Hypothesis: Concentrated incubation prior to seeding could induce the same level of proliferat with a reduced quantity of CD3/CD28 Dynabeads

There is a general desire in the therapy manufacture field to move away from non-biological and non-human elements as much as possible. The results from the initial experimental chapter (chapter 3) highlighted the importance of CD3/CD28 Dynabeads for T-cell culture, whilst the data

from the previous chapter appeared to suggest that the response to the beads was concentration related rather than bead:cell dependent. A process was set up to assess the impact of incubating cell cultures with a reduced quantity of CD3/CD28 Dynabeads at a higher concentration for a period of time prior to seeding to induce the same level of activation with fewer beads.

This investigation produced variable results on growth rate of cultures, with initial investigation appearing to show benefits of undergoing this process compared to the standard protocol. Further investigation increasing the range of conditions tested showed the opposite, with any incubation having a negative effect relative to control conditions. In addition, increasing the duration or concentration of cells during incubation was shown to have an increasingly negative effect as the value was increased. Attempting to validate these responses with specifically chosen conditions appeared to better correlate with the outputs of the initial investigation, showing similar responses to the concentration and duration of the incubation.

Once again phenotypic response was linked to proliferation in culture. In both the expanded and validation experiments the predominant trend in the response of selected subpopulations matched that of the growth rates in response to the culture conditions. Attempts to compare similarly presenting subpopulations failed to identify substantial correlation of response to the experimental variables.

Overall, it appears that concentrated incubation could have positive applications in culture with some evidence showing positive response to the process. However, further investigation would be required to identify if the variation seen in the response to variables was due to the variability of the donor material or was induced through methodology.

6.4 Conclusions on the novel optimal phenotypic resolution analytical process

Through the course of this research the novel phenotypic analytical method developed was shown to have a greater sensitivity to phenotypic variation than the standard gating process traditionally used. The key benefits of the novel process included the ability to analyse an entire dataset simultaneously. The process could analyse multiple markers simultaneously, allowing it to assess relationships between markers that may be lost in traditional stepwise analysis. In addition, this also meant that the process was able to utilise information from multiple timepoints in analysis, as well as ensuring analysis covered the full phenotypic space occupied for the duration of the experiments, where the standard process would require the application of gates to a sample and checking their validity across all other samples.

This also alludes to the benefit of having and algorithmic approach to the process of identifying clustering points in the data, the removal of user bias. The process utilises an iterative approach to

identifying focal points of the data and, apart from some initial 'cleaning' of the data to remove unwanted events, is free from user-based gating. This helps to eliminate variations in gating based on between session and between user variation, as well as reducing the impact of user bias caused by anticipating responses in subpopulations. Ultimately, this should lead to a more impartial and more consistent analytical method.

Comparing the level of variation in a whole dataset and the mean variation of repeated conditions at each timepoint, the novel method was able to identify the point at which the experimental variables had the greatest impact on defining subpopulations, and subsequently the optimal number of subpopulations to split the dataset into. As such, the process would optimally divide the total population into the most appropriate number of subpopulations, so that each was affected differently by the experimental variables in use. This would allow for a more nuanced analysis of the data than traditional gating, which would miss some of these effects due to the broader gating strategy. Additionally, the ability to visualise the events associated to each node on a 2D plot post-analysis means that it's possible to identify the 'traditional' maturity phenotypes that the node represents, and therefore identify target nodes for further analysis of performance and experimental impact.

Finally, analysis of the performance of these newly identified subpopulations over time would highlight whether the experimental variables being tested would induce selection for or against them. This part of the process could be performed as part of traditional gating, however the increased resolution and dynamic nature with which subpopulations were defined should mean that the process will give a more nuanced assessment of the experimental data and allow greater insight into the impact of the culture conditions being tested.

The intention of the research carried out was to identify the impact of culture conditions on T-cell maturity populations, specifically with a view to increase the selection for Naïve and Central Memory cells which are preferable in the transduction step of CAR T-cell therapy manufacture. The novel analysis requires the completion of several SPADE analyses, as well as complex statistical assessment of these outputs to yield the conclusions mentioned above. As such, the process can be complex and labour intensive, and would not be suitable for monitoring populations during therapy manufacture, either to predict the population balance outcome of the culture, or to influence the culture process dynamically. However, the process has been designed so that in an investigative setting it would be able to analyse data with a greater sensitivity than the standard process currently in place, and would therefore has the potential to be more informative on the effect that different culture conditions have on the selection of these T-cell maturity subpopulations. As such, it would be a suitable tool to use in process development and improvement, with a greater level of feedback

than using the common phenotypic analysis, and would improve the manufacturing process overall from a research and development standpoint rather than an in-line monitoring perspective.

The application of this analytical process also extends beyond that of T-cell culture and CAR T-cell therapies, with this process feasibly being applied to phenotypic analysis of any cell data. The key benefits of the process, mentioned above, include the ability of the process to manage and incorporate large numbers of markers simultaneously, as well as identify the smaller variations in marker expression normally lost in the broader gates of traditional analysis. Therefore, this process is most appropriate for cell cultures that use multiple markers to track cell response, and where these markers are predominantly expressed dynamically across a range, as opposed to the more binary expression of markers such as CD3, CD4 and CD8.

In particular, it was anticipated that this process could be used to analyse experimental impacts on differentiation of cells. Marker expression is a key element of identifying these transitions, subtle changes in expression levels can have larger implied significance on their biological state. The analytical method would be able to identify these variations with a higher degree of accuracy than the traditional method, as well and performing assessment free from the bias of expecting transition or response inherent to user-based analysis.

6.5 Overall summary

The investigations carried out over the course of this research were intended to highlight relationships between experimental parameters and the resultant proliferation and phenotypic response of T-cell cultures. The aim was to identify conditions which would retain the proliferation required to manufacture T-cell therapies such as CAR T-cells whilst selecting preferentially for the Naïve and Central Memory subpopulations.

Individual experiments have drawn their own conclusions based on the data obtained, however there are some conclusions which have spanned between them. Evidence has suggested that there is a slight inhibitory effect of supplementing IL-2 into culture at seeding on proliferation and maturation, despite it being a common practice in T-cell culture throughout the field. It is proposed that this due T-cells interacting with IL-2 prior to sufficient CD3/CD28 activation. It is known that stimulation in this manner will increase the expression of CD25, a component of the IL-2 receptor which increases affinity and response of binding to IL-2. Exposure to IL-2 prior to the upregulation of this protein could reduce the effectiveness of IL-2 in promoting proliferation and maturation in T-cell cultures.

It is clear to see that CD3/CD28 stimulation, via CD3/CD28 Dynabeads or another method is key to promoting proliferation in T-cell cultures. Increasing the concentration of CD3/CD28 Dynabeads has

the ability to induce a greater proliferation in cultures, however chapter 5 also highlighted the possibility of reducing the quantity of beads used by concentrating cell samples prior to supplementation of CD3/CD28 Dynabeads. This process requires further investigation, however if proven it could reduce the reliance of T-cell culture on CD3/CD28 Dynabeads.

The data obtained across experiments has frequently shown that there has been a correlation between the proliferation of T-cell cultures and the phenotypic response. Furthermore, the response has been relatively conserved across experiments, indicating a genuine link between growth of a culture and the phenotypic profile. The data indicate that with an increase in proliferation there was a greater concentration of Effector Memory subpopulation cells in culture. It was not clear from the work performed if this was due to phenotypic transition of cells from other subpopulations into the Effector Memory subpopulation or if the conditions which promoted growth in T-cell cultures selectively promoted growth in the Effector Memory subpopulation. However, understanding the *in vivo*roles of T-cells and their biological response to stimulation would suggest that transition of phenotypic profile is contributing as T-cells are being pushed towards the Effector Memory phenotype by activation of the CD3/CD28 pathway.

The key contribution of this research was the development of a novel phenotypic analysis process. The process was developed in response to the shortcomings of the traditional analytical method. It was observed the traditional process took little account of the nuanced phenotypic variation that could be observed in the maturity subpopulations of T-cell cultures. In addition, the limitations of user based phenotypic assessment were highlighted, suggesting that a non-biased algorithmic approach would be more accurate in representing the subpopulations which were present. The novel process was developed to identify the most optimal resolution for phenotypic analysis using the outputs of algorithmic data analysis tool SPADE. The data was then used to calculate the relative selection rates of cultures in target subpopulations and identify conditions which supported or inhibited the culture of target phenotype cells.

Further work in these areas could serve to answer the remaining questions and enable a greater level of control over T-cell culture for improved and more directed outputs.

6.6 Further work

Over the course of this research several key observations have been made that may inform the T-cell culture process. However, due to limitations in time, budget or practical execution by a single person not all avenues of investigation could be explored. As such, there are several instances where further work could improve on what has been completed so far.

The investigation into delayed IL-2 supplementation appeared to confirm the hypothesis that addition of IL-2 at seeding had a negative impact on growth. The experimental set up compared the supplementation of 0 or 200 UI/mL IL-2 at 0 or 96 hours. Unexpectedly, cultures supplemented with 0 UI/mL IL-2 at 0 and 96 hours, which were therefore not functionally different, showed significantly different responses in phenotypic responses. This was observed in both the traditional and novel phenotypic analyses. This investigation would benefit from being revisited without the use of non-supplemented cultures to identify the genuine nature of the response. In addition, this experiment tested the effects of delaying the supplementation of IL-2 for 96 hours. Where included in the model, data from the midpoint condition, delayed for 48 hours, showed a non-linear relationship between IL-2 delay and growth or phenotypic response. The nature of the non-linear relationship mostly identified that supplementation of 100 UI/mL IL-2 had a more positive response than supplementing 200 UI/mL IL-2 at 0 or 96 hours. Further investigation would increase the number of delay periods tested to ascertain the most optimal point of supplementation.

The investigation into concentrated incubation prior to seeding provided some inconclusive data on whether the process would benefit cultures or not. The variability seen in the response could be due to the variability of donor material, however the overall negative responses of the cultures in the largest experiment could have been caused by the number of conditions which were required to be simultaneously processed. Furthermore, the validation experiment included test conditions with the recommended quantity of CD3/CD28 Dynabeads and a negative control which used a reduced quantity of beads but was specifically selected for its reduced activity. It would be desirable to revisit this process and test a selection of conditions that included a reduced quantity of CD3/CD28 Dynabeads in combination with more favourable parameters as concluded in 5.3.2.3. The broad range of conditions tested was intended to ensure a full spectrum of data, however it appears that the size of the experiment worked against itself.

One of the main outputs of this research was the novel phenotypic analysis process, utilising the SPADE tool to analyse phenotypic data algorithmically and optimise the resolution of the phenotypic assessment. Once the Nodes of Interest had been selected, relative selection rates of these subpopulations were calculated using the relative ratios and the LINEST function in Excel. Although the progress of relative ratios over time could be suitably represented over time by a linear function, there were many instances where this was not as appropriate. Further work into the development of this novel process would be most beneficially focussed on improving this step to most accurately represent the change in relative ratio over time and therefore the relative selection rate. This is particularly pertinent were the analytical process to be applied to other cell data where expression

of markers could temporarily increase and then decrease over time, or vice versa, and an exponential analysis of population performance would be required.

The process would also benefit from the development of a framework by which outputs of different experiments could be compared. This was attempted in 5.3.3.5 by comparing event plots for similarity and comparing responses, however the limited number of comparable plots reduced the effectiveness of validation. With phenotypic resolution optimised for each experiment separately, this becomes a further issue as presentation of event plots may vary considerably. The development of this framework would be key to enabling the mass application of this novel process, as without the ability to compare to previous or contemporary work the phenotypic analysis will lose a lot of its power.

Lastly, this novel process could benefit from better accessibility and application for users. Currently this process is dependent on the SPADE analytical tool hosted by Cytobank. Annual licenses for this platform are costly and may not be feasible in all situations. Investigation into using other methods such as running the analysis via Matlab could offer cheaper alternatives, however the device-based software is then dependent on the computing power for speed of analysis. Alternatively, other clustering software such as Scalable Weighted Iterative Flow-clustering Technique (SWIFT), or automated gating in Bioconductor may offer a more cost effective, and therefore accessible, alternative. In addition, the process by which the SPADE output data are analysed requires a high degree of processing. Development of a platform where SPADE output data and basic parameters could be added to automate the analytical process would be likely to significantly reduce the time and effort required from each user to achieve the desired results.

Finally, the most prominent pattern seen in the data across all experiments was the relationship between higher proliferating cultures and the increased concentration of Effector Memory subpopulations. This relationship is likely due to the maturing effect of conditions which induce the greatest level of growth, indicating that increased proliferation comes at a cost of reduced proportions of target subpopulations. Although this would imply that the retain greater proportions as target T-cell subpopulations some level of proliferation must be sacrificed, the data were never analysed to identify if the reduced level of proliferation achieved would still be acceptable in a clinical setting. Further investigation of this relationship would be focussed around identifying if culturing T-cell populations in conditions which had a higher retention of target subpopulations would still show rates of proliferation acceptable in the manufacturing process.

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