Generating Ag-specific Human Regulatory T-cell by TCR Gene Transfer for the Treatment of Rheumatoid Arthritis.

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Abstract

Rheumatoid Arthritis (RA) is a systemic autoimmune disease that develops when the immune system loses tolerance to self, resulting in effector cells erroneously causing joint damage. Despite advances in biological therapy there is currently no cure for RA. Regulatory T-cells (Tregs) can regulate a broad range of immune effector cells when specific antigens (Ag) activate them through their individual T cell receptors (TCR). Using retroviral gene transfer, Tregs can be modified to express a chosen disease-related TCR, producing an Ag-specific Treg population that can target and suppress inflammatory arthritis in murine models. In this study the aim was to validate a reproducible method of generating Ag-specific Tregs from human peripheral blood. The results of this research demonstrates the successful TCR transduction into isolated human Treg cells. This was achieved by completing the outlined objectives to optimise the transduction process and the isolation of Tregs from peripheral blood. By sorting on the CD4+CD25+CD127dim cell population, Tregs were isolated and the purity of the population demonstrated by the high percentage of FoxP3 expressing cells. The results further show that FoxP3 is maintained after the transduction process. It is now necessary to demonstrate the working functional capacity of the transduced Tregs using a robust antigen-specific suppression assay. With the future goal of transitioning this approach into a human clinical setting, the method requires further validation to prove a reproducible method of generating Ag-specific Tregs from human peripheral blood taken from RA patients. This has implications for the advancement for adoptive Treg therapy to treat RA and other autoimmune diseases and will highlight the necessity of considering treatments in the context of an inflammatory environment. It will be important to understand the nature of any defects in the Treg subsets and how these might be corrected.

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List of Abbreviations

Ag	Antigen
Anti-CCP	Anti-Cyclic Citrullinated Peptide
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CTV	Cell Trace Violet
CIA	Collagen Induced Arthritis
CTLA4	Cytotoxic T-lymphocyte Associated Antigen 4
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
ELISA	Enzyme-Link Immunosorbent Assay
FoxP3	Forkhead Box Protein 3
GITR	Glucocorticoid-Induced TNF-R Related
GvHD	Graft versus Host Disease
GZ-B	Granzyme B
HLA	Human Leukocyte Antigen
IFN	Interferon
IL-	Interleukin
IPEX	Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked syndrome
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
NOD	Non-Obese Diabetic
nTreg	Natural regulatory T cell
PA	Phoenix Ampho Cells
PAD	Peptidylarginine deiminase
PBMC	Peripheral Blood Monocytes
PBS	Phosphate Buffered Saline
RA	Rheumatoid Arthritis

RF	Rheumatoid Factor
ROR	Retineic-acid-receptor-related Orphan Receptor
SE	Shared Epitope
T cell	Thymus derived lymphocyte
TCR	T cell receptor
TGF- β	Transforming Growth Factor β
TNF	Tumour necrosis factor
Tcon	Conventional T cell
Treg	Regulatory T cell

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Chapter 1 - Introduction

1.1 Autoimmune Disease

In a healthy individual the immune system acts to protect the body against invading pathogens, whilst remaining tolerant to, and not responding against self-structures. Autoimmune disorders develop when the immune system loses this self-tolerance and effector cells respond erroneously against self-antigens inflicting damage, inflammation and often chronic pain. The initial event which stimulates the self-attack response is difficult to identify, and often occurs years prior to the development of symptoms and once initiated cannot, at present, be stopped, hence there is no cure for autoimmune diseases.

Understanding of autoimmunity has greatly advanced, with the identification and characterisation of crucial components of the immune system, such as the major histocompatibility complex, T-cell receptors and regulatory T-cells (Lleo *et al.*, 2010). Over 80 autoimmune disorders are now officially recognised and a more detailed knowledge of autoimmunity allows diseases to be described as B-cell mediated, or T-cell mediated or both.

1.2 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a severe autoimmune disease in which dysregulation of the immune system causes the body to self-attack the bones and joints, causing persistent inflammation, chronic pain and irreversible joint destruction. The condition is typically characterised by the inflammation and proliferation of synovial cells at the joints, however RA can affect numerous different joints with a spectrum of severity.

1.2.1 Epidemiology and Genetics of RA

As the most common form of inflammatory arthritis, and the second most prevalent autoimmune disorder, RA affects 1% of the world population (Scott, Wolfe, & Huizinga, 2010). In the UK this is estimated to translate to affect approximately 700,000 individuals. The most recent epidemiological study of RA in the UK was published in 2002 (Symmons *et al.*, 2002). However, incidences reported in the study are based on diagnosing RA using an outdated classification system, and therefore may not be accurate. Despite this, it has been clearly established that RA affects woman three times more than men, and the prevalence increases with age, resulting in the highest incidence in woman over 65 years of age (Symmons *et al.*, 2002).

The greatest susceptibility for developing RA is genetic factors (Yamamoto *et al.*, 2015a). There are more than 30 genetic regions which have been identified as being associated with the RA (Yamamoto *et al.*, 2015b). Currently the most significant link to the susceptibility of RA inheritance, and therefore the biggest risk factor is considered to be the presence of HLA-DRB1 (Human Leukocyte Antigen) alleles associated with MHC Class II (Tezenas *et al.*, 2005). This links directly to the 'shared epitope hypothesis', which postulates that one possible mechanism of pathogenesis in autoimmunity is the structural similarities between HLA variables, which alter the MHC-TCR interaction during antigen presentation to T-cells (Gregersen, Silver and Winchester, 1987). A number of different HLA genes can specifically confer a high risk of RA, with all these genes having structural similarities in the peptide binding groove that has been referred to as the 'shared epitope' (SE). It has been suggested that high risk of developing the condition is therefore linked to inheritance of the shared epitope in the form of a small portion of amino acid sequences shared by particular alleles within an HLA subtype. It is now accepted that HLA DR4 type has an association with the onset of RA, in particular HLA DR4 alleles containing the identified SE (HLA-DRB1*01, HLA-DRB*04). However, as identified by Gregerson *et al* (Gregersen, Silver and Winchester, 1987) when the shared epitope hypothesis was first formulated, a number of RA patients do not possess DRB molecules, and the inheritance of DR4 has also been linked to increased risk of developing other autoimmune diseases. DR4 is therefore not considered a specific risk factor for RA, and subsequently genetic screening to predict the onset of RA is impossible, further limiting the ability to treat the condition early and slow disease progression.

It has been observed that individuals with RA often have increased levels of antibodies formed against citrullinated proteins. Citrullination is a post-translation modification in which the amino acid arginine is converted to citrulline. The mechanism which initiates citrullination has not been definitively established, although there is evidence that it may linked to the release of intracellular PAD (Peptidylargine deiminase) enzymes and Ca^{2+}, during inflammation-driven tissue damage, leading to the inappropriate extracellular citrullination of proteins. Citrullination has also been linked to risk factors such as smoking (Luban and Li, 2010). There is an association drawn between the inheritance of SE containing alleles and increased levels of anti-citrullination antibodies. It is thought that the citrullination process may result in the unintentional alteration of the shapes of antigens resulting in better conformation to bind to SE containing alleles (Luban and Li, 2010). The presence of anti-CCP antibodies (Anti-Cyclic Citrullinated Peptide), which are produced against citrullinated antigens, is relatable to the onset and severity of RA, and therefore link

citrullination and SE-alleles associated with DR4, and emphasise the increased genetic risk that the DR4 HLA type confers. It should be noted that not all incidences of RA coincide with the presence of anti-CCP antibodies, and therefore the condition is often discussed as anti-CCP positive RA and anti-CCP negative RA (Schellekens *et al.*, 2000), with different CCP-variants related to different HLA susceptibility. This is important to consider when studying the condition at the serum level.

1.2.2 Diagnosis and Treatment

Classification criteria for the diagnosis of the RA were first published in 1958, revised in 1987 and further revised in 2015 by the American College of Rheumatology and the European League Against Rheumatism (Singh *et al.*, 2015). The classifications characterise the condition based on a combination of factors, and the newest modifications were introduced in an effort to identify the onset of RA as early as possible, a difficult task as often manifestations of RA appear years after the initial development of the disease. The newest diagnostic criteria uses a score-based algorithm based on the number of joints inflamed, the type of joints, the serology and duration since the symptoms have appeared.

A crucial component used to identify and diagnose RA is the results of serological tests. Tests are used to detect the presence, concentration and levels of autoantibodies - an antibody that is produced by the body and attacks self-proteins. In RA specifically, two key autoantibodies have been identified as being present in high concentrations: Rheumatoid factor (RF) and Anti-Citrullinated Protein Antibody (ACPA). The presence of autoantibodies is considered an indicator of autoimmune disease, however there is conflicting evidence concerning the potential role of autoantibodies in the pathogenesis of autoimmunity. For example, high levels ACPA could potentially contribute to the disease, however disease remission does not necessarily correlate with a reduction in ACPA levels within a patient. RF is considered to be a marker of chronic inflammation, and can therefore be present in individuals who do not demonstrate symptoms of RA. This is a factor to be considered in the search for potential treatments. The relative concentration and presence of these proteins can be used to predict the onset and diagnose the condition. Increased concentrations of autoantibodies identified in patient samples can be indicative of RA years before clinical symptoms begin to manifest (Forslind *et al.*, 2004).

1.2.3 Current RA Treatments

There is no cure for RA, and current treatments for the condition focus on symptom management. The most up to date guidelines in the UK recommend the administration of

multiple drugs to simultaneously reduce inflammation and pain (Singh *et al.*, 2015). Disease-Modifying Anti-Rheumatic Drugs (DMARDs) are predominately prescribed, and these act to suppress the body's immune system from actively destroying the joints (Press, 2009). DMARD can refer to any chemical that can be used to treat the underlying disease condition and slow progressive joint erosion. A long-term study which examined approximately 3000 RA patients over the course of 20 years concluded that there was 30% reduction in the development of longer term disability after consistent use of DMARD drugs (Wolfe *et al.*, 1994). However, DMARDs target the general immune system and their long-term use is associated with toxic effects. As the long term continuous use of DMARDs is essential for sustained treatment, this emphasises the vital necessity of developing a more efficient therapy to treat RA. Analgesics are additionally required for the management of pain, and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are recommended to reduce inflammation. Steroids are often prescribed for short term pain relief (Ally and Hodkinson, 2016). This combination of prescriptions represents the life-long challenge for RA patients in dealing with the co-ordination of multiple pharmaceuticals for symptom management and the possibility of copious side effects which accompany them.

In recent years a new form of DMARDs are available for RA treatment, and these are known as 'biological DMARDs', or 'Biologics'. This new class of drugs is increasingly prevalent as research increases knowledge of the underlying mechanisms causing RA (Story and Far, 2013). Biologic drugs are designed to specifically block the production of the damage causing chemicals in RA, such as Tumour-Necrosis Factor (TNF) thereby reducing the inflammation and joint destruction while avoiding general immune suppression. However, since TNF plays an important role within the immune system, blocking the cytokine can have demonstrable consequences, and has the potential to increase susceptibility to infections. In 2010 the British Society for Rheumatology produced guidelines for the safe use of TNF therapies, recommending that caution is required when prescribing anti-TNF biologics due to the damaging side effects and the increased risk of infections that accompany the treatment (Ding *et al.*, 2010). As with DMARDS, the continuous and life-long use of the biologics is required.

It would be greatly advantageous to develop a treatment with the ability to halt re-occurring and chronic inflammation, restoring the tolerance of the immune system with long-term sustainability. A promising method of achieving this is the genetic modification of a particular sub-population of cells known as regulatory T-cells (Tregs) to generate an adoptive therapy.

1.3 Regulatory T-cells

In the early 1970's Gershon and Kondo proposed thymus derived lymphocyte populations contained more than one subset of T-cells (Gershon and Kondo, 1970). Early studies of autoimmune disease suggested that a suppressor T cell population with the ability to regulate and suppress other T-cells existed, with the means of mediating immune tolerance. However there was initially no definitive evidence for the existence of a distinct regulatory T-cell population as no discriminatory population markers could be identified, and a pure Treg population could not be isolated (Moller, 1988). The identification and isolation of Tregs did not occur until 1995, when Sakaguchi (Sakaguchi, 1995) demonstrated the elimination of the CD4+CD25+ subset in mice resulted in the onset of a multiple autoimmune conditions.

Regulatory T-cells are now an accepted sub-population of T-cells, composing only 2-3% of the total T-cell population. Tregs have an important role in preventing and turning off the immune response, and evidence suggests that through recognition of self, Tregs can dominantly suppress immune responses that may result in inappropriate self-targeting, followed by the turning off of the adoptive immune responses (Sakaguchi *et al.*, 2008).

1.3.1 Identification of Tregs

CD25 is the high-affinity alpha chain of the IL-2 receptor, and this receptor is essential for the development of Tregs (Furtado *et al.*, 2002). As CD25 is constitutively expressed on most Tregs it was identified as a surface marker to be used in combination with CD4 to isolate a Treg population. While the use of CD4+CD25+ surface markers is still considered the most practical method of characterising and isolating Treg cells unfortunately this approach is flawed. Due to the role of IL-2 in T cell inflammatory responses, the expression of CD25 on Tcons is upregulated after activation (Hatakeyama *et al.*, 1989), and consequently the use of this marker to isolate pure Treg populations can often include a contamination of conventional CD4 T-cells. The closest marker identified to isolate a genuine Treg population is Forkhead Box Protein 3 (FoxP3), which is a transcription factor that has been described as a 'master regulator' for Tregs, with a critical function in determining the cell lineage function and commitment (Zheng and Rudensky, 2007). It is therefore highly expressed and a reliable marker for Tregs. As a transcription factor FoxP3 persists in the nucleus of cells; this can be detected by flow cytometry however the process requires the fixing and permeabilisation of the cells, and therefore FoxP3 cannot be used to sort viable cell populations. Furthermore, the upregulation of FoxP3 expression in activated Tcons has also been observed (Gavin *et al.*, 2006).

CD127 can be used in combination with CD4 and CD5 to obtain a purer population (Liu *et al.*, 2006). CD127 is the receptor for IL-7, and is downregulated on T-cells after activation, however while T-cells re-express the receptor Tregs demonstrate a reduced expression of CD127. Liu *et al* (2006) isolated a highly purified Treg population using the markers CD4+CD25+CD127dim to identify the Treg cells, and further demonstrated a direct link between the reduced expression of CD127 and the expression of FoxP3 in Tregs.

It is recognised that more unique surface markers are required for easier identification and isolation of Tregs. Seddiki (Seddiki *et al.*, 2017) therefore assessed a large number of potential markers, such as the surface proteins CTLA-4 and GITR (Glucocorticoid-Induced TNF-R Related), which are expressed by Tregs and are essential for the functional capacity of the cells, however cannot be considered unique enough to qualify as effective Treg markers. CD4+CD25+C127 low therefore remain the best markers for the isolation of the Treg population.

1.3.2 Tregs: Mechanisms of Action

Like all T-cells, Tregs recognise their target through their individual TCR. TCRs are generated in such a way that each T-cell has a unique receptor capable of recognising a different protein structure, known as its antigen. Hence, in Tregs, it is the individual TCR that enables the Treg to recognise antigens and prevent responses that may compromise self-tolerance. Natural Tregs that develop in the thymus are a polyclonal population, already primed to activate in response to a diverse range of antigenic stimulation. The existence of tissue specific Tregs indicates that a portion of Tregs migrate to regional lymph nodes and are activated by tissue specific antigens, in addition to the exportation of Tregs to inflamed and infectious areas where they can exert their regulatory function (Bluestone and Abbas, 2003). It is known that Tregs can supress in a number of ways which can be divided into contact dependent suppression and contact independent suppression. In order to comprehend the potential impact and function of regulatory T-cells within autoimmune disorders the mechanisms through which Tregs act must first be understood.

1.3.3 Suppression by Tregs

1.3.3.1 Contact-dependent suppression

Cell contact-dependent mediated suppression by Tregs was first suggested after *in vitro* investigations indicated that when co-cultured, but separated by a semi-permeable membrane, Tregs failed to suppress Tcons (Sakaguchi *et al.*, 2001). A number possible mechanisms have been described.

One hypothesis suggests that contact-dependent suppression is mediated through the GITR receptor, which is constitutively expressed on Tregs. The activation of the GITR receptor has been acknowledged to halt suppression: Gondek *et al* (Gondek *et al.*, 2005) noted the upregulation of the molecule GZ-B (granzyme B) after activation of the GITR receptor during contact-dependent suppression. GZ-B is a serine protease which can induce apoptosis in targeT-cells, and therefore the upregulation of this molecule through cell contact with T-regs highlights a potential mechanism for suppression. This is further emphasised by the reduced ability of Tregs removed from GZ-B knockout mice to suppress effector cells. Other mechanisms of contact-dependent suppression have been described, for example, it has been suggested that Treg contact-dependent suppression utilises the molecule cyclic adenosine monophosphate (cAMP), the upregulation of which can reduce IL-2 and inhibit T-cell growth (Bopp *et al.*, 2007). The range of published research investigating Treg mediated suppression highlights the complexity surrounding the function of Treg cells.

The most interesting mechanism of contact-dependent suppression, in the context of considering the potential role of Tregs in autoimmunity, is the direct interaction of Tregs with antigen presenting cells (APCS). This is mediated through the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on the surface of Tregs: CTLA-4 binds directly with a high affinity to the surface proteins CD80 and CD86 expressed on the APCs, blocking the required co-signal for the activation of conventional T-cells. CTLA-4 knockout mice have a high mortality from the rapid develop autoimmune multi-organ failure (Klocke *et al.*, 2016). This mechanism of suppression therefore requires the Treg to be activated by the antigen presented by the APC, as Tregs will not interact with APCs in the absence of antigen activation, and is therefore an Ag-specific method of suppression. Additionally it requires the presence of mature APCs with upregulated expression of CD80/CD86. However it is interesting to note that research has indicated that immature APCs can activate naïve Tregs despite the low expression of CD80/86 (Mahnke *et al.*, 2002).

1.3.3.2 Contact-Independent suppression

There is accumulating evidence to suggest key roles of cytokines IL-10 and TGF-β in Treg mediated suppression. IL-10 is a cytokine with documented functions in immunoregulation, and suppresses the expression of MHC class II while inhibiting the production of inflammatory cytokines and interfering with the proliferation of Tcons (Bluestone and Abbas, 2003). Similarly TGF-β acts to inhibit conventional T-cells, and downregulates cytokine receptors that promote cell survival, including IL-2 receptor, which is essential for cell

survival. Interestingly CTLA-4 knockout mice demonstrate increased secretion of IL-10 and TGF-β, suggesting that Tregs utilise a combination of contact-dependent and independent-suppression (Bluestone and Abbas, 2003).

1.3.4 Linked Suppression and Infectious Tolerance

Whilst Tregs require recognition of a specific antigen to become activated and suppress, once activated, Tregs can suppress conventional T-cells (Tcons) specific for any target, provided they are co-localised. APCs play a crucial role by providing proximity required for this process through the simultaneous presentation of different antigens. This phenomenon is known as linked suppression and is essential in preventing the broad range of self-responses that may be initiated (Thornton and Shevach, 2017). This is likely to be particularly important in curing autoimmune disease where the original inciting antigen may not have been identified.

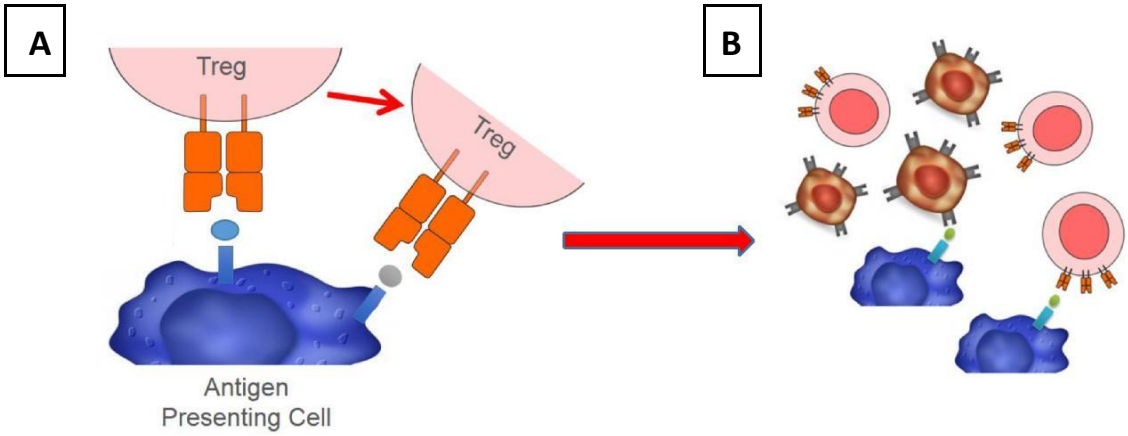

Figure 1.1: Diagram describing the Treg characteristics linked suppression and infectious tolerance. (A) describes linked suppression, where the Treg is capable of supressing the effector cell of different specificity when the two peptides are presented by the same APC. (B) shows infectious tolerance, in which tolerance to a peptide is passed onto surrounding cells.

Tregs have also demonstrated a capacity for infectious tolerance, a process in which tolerance towards specific proteins can be promoted among surrounding T-cells, and this allows for a long term sustained suppression of Tcons (Waldmann *et al.*, 2006). As with linked suppression, the role of the APC is primary to the process of infectious tolerance, and the introduction of activated APCs into a tissue graft can help to restore immune homeostasis (Sakaguchi *et al.*, 2009). Within the tissue microenvironment, Tregs specific for antigens presented by the APCS can exert influence over the APC to induce the surrounding effector cells to convert into adaptive T-regs. This increases suppression and maintains long term tolerance in a self-perpetuating fashion, a highly attractive function of Tregs in the context of exploiting the population to generate an effective autoimmune therapy.

1.4 Adoptive Treg Therapy

Adoptive therapy describes a treatment that involves the re-introduction of cells into a patient to cure a disease. Adoptive cell therapy has been actively researched with the aim of both suppressing immunopathology (using Tregs) and enhancing immune responses to eradicate disease (using conventional T-cells). Adoptive T-cell therapy is also an active area of cancer research, with the aim of targeting tumours through the re-introduction of killer T-cells that have been genetically modified to express a tumour-specific receptor (Restifo, Dudley and Rosenberg, 2012).

In the context of this project, adoptive therapy describes the re-introduction of a Treg population that has been modified to specifically suppress the inflammation caused by RA. It should be noted that this therapy could be applied to target any chosen autoimmune condition.

1.4.1 Potential of T--regs to Treat Autoimmune diseases

The transfer of a T-cell population depleted of regulatory T-cells into animal models can induce the development of autoimmune diseases. This was demonstrated by Sakaguchi (Sakaguchi *et al.*, 2001) who also observed that the onset of autoimmunity could be reversed by re-introduction of Tregs into the depleted mouse model. Moreover, a rare genetic disorder known as 'IPEX' (Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) which results in an absence of functional Treg cells is typically fatal due to early life onset of severe autoimmune conditions (Ochs, Gambineri and Torgerson, 2007). It is becoming evident that regulatory T-cells have a role in autoimmunity, and there is a potential to study and exploit this cell population in pursuit of a treatment for autoimmune disease.

Advantages of utilising Tregs as a therapeutic are evidenced throughout the literature. Promising results published from studies conducted in 2003 report the introduction of Treg cells establishes a cure of colitis in mouse models (Liu *et al.*, 2003). Inflammatory colitis was induced in mice via infection before mice were treated with an injection of effector T-cells, followed by the introduction of isolated Treg cells. The authors report that mice injected with the Treg cells at early time-points did not develop any indication of colitis, and the conclusions state that Tregs have a clear positive therapeutic effect in the treatment of the condition (Liu *et al.*, 2003). The transfer of regulatory T-cells into lymphopenic mice exhibiting Collagen-Induced Arthritis (CIA) has resulted in a significant decrease in both the

progression of the disease and levels of inflammatory proteins, with long term reduction in inflammation (Morgan *et al.*, 2005). The adoptively transferred Tregs were observed localised in the joints, indicating the ability of Tregs to localise and control inflamed areas. CIA is an autoimmune disease that has similar mechanisms and symptoms to RA, and subsequently the study of CIA in mice has previously been considered a representative model for studying RA in humans.

In 2011 the first human clinical trial utilising adoptive Treg therapy to prevent Graft-Versus-Host-Disease (GvHD) was published (Di Ianni *et al.*, 2011). Adoptive Treg transfer produced promising results, evidenced by Treg treatment that been demonstrated to prevent GvHD. This is a condition that results when T-cells present in a donor bone marrow (BM) transplant recognise the recipienT-cells as foreign, inciting a damaging response. The Treg population was isolated and purified before being re-introduced into participating patients, and after a 12 month follow up 46.1% of the patients who had been indicated to have a high chance of re-lapse prior to treatment were reported to be disease free. The paper concludes that the adoptive Treg therapy prevented GVHD and enhanced patient recovery (Di Ianni *et al.*, 2011). However, it is unlikely that a similar treatment involving the transference of a large number of polyclonal T-cells is likely to produce a successful treatment in autoimmune conditions.

The transfer of polyclonal Tregs has the potential to suppress and prevent disease progression, however this has been achieved only using a large number of Tregs and is observed only at Treg: Teffector ratios of 0.5:1 or 1:1 *in vitro* (Tang *et al.*, 2004). This low efficiency could be due to the low percentage of Tregs specific for the target antigen present within the polyclonal population, as determined by the T-cell Receptor (TCR) expressed by each Treg. The use of Tregs expressing TCR's specific for disease related peptides is therefore important for the appropriate activation of Tregs at a targeted suppression site. Tang *et al* compared the effect of polyclonal Tregs and Ag-specific Treg treatment on disease onset and progression in diabetic and non-diabetic mice. Tregs were isolated from TCR transgenic mice, and the suppressive capabilities of the Ag-specific Tregs tested against an expanded polyclonal population. Ag-specific Tregs suppressed effector T-cells with significantly higher efficiency compared to polyclonal Tregs when tested *in vivo*, with only a small number of cells required to exert a suppressive response which could be observed at ratios of 1:9 Treg: Teffector. In contrast polyclonal Tregs were required at ratios of 1:1 for an effect to be seen, requiring a large number of cells (Tang *et al.*, 2004). Furthermore the high ratios of Treg: Teffector cells that would be required for an observable effect is not representative of physiological levels, highlighting the inefficiency of polyclonal Treg transfer. In contrast Ag-

specific Tregs have been shown to exert an effect with 100-fold higher efficiency, and only small numbers of cells would be required for adoptive transfer (Tang *et al.*, 2004).

1.4.2 Generation of Ag-Specific T-reg Populations

The isolation and generation of populations of Ag-specific Tregs presents a challenge. As previously mentioned, the specificity of Treg cells is determined by the expressed TCR, and therefore the generation of a population of Tregs with uniform specificity for a targeted antigen would require precision isolation and expansion of particular Treg clones. Research investigating the use of Ag-specific Tregs in animal models have utilised genetically altered animals that express a single TCR specific for an identified antigen, known as TCR-transgenic, as a reliable source of Ag-specific Tregs. However, there is no equivalent method for the generation of human Ag-specific Treg cells.

For the realistic advancement of Treg adoptive therapy it is necessary to explore the generation of sufficient populations of Ag-specific Tregs. A retroviral gene transfer method has been used to transduce a specific TCR to a purified population of polyclonal Tregs (Wright *et al.*, 2009). The phenotype and function of the transduced Tregs was tested 3 days after the transduction protocol, and flow cytometry showed that approximately 60% of the Treg population was expressing the transduced TCR, and was therefore Ag-specific. Tests of the functional capacity of the receptor demonstrated increased suppression of effector cells in the presence of the target antigen, indicating the activation of the Ag-specific TCR. These promising results were further investigated in an *in vivo* setting. The stability of the adoptively transferred transduced Tregs was demonstrated using a partially lymphopenic mouse model, from which blood samples were taken after 2 and 4 weeks. Further analysis showed the introduced Tregs maintained their typical characteristics. To investigate the ability of the Transduced Tregs to actively demonstrate Ag-specific suppression *in vivo* an antigen-induced arthritis mice model was used, in which T-cell mediated tissue damage was induced. It was shown that transduced Tregs have the ability to supress damage and decrease inflammation when the target peptide was present in the same localised area, with swelling in the joint significantly reduced compared to negative controls. The results therefore provide compelling evidence for the efficacy of Ag-specific Treg adoptive therapy. Additionally the transduced Treg suppression of the effector cells was shown to occur even when the effector cells hold different antigen specificity, presenting clear evidence of utility of linked suppression in controlling autoimmune inflammation without targeting the pathology causing antigen. As previously discussed, this is an important characteristic of Tregs that allows for targeted suppression of inflammation caused by unknown antigens.

Brusko *et al* isolated and expanded a human polyclonal Treg population *in vitro*, before a specific TCR was introduced into the population using retroviral gene transfer method (Brusko *et al.*, 2010). The expression of the introduced TCR on the polyclonal T-cell population was tested using flow cytometry. The transduced Tregs were selectively enriched by using antigen specific activation of the newly introduced TCR, resulting in a population of Tregs with 66.1% that express the same TCR and were therefore antigen specific. Ag-specific Tregs generated in this manner demonstrated Ag-dependent suppressive function. The *In vivo* capabilities of the generated population was investigated through adoptive transfer into transgenic mice, and in comparison to polyclonal Tregs the transduced Ag-specific Treg population demonstrated much higher efficiency, requiring a lower number of cells for notable effects. Overall the method was able to generate a sufficient number of functional Ag-specific Tregs from low peripheral blood volumes, and in a time sufficient manner.

The generation of an Ag-specific Treg population needs to be optimized for human Treg cells, which will first be required to be isolated and expanded from peripheral blood. The generation of a pure Treg population presents a challenge due to the small population size combined with the difficulty in defining specific cell markers to target the correcT-cells. Furthermore, contaminating effector T-cells still present during the expansion of a Treg population has the potential to outgrow the Treg population and generate a significant contaminating and potentially pro-inflammatory T-cell population (Koenen *et al.*, 2008). Particular subtypes of Tregs have been observed to exhibit phenotypic instability, with the down-regulation of FoxP3 noted. The down regulation of FoxP3 has been directly correlated with a loss in regulatory function, but also been linked to a resulting switch to become Th17 cells. Th17 cells are a pro-inflammatory population and have been associated with pathogenic activity (Koenen *et al.*, 2008). It is therefore imperative to develop a method that allows the isolation of a Treg subset that confers both functionality and stability. Hoffman *et al* identified that a subset of Tregs, $CD4^{+}CD25^{+}CD45RA^{+}$, can be expanded in vitro to produce a homogenous Treg cell line that is ideal for use in adoptive therapy. CD45RA+ cells have been shown to maintain high suppressive capability upon stimulation to differentiate into mature Tregs and proliferate *in vitro*, and have demonstrated the ability to maintain FoxP3 expression after expansion (Hoffmann *et al.*, 2017).

1.4.3 Tregs in Rheumatoid Arthritis

An important consideration for Treg adoptive therapy is the involvement of Tregs in the initial onset and perpetuation of autoimmunity. Conflicting research has been published; a

review of the role of Tregs published in 2011 collated research investigating the number and function of peripheral Tregs in a number of autoimmune disorders including RA. For each condition investigated, the review located results that indicated a combination of increased, decreased and normal peripheral Treg numbers, with the function of cells being inconsistently found to be either normal or defective. This particular review paper referenced 17 papers regarding RA, with 6 papers stating a decreased number of peripheral Tregs, 3 suggesting increased numbers, and the remaining 8 stating normal peripheral Treg numbers. The studies date from 2004 – 2009, it is likely that the earlier studies will produce less reliable conclusions as a result of using less specific cell markers to identify the Treg population throughout the research (Miyara *et al.*, 2011). However some research has utilised more specific cell markers, and included FoxP3 purity checks alongside cytokine analysis to confirm that FoxP3 population studied is in fact Tregs and not recently activated T-cons with upregulated FoxP3. Considering results taken from such studies the evidence suggests that the peripheral blood of RA patients contains normal numbers of Treg cells. However, unpublished research from our lab has suggested that the ratio of Ag-experienced Tregs (CD45RO) in comparison to naïve Tregs (CD45RA) is different in the peripheral blood of RA patients compared to controls. The finding of the altered ratio of naïve/adaptive Tregs in the peripheral blood of RA patients therefore has implications for the advancement for adoptive Treg therapy in the treatment of RA. Recent research investigated the state of Tregs from the peripheral blood of chronic RA patients through analysis of the phenotype, cytokine expression, suppression abilities and gene expression profiles of the CD4+CD25+CD127dim CD45RO and CD45RA subsets. After analysing results from 43 RA samples, compared to 42 healthy controls, no statistically significant differences were observed (Walter *et al.*, 2016). However, while not observed in all patient samples, some CD45RO Tregs from RA patients were noted to have an impaired ability to supress certain inflammatory cytokines.

Despite the observed normal numbers of Tregs in the periphery, it will be important to understand the nature of any potential defects in the Treg subsets and how these might be corrected. There is conflicting information concerning the functional capacity of Tregs in patients with RA. Early research reported no defects in the function of Tregs, based on the observation that Tregs (CD4+CD25+) isolated from the RA patients demonstrated the suppression of Tcons *in vitro*. However, it has since been noted that Tregs from RA patients demonstrate an inability to suppress inflammatory cytokines typically produced from conventional T-cells. Ehrenstein *et al* (Ehrenstein *et al.*, 2004) analysed the functional capacity of Tregs isolated from 27 RA patients, the results indicated that the Tregs isolated

from RA patients failed to inhibit the production of inflammatory cytokines in comparison to the healthy controls which demonstrated successful suppression. Flores-Borja (Flores-borja *et al.*, 2008) investigated potential defects in CTLA-4 in Tregs isolated from the peripheral blood of RA patients, and noted significantly reduced expression of CTLA-4 on the cells compared to healthy controls. This research therefore linked the inability to suppress inflammatory cytokines with a defect in the accumulation of CTLA-4 on the surface of Tregs. The TCR associated signalling of the RA Treg population was reduced, however function could be restored by treating the cells with a reagent known to induce surface accumulation of CTLA-4. The conclusion was drawn that CLTA-4 abnormalities in Tregs may underlie issues with suppression by Tregs in RA. CTLA-4 is considered an important mechanism in contact-dependent mediated suppression. Adoptive Treg therapy will rely on Ag-specific direct contact dependent interaction with APCS, which allow the bystander suppression of effector cells also in contact with the APC, and negate the need to identify a specific antigenic target. It is therefore an important to consider the potential defects that could be encountered in the isolated cells that will be involved in this contact. This emphasises the necessity to design a robust test of the functional capacity of isolated and modified cells, but also highlights the potential for future research to further investigate and potential correct defects as part of the adoptive therapy for RA.

1.5 Project Overview

The combined characteristics of Tregs provide compelling insight into the potential of a Treg Adoptive Therapy to suppress RA induced inflammation without general suppression of the immune system. The overall aim would be the re-establishment of normal immune tolerance and cure of the disease. The crucial next step is the translation of Treg adoptive therapy research into a human setting, and the successful demonstration of the generation of an Ag-specific human Treg population that could be re-directed to target autoimmune damage. The *in vivo* demonstration of linked suppression in the mouse models encourages the hypothesis that: Ag-specific human Tregs, that have been designed to specifically target a disease-associated Ag, could successfully suppress inflammation and damage. Unpublished research has begun to investigate the best methods and targets to achieve this in the context of RA.

First a suitable TCR for transduction into Tregs has to be identified. The TCR must recognize a relevant Ag presented by an MHC class II molecule on an antigen-presenting cell. Unpublished work from my host lab has utilized the TCR MS2.3C8-TCR to demonstrate the therapeutic potential of Tregs in RA treatment as it is specific for a protein presented by HLA-DR4 which is known to be the largest genetic factor influencing RA development, and is identified in a large number of patients (Hammer *et al.*, 1995). Ag-specific targeting of a relevant RA-associated peptide will therefore allow the activation of Tregs at sites of RA induced pathology.

Aims and Objectives

Aim of the Research: To generate functional Ag-dependent suppressive human Tregs using TCR gene transfer.

Research Hypothesis: Human Tregs isolated from peripheral blood can be genetically modified to express a chosen TCR, thereby generating a functional Ag-specific Treg population.

Objectives:

1. Set-up and optimise protocols for the retroviral transduction process.
2. Set-up and optimise protocols to isolate and sort Tregs from human peripheral blood.
3. Establish robust protocols for the successful TCR transduction of Tregs.
4. Demonstrate the functional capacity of Ag-dependent Treg mediated cell suppression through the use of suppression assays.

Continuation of the research would then focus on applying the validated protocols to RA patient blood samples. This will require the isolation of CD45RA+ Tregs from peripheral blood taken from RA patient samples to be used in the transduction procedure.

Chapter 2 – Materials and Methods

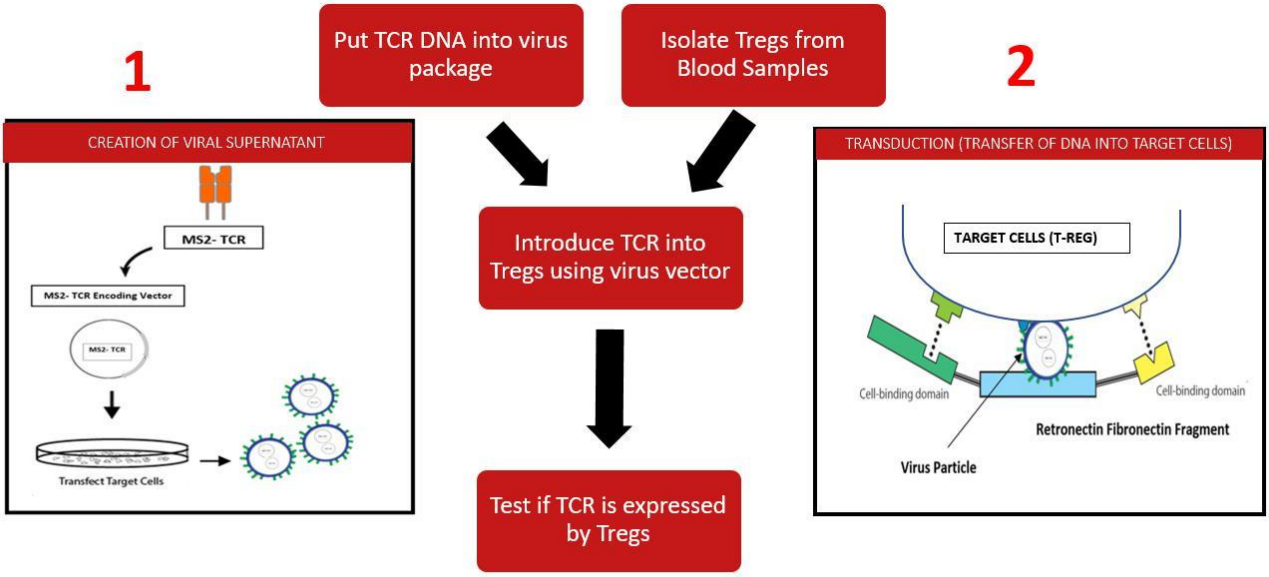

Figure 2: Work-flow diagram of the overall processes required for eventual TCR transduction. The viral supernatant used as a tool to transfer the TCR DNA into the target cells must first be generated, and this process is depicted in 1. Similarly the target cells must be isolated from fresh blood. 2 demonstrates how the target cells and generated virus are brought together in the transduction process using the retronectin fragment to facilitate DNA transfer.

2. 1 Ethics and Sample Population

Ethics forms for working with human samples were submitted and approved by the Ethics boards at Edinburgh Napier University. Blood samples from healthy controls were taken from volunteers by a trained phlebotomist and each participant was fully informed of the study requirements and asked to sign a form confirming voluntary participation. Participants included both males and females, of the age range 20-30 years.

2.2 Retroviral Vectors and DNA preparation

2.2.1 Transformation and Purification.

The TCR DNA used in the final transduction procedure had to be transformed using a high efficiency transformation protocol, utilising NEB-5-alpha Competent *E.coli* cells. The transformation was performed by following the protocol provided by NEB (New England Biolabs, UK). NEB 5-alpha Competent *E.coli* cells were stored at -80°C until use. The *E.coli* cells were thawed on ice prior to being used in the protocol. The DNA to be transformed was diluted with nuclease free water in sterile conditions down to an appropriate

concentration for the procedure, using the recommended DNA concentration of 100ng of plasmid DNA.

Briefly, 5µL of the DNA plasmid was added to and mixed through gentle pipetting with 50µL of E.coli cells before the cell-plasmid mixture were stored on ice for 30 minutes. A sample containing cells only was included as negative control. Samples were then heat shocked for exactly 30 seconds at 42°C before being incubated for a further 5 minutes on ice. 700µL of SOC (super optimal broth) media was added to each transformation tube and the samples were incubated with 250rpm shaking for 60 minutes. At the end of the incubation cells were plated on prepared LB agar plates by spreading the cells across one half of the plate and sequentially spotting the cells down the corresponding half of the plate to create a dilution. The plates were incubated overnight at 37 °C , after which a starter colony from each plate was inoculated in 5mL of LB broth and further incubated for 8 hours before being purified.

After the transformation process the plasmids were purified using the Qiagen plasmid Purification kit, and following the provided protocol (Qiagen, 2012). The kit contains Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC and Qiagen-Tip 100. In addition the kit provides RNase A and Lysate Blue which were added to Buffer P1, after which Buffer P1 was stored in the fridge as per recommendations. The rest of the kit was stored at room temperature.

500µL of each starter colony was diluted in 500mLs of LB broth and centrifuged at 6000g for 15 minutes at 4°C. After centrifugation bacterial pellets were re-suspended in 4mL of Buffer P1 (with added RNAse A and Lysate Blue). 4mL of Buffer 2 was added and the solution vigorously mixed and incubated at room temperature for 5 minutes. 4mL of Buffer P3 was then added, and again the solution mixed by inverting before being incubated on ice for 15 minutes. Samples were centrifuged at 20,000xg for 30 minutes at 4°C and the supernatant removed before samples were centrifuged again at 20,000xg for 15 minutes to ensure the removal of particulate material, and the plasmid containing supernatant carefully collected.

The Qiagen-100 tip was prepared by pipetting 4mLs of the provided QBT buffer through the column, which was allowed to empty by gravity flow. A further 2x10mLs of the Buffer QC was additionally added before the plasmid containing supernatant was applied to the Qiagen 100-tip. The DNA was then eluted through the addition of 5mLs of Buffer QF, before the addition of 3.5mL isopropanol. Samples were then centrifuged for 60 minutes, using a rotatory arm centrifuge at 5000g. The supernatant was removed and the DNA pellet re-suspended in 70% ethanol before another centrifuge at 5000g (rotatory arm) for 60 minutes.

Under sterile conditions, the supernatant was carefully removed and pellets allowed to air dry before being suspended in sterile water and analysed on the nanodrop for the purity and concentration.

Due to the limitations of the laboratory equipment, the centrifuge suggested in the Qiagen protocol was altered from the recommended 30 minutes at 15,000g, and instead samples were centrifuged for 60 minutes, at 5000g using a rotatory arm centrifuge.

2.2.2 Analysis of DNA

2.2.3.1 Gel Analysis

DNA samples were run on a 1% agarose gel (0.5 agarose, 50mL TAE buffer), with 2.5µL of Safe-view (NBS Biologics LTD). TAE buffer was composed of 48.4g Tris Base, 11.4mL glacial acetic acid and 3.7g EDTA. Samples were run in duplicate at two different concentrations: 100ng/5µL and 300ng/5µL. 3µL of loading dye was added to all samples, which were run alongside a 1 Kb DNA ladder (Bioline Hyperladder).

The gels were run at 120 volts for approximately 30 minutes before being removed from the gel tank.

2.2.3.2 Nanodrop

After the initial calibration of the Nanodrop (Thermofisher, Rockford IL, USA2000) with sterile nuclease free water, 1µL of each DNA sample was run. For DNA samples the purity is indicated by the 260/280 ratio, which was taken as acceptable if within the range of 1.8-1.9.

2.3 Cell Culture

2.3.1 Cell Culture Media

The growth medium used for each cell type is shown in **table 2.1:**

Table 2.1: Components of Media used for Cell Culture

Cell Line	**Medium**	**Supplemented**
Phoenix-Ampho	IMDM (Thermofisher)	L-Glutamine, 100Units/mL Penicillin, 100µg/mL Streptomycin, FBS
Jurkat	RPMI 1640 (Thermofisher)	L-Glutamine, 100Units/mL Penicillin, 100µg/mL Streptomycin, FBS

ThP1	RPMI (Thermofisher)	L-Glutamine, 100Units/mL Penicillin, 100µg/mL Streptomycin, FBS
Primary T-cells	RPMI OR TexMACs (Thermofisher)	L-Glutamine, 100Units/mL Penicillin, 100µg/mL Streptomycin, AB Serum (TexMACS)

2.3.2 PBMC Cell Isolation

Peripheral Blood Monocytes (PBMCs) were isolated from blood samples using Lymphoprep (Axis Shield, Biodundee, StemCell Technologies, 2017), following the protocol supplied. Blood samples were diluted at a 1:1 ratio with sterile PBS and cells isolated within an hour of initial blood collection. The Lymphoprep solution was layered with the sample by inserting a strippette to the bottom of the sample and carefully allowing the Lymphoprep solution to flow out underneath the blood sample in the tube, before taking care to remove the strippette without affecting the blood: Lymphoprep layer interface. Samples were centrifuged according to the parameters: 20 minutes, 137 RCF, 880g RPM, 9 Acceleration and 0 Break. Note it was imperative to remove the brake on the centrifuge to avoid disrupting the layers formed by the cells as they migrate through the solution at the end of the centrifuge cycle. After centrifugation the samples separate into three distinct layers according to the differential migration speed of the cell types within the blood sample. Red blood cells aggregate at the bottom of the tube as the base layer and the top layer formed is composed of granulocytes which can migrate through the Lymphoprep due to their density. PBMCs settle at the interface between the plasma and Lymphoprep layer.

Using Pasteur pipettes the PBMCs were carefully removed from the interface layer and transferred into a clean falcon tube, taking care not to disturb the formed layers. The isolated cells were washed in RPMI media before a final 15mintue centrifuge step (400g, temperature 20, and brake normal).

In order to reduce retention of platelets a final centrifuge step was included at the end of the protocol (200g for 15 minutes at 20°C).

2.3.3 EasySep Human CD4+ T-Cell Isolation

PBMCs were CD4 enriched following Stemcell technology, protocol for EasySep T-cell isolation kit (StemCell Technologies, Vancouver, Canada), which depletes non-CD4 cells using a magnetic column. Cells were counted and prepared in EasySep buffer at a concentration of 5x10^7 cells/ML. The enriched cell suspension was transferred into a FACS collection tube for immediate CD25 isolation using the Miltenyi isolation protocol (Miltenyi Biotec, Bergisch, Germany, 2012a).

2.3.4 Miltenyi Protocol for bead isolation of CD4+CD25+CD127dim T-cells.

The isolation kit used was the Miltenyi Biotec CD4+CD25+CD127dim Regulatory T-cells isolation kit (Miltenyi Biotec, Bergisch, Germany, 2012a). PBMCs were counted and suspended at a concentration of 40µL MACS buffer (500mL Sterile PBS, 0.5% Bovine Serum Albumin, 2mM EDTA) per 10^7 cells.

The procedure is completed in two steps. PBMCs were treated with the T-cell Biotin Antibody cocktail II human, which contains a monoclonal anti-human antibodies for CD8, CD19, CD123 and CD127. This acts as the primary labelling agent before the Anti-biotin Microbeads were added as the secondary agent. The microbeads are conjugated to a monoclonal mouse anti-biotin antibody, and therefore conjugates to the biotin antibody acting as the primary labelling agent to the cells. When placed in a magnetic field the labelled cells are depleted from the effluent as the magnetic microbeads conjugated to the labelled cells causes the cells to be retained within the magnetic field of the separation column. The effluent from this step should contain CD4 cells as the Antibody Cocktail contains no antibody against this marker. The first step is therefore referred to as a depletion of non-CD4 cells, resulting in a pre-enriched CD4 cell fraction. This sample is then taken forward to the next separation stage, which involves the magnetic labelling of CD4+CD25+CD127dim T-regulatory cells. The pre-enriched CD4 cell effluent is directly labelled with CD25 Microbeads II Human, which contain microbeads conjugated to a monoclonal anti-CD25 antibody. When placed in the magnetic field the CD4+CD25+ cells are caught in the column due to magnetised beads conjugated to CD25. The effluent should therefore contain CD4+CD25- cells, conventional T-cells, while the retained sample will be composed of the CD4+CD25+CD127dim regulatory T-cells.

Note T-cell Biotin Antibody cocktail II human was added at a concentration of 10µL/10^7 cells. The pre-enriched CD4 cell effluent was re-suspended in MACs buffer at a concentration of 90µL/10^7 total cells. 10µL/10^7 of CD25 Microbeads II was added.

2.3.5 Cell counting and Viability

Cells were counted by looking at T-cells under a microscope using FastRead counting slides (IMMUNE SYSTEMS LTD). The viability of the cells was assessed using 0.1% Trypan Blue viability dye, the uptake of which is blocked by healthy cell membranes, therefore only dead cells incorporate the dye and appear blue under the microscope. The cells were counted immediately after the addition of the Trypan blue dye.

2.4 Retroviral Transduction

2.4.1 Transfection

The transfection process was performed using Fugene Transfection Reagent, and following the optimised protocol provided by Promega (Promega, 2013). The phoenix-ampho packaging cells were counted and plated onto sterile plates at a density of 1.5x10^6 cells in a total volume of 10mLs IMDM media per plate. The transfection protocol was conducted the next day after the cells were allowed to reach approximately 80% confluence. Prior to transfection the media on the cell plates was changed and replaced with 5mLs of IMDM media. The exact concentrations and quantities of reagents used throughout the research is provided in **table 2.2**. The optimum media volume was kept constant throughout all experiments at 250µL and the sterile nuclease free water added to the DNA Eppendorf was adjusted to ensure a final volume of 320µL for every experiment.

Table 2.2: Concentration of the reagents used in transfection experiment

Reagent / DNA	**Concentration**
Fugene	1µg/µL
Plasmid DNA	1.5µg/µL
Vector DNA	2.6µg/µL

10µL of Fugene Reagent was mixed with 250µL of Optimum Media in a sterile Eppendorf. In a separate sterile Eppendorf, appropriate quantities of vector DNA, plasmid DNA and sterile water were mixed through pipetting before the two prepared eppendorfs were combined and allowed to incubate at room temperature for 15 minutes, after which incubation the Fugene-DNA mixture was carefully pipetted drip-wise onto the prepared plated cells.

Plates were incubated at 37°C overnight, and the IMDM media was replaced with RPMI or TexMACS media the following day. Approximately 48 hours after the initial transfection the viral supernatant was harvested by carefully collecting the plate media and using a centrifuge

cycle to remove any remaining packaging cells (5 minutes, R137, 400G, 20°C). Viral Supernatant was stored in -20 in 1mL aliquots until use.

2.4.2 Transduction

The DNA vectors were transfected into packaging cells (Phoenix Ampho cell line (AllelleBiotec, 2012)) to create a viral supernatant containing the genes to be introduced and incorporated into cells. Retronectin coated plates were prepared as described in the Retronectin Product Manual (Clontech).

The required number of wells in a sterile untreated 24 well plate were coated with 1mL of Retronectin. This was incubated at room temperature for 2 hours in sterile conditions, before the retronectin was removed and stored for future use. The Retronectin reagent can be re-used up to 6 times, and the number of times used was recorded at each use. To limit contamination Retronectin was aliquoted into 25mL falcon tubes, and after use was transferred into a clean sterile tube. Immediately after removal of Rectronectin the wells were coated with sterile 2% BSA (filtered) for 30 minutes at room temperature. After the inucabation the BSA was removed, and wells coated in RPMI media to avoid drying out.

1.5mL of prepared viral supernatant was added to each well of the RN coated plate. The plate was centrifuged for 1 hour 45 minutes (R124, RCF 2000, T-30). 0.5mL of target T-cells were added to each well before a final centrifuge for 3 minutes (R124, RCF 300, T-30). The plate was stored in the incubator for 48 hours before analysis.

2.4.3 Bead Activation of Cells

T-cells were activated using Dynabeads T-Cell Expander αCD3/CD28 activation beads and following the provided protocol (Technologies, 2011). Immediately after sorting CD4+CD25+CD127dim and CD4+CD25-CD127high cells were counted and plated in a 6 well plate at a concentration of 0.5x10^6 cells per mL in standard culture medium. Activation beads were added to cells at 1:1 ratio. Cells were incubated for 2 days (37°C, Co^2 5%) before being transduced. Beads were removed after transduction by placing the cell suspension on a magnetic source (Dynal MPC-L) and retaining the supernatant.

2.5 Flow Cytometry Procedures and Analysis

Flow cytometry data was collected using BD Celesta and analysed using FACSDiva software (BD Biosciences) and FlowJo v.9 (FlowJo LLC BD Biosciences). A full 30min clean was performed prior to any analysis, and the calibration conducted using CST beads (BD Biosciences).

2.5.1 Cell surface staining

0.2-0.5x10^6 cells were counted and washed in FACS buffer (500mL PBS, 0.5% BSA, 2mM EDTA) before being re-suspended in FACS tubes with 50µL of FACS buffer. 5µL of target antibody was added to the cell suspension and incubated for 15 minutes at room temperature, protected from light. Cells were washed twice more before either being suspended in 400µL FACs buffer for Flow analysis, or suspended in residual volume by vortexing before intracellular staining. A list of the antibodies used can be found in section 2.5.5.

2.5.2 Intracellular FoxP3 staining

Intracellular FoxP3 staining was completed using the FoxP3 Intracellular Buffer Set (ebiosciences, 00-5523-00), and following the Thermofisher, Rockford IL, USAprotocol for intracellular staining (Thermo Fisher Scientific). The incubation period for intracellular staining was 60 minutes, at room temperature in the dark. After staining the samples were suspended in 400µL FACS buffer for analysis.

2.5.3 FACS sorter Staining

Cells were counted and washed in FACS buffer and supernatant aspirated before cells were re-suspended in the residual volume. 0.5µL of antibody per 1x10^6 cells was added and samples incubated at room temperature for 15 minutes. Cells were washed in FACS buffer and suspended at a concentration of 20x10^6/mL before samples were passed through 40µm cell mesh strainers.

2.5.4 Cell trace Violet staining

The protocol supplied with CellTrace Violet (CTV) Proliferation Kit (Invitrogen, Carlsbard CA, USA) was followed (Invitrogen, 2010). Cells were counted and suspended in PBS, and CTV stock solution was added at a concentration of 1µL/mL of cell solution.

2.5.5 Antibody List

Table 2.3: Antibodies used throughout the research project

Antibody/Flourochrome	**Clone**	**Required concentration**
CD4 FITC		0.25µg / test
CD25 PE	BC96	0.25µg / test
CD127 PE-Cy5	HIL-7R-M2I	0.25µg / test
mTCRβ B711	H57-597	0.25µg / test

FoxP3 CF694	259D/C7	0.25µg / test

2.6 *In Vitro* Cell Assays

2.6.1 T-Reg Inspector

Sorted Tcons and Tregs were counted and re-suspended in appropriate volumes of RPMI media as indicated by the Treg Suppression Inspector protocol (Miltenyi Biotec, Bergisch, Germany,). T-cons were stained with CellTrace Violet. The cells and beads were added to a 96 flat-bottomed plate as described in the protocol. After 5 days the cells were harvested and analysed using flow cytometry.

2.6.2 CD3/CD28 Activation Bead Proliferation Assay

Cells were stained following the CellTrace Violet protocol (Invitrogen, Carlsbard CA, USA, 2010), and activated using the CD3/CD28 Dynabeads (Technologies, 2011). Cells were counted and plated in a 6 well plate at a concentration of 0.5x10^6 cells per mL in standard culture medium. Activation beads were added to cells at 1:1 ratio. One well of cells were harvested and analysed using flow cytometry every 24 hours, for 5 days.

2.6.3 Monoclonal Antibody Suppression Assay

Tcons were stained according to the CellTrace Violet protocol (Invitrogen, Carlsbard CA, USA, 2010)). 1x10^5 Tcons was added to each well of the 96 well plate in a volume of 100µL, as shown in **table 2.4**. Tregs were counted and suspended in the appropriate volume, before being added to the wells to achieve the Tcon:Treg ratios shown below. Monoclonal antibody CD3 (HIT3a ebiosciences. Or OKTαCD3 ebiosciences) and CD28 (CD28.2 ebiosciences) were added at a concentration of 1µg/mL, and the total volume of each well was made up to 200µL with RPMI medium where necessary. The plate was incubated for 5 days in standard incubation conditions before wells were harvested and analysed by flow cytometry.

Table 2.4: 96 well plate set-up for the Treg suppression assay using monoclonal antibodies to activate the T-cells.

Ratio/Conditions (Tcons:Tregs)	**Tcon Number**	**Treg Number**	**Tcon Volume (µL)**	**Treg Volume (µL)**
Unactivated Tons (No antibodies)	1x10^5	-	100	-
Unactivated Tregs (No antibodies)	-	1x10^5	-	100
Tcons alone	1x10^5	-	100	-
Tregs alone	-	1x10^5		100
1:1	1x10^5	1x10^5	100	100
2:1	1x10^5	0.5x10^5	100	50
4:1	1x10^5	0.25x10^5	100	25
8:1	1x10^5	0.125x10^5	100	12.5
Unstained Tcons	1x10^5	-	200	-

Chapter 3 – Results

3.1 Isolated Cells from Healthy Control Samples

3.1.1 Isolation of Tregs using CD4+CD25+CD127- Miltenyi Kit

The T-cells were isolated from PBMCs using a magnetic sorting method, performed using the manual magnetic separation columns. PBMCs were stained with magnetic conjugated antibodies targeted to specific cell markers, the aim of which is to cause the retention of the cells within a magnetic field, allowing unlabelled cells to be washed through. Magnetically labelled cells can then be eluted, allowing recovery of multiple cell types (Miltenyi Biotec, Bergisch, Germany, 2012b).

The procedure was completed in two steps: PBMCs were treated with the T-cell Biotin Antibody cocktail II human, which contains monoclonal anti-human antibodies for CD8, CD19, CD123 and CD127. Depletion of cells with these surface markers removes cytotoxic T-cells (CD8), B cells (CD19), dendritic cells (CD123). The antibody cocktail acts as the primary labelling agent before the Anti-biotin Microbeads were added as the secondary agent. The microbeads are conjugated to a monoclonal mouse anti-biotin antibody, and therefore conjugates to the biotin antibody acting as the primary labelling agent to the cells. When placed in a magnetic field the labelled cells are depleted from the effluent as the magnetic microbeads conjugated to the labelled cells causes the cells to be retained within the magnetic field of the separation column (LD column). The effluent from the LD column should therefore contain CD4 cells as the Antibody Cocktail contains no antibody against this marker. The LD effluent was then taken forward to the next separation stage, which involves the magnetic labelling of CD4+CD25+CD127dim T-regulatory cells. The pre-enriched CD4 cell effluent was directly labelled with CD25 Microbeads II Human, which contain microbeads conjugated to a monoclonal anti-CD25 antibody. When placed in the magnetic field (MS column) the CD4+CD127dimCD25+ cells are caught in the column due to magnetised beads conjugated to CD25.

Samples were taken at each stage of the process, as indicated in **table 3.1**. These samples were stained for surface markers CD4, CD25 and CD127 and analysed by flow cytometry in order to monitor the progress of the cell isolation. The PBMC sample was accepted as the positive control population, and an unstained PBMC sample was included as a control to demonstrate the success of the staining process and to establish gating for flow cytometry.

Table 3.1: Expected cell surface marker levels on isolated cell populations using the Miltenyi Biotec CD4+CD25+ isolation kit.

Sample	Fraction
PBMC	CD4, CD25 and CD127 present
LD Effluent population	CD4 Enriched (CD25 and CD127 present but not enriched)
LD Retained population	Absence of CD4
MS Effluent population	CD4+, CD25-, CD127high
MS Retained population	CD4+, CD25+, CD127dim

CD4 Enrichment

Figure 3.1 shows a comparison of the results obtained from the cell surface staining of the LD effluent (CD4 enriched) and LD retained (CD4 depleted) cell populations. After repeating the depletion of non-CD4 cells, the LD Column of Miltenyi Cell Isolation Kit was observed to consistently fail to efficiently enrich CD4.

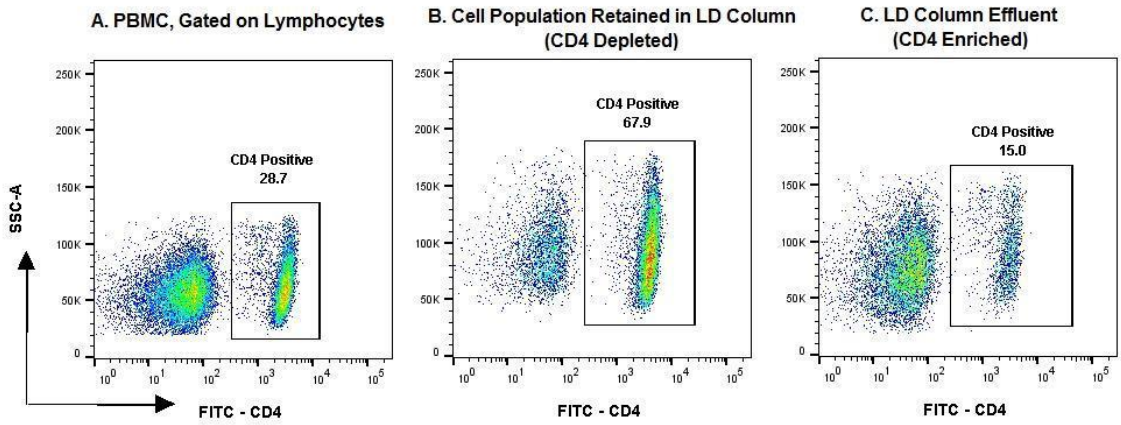

***Figure 3.1: Bead sorting of PBMCs using Miltenyi Biotec CD4+CD25+ isolation kit fails to enrich CD4 portion of isolated PBMC**. Magnetic beads conjugated to a cocktail of antibodies is added to the PBMCs, before the PBMCs are run through a magnetic column intended to retain the non-CD4 cells and allow a CD4 enriched fraction to flow through as the effluent. CD4 FITC stained PBMC fractions were analysed by flow cytometry. (A) Shows the fraction of cells retained within the magnetic column is high in CD4, (B) shows the column effluent is low in CD4, suggesting the isolation kit has failed to enrich CD4 cells. (C) demonstrates the proportion of CD4 positive cells in the fluid that has been eluted from the LD column, and is therefore supposed to represent a CD4 enriched fraction. Plots represent three independent experiments.*

Figure 3.1A shows that 29.9% of the lymphocyte population from the isolated PBMCs are CD4 positive. The expected range is 25-60% (StemCell Technologies, 2017a), and therefore

the percentage of CD4 positive lymphocyte cells across the healthy volunteers used throughout the research was as expected. **Figure 3.1B** demonstrates the percentage of CD4 positive cells within the depleted cell population retained in the LD column after the cell depletion process. The primary labelling of the PBMC's utilised monoclonal anti-human antibodies for CD8, CD19, CD123 and CD127, however no antibody is included to target the CD4 positive cells at this stage, and therefore it is expected that the LD column retained fraction should contain very few CD4 cells. The high percentage of 68.3% positive CD4 cells within this population is therefore an unexpected result, which was witnessed across multiple repeats. The failure of the LD column to deplete the non-CD4 cell population is further emphasised by the low percentage of the CD4 population in the LD column effluent, as shown in figure 2.1C, where only 14.4% of the population stains positive for the CD4 surface stain marker. This population would be expected to be over 90% CD4 positive, as published by Miltenyi Biotec (Miltenyi Biotec, Bergisch, Germany, 2012a). To rule out that the depletion failure was due to the use of a faulty isolation kit the protocol was repeated using two different Miltenyi Biotec CD4+CD25+CD127dim Regulatory T-cells isolation kits simultaneously, and samples taken and stained throughout the procedure as before. **Figure 3.2** shows the comparison of CD4 percentage present in the enriched CD4 effluent and the depleted CD4 population after isolation using the original isolation kit A (lot no.5170317131), and a second isolation kit B (lot no.5170309469).

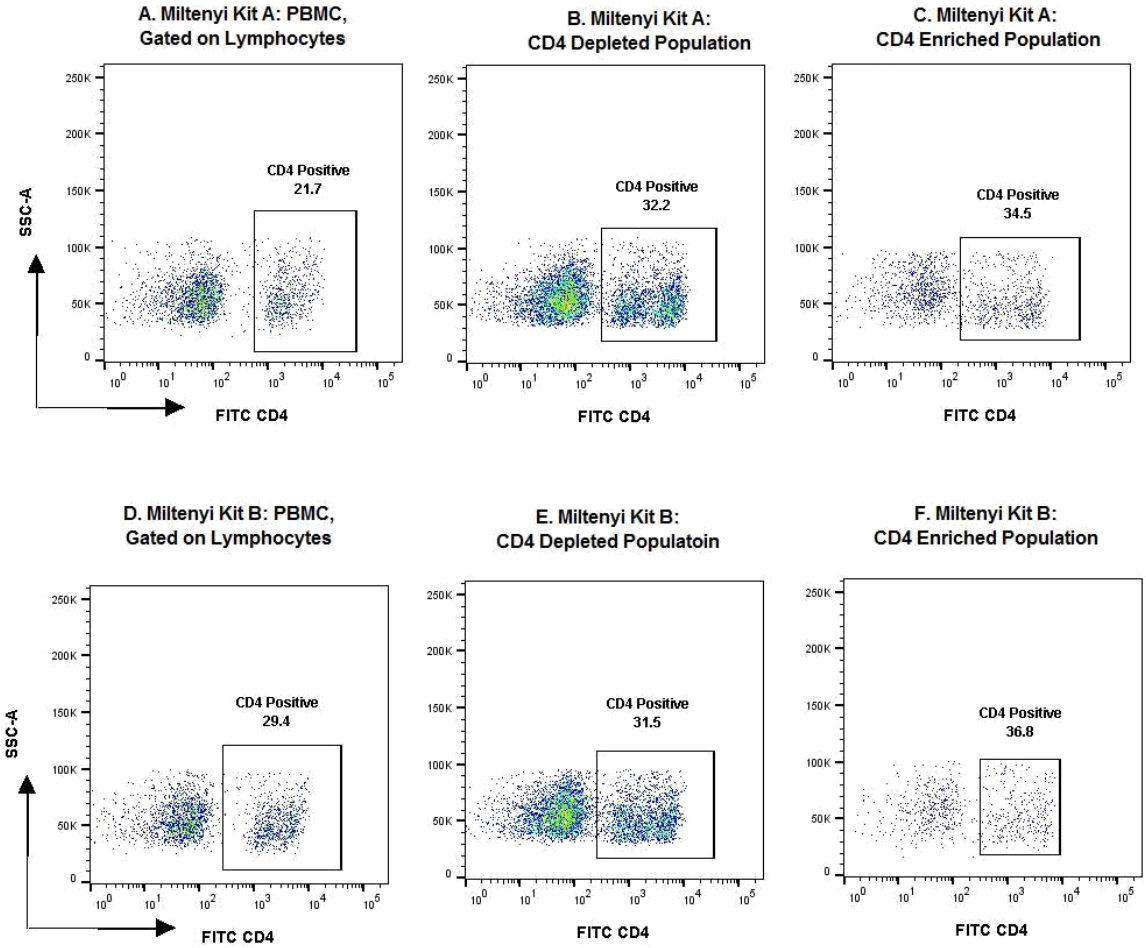

Figure 3.2: Comparison of the ability of two different Miltenyi Biotec CD4+CD25+ cell Isolation Kits shows both do not enrich CD4 cells from isolated PBMC samples. *CD4 FITC stained samples were analysed by flow cytometry. Figures A,B and C show the results of Kit A after staining the PBMC (A) CD4 depleted cells (B) and CD4 enriched population (C). Figures D, E and F show the results of Kit B after staining the PBMC (D), CD4 depleted cells (E) and CD4 enriched population (F). The cell isolation using both kits was done simultaneously. The low percentage of CD4 cells shown in figures (C) and (F) indicate that neither column have enriched the CD4 cell population. Plots represent a single comparison experiment.*

It is clear from the results shown in **figure 3.2** that both Miltenyi isolation kits failed to enrich the CD4 cell population after depletion through the LD column. The decision was made to instead enrich the CD4 cells through the use of Stemcell Technology EasySep CD4 enrichment kit, which works with a similar mechanism to the Miltenyi kit, involving the addition of an antibody cocktail conjugated to magnetic beads causing the retention of non-CD4 cells when the sample is placed in a magnetic field. After the EasySep enrichment, a portion of the sample was stained for CD4 and analysed by flow cytometry, with an unstained portion of cells was used as a negative control, and a stained sample of un-enriched PBMC's included as a positive control. The results are shown in **figure 3.3**:

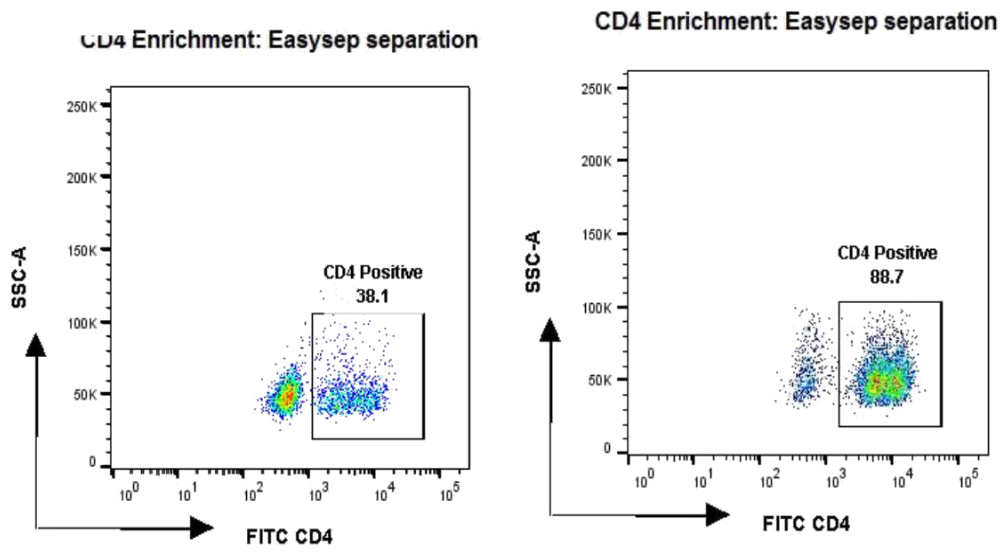

Figure 3.3: CD4 T-cells are enriched from PBMC samples by using the Stemcell EasySep CD4 Bead Enrichment kit. *The EasySep kit depletes non-CD4 cells using an antibody cocktail conjugated to magnetic beads, before PBMCs are run through a magnetic column intended to retain all non-CD4 cells and allow a CD4 enriched fraction to flow through as effluent. CD4 stained PBMC fractions were analysed by flow cytometry. A. shows the flow cytometry result shown after the initial PBMC isolation, and presents the proportion of CD4 cells in this population. B. shows the same population after the CD4 enrichment using the Easysep Kit. Plot represents three independent experiments, after which the fractions were not stained and analysed before use in further experiments.*

The EasySep CD4 enrichment kit consistently produced an enriched cell population containing over 80% CD4 positive cells. This isolation kit was therefore used in place of the Miltenyi kit throughout the research.

Isolation of CD25+CD127dim Cell Population (Tregs)

Figure 3.4 shows the flow cytometry data obtained from MS column effluent and the MS retained cell population. The MS effluent should contain CD4+CD25- cells, conventional T-cells, while the sample retained in the MS column will be composed of the CD4+CD25+CD127dim regulatory T-cells.

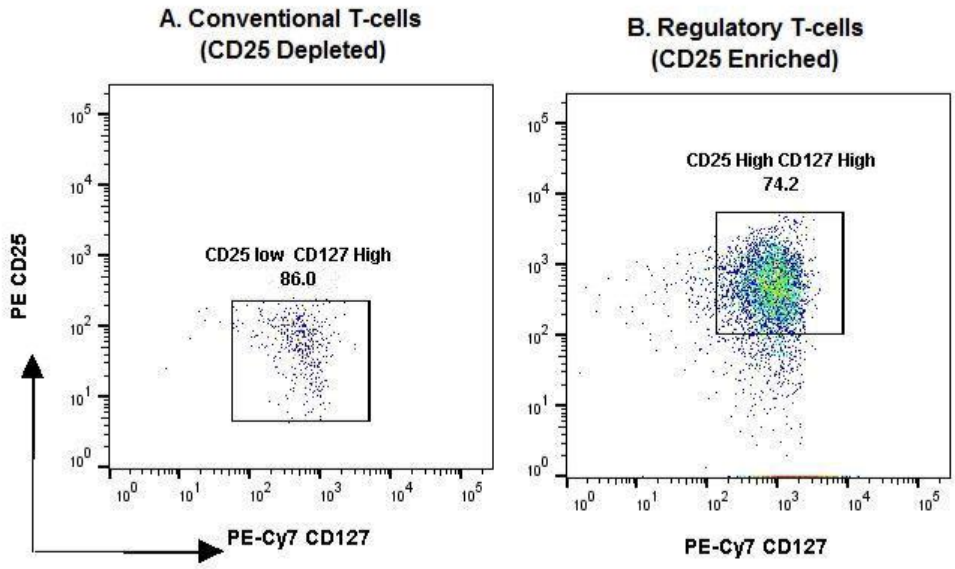

Figure 3.4: Bead sorting of a CD4 enriched cell population using the Miltenyi Biotec, magentic column retains a CD25high CD127dim cell fraction. *Magnetic beads conjugated to CD25 antibodies is added to the enriched CD4 cell fraction before cells are run through a magnetic column intended to retain the CD25high CD127dim (Tregs) cell fraction and allow CD25low CD127high (Tcons) to flow through. (A) Represents the data obtained through flow analysis of the MS column effluent, and therefore the conventional T-cell fraction. (B) Demonstrates the data from flow analysis of the cell fraction retained in the MS column, and should therefore show regulatory T-cells. Samples were stained with CD25 PE, and CD127 PE-Cy7 for analysis by flow cytometry. Plots represent three independent experiments. Cell populations were not stained before use in further experiments.*

The MS column did enrich the CD25 population in the retained cell population, as shown by figure 3.4. However, utilising the EasySep CD4 enrichment kit instead of the Miltenyi LD depletion means that there is no depletion of CD127high cells and therefore this leads to the presence of a population of CD25, CD127high cells present in the MS retained population. Furthermore, the poor efficiency of the CD25 enrichment led to a small population of CD25 positive cells within the MS effluent population.

After the CD25+ and CD25low populations were isolated the samples were stained for FoxP3 to indicate the purity of the Treg population. An isolated Treg population would be expected to contain over 90% FoxP3 positive cells, while the Tcon population would be expected to

demonstrate a lower percentage. The expression of FoxP3 within the Tcon population can still be expected, either due to the upregulation of FoxP3 which can occur after Tcon activation, or due to anomalies within the FoxP3 staining process, which requires the permeabilisation and fixation of cells. **Figure 3.5** shows the FoxP3 purities achieved after cell isolation using the MS columns:

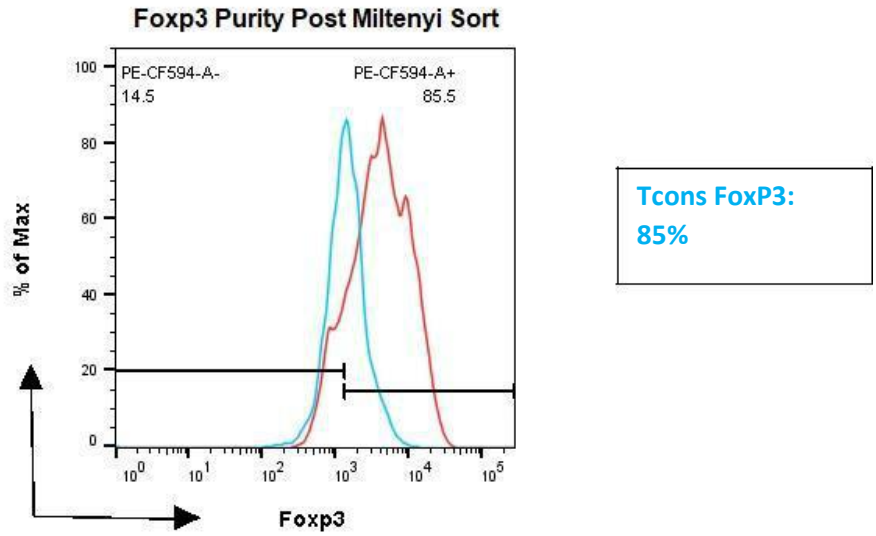

Figure 3.5: Treg and Tcon cells isolated from PBMCs demonstrate high expression of FoxP3, which is used to indicate the purity of the Treg population. *Purity of the isolated populations was determined by staining cell populations for internal marker FoxP3. FoxP3 PE-cyf596 stained cells were used for flow cytometry analysis. The analysis indicates that the Treg population are expressing high levels of FoxP3. Plots represent three independent experiments.*

The percentage of FoxP3 within the isolated Treg cell population was consistently observed over 80%. However, the high FoxP3 levels in the Tcon fraction is unexpected and introduces uncertainty as to the exact cell types in each fraction. To ensure the optimum purity of the Treg population the decision was made to sort the enriched CD4 population into CD25+CD127dim Tregs and CD25-CD127high Tcons through the use of Fluorescence Activated Cell Sorting (FACS) on the FACS Aria II.

3.1.2 Isolation of Tregs using FACS sorting

After the CD4 enrichment of the PBMC's using the EasySep method, samples were sorted into Tregs (CD25+CD127dim) and Tcons (CD25-CD127high) by gating on CD4 positive cells, and then gating on the two populations to be sorted, as shown in **figure 3.6**.

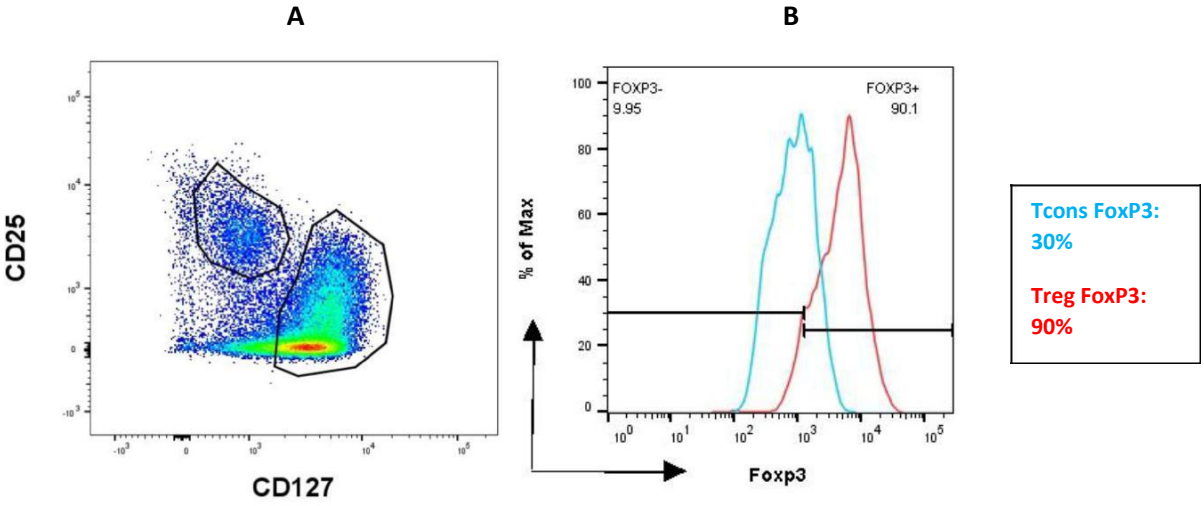

Figure 3.6: FACS sorting using FACS Aria II and gating on CD4+CD25+CD127dim, and CD4+CD25lowCD127high isolates two distinct populations. FoxP3 staining of the cell fractions distinguishes the populations as Tregs and Tcons. *(A) shows the FACs sorting of CD4 enriched cells into Tregs (CD25highCD127dim) and Tcons (CD25lowCD127high), using CD25 PE and CD127 PE-Cy7 to identify the cell population by flow cytometry analysis. (B) A post sort purity stain of FoxP3 levels was completed using FoxP3 PE-C7956 for flow cytometry analysis, and indicates the high levels of FoxP3 present in the isolated Treg population. Plots represent three independent experiments.*

After the sort the samples were stained for FoxP3 to indicate the purity of the samples, the results are shown in **figure 3.6B**. The high FoxP3 levels achieved for the sorted CD25+CD127dim cell population indicates the high purity of the sample and indicates that the sorted population is Tregs. It can be seen that the purity of the Tcon population is higher than expected, and this anomaly was consistently observed. It is possible that the FoxP3 intracellular staining procedure results in a high degree of non-specific binding – this could be confirmed by using a FoxP3 isotype stain as a control, however this was not included in the experiment. It must also be considered that Tcons have the potential to upregulate FoxP3 after activation, however this is unlikely to be the cause of the increased FoxP3 percentage in this particular experiment as the cells have not been stimulated at the time of staining. Both sorted cell populations were taken forward to the transduction experiments.

3.2 Optimisation and Troubleshooting of Transduction

3.2.1 Transduction Jurkats: Positive Control Validation

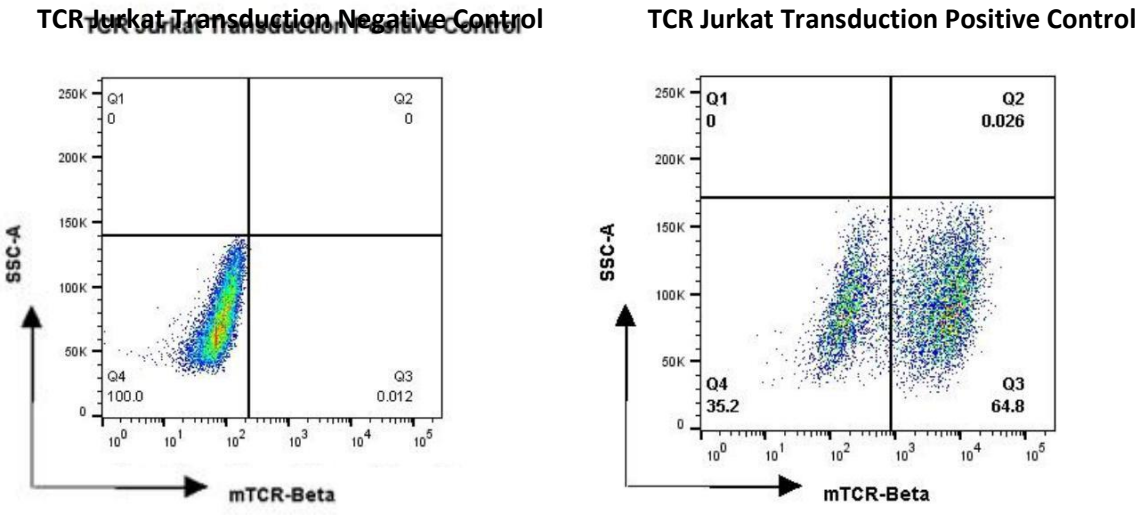

Figure 3.7: JurkaT-cells transduced with mTCRβ demonstrate the expression of the TCR on 64.8% of the population. *JurkaT-cells were transduced with TCR specific for MBP and restricted to MHC class II. Transduced cells were stained for mTCRβ- chain expression with PE mTCR. Plot represent twelve independent experiments.*

JurkaT-cells were used as a positive control to indicate the success of the transduction process. The transduction of JurkaT-cells using Retronectin was validated by clontech labs, who indicated an expected efficiency of 80%. After achieving consistent high transduction efficiencies **(figure 3.7),** a Jurkat transduction was included in every transduction experiment.

The transduction of JurkaT-cells provided an indication of issues with the experimental process. Over time the transduction efficiency dropped until no transduction was achieved **(figure 3.8).** Transduction is a multi-factorial process relying on the success of multiple experiments to produce results. Therefore, in the case of reduced transduction efficiency or the failure of transduction it is difficult to isolate the cause of the issue within the entire transduction process. Furthermore the transduction efficiency does not indicate whether the issues arises with the initial transfection experiment, or the retroviral transduction process itself. The reduction in transduction efficiency over time, as seen in **figure 3.8**, led to a series of investigative experiments to determine any issues within the transfection and transduction process. Steps were taken to first investigate the transfection efficiency, and then further correlate the transfection efficiency to transduction efficiency through the inclusion of a GFP marker that could be detected by flow cytometry.

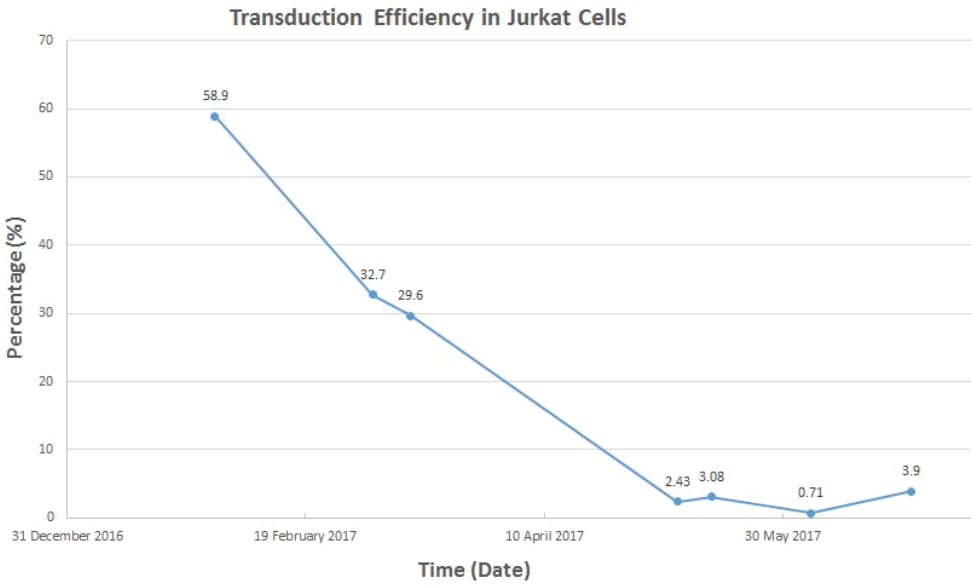

Figure 3.8: The transduction efficiency of TCR transduction into JurkaT-cells over time, as determined by flow cytometry.

After troubleshooting the transfection and transduction processes the consistent transduction efficiency at the expected 80% was restored, as seen in **figure 3.7**.

3.2.2 Transfection efficiency

Green Fluorescence Protein (GFP) is a reporter gene, and the expression of the protein is easily tracked and monitored. GFP was incorporated into the transfection to test the efficiency of the process. GFP was used in place of plasmid DNA at a concentration of 2.6µL, following the transfection protocol. Upon successful transfection the PA cells incorporate and express GFP, and the fluorescence of this can be detected using Flow Cytometry. After the two days incubation, PA cells were scraped from the plates and suspended in FACS buffer before being analysed on FITC channel. The percentage of cells expressing GFP is taken as the percentage efficiency of the transfection process.

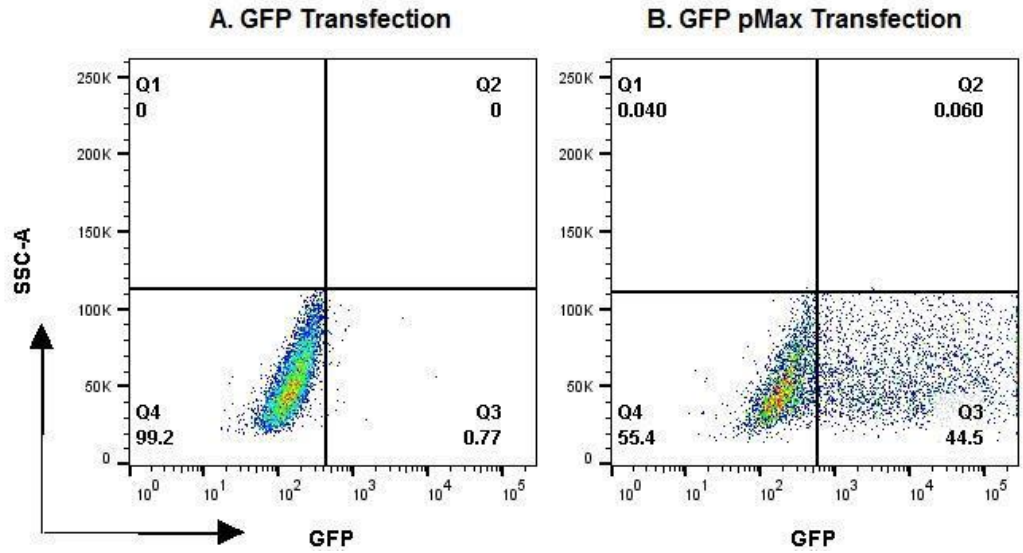

Figure 3.9: The transfection efficiency as determined by the expression of GFP protein in phoenix ampho cells after the transfection process indicates an issue with the transfection process. *The expression of GFP is seen on using the FITC channel. A. Shows the GFP transfection using the GFP vector, B shows the GFP expression in the ph-ampho cells after transfection using GFP pmax control (Bio.lonza). Plots represent a single experiment.*

Initially GFP generated by plasmid purification in the lab was used. Unfortunately, as shown by **figure 3.9A** the initial results produced low or absent fluorescence, indicating the absence of GFP uptake in the PA cells, and the failure of the transfection procedure. The quality of DNA used in the transfection process is documented to be a contributing factor to the success of the process, and so to eliminate this variable the transfection efficiency was re-tested using control pMax GFP DNA (Bio.Lonza). **Figure 3.9B** shows the results of the GFP pMax Transfection; the low efficiency suggest that the DNA quality was not the only factor influencing the transfection efficiency. In light of this an experiment was designed to test other factors known to affect the transfection efficiency: quality of the PA cells and the transfection reagent (Fugene). Four different transfections were completed simultaneously

with varying conditions, as shown in **table 3.2**. Controls were included in the form of unstained and un-transfected PA cells.

Table 3.2: Experimental conditions for transfection troubleshooting and optimisation experiments.

Experiment ONE	Conditions	Experiment TWO	Conditions
1A	Ph-Ampho cells A (Date defrosted: 03/04/2017) Fugene Reagent A (lot 12572700)	2A	DNA Vector A (transformed 1/03/2017) Fugene A
1B	Ph-Ampho cells A Fugene Reagent B (lot 000174624)	2B	DNA Vector A Fugene B
1C	Ph-Ampho cells B (Date defrosted:15/05/2015) Fugene Reagent A	2C	DNA Vector B (transformed 19/04/17) Fugene A
1D	Ph-Ampho cells B Fugene Reagent B	2D	DNA Vector B Fugene B

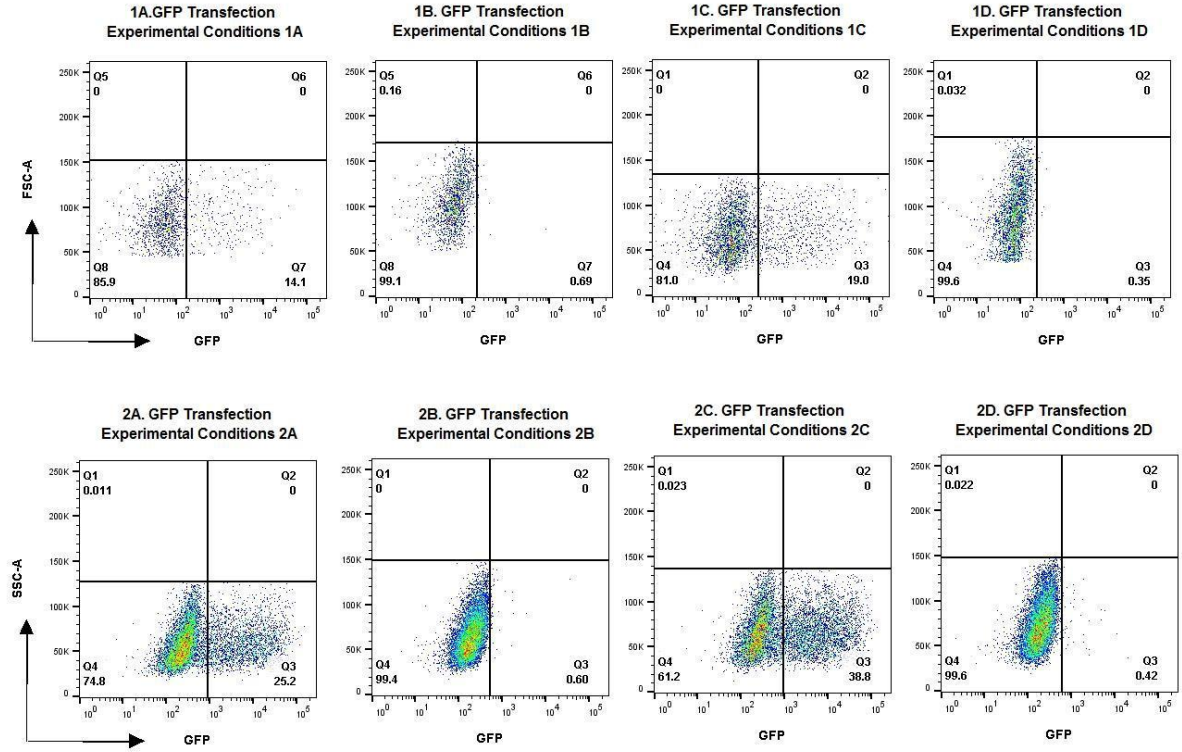

Figure 3.10: Transfection of Phoenix-Ampho cells with GFP under varying experimental conditions suggests that the transfection reagent and cell health affects the transfection efficiency. *Experiments 1A-D were designed to test the transfection reagent with reagent A used in 1A and 1C, and reagent B used in 1B and 1D. Absence of expression of GFP in 1B and 1D suggests the failure of the Fugene transfection reagent. Experiment 2 shows a repeat experiment using new PA cells to increase the efficiency. The absence of expression of GFP in 2B and 2D further suggests the poor working of the Fugene reagent. The expression of GFP was analysed using the FITC channel. Plots represent a single experiment.*

Figure 3.10 shows the transfection efficiencies achieved for all transfections in experiments one and two with the various conditions. The absence of any transfection in experiment **1B** and **1D** indicates that the transfection reagent (B) is responsible for the failure of the transfection experiment. The drop in transfection efficiency between conditions **1A** and **1C** suggest that the health of the PA cells is also affecting the final efficiency.

The transfection efficiency achieved for conditions **1A** and **1C** is still significantly lower than expected, and therefore experiment two was designed to confirm the effect of the transfection reagent, and further test the effects that the potential DNA degradation of the vector DNA may have on the final transfection. As expected the conditions using Fugene transfection reagent B achieved no evidence of transfection (**2B** and **2D**)**. Figure 2A** and **2C** demonstrated increased transfection efficiency compared to the results from experiment one, with the best results shown by condition **2C**.

While optimising the transfection an experiment was conducted using a GFP-FoxP3 vector construct. This is a retroviral vector, comparable to the TCR vector used in the transduction experiment, and therefore can be used to create a viral supernatant which can be further used to complete a transduction experiment. The efficiency of the transduction can then be correlated to the efficiency of the transfection. This means that a GFP transfection can be conducted alongside future transfection experiments as a positive control that provides an indication as the final success of the transduction experiment. **Figure 3.11** shows the correlation between the transfection and transduction efficiencies:

Table 3.3: Conditions for a transfection experiment to test the effects of changing the DNA and the Fugene reagent on the final transfection efficiency.

Experiment Three (GFP-FoxP3)	**Conditions**
3A	DNA Vector A Pcl Ampho A Fugene A
3B	DNA Vector A Pcl Ampho A Fugene B
3C	DNA Vector B Pcl Ampho B Fugene A
3D	DNA Vector B Plc Ampho B Fugene B

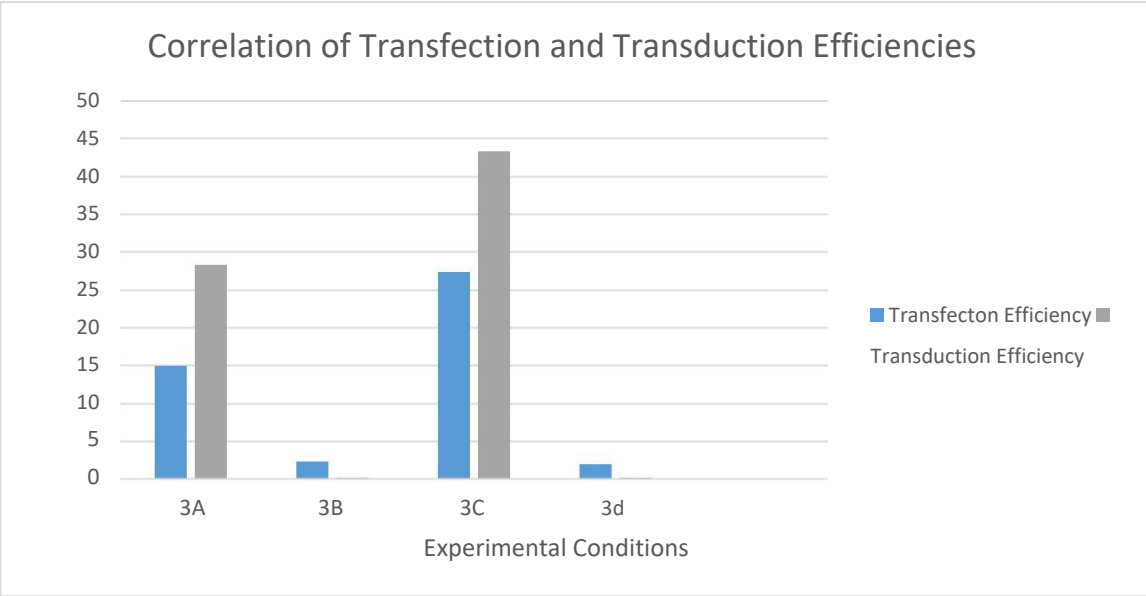

Figure 3.11: Comparison and correlation of the transfection efficiencies to the transduction efficiencies using the viral supernatant generated with transfection of GFPFoxP3 vector suggests that the transfection efficiency can predict the transduction efficiency. .

A lower transfection efficiency of is expected when using the GFP-FoxP3 construct owing to the increased size of the DNA vector. It is thought that an increased vector size results in a reduced rate of intracellular movement, allowing increased time for DNA plasmid degradation and subsequently reduced transfection efficiency (Lukacs *et al.*, 2000)(McLenachan *et al.*, 2013). However the experiment indicates how a failed transfection relates to a failed transduction, as seen in conditions 3B and 3D. The results suggest a correlation between transfection and transduction, suggesting that the transduction efficiency can be estimated from the transfection efficiency.

After testing the variables affecting the transfection, the highest achieved efficiency was 52% (2C). Whilst 52% efficiency is lower compared to the transduction efficiency achieved with standard TCR vectors, 52% is a much higher efficiency than has previously been achieved with this vector. These conditions were therefore continued moving onwards: PA cells A were frozen in liquid nitrogen to create stocks which could be used throughout the rest of the project in order to optimise results. Furthermore, pcl-ampho B DNA was used as the vector DNA. The factor with the biggest influence was determined to be the transfection reagent, and therefore after the optimisation experiments a new transfection reagent (Fugene C, lot 0000245256) was ordered. After optimising the conditions of the transfection process, the transduction efficiency returned to over 70%, as shown in **figure 3.7.**

3.3 Transduction of Isolated Cells form Healthy Control Samples

3.3.1 Activation of Cells

In order for the retroviral transduction to be successful, it is important for the target population of cells to be actively dividing. Further to this, Tregs are a small population and it is necessary to first expand the cells after isolation. Prior to the transduction process cells were first activated through the use of CD3/CD28 Dynabeads and the addition of IL-2. The use of Dynabeads to activate T-cells has a number of advantages over the use of feeder cells or APCs, which can be difficult to sufficiently maintain and regulate. Published research has shown the beads can expand T-cells in combination with IL-2, and have been demonstrated to efficiently expand Tregs with maintained suppressive characteristics.

To ensure the sufficient cell activation cell numbers were tracked over a course of two weeks. The average expansion rate was determined to be 2.8x10^4 cells/day across a ten day period. This was conducted only once prior to the first transduction experiment to confirm the expansion of the cells, however due to the small numbers of the Treg population the decision was made not to reduce cell numbers by continuous counting during future experiments.

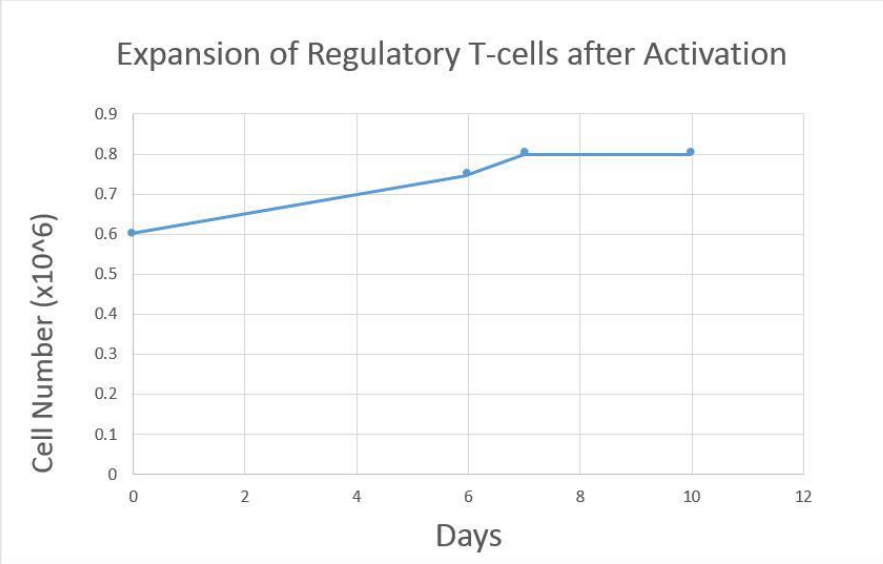

Figure 3.12: Treg cell numbers increase after in vitro activation with αCD3 and IL-2. *Cells were counted on days one, six, seven and ten before being used in experiments.*

The proliferation of Tcons cultured alone and activated with CD3/CD28 beads was tracked by staining the Tcons with CellTrace Violet (CTV) and analysing one well of activated cells a day over a time course of 5 days. CTV is a fluorescent dye excited by violet wavelength range emissions, and can be used demonstrate proliferation of a cell population based on the premise that a cell division event will equally divide the fluorescent dye between daughter cells, simultaneously demonstrating a half in detected fluorescence and a double in cell number (Lyons and Doherty, 2004). The use of CTV has been reported to be sensitive to detect up 8 cell divisions (Quah and Parish, 2012). **Figure 3.13** shows the proliferation of Tcons after activation with the CD3/CD28 Dynabeads.

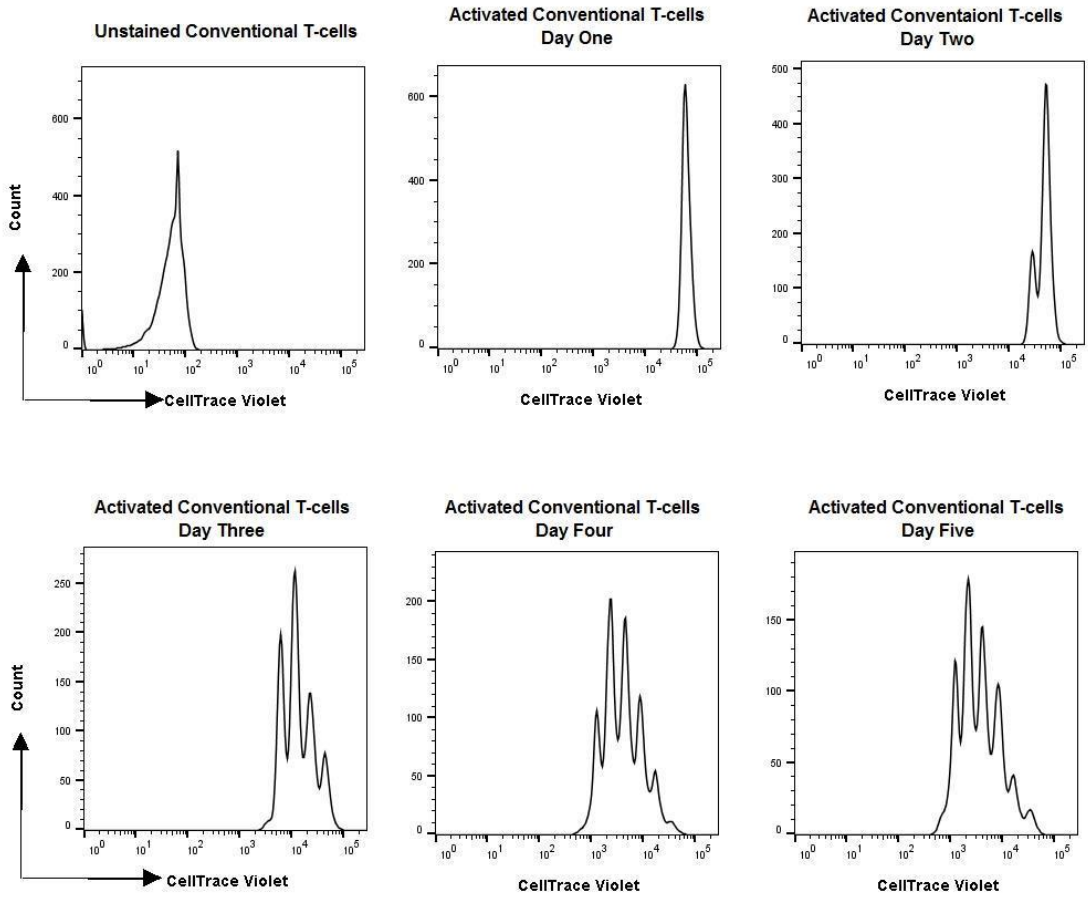

Figure 3.13: Proliferation Assay of Conventional T-cells indicates the successful activation and proliferation of T-cells*. T-cells were stained with CellTrace Violet and activated using αCD3/CD28 beads. The proliferation of the cells was analysed using flow cytometry every day for a 5 day incubation period. Plots represent three independent experiments.*

3.3.2 Transduction of Cells

After the optimisation of the transduction process, the following results were obtained for the transduction of mTCRβ into the sorted Tcons and regulatory T-cells.

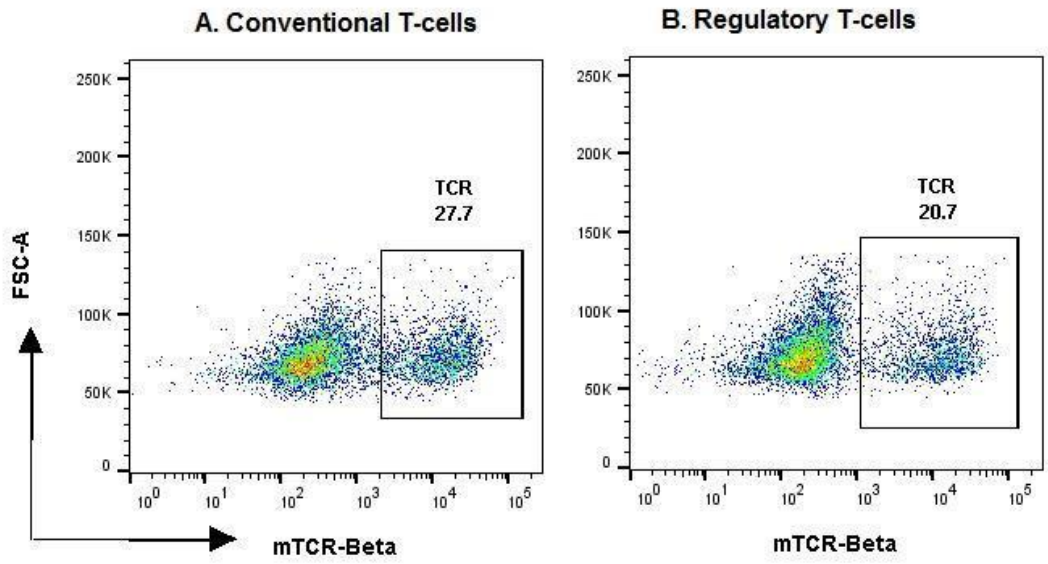

Figure 3.14: TCR expression on conventional T-cells and regulatory T-cells indicates the success of the TCR transduction process. *Cells were transduced with TCR specific for MBP and restricted to MHC class II. Transduced cells were stained for mTCRβ- chain expression and analysed by flow cytometry. Plots represent three independent experiments.*

Over 20% of the cell populations were consistently transduced. It was observed that the Tcons transduced with a higher efficiency in comparison to the Treg subset. As the samples were transduced at the same time using the same viral supernatant and stained with identical conditions, it is possible that the increased transduction efficiency observed within the Tcons is due to a more efficient activation of the cells, which would subsequently allow for increased proliferation and increased uptake and expression of the transduced DNA. After transduction the cells were analysed for FoxP3 in order to ascertain if the purity of the cell population is affected by the transduction process. **Figure 3.15** shows the purity of the sorted cells was maintained after the transduction of the TCR.

The purity of the T-cell population was seen to increase after the transduction procedure; this observation could be the result of a number of factors. One variable could be the effectiveness of the intracellular staining procedure, as during the initial FoxP3 staining the cell were permeabilised overnight, but during the post transduction staining the cells were permeabilised for only 30 minutes. A second variable is the health of the cells - post-sort the

cells were transported on ice prior to the FoxP3 staining, while post transduction the cells were allowed to incubate in optimum conditions. Furthermore the purity of the sorted cells was assessed using un-activated cells, while the post-transduction purity stain was conducted 5 days after the activation of the cells using CD3/CD28 Dynabeads. The activation of the cells is likely to increase the levels of FoxP3 as the cells increase in size and proliferate.

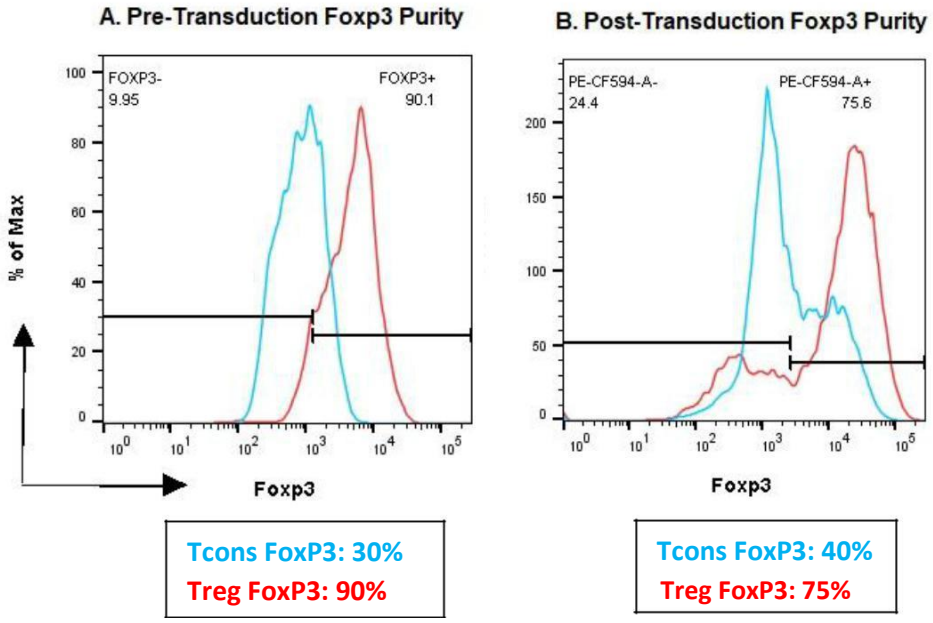

Figure 3.15: Isolated Treg and Tcon cells stained for FoxP3 to indicates the purity of the populations is maintained after the transduction process. *Cell fractions were stained with FoxP3 PE-Cf694 to be analysed by flow cytometry. (A) Shows the FoxP3 expression of cells before the transduction process, (B) shows the FoxP3 expression of cells after the TCR transduction. Plots represent three independent experiments.*

3.4 Suppression Assays

3.4.1 Suppression Assay: Treg Inspector

To determine the functional suppression of effector T-cells by Tregs the proliferation of the effector T-cells can be measured after co-culture with varying ratios of Tregs. Sorted Tcons were stained with CTV and co-cultured with titrated numbers of sorted Tregs (unstained). The cells were activated using the Miltenyi Treg Inspector kit, which has been optimised for use in Treg suppression assays. CD2, CD3 and CD28 biotinylated antibodies are loaded onto anti-biotin particles which activate Tcons while the Tregs remain un-activated. When co-cultured in the presence of Treg Inspector Particles any reduction in Tcon proliferation is due to the suppressive capacity of the Tregs. However, as shown in **figure 3.16** the Tcons cultured alone with the Treg inspector beads failed to show evidence of proliferation. Consequently, it is impossible to determine if the Tregs exerted any suppressive capabilities despite the Tcon:Treg co-culture at a one:one ratio demonstrating little evidence of proliferation.

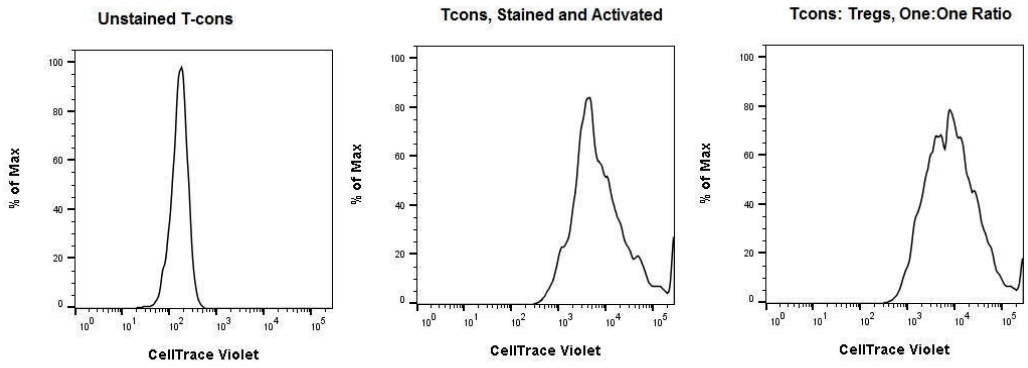

Figure 3.16: Suppression assay set up using Treg Inspector Beads shows no evidence of cell activation. *Tcons cells were stained with CellTrace Violet and co-cultured with Tregs at various ratios for 5 days in standard incubation conditions. The figure shows that after 5 days there was no evidence of Tcon proliferation in the Tcons activated and cultured alone, suggesting the cells have not been appropriately activated. Plots represent three independent*

The experiments absence of. Tcon activation using the Treg Inspector kit was unexpected; the consistency of the result lead to the hypothesis that the particular Treg Inspector Kit used in the experiments was not fully functional. The decision was made to conduct a suppression assay using an alternative approach validated in the lab using monoclonal antibodies.

3 .4.2 Suppression Assay: Monoclonal Antibodies

The decision was made to design an assay for activation using monoclonal antibodies (αCD3 (HITa) and αCD28). Sorted Tcons were stained with CTV and co-cultured with titrated

amounts of Tregs in a 96 well plate, and antibodies added at a concentration of 1µg/mL. Unactivated cells (Treg and Tcon) and unstained Tcons were included as controls. After 5 days incubation the samples were analysed by flow cytometry, the results are shown in **figure 3.17**:

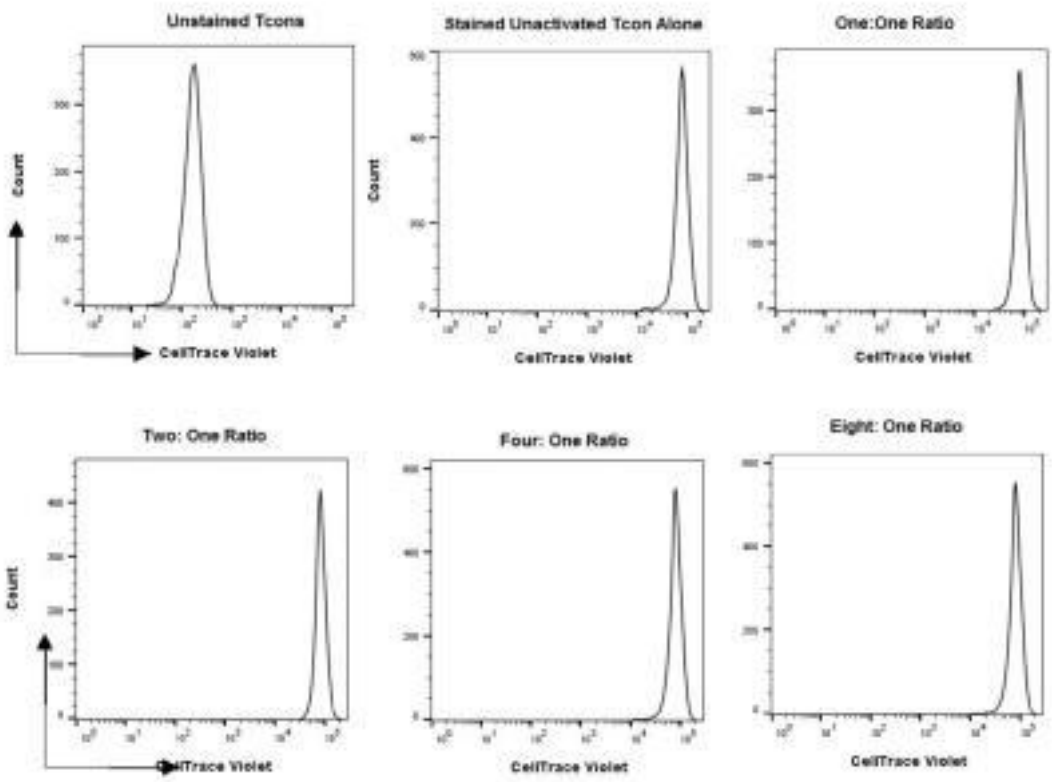

Figure 3.17: Suppression assay of conventional T-cells activated with monoclonal antibodies αCD3 and αCD28 and co-cultured with Treg cells at various ratios shows no evidence of cell proliferation. *Cells were stained with CellTrace Violet and co-cultured with Tregs in standard culture conditions for 5 days. No cell proliferation is seen in any condition. Plots represent two independent experiments.*

The absence of proliferation seen in Tcons when activated and cultured alone suggest the cell population has not been sufficiently activated. The absence of proliferation could be the result of a number of variables: the health of the cells, improper staining, unexplained cell death, or failure of the monoclonal antibodies to stimulate cell activity. As this was an unexpected result, the cell health was not examined prior to the assay and no viability dye was included. The inclusion of an unstained control indicates it is unlikely to be an issue with the staining. As both the assays conducted were performed on sorted cells directly after being held for a number of hours in an ice box for travelling purposes, it is possible that cell health has adversely affected the ability of the cells to proliferate. However it would be unlikely to completely halt the proliferative processes in the way that was observed across the repeats of the experiment.

3.4.3 Troubleshooting and Optimisation of Suppression Assays

An experiment was set up to compare the efficacy of the monoclonal antibodies ability to activate Tcons with the CD3/CD28 Dynabeads. The CD3/CD28 bead activation was included as a positive control, as **figure 3.13** demonstrates the ability of the beads to produce a proliferative response. The ability of the beads and antibody's to activate cells in a PBMC sample compared to a CD4 enriched sample was further tested and over a 5 day period cells were analysed for evidence of proliferation. A 24 well plate was set up with the following conditions:

Table 3.4: Conditions of the 24well plate used for troubleshooting suprresion assay's.

PBMC Activated by CD3/CD28 Dynabeads	5 Wells
PBMC Activated by Monoclonal AB	5 Wells
CD4 enriched cells Activated by CD3/CD28 Beads	5 wells
CD4 enriched Activated by Monoclonal AB	5 wells
Unactivated PBMC	1 Well (control)
Unactivated CD4 enriched	1 Well (control)
Unstained PBMC	1 Well (control)
Unstained CD4 enriched	1 Well (control)

The CD3/CD28 provided efficient activation with clear proliferation seen in both the PBMC and CD4 samples, as shown in **figure 3.18.**

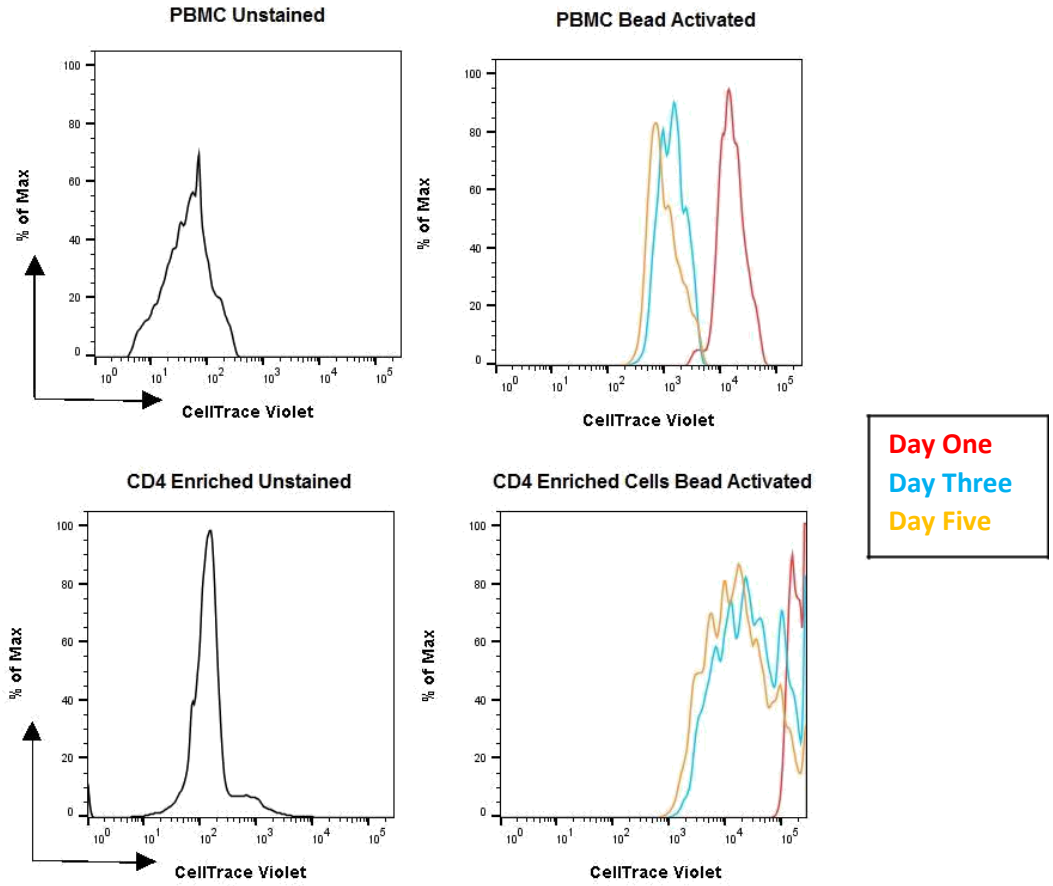

Figure 3.18: Proliferation assay of PBMC and CD4 enriched cells indicates that both cell fractions can be activated and induced to proliferate using αCD3/αCD28 beads. *Cells were stained with CellTrace Violet before being cultured in standard conditions. Both PBMC and CD4 enriched cells demonstrate proliferation across five days of culture. Plots represent a single experiment.*

The monoclonal antibodies produced clear proliferation in the PBMC sample, however there was little evidence of proliferation in the CD4 enriched sample. This is highlighted further by comparing the unactivated CD4 enriched sample to the CD4 enriched sample analysed 5 days post activation (Figure 3.19). The proliferation of the PBMC sample after activation with the monoclonal antibodies indicates that the antibodies are functional, and capable of activating the cells. It may be hypothesised that the absence of proliferation in the CD4 enriched samples is due to the low affinity signalling of the antibodies, which may be incapable of providing a strong enough activation signal.

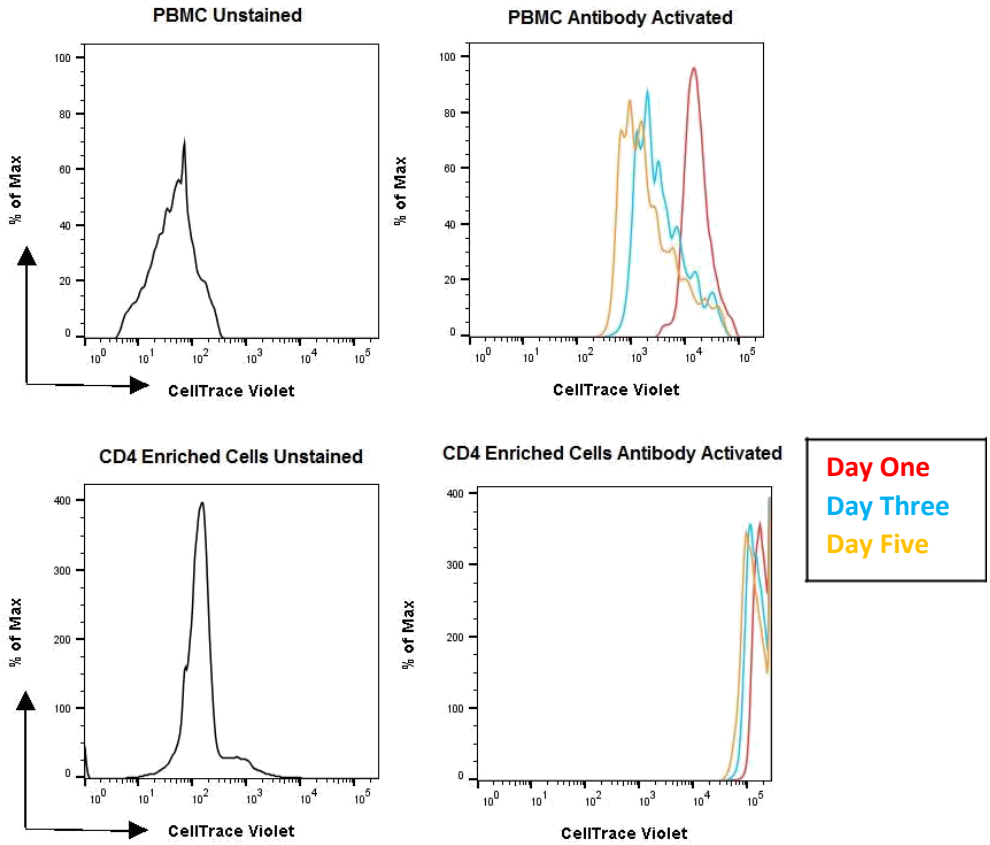

Figure 3.19: Proliferation assay of PBMC and CD4 enriched cells shows that only the PBMC fraction is stimulated by αCD3/αCD28 monoclonal antibodies. *Cells were stained with CellTrace Violet prior to culture in standard conditions. PBMC demonstrate proliferation across five days but there is little evidence of proliferation of the CD4 enriched cells. Plots represent a single experiment.*

The particular antibodies used were chosen specifically for the low affinity signalling in order to avoid over-stimulation of the Tcon which would both reduce the efficiency of suppression, and more accurately represent the physiological levels of activation. In PBMC samples the activation signal would be strengthened by the presence of monocytes which would cluster the antibodies allowing greater interaction with the T-cells. To further investigate this hypothesis the experiment was repeated utilising monoclonal antibodies with a documented stronger activation signal. **Figure 3.20** presents the results of the assay set up with the OKT3 antibodies, which were chosen for this purpose.

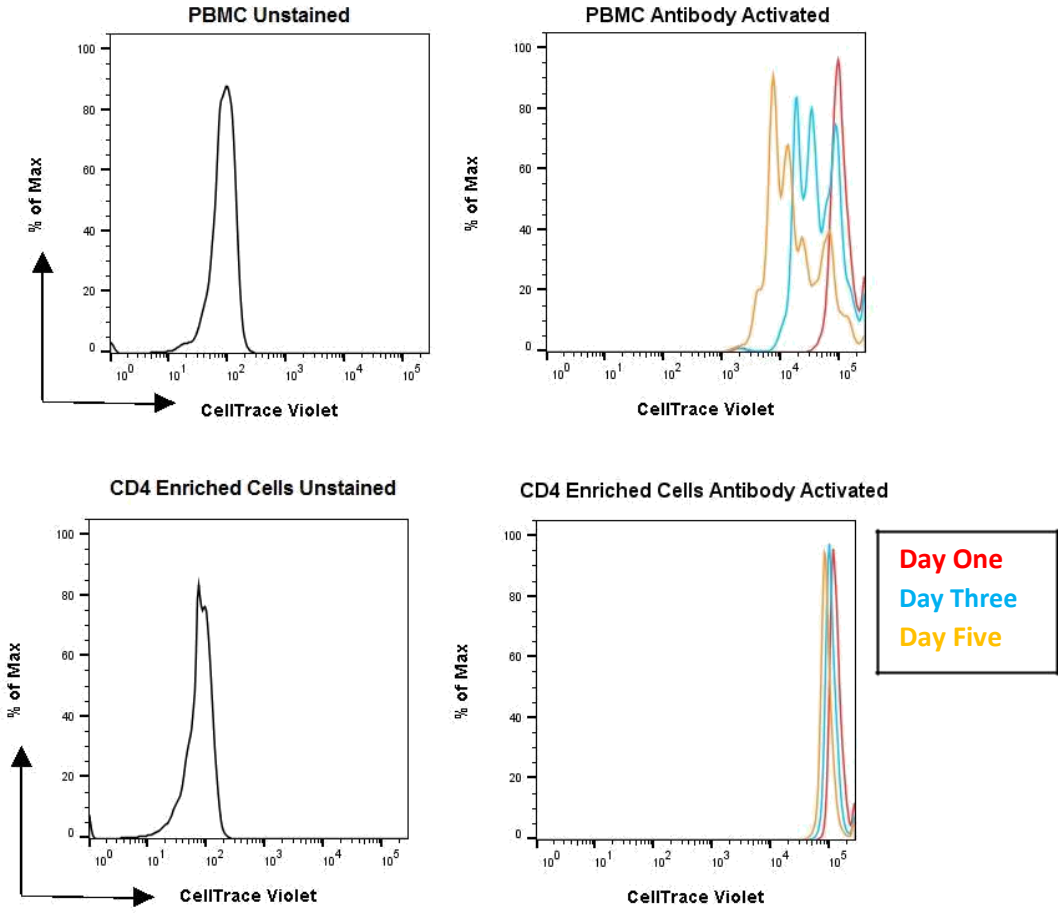

Figure 3.20: Proliferation assay of PBMC and CD4 enriched cells shows that only the PBMC fraction is stimulated by OKT3/ αCD28 monoclonal antibodies. *Cells were stained with CellTrace Violet prior to culture in standard conditions. PBMC demonstrate proliferation across five days but there is little evidence of proliferation of the CD4 enriched cells. Plots represent a single experiment.*

Unfortunately, as can be seen from **figure 3.20**, the OKT3 antibodies did not appear to provide strong enough stimulation to activate the CD4 enriched cells. The activation of the PBMC's seen under the same experimental conditions indicates that the antibodies are functional, while the activation of CD4 cells isolated from the same PBMC sample with the CD3/CD28 coated dynabeads suggests that the population is capable of proliferating. It would be ideal to repeat this experiment with the monoclonal antibodies pre-plated onto the assay plate; activation of the CD4 enriched cells under these conditions would indicate of the lack of stimulation is caused by the low signalling in the absence of clustering factors, such as monocytes, which allow for greater numbers of antibodies to simultaneously interact with the T-cells.

3.5 Ag-specific Suppression Assays Preparations

Antigen specific suppression assays require the co-culture of effector cells with titrated concentrations of Treg cells in the presence of a peptide loaded antigen presenting cell. For the activation of modified T-reg cells by the targeted specific antigen, and the subsequent suppression of localised effector cells through a contact dependent mechanism, it is essential that the APC can present the peptide to the Treg population while simultaneously interacting with the effector cell population. It is necessary for the APC to contain the appropriate HLA type. To perform *in vitro* assay's designed to test the antigen specific activation of modified Tregs an appropriate APC cell line can be chosen and transduced with the HLA-DR receptor before being loaded with the peptide and added to the suppression assay.

The appropriate cell line has to be identified for the vector to be transduced into. Previous work has successfully transduced the HLA-DR-0401 vector into T2 cells. T2 cells are a human lymphocyte cell line that has been mutated to not express most HLA types, including HLA DR and therefore the modified cells can be isolated through the use of anti-HLA DR antibodies. The vector expressing T2 population was sorted using magnetic columns using bead conjugated anti-HLA DR antibodies. The T2 cell line Flow cytometry using PE conjugated to HLA-DR shows the T2 population expressing HLA-DR-0401.

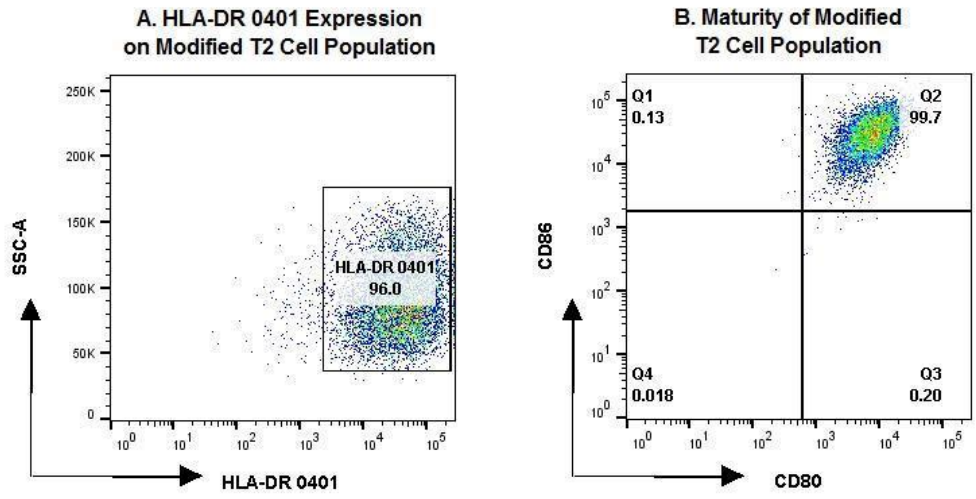

Figure 3.21: A Transduction of T2 cells with HLA-DR 0401 demonstrates high expression of HLA-DR*. B. T2 cells stained for CD80 and CD86 demonstrates high levels of both surface markers indicating maturity of the cell line. Plots represent a single experiment.*

This demonstrates the generation of an HLA-DR-0401 expressing APC population. The modified T2 cells were further stained for the expression of CD80 and CD86 to determine the maturity of the cell population. CD80/86 are essential protein receptors that provide the co-

stimulatory signal for T-cell activation through direct interaction with CD28. High expression of these surface proteins indicates the increasing maturity of the APC. Upon APC interaction with Tregs CD80/86 surface proteins are downregulated through interaction with CTLA-4, thereby blocking activation signals to the effector cell population. Mature APC's demonstrate strong activation of Tcons, as the increased expression of CD80/86 on the cell surfaces reduces the competition for the ligands and subsequently increasing levels of Tcon activation signals are received. An ideal APC for use in suppression assays would therefore exhibit a reduced level of CD80/CD86 expression equivalent to immature or unactivated APCs. **Figure 3.21B** shows the CD80/86 expression on the modified T2 cells in comparison to the expression with a PBMC population. As can be seen, the T2 cells express high levels of CD80/86, indicative of a mature APC population with strong Tcon activation capabilities. In unpublished work a suppression assay utilising modified T2 cells failed to show any suppression by the Treg cells, and it was hypothesised that this was due to the high maturity of the T2 cell line. Further work is required to identify an appropriate cell line with lower maturity levels. This is critical to achieving the designed robust antigen-specific suppression assay.

Chapter 4 - Discussion

4.1 TCR Expression in Transduced T-cells

The results show the successful transduction of the chosen TCR into isolated human T-cells, with an expression of 19.3-20.7% in Treg cells, and 17.5 - 27.7% in Tcons. Using the same retroviral transduction method that was used in this project, Wright *et al* (Wright *et al.*, 2009) transduced a TCR receptor into isolated murine T-cells, with 56.3% in Treg, and 61.8% in Tcon. However in unpublished work from the lab working on human RA derived Tregs, there has already been success in introducing a TCR with up to 37.9% surface expression. Brusko *et al* (Brusko *et al.*, 2010) generated human Ag-specific Tregs through the transduction of a HLA-A*0201 TCR into human cells, with achieved TCR expression of 33.5% in Tregs and 16.8% in Tcons. While these results are comparable to the transduction efficiencies achieved in this particular project, it was observed that in contrast to the results published by Brusko *et al*, the transduction of the Tcons was observed to be consistently higher compared to the Tregs. The retrovirus used during the transduction process in this research requires cells to be actively dividing for gene transfer to occur (Miller, Adam and Miller, 1990). Therefore the cells first had to be activated prior to transduction – the increased efficiency seen in the Tcons was hypothesised to be correlated to more efficient activation of the Tcon population, which allows for increased proliferation of cells and results in greater uptake and incorporation of the retroviral vector containing the TCR DNA. It has been noted by published research that alterations to the retroviral titre in an attempt to increase the efficiency of the transduction process does not appear to significantly increase the resulting TCR expression, and therefore this was not attempted in this research (Brusko *et al.*, 2010).

In order for Ag-specific therapy to stand the best chance of success, the maximum TCR expression is important. Therefore after a successful transduction the modified T-cell populations can be further selectively expanded. A method for doing this is described by Maus *et al* (Maus *et al.*, 2002) who developed artificial APCs which can be used in culture to specifically expand antigen specific populations. Brusko *et al* (Brusko *et al.*, 2010) utilised this methodology to expand the transduced Treg population by co-culturing the modified Tregs with an artificial APC and the TCR specific peptide, allowing the activation and proliferation of the TCR-transduced Tregs. The relative expansion of the modified populations was dependent on the initial transduction efficiency. Using this method Brusko *et al* observed a >5200-fold expansion of the TCR-transduced Tregs, increasing the percentage of modified Tregs in the population to 66.1%. It would be advantageous to similarly expand the modified

Tregs in this research, which would require the design of an artificial APC restricted to HLA-*0401.

Prior to attempting TCR transduction, Ahmadi *et al* (Ahmadi *et al.*, 2011) codon optimised the gene expression of the chosen TCR, and further modified the structure of the TCR through the addition of di-sulphide bond, in addition to murinising the constant alpha and beta chains. The introduction of the di-sulphide bond was to reduce the chances of mis-pairing with the endogenous TCR, while the codon optimisation can increase the gene expression of the newly introduced TCR construct. This was conducted on the basis of research by Staus *et al* (Stauss *et al.*, 2008) which investigated the potential to modify TCR gene constructs with the aim of improving TCR gene therapy, and stated that by modifying the TCR structure dominant TCR, constructs could be generated which suppress the expression of endogenous TCR's present on the targeT-cells. Evidence of the efficacy of this approach is provided by Hart *et al* (Hart *et al.*, 2008), who were attempting to conduct TCR transduction to generate a modified T-cell population capable of treating Hodgkin Lymphoma. Hart *et al* observed that their initial transduction resulted in poorly expressed TCR's on the target cell population (human primary t-cells), and it was hypothesised that this was directly caused by the introduced TCR's poor ability to compete for cell surface expression compared to the endogenous TCR. After the codon optimisation and modification of the TCR, efficient expression on the transduced cells was observed. This highlights the importance of choosing and designing an appropriate TCR for the transduction process. With the aim of achieving good TCR expression on the modified Tregs after the transduction process the TCR used in this project has been both codon optimised, murinised and had the addition of di-sulphide bonds.

With the intention of increasing the TCR expression on the modified cell population it is necessary to consider the potential factors which can affect low TCR expression, and how these may be addressed or overcome. For example, it has been observed that CD3 is a limiting factor in the expression of TCR; TCR subunits form a complex with CD3, and this complex is required for the surface expression of the TCR receptor. Ahmadi *et al* hypothesized that after TCR transduction, the transduced TCR's will be in direct competition for CD3 with the endogenous TCR, and this was demonstrated in a murine model system by comparing the transduction of a TCR alone to the co-transduction of a TCR alongside CD3 complex genes. The co-transfer of CD3 increased TCR expression by 16-20 fold, indicating that the addition of CD3 can enhance the TCR transduction expression, and subsequent

activity of TCR modified T-cells (Ahmadi *et al.*, 2011). It would be valuable to validate this process using human primary T-cells *in vitro*.

4.2 FoxP3 Purity of cell Populations

The FoxP3 purity of the transduced population was maintained after transduction with comparable levels to mock transduced cells. This is consistent with previously published research, Wright *et al* (Wright *et al.*, 2009) reported 88.7% FoxP3 levels in transduced Tregs, and Brusko *et al* (Brusko *et al.*, 2010) reported 98.2%. In this project levels of FoxP3 in Tregs were seen to remain consistent, with the average FoxP3 percentage before transduction at 85.2%, and post transduction FoxP3 at 85.6% **(figures 3.5, 3.6 and 3.15).** It was noted that the FoxP3 purity of the conventional T-cell population was observed to be present at higher levels than would be expected, both in the initial measurements and post transduction analysis. The initial pre-transduction FoxP3 stain was conducted on unactivated cells, and in contrast the post-transduced Tcons were activated 3 days before the transduction protocol was completed. The increased proliferation in this time could account for observed upregulation in FoxP3 post transduction. Published results have reported FoxP3 levels in Tcons at 33.8+-17.9% (Brusko *et al.*, 2010), which is lower than the 52.3+-19.6% observed in this research. After single cell analysis of FoxP3 expression in human T-cells it was observed that Tcons can upregulated FoxP3 without demonstrating any regulatory capabilities or converting to Treg cells (Gavin *et al.*, 2006). It is therefore known that levels of FoxP3 can be upregulated in response to cell activation, and indeed Brusko *et al* observed an upregulation of FoxP3 when the purity of the transduced population was measured after following an expansion protocol. In this particular research the initial pre-transduction FoxP3 purity stain was conducted on unactivated cells and therefore this is unlikely to be the cause of the observed high FoxP3 expression. It is possible that the high purity is a consequence of the particular FoxP3 antibody in this research demonstrating high levels of unspecific background staining. To test this hypothesis it would be ideal to utilise an isotype control for the FoxP3 antibody; this would provide an indication of the level of non-specific background staining, which could then be removed from the analysis so that only antibody specifically bound to FoxP3 is shown by the flow cytometry results.

4.3 Treg Suppression Assay's

Unfortunately a successful suppression assay was not achieved in this project as the monoclonal antibodies failed to produce proliferation. The same concentration of antibodies stimulated a proliferative response in PBMC samples, and it was hypothesised that the

monocytes present in the PBMC sample aided in the clustering of the antibodies to present to the T-cells, allowing increased signal transduction leading to activation and proliferation. A proliferation assay was conducted using stronger CD3 antibodies in order to account for this, however the CD4 cells activated in isolation still failed to show evidence of proliferation. The next step would be to coat the antibodies onto the surface of the assay plate, allowing for greater contact between the targeT-cells and antibodies, but unfortunately this experiment was not conducted due to time constraints.

An appropriate suppression assay with Tregs could not be set up in the absence of conventional T-cell proliferation. Upon demonstrating successful Tcon proliferation with sufficient stimulation, the next step would be to set up a Treg suppression assay: the effector T-cells would be co-cultured with varying ratios of Tregs to determine the ability of the Treg cells to suppress the Tcons in a dose dependent manner. This is a crucial step which demonstrates whether the Treg cell population is functioning as expected – while the expression of FoxP3 indicates a subset of T-cells with regulatory function, the presence of FoxP3 alone does not prove the functioning capacity of the cells. Furthermore, as already discussed, the expression of FoxP3 can be upregulated in Tcons. By setting up an assay with co-cultured Tregs and Tcons, the suppression of the Tcons serves as proof of the working functionality of the Treg population. Achievement of this assay will demonstrate whether the transduction process has had a negative impact on the suppressive capabilities of the Tregs. Altering the ratios of Treg: Tcons allows a more comparable consideration of the suppressive abilities of the modified Tregs when present at ratios simulating expected *in vivo* physiology. When preparing the assays it is also crucial to understand the precise treatment of each cell population and consider any affects this may have on the results. For example, over stimulation of an effector T-cell population with IL-2 can lead to overgrowth consequently increasing cell death, which may mistakenly present in an assay with co-cultured Tregs as suppression (Yarkoni *et al.*, 2008; Boyman and Sprent, 2012). The assay would therefore by designed to include controls of each treated cell line in isolation to account for this. Furthermore, it has been observed that Tregs are a particularly vulnerable population to culture *in vitro* and are susceptible to cell death (Taams *et al.*, 2001). This occurrence risks presenting as inefficient suppression, and care has to be taken to assess the viability of the Treg population prior to inclusion in assays. However, this particular assay does not measure the Ag-specific function of the population as the cells are provided with stimulation through any TCR on the cell surface, and not specifically activated through the introduced TCR on the modified Tregs. The next stage is to validate that the modified Tregs

can exert suppressive capacity when activated through the Ag specific stimulation of the introduced TCR, and this will require the design of a robust antigen specific suppression assay.

Cell proliferation provides only a single measure of T-cell function, and cannot suggest any evidence that the actively dividing Tcons are producing pro-inflammatory signals. It would therefore be advantageous to measure the level of cytokines in the supernatant harvested from prepared assays using ELISA. This analysis has the potential to indicate the ability of the modified Tregs to suppress inflammatory cytokines released by effector T-cells. The measurement of inflammatory cytokines and IL-2 can produce a clear measurement of inflammation. The suppression of Tcon cytokine production by Treg cells is less variable than suppression of cell proliferation, and therefore the measurement of cytokine level across multiple experiments can provide a clear and consistent indication of Treg function (Mcmurchy and Levings, 2012). This can be seen from the results of Wright *et al* who utilised measurements of IL-2 secretion to demonstrate the proliferation of the Tcon population (Wright *et al.*, 2009). IL-2 is a key cytokine in relation to T-cell activity, forming negative feedback regulation of T-cells: IL-2 drives the expansion of Tcons, which further produce IL-

2. The function and development of Tregs requires the presence of IL-2, however Treg cells cannot produce the cytokine. The increase in Tcon proliferation therefore increases IL-2 concentration, driving the action of the suppressive Treg cells, forming the negative feedback regulation system. Therefore the reduction in IL-2 levels in suppression assays with Treg:Tcon co-culture is an important measure, and may be considered to indicate the Treg cells actively utilising the IL-2 present in the assay, suggesting the functional capacity of the Tregs. Brusko *et al* also measured concentrations of IFN-γ and IL-2 after the co-culture of Treg and Tcons, and observed the suppression of IFN-γ and IL-2 as evidence of Treg suppression of Tcons (Brusko *et al.*, 2010).

4.3.1 Design of Antigen Specific Assay's

The next crucial step is to test the functional capacity of the modified Tregs when specifically activated through the stimulation of the introduced TCR by interaction with the target antigen. This can be achieved through the design of robust antigen-specific assays in which the co-culture the Tregs and Tcons in the presence of the target antigen and an appropriate antigen presenting cell (APC) is set up. The modified Tregs should be co-cultured with Tcons in the presence of an APC pre-pulsed with the target peptide. When the APC presents the peptide that the transduced TCR is specific for, the modified Tregs will be stimulated and

able to exert suppressive functions. The Tcons must be able to also be activated through interaction with a peptide presented by the APC, allowing for Tcon proliferation. As a control the assay would be required to be repeated with mock-transduced Tregs: in the absence of the transduced TCR the mock Tregs would not be able to be activated, and hence unable to exert full suppression.

There are a number of key considerations to account for while designing this assay. For bystander suppression to take place, it is essential for the Treg and Tcon to both be able to interact with the APC in order to ensure that both subpopulations are sufficiently activated. Therefore the Tcons used in the assay would have to be additionally transduced with the TCR. For the formation of the TCR: MHC complex required for Treg activation, the APC must present the peptide with the same HLA class that the transduced TCR is restricted to. A proposed method of achieving this is by transducing the appropriate HLA molecule in a retroviral vector into a chosen APC cell line. Previous work has already validated the vector HLA*DR*0401, which has been identified as a risk factor in RA, and is therefore a large proportion of RA patients possess this HLA type, making it an appropriate HLA type to be exploited in this work. The results in section 3.5 demonstrate the success in transducing the HLA DR *0401 vector into a T2 cell line. T2 cells are a fusion of a B-lymphoblastoid cell line and are MHC class II negative, with no expression of HLA DR (Henderson *et al.*, 1992) the presence of HLA DR after the transduction process is therefore a strong indication of the successful introduction and uptake of the HLA*DR*0401 vector. However, whilst we were unable to validate this new reagent in Ag-specific Treg suppression assays, one concern was that the T2 cells demonstrate high levels of the surface markers CD80 and CD86 indicating the maturity of the cell line and subsequent ability to be strong stimulators of T-cells. For this particular assay, the strong stimulation of Tcons has the potential to limit the efficiency of Treg suppression and could mask the genuine suppressive capacity of the modified Tregs. Further work may be required to choose a more appropriate cell line for generating an APC. A potential cell line for this purpose is the K562 cell line: this is a human eyrhroleukamic cell line and demonstrates no expression of either MHC class I or II (Tanaka *et al.*, 2010). Similar to the T2 cell line, this means that the success of the HLA*DR*0401 transduction can be easily analysed by flow cytometry. Research has already been done to confirm the ability of transduced K652 cells to simulate HLA-DR restricted CD4 T-cells (Tanaka *et al.*, 2010), however further work is required to validate this cell line for use as an APC for testing the functional capacity of Tregs within a suppression assay.

4.4 Selecting an Appropriate TCR

The mTCR used in this project is restricted to the HLA type HLA-DR*0401, and specific for Myelin basic protein (MBP). The protein target of this particular TCR is not specific for the RA disease environment, and is being used only as a model to validate the generation of a population of functional Ag-specific human Tregs *in vitro.* One of the crucial challenges of this research would be choosing a TCR specific for an appropriate peptide target. One advantage of TCR gene transfer to generate the Ag-specific population is that the population is not limited to be specific to a TCR already present within the endogenous T cell receptor repertoire, but instead a new TCR not present can be introduced. There is therefore no limit to the specific peptide which can be chosen as a target. It would be ideal to utilise a TCR specific for a disease related peptide that will allow the modified Tregs to activate in the RA inflammation sites. It is realistic to anticipate the generation of more than one disease specific TCR which can be utilised to treat a wider range of patients, or further applied to the treatment of a range of autoimmune disorders. The number of TCRs required can be reduced by exploiting the strong association that has been drawn between the onset of autoimmunity and particular MHC II class sub types. As mentioned in section 1.5, there is a genetic association between the inheritance of HLA type DR*04 and the onset of RA, and therefore there is a high chance of RA patients being HLA-DR4 positive. By choosing a TCR restricted to this particular HLA type it therefore increases the number of individuals that are eligible for the treatment, and only a small number of different disease-related TCRs would be required to generate a powerful adoptive therapy to treat the condition.

A further factor for consideration when choosing an appropriate TCR for the gene therapy process is the affinity of the TCR for its antigenic target. Lowered T-cell sensitivity may be seen as a consequence of reduced TCR expression, but it would also be pertinent to consider that the transduced TCR may be demonstrating reduced affinity for the TCR-peptide-MHC complex. It may be possible to modify the structure of the TCR to increase the affinity of the receptor – Robbins *et al* demonstrated that amino acid substitutions can enhance T-cell function as mediated through the receptor, and suggested that key substitutions in TCR structure has the potential to increase the efficacy of the TCR (Robbins *et al.*, 2008). However, care has to be taken when attempting to increase the affinity of the TCR: Linette *et al* reported severe consequences of adoptive T-cell therapy in a clinical trial after engineering T-cells with an affinity enhanced TCR against a HLA-A*A01 restricted peptide. The treatment unexpectedly caused severe myocardial damage and cardiogenic shock, resulting in the death of two trial participants (Linette *et al.*, 2013). The cause was

determined to be an unexpected cross-reactivity of the TCR-enhanced T-cells with an unrelated peptide expressed in healthy cardiac tissue. A further risk of increasing TCR affinity is the unintended consequence of reducing the activation potential of the TCR. This can occur if the binding between the TCR: MHC complex is too strong, and unable to break easily, leaving the T-cell unable to engage with multiple receptors sequentially. This was described by Thomas *et al*, who analysed the functional capacity of modified TCRs after transduction into human T-cells and noted that while the binding of the modified cells improved, the function was not improved (Thomas *et al.*, 2007).

4.5 Potential Challenges of Treg Adoptive Therapy

Tregs persisting in inflammatory conditions do not always maintain a stable phenotype, and the downregulation of FoxP3 has been observed coinciding with a switch to a potentially pathogenic Th17 phenotype. This differentiation of Treg into IL-17 producing cells is thought to be reliant on epigenetic modification, and in the presence of pro-inflammatory cytokines such as IL-1beta and IL6. Ayyuab *et al* (Ayyoub *et al.*, 2009) examined Treg clones and noted that the IL-17 cells derived from Treg co-express FoxP3 and transcription factor RORC, and demonstrate a loss of suppressive activity. There is a suggestion that the instability of FoxP3 Tregs converting into pro-inflammatory IL-17 secreting cells play a role in autoimmunity; this was investigated by Zhou *et al* (Zhou *et al.*, 2011) using *in vivo* mouse models, who noted that a high percentage of FoxP3 Tregs exhibited a downregulation of FoxP3, and these cells could produce pro-inflammatory cytokines and drive the onset of diabetes in NOD mice. As this phenotype switch presents in an inflammatory environment, a crucial consideration of this particular research, which is looking to introduce Tregs into an inflammatory setting, is to identify methods of maintaining the stability of the Tregs. One potential route is to focus on blocking the epigenetic modifications that lead to the downregulation of FoxP3. Lal *et al* (Lal *et al.*, 2009) investigated the epigenetic regulation of FoxP3 by DNA methylation, and reported that a specific site present within the upstream enhancer of FoxP3 in Tregs remains un-methylated, but in contrasts is observed to be heavily methylated within conventional T-cells. The de-methylation of this site resulted in the stable induction of FoxP3, while the methylation of this site in Tregs resulted in the downregulation of FoxP3. Protecting this site DNA site from methylation is therefore an example of how the stability of Treg cells could be enhanced and the suppressive phenotype protected.

Additionally particular subsets of Treg cells have been identified which demonstrate increased stability; as discussed in section 1.4.1, Hoffman *et al* (Hoffmann *et al.*, 2017) identified that a subset of Tregs, $CD4^{+}CD25^{+}CD45RA^{+}$, can be expanded in vitro to produce a

homogenous Treg cell line that have demonstrated the ability to maintain FoxP3 expression after expansion. In contrast the Ag-experienced CD45RO+ Treg subset have been noted to downregulate FoxP3 after 2-3 rounds of TCR stimulation *in vitro* (Edinger *et al.*, 2003). In *in vitro* experiments, Hoffman *et al* demonstrated that CD45RA+ subsets from the same donor maintained FoxP3 expression throughout the 3 week long experiment. This research highlights the importance of identifying the most appropriate population to target for modification when attempting to create an effective sustainable adoptive therapy.

4.5.1 Adoptive Therapy in an Active Disease Setting

After the identification of naïve $CD4^{+}CD25^{+}$CD45RA Tregs as a stable population ideal for adoptive therapy, the suitability of the isolating and utilising that specific population has be considered within an active disease setting. It has to be considered further that the endogenous Treg population in an active autoimmune setting may be defective. Unpublished work from our lab has observed that in patients with RA there is a significantly reduced number of Ag-naïve Tregs compared to healthy controls, and a resulting higher proportion of Ag-experienced Tregs. After TCR-transduction it was identified that experienced Tregs were defective in suppressing IL-2 production by effector cells in a manner not observed in naïve Tregs from the RA patients or either subset from healthy controls. This has implications for the advancement for adoptive Treg therapy in the treatment of RA and other autoimmune diseases as it highlights the necessity of identifying the appropriate Treg population for use in therapy. Furthermore, it will be important to understand the nature of any defects in the Treg subsets and how these might be corrected.

One potential method of dealing with potential defects in the endogenous Treg population would be to introduce additional genes into the TCR vector, opening up the potential to genetically correct any characterised Treg defects. **Figure 4.1** shows an overview of the vector structure: the presence of the P2A peptide sequence in the vector causes the ribosome to skip the synthesis of the peptide bonded at the C-terminus of the 2A peptide, therefore allowing the continuous synthesis of the next downstream peptide. This therefore allows for the incorporation of an increased number of genes into the vector, without increasing the vector size to an unfeasible level (Szymczak and Vignali, 2005).

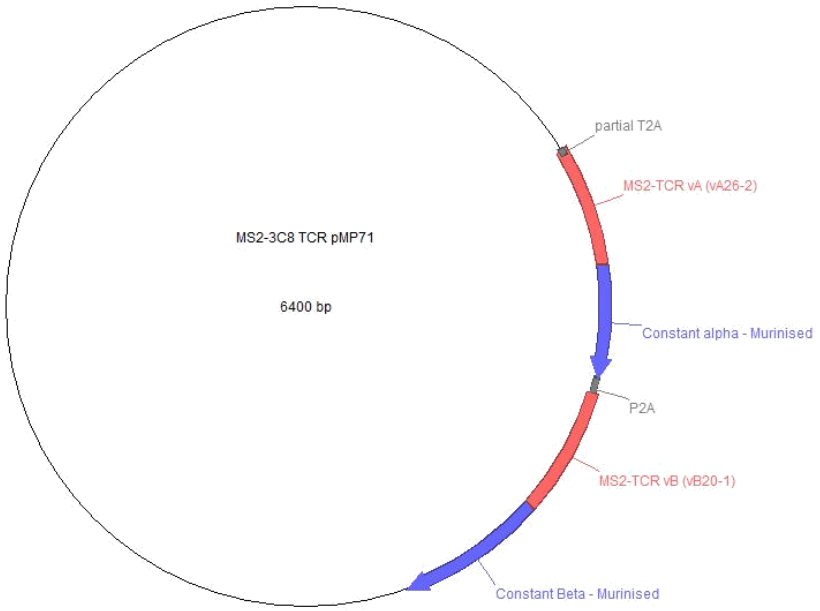

Figure 4.1: Structure of the TCR vector used in the research.

It would be greatly advantageous to exploit this and introduce genes with the potential to augment and improve the function of the Ag-specific Treg population. For example, it has been observed that while Tregs from RA patients can suppress Tcon proliferation through direct contact mechanisms, they appear to be defective at producing anti-inflammatory cytokines (Flores-borja *et al.*, 2008). It has been suggested that this is associated with a defect in CTLA4 expression and function. To test this hypothesis Flores-bora *et al* compared the surface expression of CLTA4 on RA Tregs to normal control Tregs, and noted that CTLA4 was reduced on the surface of RA Tregs, with an increased rate of internalisation. Furthermore CTLA-4 mediated signalling was impaired, but full suppressive function was restored after the artificial induction of CTLA4. It could be possible to include the addition of genes into the vector with the ability to correct this defect – this is just one example of how defects may be corrected, or the Treg population enhanced.

However, while there is conflicting evidence regarding the function of Tregs within RA, it is necessary to also consider the possibility that the Tcons are not fully functional. In light of the recent evidence presented by Walter *et al* (Walter *et al.*, 2016), which indicated that there is no defect in the naive or Ag-experienced Tregs from RA patients, it is crucial to consider that in certain conditions Tcons may have developed a defect which allows them to by-pass or resist Treg suppression. This idea is supported by evidence which indicates that the number of Tregs actually increases in RA. Monte *et al* investigated the interactions between Tregs and Tcons in an *in vitro* RA mouse model, in which the proliferation and

function of both cell types at various stages of arthritis was examined (Monte, Wilson and Shih, 2008). They observed that while the Treg number increased in an environmental setting, this was balanced by a simultaneous increase in the resistance to suppression by the conventional t-cells. The authors noted that the APC contributed to the inflammatory environment through the release of cytokines which could enhance Tcon resistance to Treg suppression, in addition to the promotion of Treg apoptosis. This research emphasises the complexity of the inflammatory autoimmune environment. This is in agreement with Walter *et al,* who despite concluding that RA Tregs are not defective, emphasise that their findings do not take into account the potential effects that a local inflammatory environment may have on the function of Tregs. It is clear that that it will be necessary to consider the effects a local inflammatory environment might have on the function of modified Tregs introduced as an adoptive therapy.

Chapter 5 – Suggestions for Future Study

For the completion of this project it would be ideal to demonstrate the working functional capacity of the transduced Tregs. This would require the modified Tregs need to be tested with a robust antigen-specific suppression assay. To achieve this further work is required to optimise the suppression assay conditions, and this may achieved in a number of ways: it would be ideal to coat the required antibodies onto the suppression plate surface, or beads, in order to increase the cell contact with antibodies, thereby increasing the activation signal. Further to this work is required to generate an artificial APC capable of activated HLA-DR*04 restricted cells with an appropriate signal strength. As discussed in section 4.3, it would be greatly advantageous to further test the suppression assay supernatant and determine the concentration of and presence of particular cytokines, with the aim of providing a clear indication of the suppressive capacity of the modified Tregs *in vitro.*

After the validation of the Treg transduction process it would be necessary to validate a reproducible method of generating Ag-specific Tregs from human peripheral blood taken from RA patients. This will require work to identify the most appropriate population to target for modification in an active disease setting. In patients with RA there is a significantly reduced number of Ag-naïve Tregs compared to healthy controls, and a resulting higher proportion of Ag-experienced Tregs. After TCR-transduction it has already been identified in unpublished work that experienced Tregs were defective in suppressing IL-2 production by effector cells in a manner not observed in naïve Tregs from the RA patients or either subset

from healthy controls. This has implications for the advancement for adoptive Treg therapy in the treatment of RA and other autoimmune diseases as it highlights the necessity of identifying the appropriate Treg population for use in therapy. Furthermore, the application of this approach to a human clinical setting will require close consideration of the potential effect of an inflammatory environment on the modified Tregs. It will also be important to understand the nature of any defects in the Treg subsets and how these might be corrected.

Chapter 6 – Conclusions

This research has demonstrated the successful validation of the TCR transduction process into isolated human Treg cells at the labs at Edinburgh Napier University. This was achieved after completing the outlined objectives to optimise the transduction process and the isolation of Tregs from peripheral blood. By sorting on the CD4+CD25+CD127dim cell population, Tregs were isolated and the purity of the population demonstrated by the high percentage of fopx3 expressing cells. The results further show that FoxP3 is maintained after the transduction process.

Ag-specific suppression assays are required to generate repeatable data demonstrating the functional capacity of the modified Treg cells. Continuation of the research would then focus on applying the validated protocols to RA patient blood samples. This will require the isolation of CD45RA+ Tregs from peripheral blood taken from RA patient samples to be used in the transduction procedure. Unpublished work has previously demonstrated this, with data showing successful transduction and suppression assays have been conducted to demonstrate the functional capacity of the modified cells.

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