REDIRECTING TREGS TO THE HLA-A2 ANTIGEN FOR TRANSPLANT TOLERANCE

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A thesis submitted to fulfil requirements for the degree of Bachelor of Medical Science (Honours)

1 November 2017

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Abstract

Background

Kidney transplantation is currently the preferred solution for organ replacement in patients with end-stage renal disease (ESRD). While nearly all transplanted grafts will survive in a short-term (up to one year post-transplant) period, graft survival rates decline at a linear rate with increasing time. This is largely contributed by chronic allograft rejection against alloantigens in the transplanted graft. One of the most commonly mismatched alloantigen is HLA-A2. Immunosuppressants have proven to be effective in promoting short-term graft survival, but are ineffective at improving long-term tolerance due to their associated toxicities and adverse events. As such, a novel therapeutic solution is necessary to overcome chronic allograft rejection. This study proposes that chimeric antigen receptor (CAR)-expressing regulatory T-cell (Treg) therapy specific to the HLA-A2 antigen (CAR-A2 Tregs) may be the solution in overcoming the immunological barrier of long-term graft tolerance.

Objectives

This study aims to generate CAR-A2 Tregs and to assess their function in suppressing allogeneic immune responses compared to polyclonal Tregs.

Methods

CAR-A2 and Foxp3 (master regulator of Tregs) plasmids were generated and amplified in *Escherichia coli* cells, and were packaged into retroviruses using EcoPack™ 293 cells. The retroviruses were then used to co-transduce $CD4^+$ T-cells from C57BL/6J(ARC) (B6) mice to generate CAR-A2$^+$ Foxp3$^+$ CD4$^+$ T-cells (CAR-A2 Tregs). CAR-A2 Treg suppression

function was assessed by mixed lymphocyte reactions (MLR) with polyclonal CD4$^+$ T-cell responders and HLA-A2$^-$ (B6) or HLA-A2$^+$ (A2Kb) splenocytes as stimulators. Suppression will be assessed by CellTrace™ Violet proliferation dye dilution of CD4$^+$ T-cell responders.

Results

Higher transfection efficiency was observed for the Foxp3 plasmid compared to the CAR-A2 plasmid in EcoPack™ 293 cells. Albeit at a low yield (0.15%), CAR-A2 Tregs were successfully generated. CAR-A2 Tregs were also more potent in suppressing CD4$^+$ T-cell allogeneic responses against the HLA-A2 antigen compared to polyclonal Tregs or CAR-HER2 Tregs.

Discussion and Conclusion

Consistent with previous studies, CAR-A2 Tregs were more potent than polyclonal Tregs at suppressing allogeneic responses to the HLA-A2 antigen. Interestingly, the chimeric HLA-A2/H2Kb molecule expressed on A2Kb splenocytes was able to elicit a suppression response from CAR-A2 Tregs, showing that only the epitope of HLA-A2 is sufficient to activate CAR-A2 Tregs. In the future, an *in vivo* study of a kidney transplant model will be necessary to assess CAR-A2 Treg efficacy in a kidney transplant setting. Further optimisation and modifications to the techniques used in the current study may be necessary to improve yield.

Acknowledgements

I would like to thank Dr. Stephen Alexander for giving me direction and for his valuable feedback, despite his busy schedule, and also Dr. Yuan Min Wang for her patient assistance in the experiments. Without them, this project would not have run efficiently. I would like to thank Dr. Samantha Ginn for providing excellent advice for our 293 cells, Cindy Zhu for providing expert help in optimising the pH of our transfection reagents, and Kenneth Hsu for his pH meter. Their contribution was essential in advancing the project. I would like to thank Dr. Min Hu for her expert assistance in optimising my mixed-lymphocyte reactions and flow cytometry experiments. Her help was crucial in obtaining the most important results of this project. I would like to thank Dr. Geoff Yu Zhang, for his advice in experimental design. I would like to thank the Flow Cytometry Core Facility of the Westmead Research Hub for the flow cytometry experiments performed, and the Cell Imaging Facility of the Kids Research Institute for the microscope images obtained.

I would like to thank Jevin Karunia, Mahnoor Bakhtiar, and Sarah Tan, my peers, for their unselfish help and support, Jimmy Zhou for instructing me in plasmid amplification techniques, and Catherine Li Lai and Thaya Shanmuganathan for their encouragement throughout the year.

Above all, I would like to thank God for giving me this learning opportunity to undergo my Honours project in a supportive environment, and for Mum and Dad, for their relentless hard work in supporting my overseas study.

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List of Abbreviations

6-MP	Mercaptopurine
7-AAD	7-Amino Actinomycin D
A2-K562	HLA-A2-expressing K562 cells
A2Kb	C57BL/6J-TgN(A2KbHLA)6HsdArc mouse
AAPC	Artificial Antigen-Presenting Cells
ACK	Ammonium-Chloride-Potassium
ALL	Acute Lymphoblastic Leukaemia
ANOVA	Analysis of Variance
ANZDATA	Australia and New Zealand Dialysis and Transplant Registry
APC	Antigen-Presenting Cell
ARC	Animal Resources Centre
B6	C57BL/6J(Arc) mouse
Bcl-xL	B-Cell Lymphoma Extra Large
blast	Basic Local Alignment Search Tool
CAA	Cancer-Associated Antigen
CAR	Chimeric Antigen Receptor
CAR-A2	Chimeric Antigen Receptor against the HLA-A2 antigen
CAR-HER2	Chimeric Antigen Receptor against the HER2 antigen
CD	Cluster of Differentiation
CKR	Centre for Kidney Research
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myelogenous Leukaemia
CNI	Calcineurin Inhibitors
ConA	Concanavalin A

CP-DNA	Calcium Phosphate-DNA
CTL	Cytotoxic T-cells
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
dATP	Deoxyadenosine Triphosphate
DC	Dendritic Cell
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
dTTP	Deoxythymidine Triphosphate
EAE	Experimental Allergic Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ESRD	End-Stage Renal Disease
FCS	Foetal Calf Serum
Foxp3	Forkhead Box P3
GFP	Green Fluorescent Protein
GVHD	Graft-Versus-Host Disease
HBS	HEPES-Buffered Saline
HEK	Human Embryonic Kidney
HLA	Human Leukocyte Antigen
IL	Interleukin
iTreg	Induced T-regulatory Cells
KRI	Kids Research Institute
lag3	Lymphocyte Activation Gene 3
LB	Lysogeny Broth

LNGFR	Low-Affinity Nerve Growth Factor Receptor
MACS	Magnetism-Activated Cell Sorting
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MLR	Mixed-Lymphocyte Reaction
MMF	Mycophenolate Mofetil
MMTV	Mouse Mammary Tumour Virus
mRNA	Messenger Ribonucleic Acid
mTOR	Mechanistic Target of Rapamycin
NFAT	Nuclear Factor of Activated T-cells
NK	Natural Killer
NS	Non-significant
nTreg	Natural T-regulatory Cells
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
qPCR	Quantitative Polymerase Chain Reaction
rATG	Rabbit Anti-Thymocyte Globulin
RCC	Renal Cell Carcinoma
RPMI	Roswell Park Memorial Institute
scFv	Single-Chain Variable Fragment
SOC	Super Optimal Broth with Catabolite Repression
TAA	Tumour-Associated Antigen
TAE	Tris-Acetate-EDTA
TCR	T-cell Receptor
TE	Tris-EDTA

Teffs	Effector T-cells
TGF-β	Transforming Growth Factor β
TNP	2,4,6-Trinitrophenyl
Treg	T-regulatory Cells
V$_H$	Variable Heavy Chain
V$_L$	Variable Light Chain

Chapter 1 Introduction

1.1 Renal Transplantation

1.1.1   Overcoming the Barrier of Graft Rejection

The advent of organ transplantation as a clinical service is one of the biggest breakthroughs in medicine. For millennia, people have teased with the idea of being able to replace parts of a body that were non-functional, however, due to the lack of understanding of the mechanisms of rejection, many grafts in transplant experiments pre-1950s were quickly rejected. At that time, the consensus was that graft rejection occurred due to humoral (antibody-mediated) immune responses, and cellular (T-cell-mediated) immune responses were largely ignored. It was only in 1957 that the cellular immune response was brought into importance in transplantation when it was found that cellular immunity, and not humoral immunity, caused graft-versus-host disease (GVHD) (Barker & Markmann 2013). Today we understand that both cellular and humoral responses contribute in graft rejection (Wood & Goto 2012).

Although skin grafts have been successfully performed between identical twins, the first documented successful human organ transplantation occurred in 1954 by Joseph Murray and his team, where they transplanted a kidney from the patient's identical twin (the graft survived and was functional for at least several months) (Murray, Merrill & Harrison 2001). Following the exciting success, attempts to overcome the immune barrier to transplantation was done by irradiation and immunosupprerssants such as 6-MP and azathioprine, yielding significantly prolonged graft survival, but only in less than 10% of the subjects. The plateau was broken through by Tom Starzl, who came up with an

immunosuppressive cocktail combining azathioprine with prednisone, allowing more than 70% of patients graft survival for more than a year (Barker & Markmann 2013).

Building upon the centuries-long work in transplantation and immunology, new immunosuppressants with better potency have been developed and are now used today. Induction agents (cellular and humoral immunity suppressors) such as alemtuzumab (anti-CD52 antibodies), anti-thymocyte globulins (rATG), and basiliximab (anti-IL2 receptor antibodies) and maintenance immunosuppressants such as cyclosporine and tacrolimus (both calcineurin inhibitors) have helped prevent and treat acute rejection and promote short-term graft survival (survival of up to one year) (Ghanta *et al.* 2013). From 2011 to 2015, the average annual incidence of end-stage renal disease (ESRD) in Australia and New Zealand was 2,623 patients, and of these, only about 860 patients received renal transplants per year. In these patients, short-term graft survival was successfully achieved in 96.5% of the grafts. However, about a fifth of them experienced renal transplant failure after 5 years, as shown in figure 1.1 (ANZDATA 2016). Graft survival becomes less likely as time goes on. At 10 years, graft survival is down to 58.75% for patients from 1990 to 2009, and a poor 27% at 20 years for patients from 1990 to 1999 (figure 1.1). This low rate of long-term graft survival (survival of more than one year) is a major problem especially for young children with ESRD, as they will be more likely to require multiple transplants and functional renal grafts for longer than advanced-age patients.
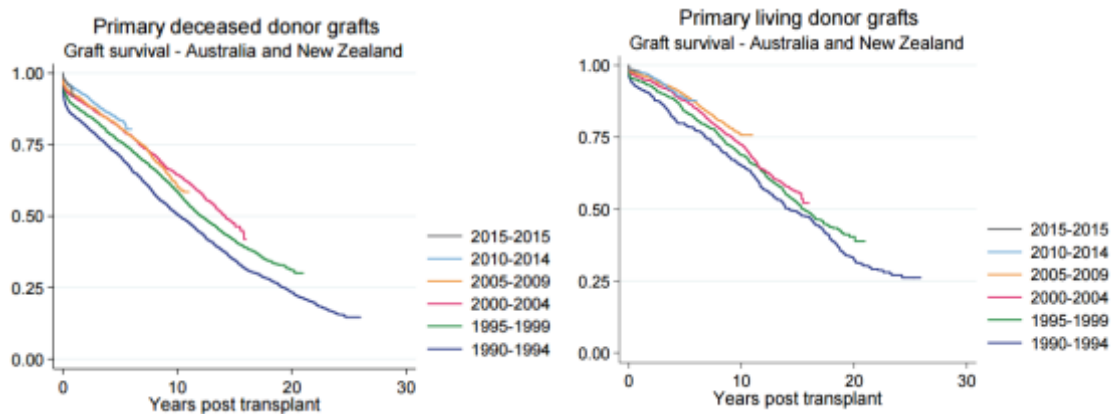
Figure 1.1. Rates of graft survival in Australia and New Zealand from deceased and living donors.
From the ANZDATA Registry, 2016 (ANZDATA 2016).

1.1.2 The Problem with Drug Immunosuppression

Drug immunosuppression has helped overcome the barrier of short term rejection. However, while drug immunosuppression has advanced transplantation outcomes and services to successes unachievable mere decades ago, there are some detrimental side effects with constant usage. These side effects include: susceptibility to infection, myelosuppression, and nephrotoxicity.

Induction agents (e.g. alemtuzumab, rATG, and basiliximab) are used in the early stages post-transplant to provide strong immunosuppression to prevent acute rejection of the graft (Gabardi *et al.* 2011). They are usually monoclonal or polyclonal antibodies that function to deplete lymphocytes to profoundly suppress the immune response. Due to their strong immunosuppressive effect, patients become more susceptible to bacterial infections (such as *Mycoplasma*, *Salmonella,* and *Legionella*), primary viral infections or reactivation of latent viruses (cytomegalovirus, herpes simplex virus, and varicella zoster virus), and fungal infections (such as *Candida*) (Orlicka, Barnes & Culver 2013).

Calcineurin inhibitors (CNIs) are the mainstay of staple maintenance immunosuppression in transplantation. They work by inhibiting the calcineurin-mediated pathway of cytokine

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gene expression to deregulate T-cell activation and response. Similar to induction agents, they have helped promote short-term allograft survival and in reducing acute rejection incidences (Ghanta *et al.* 2013). However, their efficacy in long-term maintenance is poor, mostly because of its associated nephrotoxicity. Nearly 100% of renal transplant patients with CNI maintenance experience CNI-induced nephrotoxicity within 10 years (Nankivell 2003). Alternatives to CNIs, such as sirolimus and mycophenolate mofetil (MMF), have been developed to minimise the associated nephrotoxic effects, however MMF and Sirolimus can also have adverse side effects. MMF is associated with gastrointestinal toxicity, and Sirolimus can have tubulotoxic and myelosuppressive effects, such as leukopenia and thrombocytopenia (Ghanta *et al.* 2013).

While drug immunosuppression is necessary for short-term graft acceptance, they have not been shown to improve long-term tolerance, and chronic immunosuppressive therapy is associated myelosuppression and nephrotoxicity. Alternative methods to promote graft tolerance need to be developed without compromising host immunity, and one of the most promising techniques is by adoptive transfer of antigen specific regulatory T-lymphocytes (Tregs).

1.2 Understanding the Mechanism of Allograft Rejection

1.2.1 Allorecognition

To put into context the principles of Treg-mediated tolerance induction, the mechanism of allograft rejection has to be understood. Allograft rejection is complex and involves a network of interplay between the subsets of immune cells (Ingulli 2010; Moreau *et al.* 2013), which begin with allorecognition. Rejection occurs when alloantigens, antigens of the same gene but different alleles, are recognised by naïve T-cells and stimulate their effector response. Allorecognition occurs in two ways: direct allorecognition and indirect

allorecognition, and differ in the source of the antigen-presenting cell (APC) that stimulates the immune response.

Direct allorecognition happens when peripheral antigen-presenting cells (APCs) from the donor (expressing donor major histocompatibility complex (MHC) molecules), contained in the allograft, becomes activated to stimulate host naïve T-cells following inflammatory signals. These APC-activating inflammatory signals, such as IL-1, IL-6, and TGF- β are observed to appear in kidneys following surgical stress or ischemic reperfusion injury (I/R) (Bonventre & Zuk 2004; Takada *et al.* 1997), which commonly happen following kidney transplantation. Naïve T-cells that recognise the allogeneic MHC-molecule from the donor APCs will activate and proliferate, and exert an acute and directly cytolytic immunological rejection response.

Indirect allorecognition is still yet to be clearly defined in literature, but is essentially mediated by APCs originating from the host. It is thought that host APCs can pick up alloantigens from the graft and present them to host naïve T-cells, which, if reactive, will activate and migrate to attack cells that present the alloantigen. This model of allorecognition came about from the works of Auchinloss, *et al.* (1993) where they observed that CD4^{+} responses were still able to be triggered by skin grafts that were depleted of MHC-class II, implying that the host APCs (that do express MHC-class II and can activate CD4^{+} T-cells) were somehow able to present antigens from the skin graft. Moreover, it has been recently observed that dendritic cells (DCs) were able to acquire whole MHC-molecules from other APCs or endothelial cells, and effectively present them to reactive T-cells (Jiang, S, Herrera & Lechler 2004), showing another possible model for indirect allorecognition.

1.2.2   The MHC Molecule: Alloantigens and Antigen-Presenting Molecules

In both mechanisms of allorecognition, MHC (human leukocyte antigen, HLA, in humans) molecules play important roles, either acting as the alloantigen or as the antigen-presenting molecule.
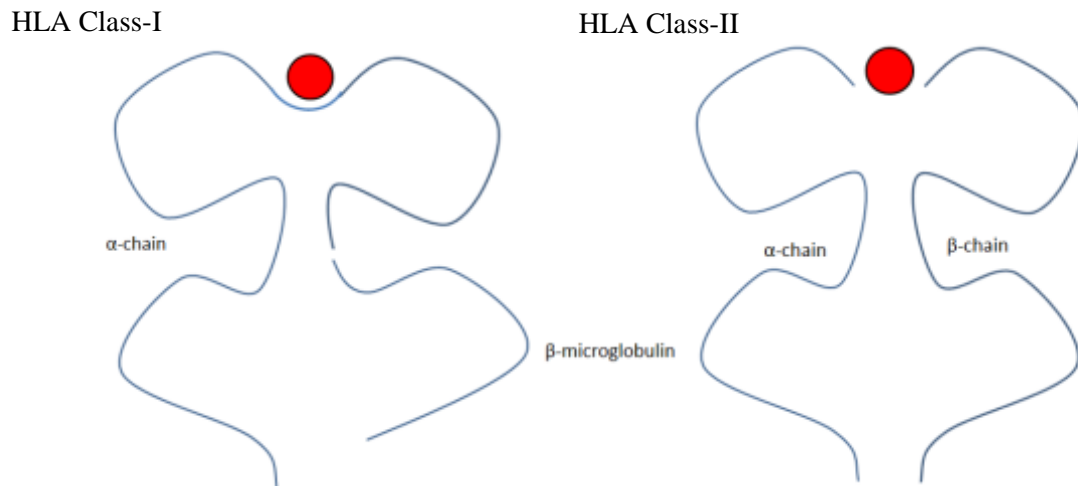
Figure 1.2. Simplified schematic of HLA class-I and class-II structures. The red spheres represent antigens.

The HLA locus in chromosome 6 contains three genetic regions. The class I HLA gene region contains 3 major class I HLA heavy chain genes: HLA-A, HLA-B, and HLA-C, which are expressed in most nucleated cells as presenters of endogenous peptide antigens for recognition by natural killer (NK) cells and CD8$^+$ T-cells. The class II HLA gene region contains 3 gene families: DR, DP, and DQ, for both α and β chains of HLA class II. These are expressed only in antigen-presenting cells, CD4$^+$ T-helper cells, and in B-cells to present exogenous peptides. Finally, the class III HLA gene region houses genes for complement expression, and not HLA molecules themselves (Choo 2007).
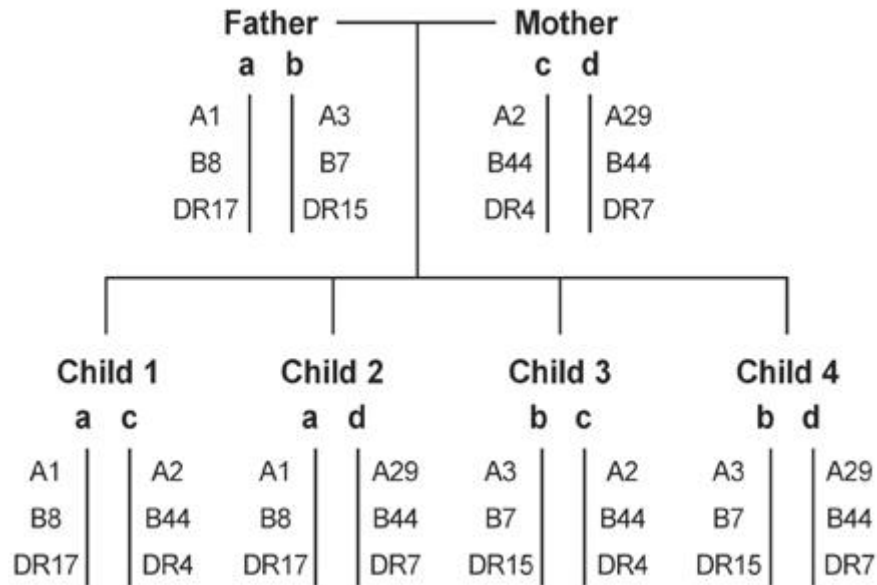
Figure 1.3. HLA inheritance happens in a Mendelian fashion. From Choo, S. Y., Yonsei Medical Journal, 2017 (Choo 2007).

These HLA genes are highly polymorphic. By 2008, 2215 class I and 986 class II HLA alleles had been discovered (Shiina *et al.* 2009). Their polymorphic nature makes them very effective as alloantigens that trigger immunological rejection. An individual receives their HLA repertoire in a Mendelian fashion from their parents, and each cell can express each of the HLA alleles in co-dominance (figure 1.3 and 1.4) (Abbas, Lichtman & Pillai 2016a). This is implicated in HLA-matching for organ transplantation, as the non-host HLA molecules can trigger allogeneic immune responses against the graft (Ingulli 2010; Lee, SJ *et al.* 2007).
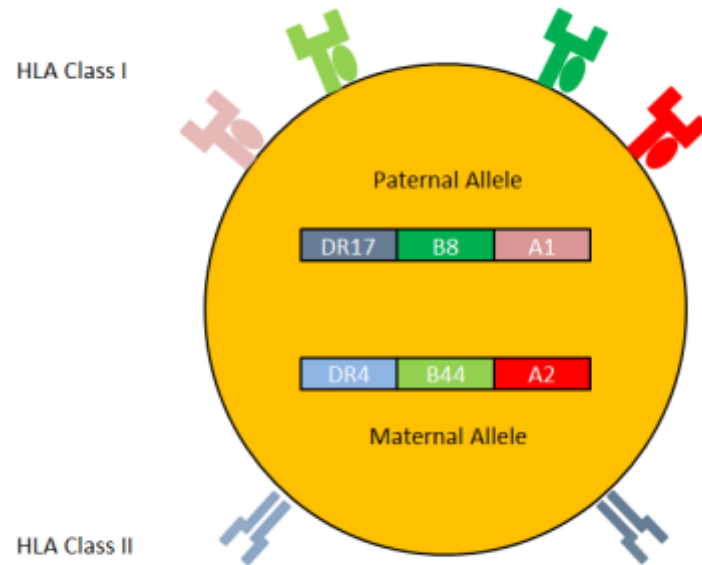
Figure 1.4. Simplified diagram of co-dominant expression of HLA molecules.

One of the most commonly expressed HLA molecules in the Australian population is HLA-A2. HLA-A2 is a subtype of class I HLA-A which is found in 50% of Caucasians (Rees & Kim 2015), and is found to have higher binding to three peptides of cytomegalovirus pp65 (Solache *et al.* 1999). If a mismatch between donor and the recipient occurs with this HLA subtype, it is much more difficult to obtain a new donor organ for re-transplantation as their immune system will be sensitised to an antigen that is very commonly expressed in the donor pool. This is our antigen of interest. If tolerance can be induced specifically towards HLA-A2, the chances of graft acceptance for Australian patients will increase phenomenally.

1.3 Regulatory T-cells

1.3.1 Immune Tolerance

In the mammalian immune system, T-cells with autoantigen specificity will be generated as they mature, by T-cell receptor (TCR) gene recombination, involving the V, D, and J regions of the genes (Abbas, Lichtman & Pillai 2016b). The body negatively selects for

these cells in two major ways: central tolerance in the thymus and peripheral tolerance in the rest of the circulation. In central tolerance, naïve T-cells that recognise autoantigens expressed in the thymus epithelia are either inactivated by apoptosis induction or differentiate into T-regulatory cells, which act to promote peripheral tolerance of autoimmune effector T and B-cells through immunosuppressive cytokines, cytolytic molecules, proliferative cytokine deprivation, and APC inactivation (figure 1.5) (Vignali, Collison & Workman 2008). In mice, these immune regulatory cells are characterised by their Foxp3 expression, but their characterisation is more controversial in humans (Vignali, Collison & Workman 2008). They are often characterised by high expression of CD4 and CD25 in mice, whereas in humans, with high expression of CD4 and CD25 together with low expression of CD127 (Vignali, Collison & Workman 2008). It has been suggested that Tregs already have a baseline suppression function without antigen-specific activation (Szymczak-Workman, Workman & Vignali 2009), but MHC-dependent activation strengthens their suppressive phenotype (Corthay 2009; Szymczak-Workman, Workman & Vignali 2009).
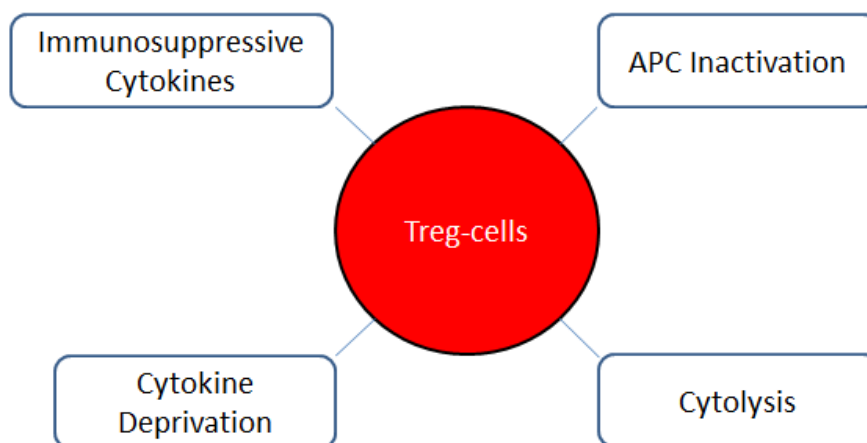
Figure 1.5. Treg suppression mechanisms.

The importance of Tregs in immune tolerance has been clearly shown in terms of self-tolerance (Hadaschik *et al.* 2015), autoimmune disease (Dejaco *et al.* 2006), and

unfortunately, also in tumour survival (Ha 2009). They have also been implicated in graft tolerance. Depletion of Tregs is associated with onset of acute graft rejection, and Treg adoptive transfer promotes longer-term graft survival (Gorantla *et al.* 2010; Joffre *et al.* 2008). As immune regulators in the mammalian circulation, successful manipulation and application of Tregs may be the key to tolerance induction.

1.3.2   Treg Therapy in Graft Tolerance Induction

Autologous cell therapies using hematopoietic cells or lymphocytes have been used as a curative solution for autoimmune diseases, but not in the context of allogeneic immune responses yet. One of the first successful Treg (defined as CD4$^+$ CD25$^+$ cells) adoptive transfer studies for suppression of allogeneic responses was undertaken by Sakaguchi and colleagues (Sakaguchi *et al.* 1995). They used a nude mouse model receiving allogeneic skin grafts, and performed adoptive transfer of lymphocytes containing CD25$^+$ cells. This significantly prolonged skin graft survival for up to 6 days (Sakaguchi *et al.* 1995). Taylor and colleagues has also shown that adoptive transfer of *ex vivo* activated and expanded CD4$^+$ CD25$^+$ cells decreased the severity of GVHD in mouse bone marrow transplant models (Taylor, Lees & Blazar 2002). Their work showed that Treg adoptive transfer is a feasible solution for tolerance induction in organ transplants. However, many issues are still present in Treg adoptive transfer, particularly in terms of Treg isolation, Treg stability, and Treg antigen specificity. Problems with Treg isolation and stability are associated with protocol optimisation and Treg interactions with cells in lymphocyte mixtures (Safinia *et al.* 2010), but Treg antigen specificity has to be dealt with by means of manipulation and redirection, which requires expansion of alloantigen-specific Tregs or gene transfer of alloantigen-specific receptors.

1.3.2.1 Obtaining Tregs for Treg Therapy

Generally, Tregs can be obtained in three ways: by isolation from peripheral blood mononuclear cells (PBMCs), by induction of T-cells to skew towards the Treg phenotype using cytokines, or by forced expression of Foxp3, a nuclear regulator for Treg function. Isolation of Tregs from PBMCs is done using specific Treg markers. Both murine and human Tregs have been classically defined as T-cells that express both CD4 and CD25 (Corthay 2009; Mandapathil *et al.* 2009), and these have been the basis of Treg isolation until recent years. Recently, other markers, such as Foxp3 (engineered to couple its expression with fluorescent proteins), and a high level of CD25 expression have also acted as Treg-specific markers for isolation (Corthay 2009; Mandapathil *et al.* 2009). Even so, there are still many challenges that are present in purifying Tregs from PBMCs, with the first of all being the low number of Tregs in peripheral blood. It is estimated that Tregs only make up 5-10% of T-cells (Duggleby *et al.* 2007). Furthermore, not all Foxp3-expressing cells have a Treg suppressive phenotype; effector CD4$^+$ T-cells can express Foxp3 at low levels as well (Corthay 2009). This expression in effector T-cells has impaired studies that have used Foxp3-reliant fluorescent sorting for Treg isolation. Fortuitously, it has been suggested that high CD39 and low CD127 surface expression can be a better marker in terms of isolating suppressive T-cells (Liu, W *et al.* 2006; Mandapathil *et al.* 2009). These markers allow for purification of naturally-occurring Tregs (nTregs) without relying on Foxp3.

Tregs can also be induced from effector CD4$^+$ T-cells by using a concoction of cytokines and stimulatory factors to create induced Tregs (iTregs). TGF-β1 and IL-2 are both key inducers of the Treg phenotype, as they function by promoting the expression of Foxp3. Other molecules, such as mTOR antagonists and PD-1 agonists, can also promote Treg induction (Ellis *et al.* 2012; Schmitt & Williams 2013). This method is favourable over

isolation from PBMCs as it can have 2-5 times more yield of suppressive Tregs than isolating nTregs (Hippen *et al.* 2011).

The other way to obtain Tregs is by forced expression of Foxp3, through viral transduction of the gene into CD4$^+$ T-cells. In this manner, it has been shown that human effector CD4$^+$ CD25$^-$ T-cells can be induced to have a Treg-type suppression phenotype (Aarts-Riemens *et al.* 2008). Furthermore, in contrast to sorting from PBMC's, viral transduction of Foxp3 to CD4$^+$ T-cells can yield up to three times more Foxp3-expressing T-cells (Wang, YM *et al.* 2006). The life span of Tregs can also be prolonged with the addition of anti-apoptotic genes such as Bcl-xL in the viral construct and can improve their therapeutic efficacy (Haque *et al.* 2010). Obtaining Tregs through viral transduction opens up greater degrees of manipulation as opposed to isolation of nTregs from PBMCs or creating iTregs. This will be the method of Treg generation in the current study.

1.3.2.2 Alloantigen-Specific Tregs versus Polyclonal Tregs

The problem with antigen specificity relates to the potency of Treg adoptive transfer. Alloantigen-specific Tregs are found to have better potency than polyclonal Tregs for immunosuppression in the context of allogeneic transplants (Lee, K *et al.* 2014; Tang & Bluestone 2013; Veerapathran *et al.* 2011). This may be due to higher cell numbers localising to sites of allogeneic inflammation (Dijke 2008). It has been shown previously that failure of Treg migration into inflammatory sites allows for the worsening of inflammation in mouse models (Huehn *et al.* 2004; Siegmund *et al.* 2005). While alloreactive Tregs are better immunosuppressors in graft tolerance than polyclonal Tregs, their population is very low in the blood, and *in vivo* stimulation with alloantigens only produce around 1% alloreactive Tregs (Veerapathran *et al.* 2011). In order to obtain high amounts of alloreactive Tregs, polyclonal Tregs need to be isolated, manipulated, and expanded *ex vivo*.

1.3.2.3 Generating Alloantigen-Specific Tregs

There are several ways to generate alloantigen-specific Tregs. One method is to stimulate isolated Tregs with allogeneic peripheral blood mononuclear cells (PBMCs) or alloantigen-expressing APCs, leading to the expansion of alloantigen-specific Tregs. Another method is by viral transduction of Tregs with engineered receptors to redirect their specificity to alloantigens. This can be done using engineered T-cell receptors (TCRs) or chimeric antigen receptors (CARs). Both methods are able to generate alloantigen-specific Tregs with superior tolerance induction to allografts compared to polyclonal Tregs. They can then be expanded using CD3/CD28 beads or using alloantigen-expressing APCs. The alloantigen-specific Tregs in the current study will be generated by CAR transduction.

1.4 Chimeric Antigen Receptor (CAR)

1.4.1 History of the CAR

In 1989, Eshhar, Gross, and Waks (1989) described the engineering of T-cells expressing an immunoglobulin light or heavy chain variable domain against 2,4,6-Trinitrophenyl (TNP) conjugated to the constant region of TCR genes, C_α or C_β, giving them antibody-like specificity against TNP along with the ability to activate their effector functions. This was the first description of CAR T-cells, T-cells with specificities that can be manipulated by design. This group then modified their construct by producing a linked light chain and heavy chain variable domain, termed the single chain variable fragment (scFv), and fused them with either the γ-chain of the Fc receptor or the ζ-chain of CD3 as co-stimulatory molecules for use in NK cells and T-cells respectively, producing scFvRγ and scFvζ (Eshhar, Z. *et al.* 1993). The chimeric scFvζ became the first generation of CARs that became the pioneering construct for CAR-T-cell research.

Since then, more advanced CARs have been developed. Second-generation CARs (figure 1.6) were designed and created in sequence (Alvarez-Vallina & Hawkins 1996; Finney *et al.* 1998; Krause *et al.* 1998) by the addition of a co-stimulatory molecule, such as CD28, proximal to the ζ-chain, and provided up to 20 times better activation efficiency than first-generation CARs (Finney *et al.* 1998). The modular characteristic of CAR design has allowed for additional co-stimulatory molecules to be conjugated, such as 4-1BB and OX40, and these are the third-generation CARs (Hombach & Abken 2011; Zhao *et al.* 2009). Most recently, CARs have been used as a molecular switch for the targeted expression of a "payload" gene controlled by CAR-induced activation factors, such as nuclear factor of activated T-cells (NFAT) (Chmielewski *et al.* 2011; Hombach & Abken 2011; Uchibori *et al.* 2015). This modularity of CARs allows for design modifications to be tailored for specific purposes.
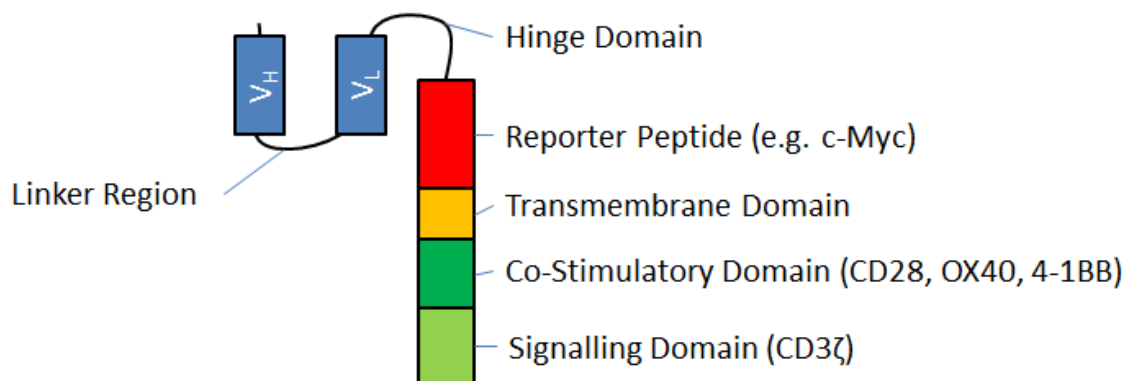
Figure 1.6. Schematic of a typical CAR structure.

1.5 Applications of CAR-T-Cells

1.5.1   CAR-T-Cell Therapy in Cancer

Due to their specificity and ability to activate T-cells independently of MHC molecules, CARs were first developed as a solution for tumours or cancers that evade host immunity. By the 1980s, most immunotherapeutic solutions to target cancers involved the use of anti-tumour associated antigen (TAA) antibodies, which were not able to match the tumour penetration ability of cytotoxic T-cells (CTLs). However, CTLs that are anti-TAA are not common, and so Eshhar and his colleagues developed a method to engineer T-cells to acquire antibody-like specificity against TAAs, using CARs (Eshhar, Zelig 1997). Within the next 5 years after the first CAR was designed, modifications were done to target cancer antigens. Eshhar and his group (1993) modified the specificity of their CAR-T-cells to recognise Neu/HER2, an adenocarcinoma antigen, and Moritz and colleagues (1994) have used a similar construct targeting ERBB2 (a breast cancer antigen). Both CAR-T-cell designs caused antigen-specific cell lysis; and the anti-ERBB2 T-cells were able to localise to sites overexpressing its target antigen. Following this work in pioneering engineering T-cells against cancer associated antigens (CAA), many have followed in their footsteps and modified T-cell specificities via CARs against a variety of other CAAs. CAR-T-cells are being developed in clinical trials for treatment against haematological cancers, such as acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL) (Grupp *et al.* 2013; Porter *et al.* 2011), and for solid tumours, for instance, renal cell carcinoma (RCC), ovarian cancer, and neuroblastoma (Haji-Fatahaliha *et al.* 2016; Kershaw *et al.* 2006; Lamers, CHJ *et al.* 2006; Louis, Chrystal U. *et al.* 2011; Louis, C. U. *et al.* 2011; Park *et al.* 2007). Anti-CD19-CAR-T-cell therapy against ALL and CLL have been reported by several groups to produce successful clinical responses in patients (Brentjens *et al.* 2011; Kalos *et al.* 2011; Kochenderfer *et al.* 2010), with Brentjens (2011) reporting

specific migration of anti-CD19-CAR-T-cells at CD19$^+$ tumour sites. While solid tumours present much more difficult hurdles for CAR-T-cells to overcome, preliminary studies in RCC, ovarian cancer, and neuroblastoma have shown that CAR-modified T-cells were able to specifically lyse cells expressing target antigens, despite the variable responses observed in patients (Hwu 1993; Lamers, CHJCH 2007; Louis, Chrystal U. *et al.* 2011). These studies in the modification of T-cell specificity towards binding cancer antigens have shown the power of CARs to function as a homing molecule for T-cells towards the antigens of interest and their ability to act as a mediator for T-cell activation following antigen stimulation in an MHC-independent manner.

1.5.2 CAR-Treg Therapy in Autoimmune Disease

The benefits of conferring antibody-like specificity to T-cells for targeted cell destruction can also be used to develop immune tolerance to target tissues; by applying CAR technology in Tregs. While CAR-redirection of T-cells has been described for nearly three decades, it has only been applied in Tregs in the past 12 years. In 2005, Mekala *et al.* had redirected CD4$^+$ CD25$^+$ T-cells against myelin basic protein (MBP) and was able to suppress the pathogenicity of MBP-reactive T-cells in an experimental allergic encephalomyelitis (EAE) mouse model (Mekala 2005). Success was also observed by Eshhar's group in 2008 in improving survival of mice with TNP-mediated experimental colitis using TNP CAR-redirected Tregs (Elinav 2008). More significantly, this improvement in survival was specific to the type of antigen used to induce colitis in the mice. Survival was only improved when colitis was induced by TNP, and not by oxazolone (Elinav 2008). These experiments provided proof of concept that CAR-redirected Tregs were able to specifically suppress unwanted immune responses in auto-inflammatory diseases.

1.5.3 Potentials of CAR-Treg Therapy in Allograft Tolerance

Building on the potential shown in autoimmune studies, CAR-Tregs are also able to induce tolerance in proof-of-concept transplantation studies. The idea of using CAR technology to induce alloantigen tolerance is still relatively new, only in the past year have CARs been used to redirect Tregs to the HLA-A2 antigen (MacDonald *et al.* 2016; Noyan *et al.* 2017) for transplantation studies, but they have shown promising results. In the experimental GVHD model described by MacDonald, and also the skin-graft model described by Noyan, alloantigen-specific CAR-A2 Treg-(CAR Treg against the HLA-A2 antigen) infusion ameliorated immune responses against the allografts. These findings show promise in using CAR-Tregs in helping induce tolerance in haematopoietic cell and solid organ transplantation.

1.6 Aim and Hypothesis

This project has two aims:

1. To use CAR technology to generate CAR-A2 Tregs with a stable suppressive phenotype.
2. To compare their antigen-specific suppressive function of CAR-A2 Tregs with the function of polyclonal Tregs with HLA-A2 antigen stimulation.

In contrast to MacDonald and Noyan's studies, Tregs will not be isolated from host mice, but CD4$^+$ T-cells will instead be *ex vivo*-induced to express Foxp3 to promote a more stable suppressive phenotype. Antigen-specific and polyclonal Tregs will be generated from CD4$^+$ T-cells obtained from B6 mice. A2Kb mice (mice expressing a chimeric MHC consisting of parts of HLA-A2) and C57BL/6 (B6) mice will be used as sources of stimulator cells in mixed-lymphocyte reaction (MLR) studies.

We hypothesise that CAR-A2 Tregs will have better suppressive ability in a mixed lymphocyte culture consisting of HLA-A2 antigen stimulation.

1.6.1 Creating CAR-Tregs against the HLA-A2 Antigen

1.6.1.1 Designing the anti-HLA-A2 CAR

The versatility of CARs allows us to insert any scFv gene into a designated CAR backbone to redirect T-cells against the antigen. The CAR backbone has already been established previously in many contexts (Alvarez-Vallina & Hawkins 1996; Chmielewski *et al.* 2011; Finney *et al.* 1998; Gross, Waks & Eshhar 1989; Hombach & Abken 2011; Krause *et al.* 1998; Uchibori *et al.* 2015; Zhao *et al.* 2009), but the anti-HLA-A2 scFv gene has to be sequenced and inserted into the CAR backbone. Methods to generate scFv's include hybridoma selection (MacDonald *et al.* 2016) and phage display (Noyan *et al.* 2017) to screen for antibodies against HLA-A2 and obtain the variable heavy chain (V_H) and light chain (V_L) sequences. They will then be combined to generate an anti-HLA-A2 scFv gene. Previous work has been done by Watkins, *et al.* (Watkins *et al.* 2000) in screening antibodies against HLA-A2 and sequencing them. The V_H and V_L sequences from Watkins, *et al.* were adopted into a scFv gene and inserted it into a second-generation CAR construct obtained from Kershaw, *et al.* of the Peter MacCallum Cancer Centre of Melbourne (John *et al.* 2013). These CAR constructs express c-Myc in the extracellular domain, and this will be used as a marker for selection.
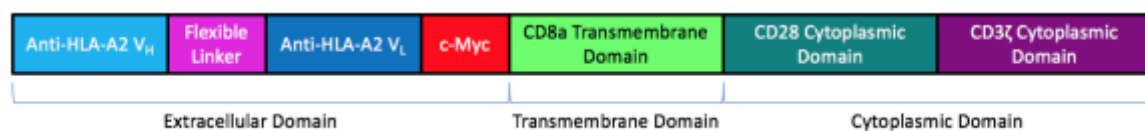
Figure 1.7. CAR-A2 gene design schematic.

1.6.1.2 Generating the Tregs

The Tregs will be created by forced expression of Foxp3 (with a GFP marker) in CD4$^+$ T-cells using retroviral vectors, instead of isolation of nTregs. This is because previous work in this laboratory has shown that CAR-A2-transduced nTregs did not have a stable phenotype when exposed to HLA-A2 *in vivo* as opposed to control CARs (non-specific to any antigen) or polyclonal Tregs. This happens due to the presence of a minor population of phenotypically plastic Foxp3$^+$ Tregs which can have lowered Foxp3 expression (Komatsu *et al.* 2009). We propose that forced Foxp3 expression will prolong the stability of the Treg phenotype and avoid loss of their suppressive capacity *in vivo*. These *ex vivo* induced Foxp3$^+$-GFP Tregs will be co-transduced with CAR-A2-containing retroviral vectors to create CAR-A2 Tregs.

1.6.2 Functional Assessment by MLR

CAR-A2 Tregs will have its general suppressive phenotype compared with polyclonal Tregs by co-culturing with CD4$^+$ CD25$^-$ effector T-cells (Teffs). Non-specific stimulation will be done using splenocytes from B6 or A2Kb mice and suppression will be measured by the proportion of proliferating Teffs, shown by dye dilution of the CellTrace Violet proliferation dye. The more CellTrace Violet is retained in CD4$^+$ Teffs, the less proliferation there is.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Mice

C57BL/6J(ARC) Mice (from the Animal Resources Centre at Perth)

C57BL/6J(Arc) (B6) mice are inbred mice obtained from the Animal Resources Centre (ARC) at Perth. They were the source of T-cells used in the study and were housed in the animal facility within the Westmead Research Hub.

C57BL/6J-TgN(A2KbHLA)6HsdArc Mice (from the Animal Resources Centre at Perth)

C57BL/6J-TgN(A2KbHLA)6HsdArc (A2Kb) mice are transgenic mice with a C57BL/6J(Arc) background expressing the chimeric MHC class I molecule made up of the α1 and α2 domains of HLA-A2.1 and the α3 to the cytosolic domain of H-2Kb on all MHC class I-expressing cells. They were obtained from the ARC and were housed in the animal facility within the Westmead Research Hub.

2.1.2 Cell Lines

EcoPack™ HEK293 Cells

EcoPack™ HEK293 cells (293 cells) are a transformed human embryonic kidney cell line that functions to package transfected plasmid DNA into mouse mammary tumour virus (MMTV) packaging proteins. They were obtained from BD Biosciences.

HLA-A2-GFP K562 Cells

HLA-A2-GFP K562 cells (A2-K562 cells) are a cell line obtained from a female Caucasian suffering from chronic myelogenous leukaemia (CML) transformed to express HLA-A2 that is reported by GFP. They were kindly given to the Centre for Kidney Research (CKR) by Dr. Megan K. Levings of the Levings Lab in the British Colombia Children's Hospital Research Institute.

2.1.3   Reagents

2.1.3.1 Bacteria Culture

Lysogeny Broth (LB)

Lysogeny broth (LB) was made by dissolving 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 5 g of NaCl, and 1 g of D-glucose up to 1 L in Milli-Q water. The solution was autoclaved before use and stored at room temperature.

LB Agar

LB agar was made by dissolving 3.75 g of bacto-agar up to 250 mL in LB. Agar was dissolved by heating the solution with a microwave. 250 μL of 100 mg/mL ampicillin was added before the solution solidified. Agar plates were sealed with parafilm and stored at 4°C.

2.1.3.2 Plasmid Isolation and PCR Reagents

Agarose Gel

The agarose gel for gel electrophoresis was made up of 1.7% agarose in 1X Tris-Acetate-EDTA (TAE) buffer. The mixture was heated in a microwave to completely dissolve the agarose and was cooled down to room temperature before use.

Anti-HLA-A2 Variable Heavy Chain Primers

Anti-HLA-A2 variable heavy chain primers were designed by Dr. Yuan Min Wang and Hamish Wan, with the sequence attached in Appendix 2 – Anti-HLA-A2 Variable Chain Primer Sequence. The primers were stored at -20°C.

Deoxynucleotide Solution Mix (dNTP)

Stock solutions (100 mM) of dATP, dTTP, dCTP, and dGTP were combined together in equal volumes to make a dNTP mixture with 25 mM of each dNTP. The mixture was then diluted in 10 in autoclaved water to make a stock solution of 2.5 mM of each dNTP for regular PCR. For reverse transcription PCR, they were diluted by 2.5 to make a stock solution of 10 mM of each dNTP.

99% Isopropanol (2-Propanol)

Isopropanol was used as a precipitating agent for plasmid DNA.

10X PCR Reaction Buffer with MgCl₂ (Roche – 11271318001)

The 10X stock PCR reaction buffer was stored in -20°C.

QIAGEN Plasmid Maxi Kit (QIAGEN – 12162)

Extraction of amplified plasmids was done using the QIAGEN Plasmid Maxi Kit following their protocol. The kit was stored at room temperature.

1X Tris-Acetate-EDTA (TAE) Buffer

1X TAE buffer was made by diluting 50X TAE buffer by 1:50. 50X TAE buffer was made by dissolving 242.28 g Tris free base, 18.61 g of disodium EDTA, and 57.1 mL of glacial acetic acid in Milli-Q water. It was stored at room temperature.

Taq DNA Polymerase (Roche – 11596594001)

Taq DNA polymerase was supplied in 250 U/bottle with a stock concentration of 5 U/μL. It was stored at -20ºC.

20X TaqMan™ qPCR Mixture for Anti-HLA-A2 Variable Heavy (V_H) Chain (Applied Biosystems)

The qPCR primer and probe mixture for the anti-HLA-A2 V_H chain gene was designed by Yuan Min Wang and Hamish Wan and was made by Applied Biosystems by referring to the gene sequence attached in Appendix 2 – Anti-HLA-A2 Variable Chain Primer Sequence. The mixture was stored at -20ºC.

2.1.3.3 Calcium Phosphate Transfection Reagents

2 M CaCl$_2$ (working concentration: 0.125 M)

The 2 M CaCl$_2$ solution was made by dissolving 22.196 g of CaCl$_2$ up to 100 mL in Milli-Q water. The solution was filtered through a 0.22 μm membrane before use and stored at room temperature.

2X HEPES-buffered saline (HBS) (working concentration: 0.99X)

The 2X HBS solution was made by dissolving 280 mM of NaCl and 50 mM of HEPES in Milli-Q water. The solution was pH adjusted to 7.1 and was filtered through a 0.22 μm membrane before use. The solution was stored at room temperature.

0.15 M Na$_2$HPO$_4$ (working concentration: 0.75 mM)

The 0.15 M Na$_2$HPO$_4$ solution was made by dissolving 10.647 g of Na$_2$HPO$_4$ up to 500 mL in Milli-Q water. The solution was pH adjusted to 7.1 and filtered through a 0.22 μm membrane before use. The solution was stored at room temperature.

10% Tris-EDTA (TE) Buffer

The 10% TE buffer was made by diluting 100% TE buffer in Milli-Q water by a factor of 10. The 100% TE buffer was made by combining 1 M Tris-Cl (pH adjusted to 7.5) and 0.5 M Na-EDTA (pH adjusted to 8.0) at a 5:1 ratio, and diluted with Milli-Q water to reach a final concentration of 1 mM Tris-Cl and 0.1 mM Na-EDTA. The final solution was filtered through a 0.22 μm membrane before use and stored at room temperature.

2.1.3.4 Cell Culture Media

2.1.3.4.1   Media Reagents

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco™ – 11995-065)

DMEM was stored at 4°C and was used to make 293 cell media.

Foetal Calf Serum (FCS, Heat-Inactivated) - Qualified for USDA-approved regions (Gibco™ - 10437028)

FCS was made into 50 mL aliquots and heat-inactivated by subjecting the aliquots to 52°C for 30 minutes and stored in -80°C.

HEPES Buffer (Gibco™ – 15630-106)

HEPES buffer was supplied at 1 M and was stored at 4°C.

Human Recombinant IL-2

Human recombinant IL-2 was supplied at 20 U/μL and was stored at -80°C. After thawing, they were stored at 4°C.

L-Glutamine

L-glutamine was supplied at a stock concentration of 200 mM and was stored at -80°C. After thawing, the solution was stored at 4°C.

2-Mercaptoethanol (Gibco™ – 21985-023)

2-mercaptoethanol was supplied at 1000X concentration and was stored at 4ºC.

Penicillin-Streptomycin

Penicillin-streptomycin was supplied at a stock concentration of 10,000U/mL and was stored at -80ºC. After thawing, the solution was stored at 4ºC.

Roswell Park Memorial Institute-1640 (RPMI) Medium (Lonza – BE12-167F)

RPMI medium was stored at 4ºC and was used to make T-cell and K562 cell media.

2.1.3.4.2   Culture Media Solutions

EcoPack™ 293 Cell Medium

The 293 cell medium was a solution of DMEM with 10% heat-inactivated FCS. They were stored at 4ºC and kept sterile.

T-cell Medium

The T-cell medium was a solution of RPMI medium, with the following in their final concentrations: 10% heat-inactivated FCS, 16.5 mM HEPES buffer, 100 U/mL of penicillin-streptomycin, 2 mM of L-glutamine, and 55 μM of 2-Mercaptoethanol. The solution was filtered through a 0.22 μm membrane before use and stored at 4ºC.

K562 Cell Medium

The K562 cell medium was a solution of RPMI medium, with the following in their final concentrations: 10% heat-inactivated FCS, 100 U/mL of penicillin-streptomycin, and 2 mM of L-glutamine. The solution was filtered through a 0.22 μm membrane before use and stored at 4ºC.

2.1.3.5 Viral Transduction

Retronectin

Retronectin was supplied as a 1 mg/mL solution and was diluted to 50 μg/mL with PBS. The solution was then stored in -20°C.

Vectofusin-1 (Miltenyi Biotec - 130-111-163)

Vectofusin-1 was supplied as a lypophilised peptide and reconstituted with autoclaved Milli-Q water making a stock concentration of 1 mg/mL. Its working concentration is 10 μg/mL. The reconstituted solutions were stored in -20°C.

2.1.3.6 Antibodies, Fluorescent Markers, and Magnetic Separation Beads

The following antibodies, fluorescent markers, and magnetic separation beads were used throughout this project:

- **7-AAD (BD Pharmingen™ - 559925)**

- **Anti-mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec - 130-049-201)**

- **Hamster IgG$_1$ anti-mouse CD3ε (purified) (BD Pharmingen™ - 553058)**

- **Hamster IgG$_2$ anti-mouse CD28 (purified) (BD Pharmingen™ - 553294)**

- **Rat IgG$_{2a}$ anti-mouse CD4 (APC) (BD Pharmingen™ - 553051)**

- **Rabbit polyclonal anti-Myc tag antibody (Biotin) (Abcam - ab34773)**

- **Streptavidin (APC) (BD Pharmingen™ - 554067)**

2.1.3.7 Other Reagents

Ammonium-Chloride-Potassium (ACK) Lysis Buffer

The ACK buffer was a solution of 150 mM NH_4Cl, 10 mM of K_2HCO_3, and 0.1 mM of Na-EDTA dissolved in Milli-Q water, pH adjusted to 7.23. The solution was autoclaved before use and stored at room temperature.

Ampicillin

Ampicillin was supplied in a solution of 100 mg/mL, aliquoted into 1.5 mL Eppendorf tubes, and was stored at -20°C.

CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher - C34557)

The CellTrace™ violet proliferation detection kit was supplied as lyophilised powder and was reconstituted in DMSO to give a stock solution of 5 mM. It was stored at -20°C. The working concentration of 5 μM was achieved by diluting the dye in 1X PBS.

Dimethyl Sulfoxide (DMSO)

DMSO was used along with foetal calf serum (FCS) as cryoprotectants for cell storage in liquid nitrogen. DMSO was used at 10% working concentration and was stored at room temperature away from direct light.

Mouse Treg Expansion Kit (Miltenyi Biotec - 130-095-925)

The Mouse Treg Expansion Kit is a solution of CD3/CD28 MACSiBead particles. They were stored at 4°C. The solution contained 60,000 beads/μL.

1X Phosphate-Buffered Saline (PBS)

The 1X PBS solution was made by diluting a 20X PBS solution in Milli-Q water. The 20X PBS solution was made by dissolving 160 g of NaCl, 4 g of KCl, 4 g of KH_2PO_4, and 40 g of Na_2HPO_4 up to 1 L in Milli-Q water. The solution was autoclaved before use and stored at room temperature.

Super Optimal Broth with Catabolite Repression (SOC) Medium (Invitrogen™ – 15544-034)

The SOC medium was used for bacterial transformation and was stored at room temperature.

Trypsin-EDTA (0.25%) with Phenol Red (Gibco™ - 25200114)

The 0.25% trypsin-EDTA reagent was supplied in solution form and was stored at -80°C. After thawing, they were stored at 4°C.

2.2 Methods

2.2.1 Plasmid Amplification

2.2.1.1 Bacterial Transformation

Escherichia coli strain JM109 was used to amplify CAR-A2-myc, CAR-HER2-myc (a CAR against the HER2 antigen), and Foxp3-GFP plasmid DNA. The *E. coli* was transformed with plasmid DNA by heat shock. Frozen *E. coli* was recovered from -80°C and was mixed with 50 ng of plasmid DNA for every 50 µL of competent cells in ice for 10 minutes and was heated at 42°C for 45 seconds. They were immediately cooled down in ice. Super Optimal broth with Catabolite repression (SOC) medium was supplemented to 1 mL and the mixture incubated at 37°C for 1 hour. 100 µL of *E. coli* was then plated on an LB agar (1.5% w/v of agar) supplemented with ampicillin at 0.1 mg/mL of agar solution. The *E. coli* was left to culture overnight at 37°C and positive colonies were selected for further culture.

2.2.1.2 Plasmid Maxiprep

Positive colonies were cultured in 7 mL of LB broth supplemented with 0.1 mg/mL of ampicillin and shaken for 6-8 hours at 225 rpm at 37°C for the plasmid miniprep. The miniprep was then added into 400 mL of LB broth supplemented with 0.1 mg/mL of ampicillin and shaken overnight at 225 rpm at 37°C. The resulting maxiprep was harvested

the next morning and the plasmids extracted by following QIAGEN's maxiprep plasmid extraction protocol with modifications in centrifugation speeds. The maxiprep was centrifuged at 4560 g for 15 minutes at 4°C and the supernatants replaced with 10 mL of the P1 resuspension buffer. After *E. coli* have been completely resuspended, 10 mL of lysis buffer P2 is added and mixed thoroughly. The solution is set at room temperature for 5 minutes. 10 mL of the pre-chilled neutralisation buffer P3 was then added and mixed thoroughly and rested for 10 minutes at room temperature while the DNA isolation column was equilibrated with equilibration buffer QBT. The whole *E. coli* mixture was then compressed in a syringe to allow for only the supernatant to flow through the DNA isolation column. The column was washed with 2 X 30 mL of wash buffer QC, and DNA was finally eluted with 15 mL of elution buffer QF. DNA was then precipitated with 10.5 mL of room temperature isopropanol, and centrifuged at 4060 g for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed with 5 mL of 75% ethanol, and centrifuged again at 4060 g for 10 minutes at 4°C. The supernatant was discarded and the pellet was air-dried for 10 minutes, and finally resuspended in 0.25-0.5 mL of autoclaved water. The concentration and purity of the purified plasmid DNA was analysed using a nanodrop spectrophotometer.

2.2.1.3 PCR of Plasmid DNA

Plasmid DNA products were tested by PCR. A solution of 1X PCR reaction buffer, 0.5 µg/µL of dNTP mixture, 2-3 ng/µL of forward and reverse primers, 4-12 ng/µL of sample DNA, and 0.058 U/µL of Taq polymerase was amplified in a PCR thermocycler with the following reaction cycle:

Denaturation: 94°c for 5 minutes for 1 cycle.

Annealing: 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 minute for 35 cycles.

Extension: 72°C for 10 minutes for 1 cycle.

Storage: 4°C indefinitely.

The amplified product was then mixed with DNA loading buffer at a 1:1 ratio and was loaded into a 1.7% agarose gel immersed in 1X TAE buffer for gel electrophoresis. Gel electrophoresis was run at 140V for 40 minutes and was stained with ethidium bromide for 15 minutes while rocking. The stained product was then brought to image using the Bio-Rad ChemiDoc MP Imaging System in the KRI.

2.2.1.4 qPCR of Plasmid DNA

Plasmid DNA products were also tested by qPCR. A solution of 0.8X PCR reaction buffer, 0.4 μg/μL of dNTP mixture, 0.24X of the TaqMan qPCR mixture, 4-12 ng/μL of sample DNA, and 0.02 U/μL of Taq polymerase was amplified into the QIAGEN Rotor-Gene Q qPCR thermocycler following their pre-set reaction cycle:

Activation: 95°C for 5 minutes for 1 cycle.

Cycling: 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles.

Melt curve analysis: 90 seconds at 60°C and rising by 1°C every 5 seconds up to 99°C.

2.2.2   Retroviral Packaging Cells

2.2.2.1 Recovering the EcoPack™ 293 Cells from Storage

EcoPack™ 293 cells were used to produce retroviral vectors of our CAR-A2, CAR-HER2 and FoxP3 plasmid DNA in mouse mammary tumour virus (MMTV) packaging proteins. 293 cells were thawed from liquid nitrogen in a 37°C water bath and washed with DMEM. 293 cells were then centrifuged at 350 g at 4°C for 5 minutes, and the supernatant was replaced with DMEM supplemented with 10% foetal calf serum (FCS). Cells were

cultured in a 10 cm culture dish at 2.5×10^6 cells in 10 mL of medium, or about 15-20% confluence. They were incubated in a 37°C and 5% CO_2 incubator.

2.2.2.2 EcoPack ™ 293 Cell Passaging

293 cells were subcultured into multiple plates using 0.25% w/v trypsin-EDTA every 2-3 days and 48 hours before transfection. 2-3 mL of Trypsin solution was used to cover one 10 cm plate of 293 cells, and was incubated at 37°C for 5 minutes. The cells were then washed with 10 mL of sterile DMEM (no serum), and centrifuged at 350g for 5 minutes at 4°C. The resulting pellet was resuspended in complete DMEM media and then diluted 1:7 in culture plates. For plasmid transfection, cells were cultured in a 10 cm culture dish at about 10-15% confluence in 10 mL of medium 48 hours prior to transfection (reaching between 40-60% confluence for transfection).

2.2.2.3 Calcium Phosphate Transfection

Plasmid DNA transfection was performed with calcium phosphate by combining the following solutions:

Solution A: 125 mM $CaCl_2$, 7% Tris-EDTA Buffer (1 mM Tris and 0.1 mM EDTA, pH 7.5), and 60 μg/mL of solution A per 10 cm plate of plasmid DNA were mixed together to make solution A.

Solution B: 2X HEPES-buffered saline (HBS, 50 mM HEPES and 280 mM NaCl, pH 7.1) and 1.5 mM Na_2HPO_4 (pH 7.1) were mixed together to make solution B.

Both solutions were mixed together at a 1:1 ratio by pipetting 1/8th of the volume of solution A to B at a time (Jiang, M), and then incubated for 20 minutes at room temperature. One mL of the mixture was used to transfect one 10 cm culture dish of 293 cells. 293 cells were further cultured for 3 days before the supernatants were collected and

filtered through a 0.45 μm filter. Supernatants were either stored at 4°C (short term) or -80°C (long term).

2.2.2.4 EcoPack™ 293 Cell Storage

293 cells were stored by subjecting them to 2 mL of 0.25% w/v trypsin-EDTA at 37°C for 5 minutes. They were washed with sterile DMEM, centrifuged at 350 g at 4°C for 5 minutes, and resuspended cells in 90% FCS and 10% DMSO up to 1 mL. They were then stored in -80°C overnight in a CoolCell® freezing container and moved to liquid nitrogen storage the next day.

2.2.2.5 EcoPack™ 293 Cell Transfection Analysis

The transfection efficiency of different plasmid combinations on 293 cells were tested by flow cytometry. Transfected 293 cells were trypsinised with 2 mL of 0.25% trypsin for 5 minutes at 37°C and filtered through a 35 μm filter cap, washed with 1-3 mL of 1X PBS (centrifuged at 350 g at 4°C for 5 minutes and discarded the supernatant) and stained with anti-Myc tag (biotin) antibodies detected using of APC-Streptavidin. 1 μL of the primary anti-Myc tag antibody was incubated into the cells for 1 hour away from light at 4°C, washed with 1-3 mL of 1X PBS, and stained with 1 μL of APC-Streptavidin for 30 minutes away from light at 4°C. The cells were then washed with 1-3 mL of 1X PBS and resuspended in 200 μL of 1X PBS and analysed by flow cytometry.

2.2.3 T-cell Culture and Transfection

2.2.3.1 Lymphocyte Preparation from Mouse Spleens

B6 mice were culled and were harvested for spleens. Spleens were homogenised in 1X PBS and the red blood cells were lysed by replacing the PBS supernatant with 1 mL of ACK buffer per spleen through centrifugation (350 g at 4°C for 5 minutes) and incubating at room temperature for 2 minutes. Splenocytes were then centrifuged at 350 g at 4°C for 5

minutes, and the supernatant was replaced with 1 mL of 1X phosphate buffered saline (PBS) per spleen used. Lymphocytes were then counted using a haemocytometer. Experiments were carried out in accordance to protocols approved by the Animal Ethics Committee of the Western Sydney Local Health District.

2.2.3.2 Separating CD4⁺ T-cells from CD4⁻ T-cells

$CD4^+$ T-cells were separated from $CD4^-$ T-cells by magnetism-activated cell sorting (MACS). 60 μL of anti-CD4 antibodies conjugated to magnetic MicroBeads™ from Miltenyi Biotec was used to separate $CD4^+$ T-cells from 10^8 lymphocytes. Anti-CD4 MicroBeads™ were incubated in the lymphocytes for 30 minutes at 4°C away from light, and the solution was washed with 5X the volume with 1X PBS and centrifuged at 350g at 4°C for 5 minutes. The supernatant was replaced with 1X PBS and resuspended. Separation was done by using a MACS separation column applied to a magnet. The MACS column was first prepared by rinsing 1X PBS through the column that is attached to the magnet. The lymphocytes were then applied through the column, and rinsed with 1X PBS. This first flowthrough contained $CD4^-$ cells. The MACS column was then removed from the magnet, and then rinsed with 1X PBS, and pressure applied with a plunger. This second flowthrough contained $CD4^+$ cells.

2.2.3.3 T-cell Expansion

Wells (one well for one spleen) of a 24-well plate were coated with 0.3 mL of 5 μg/mL anti-CD3ε antibodies at 4°C the day before $CD4^+$ cell isolation and washed with 1X PBS before use. $CD4^+$ and $CD4^-$ cells were resuspended in T-cell medium at 10^7 cells/mL. One well contained 1 mL of $CD4^+$ cells and the other contained 1 mL of $CD4^-$ cells. Anti-CD28 antibodies (final concentration of 5 μg/mL) were added along with human recombinant IL-2 (final concentration of 100 U/mL) to each well, and plates were left to incubate at 37°C and 5% CO_2 for 24 hours before retroviral transduction.

2.2.3.4 Retroviral Transduction of CD4+ T-cells

CD4+ T-cells were transfected with retroviruses produced in 2.2.2.3. CD4+ T-cells were transferred to wells of another 24-well plate coated with 50 μg/mL of Retronectin at 4°C 24 hours prior (washed with 1X PBS before use). The supernatant was first concentrated at 3000 g for 30 minutes at 20°C in a Vivaspin® concentrator tube. Concentrated retroviral supernatants were mixed with a Vectofusin solution dissolved in DMEM at 10 μg/mL of total culture volume at a 1:1 volume ratio. The mixture was incubated at room temperature for 5-8 minutes and then transferred to the CD4+ T-cells. The culture plate was then centrifuged at 1000 g for 1 hour at 32°C. They were then incubated at 37°C and 5% CO_2 for 3-4 days.

2.2.3.5 Flow Cytometric Detection and Sorting of Transduced T-cells

To detect transduced CD4+ T-cells, CD4+ T-cells were stained with anti-Myc tag (biotin) detected by APC-Streptavidin. Cells were collected into FACS tubes and washed with 1-3 mL of 1X PBS (depending on cell number), centrifuged at 350g at 4°C for 5 minutes (this speed is used for washing), and had their supernatants discarded. Cells were then incubated with 1 μL anti-Myc tag (biotin) antibody at 4°C away from light for 1 hour. They were then washed once and incubated with the detecting 1 μL APC-Streptavidin at 4°C away from light for 30 minutes to 1 hour. They were washed and resuspended in 200-300μL of 1X PBS and analysed by flow cytometry.

For cell sorting, 5 μL of each antibody was used, and an additional straining step was done through a 35μm cell-strainer cap into sterile FACS tubes before the final wash step. Sterile sort tubes were prepared by adding 5 drops of FCS for each population sorted.

2.2.3.6 CD4+ T-cell Maintenance

Following sorting, Foxp3- cells were centrifuged at 350g at 4°C for 5 minutes and their supernatants were discarded. They were then resuspended in T-cell medium at 1-10 x 10^6

cells/mL and each population was cultured in a u-bottom 96-well plate. CD4+ T-cells were maintained every 3-4 days by topping up with 100-150 μL of T-cell media and 100 U/mL of IL-2 and resuspending them. They were incubated in a 37°C and 5% CO_2 incubator.

2.2.3.7 Treg Expansion

Following sorting, Foxp3+ cells were centrifuged at 350g at 4°C for 5 minutes and their supernatants were discarded. They were then resuspended in T-cell medium at 1×10^6 cells/mL. Foxp3+ cells were expanded in culture with CD3/CD28 Treg expansion MicroBeads (Miltenyi Biotec) at a 4:1 bead to cell ratio and topped up with T-cell media to a total of 150μL with 2000 U/mL of IL-2 at 37°C and 5% CO_2 in a u-bottom 96-well plate. On the third day, 100-150 μL of T-cell medium with 2000U/mL of IL-2 was topped up for each well.

2.2.4   Preparation of Stimulator Cells for MLR

2.2.4.1 Recovering A2-GFP K562 Cells from Liquid Nitrogen

K562 cells were thawed from liquid nitrogen in a 37°C water bath and washed with K562 cell medium. K562 cells were then centrifuged at 350 g at 4°C for 5 minutes, and the supernatant was replaced with 10 mL of K562 cell medium. Cells were cultured in a 50 mL flask at 37°C and 5% CO_2.

2.2.4.2 Maintenance of A2-GFP K562 Cells

A2-GFP K562 cells were maintained in culture by splitting cells into two flasks or for storage (2.2.4.3) and topping up with K562 medium up to 10 mL. This was done every 2-3 days depending on the growth rate of the cells (observed by colour change of medium).

2.2.4.3 A2-GFP K562 Cell Storage

K562 cells were stored by centrifugation (350 g at 4°C for 5 minutes) and resuspension in 90% FCS and 10% DMSO up to 1 mL. They were then stored in -80°C overnight in a CoolCell® freezing container and moved to liquid nitrogen storage the next day.

2.2.4.4 Processing B6 and A2Kb Murine Spleens

B6 and A2Kb splenocytes were also used as stimulator cells for the mixed lymphocyte reaction in this project. Lymphocyte preparation was performed following the protocol from 2.2.3.1. 1/5 of the total splenocytes were then collected into round-bottom tubes, centrifuged at 350 g at 4°C for 5 minutes, and were taken to be irradiated. The rest of the splenocytes were stored by centrifugation (350 g at 4°C for 5 minutes) and resuspension in 90% FCS and 10% DMSO, and aliquoted to contain 1/5 of total splenocytes in each cryogenic tube. They were then stored in -80°C overnight in a CoolCell® freezing container and moved to liquid nitrogen storage the next day. Each tube of frozen splenocytes was recovered on the day of performing mixed lymphocyte reactions (MLR). These cells were thawed from liquid nitrogen in a 37°C water bath and washed with T-cell medium, centrifuged at 350 g at 4°C for 5 minutes, and the pellet was irradiated. Cells were kept at 5-10 x 10^6 cells/mL at a 37°C and 5% CO_2 incubator. Experiments were carried out in accordance to protocols approved by the Animal Ethics Committee of the Western Sydney Local Health District.

2.2.4.5 Irradiating Stimulator Cells

Stimulator splenocytes were collected in a tube and pelleted with the supernatant removed, and irradiated with ionizing radiation on the day MLRs are performed. 25 Gy of radiation was given for 4.74-4.78 minutes before they were ready to be put into the MLR. After irradiation, splenocytes were resuspended with 1 mL of T-cell medium.

2.2.5   Mixed Lymphocyte Reaction (MLR)

2.2.5.1 Proliferation Tracing

Proliferation was tracked by using the CellTrace™ Violet Proliferation Kit by ThermoFisher Scientific. This dye labels by covalent binding of free amines on the surface and inside the cell and traces proliferation of successive generations by dye dilution. Double negative (Control), CAR-A2$^+$, and CAR-HER2$^+$ CD4$^+$ Foxp3$^-$ T-cells (Teffs) were labelled with CellTrace™ Violet at 5 µM for 10^6 cells/mL by incubating the cells with the right amount of the dye at 37°C for 20 minutes away from light. Excess dye was washed by adding 5 volumes of complete T-cell medium and incubating at room temperature away from light for 5 minutes. They were then pelleted and resuspended in T-cell medium.

2.2.5.2 Proliferation Assay

The proliferation assay was performed to test the response of CAR-A2$^+$ T-cells to the HLA-A2 antigen. It was done by incubating labelled CD4$^+$ Foxp3$^-$ Teffs (either Control, CAR-A2$^+$, or CAR-HER2$^+$) with irradiated B6 or A2Kb splenocytes at a 1:1 ratio to a total of 12,000-100,000 cells in u-bottom wells of 96-well plates. Each well was then topped up with T-cell medium containing 100 U/mL IL-2 to 150 µL. All cells were stained with 1 µL of anti-mouse CD4-APC antibodies and 5 µL of 7-AAD on the fifth day of co-culture and analysed by flow cytometry. The proliferation baseline was a well of Violet-labelled Control CD4$^+$ Foxp3$^-$ T-cells without any additional stimulus.

2.2.5.3 Suppression Assay

The suppression assay was performed to test the suppression response of CAR-A2$^+$ Foxp3$^+$ Tregs to the HLA-A2 antigen to suppress the allogeneic proliferation of CD4$^+$ Teffs. It was done by incubating Violet-labelled Control CD4$^+$ Foxp3$^-$ Teffs with irradiated B6 or A2Kb splenocytes together with either polyclonal, CAR-A2$^+$, or CAR-HER2$^+$ CD4$^+$ Foxp3$^+$ Tregs at a 1:1:1 ratio to a total of 30,000-150,000 cells in u-bottom wells of 96 well plates.

Each well was then topped up with 150 μL of T-cell medium. All cells were stained with 1 μL of anti-mouse CD4-APC antibodies and 5 μL of 7-AAD on the fifth day of co-culture and analysed by flow cytometry.

2.2.6 Statistics

The ANOVA statistical test was done using GraphPad Prism 7 with the "Analyze" tool.

Chapter 3   Results

3.1 CAR-A2 and Foxp3 Plasmids

3.1.1   CAR-A2 Insert in the pSAMEN Vector

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TAACAGCCCCTCTCTCCAAGCTCACTTACAGGCTCTCTACTTAGTCCAGCACGAAGTCTGGAGACC
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CCTGCAGACCACCCCCACCGCCCTCAAAGTAGACGGCATCGCAGCTTGGATACACGCCGCCCACGT
GAAGGCTGCCGACCCCGGGGGTGGACCATCCTCTAGACTGACGCGGCCGCTACGTACCATGGATTT
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AGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGATCCCTTTTGGGTGCTGGTGGTGGTTGGTGG
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TTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGA
TACACCTGCAAAGGCGGCACAACCCCA
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Figure 3.1. CAR-A2-pSAMEN plasmid sequence. Highlighted in yellow are the restriction enzyme sites to replace the CAR-HER2 scFv gene to CAR-A2 scFv. The turquoise region is the heavy chain, the pink region is the linker sequence, and the blue region is the light chain. The red region is the c-Myc reporter tag to detect the expression of the protein. The green region is the CD8 linker sequence, the teal region is the CD28

sequence, and the violet region is the CD3ζ sequence. Grey regions are sections of the pSAMEN vector backbone. This sequence was modified from Dr. Yuan Min Wang.

3.1.2   CAR-A2 and Foxp3 Plasmids and Their Gene Products
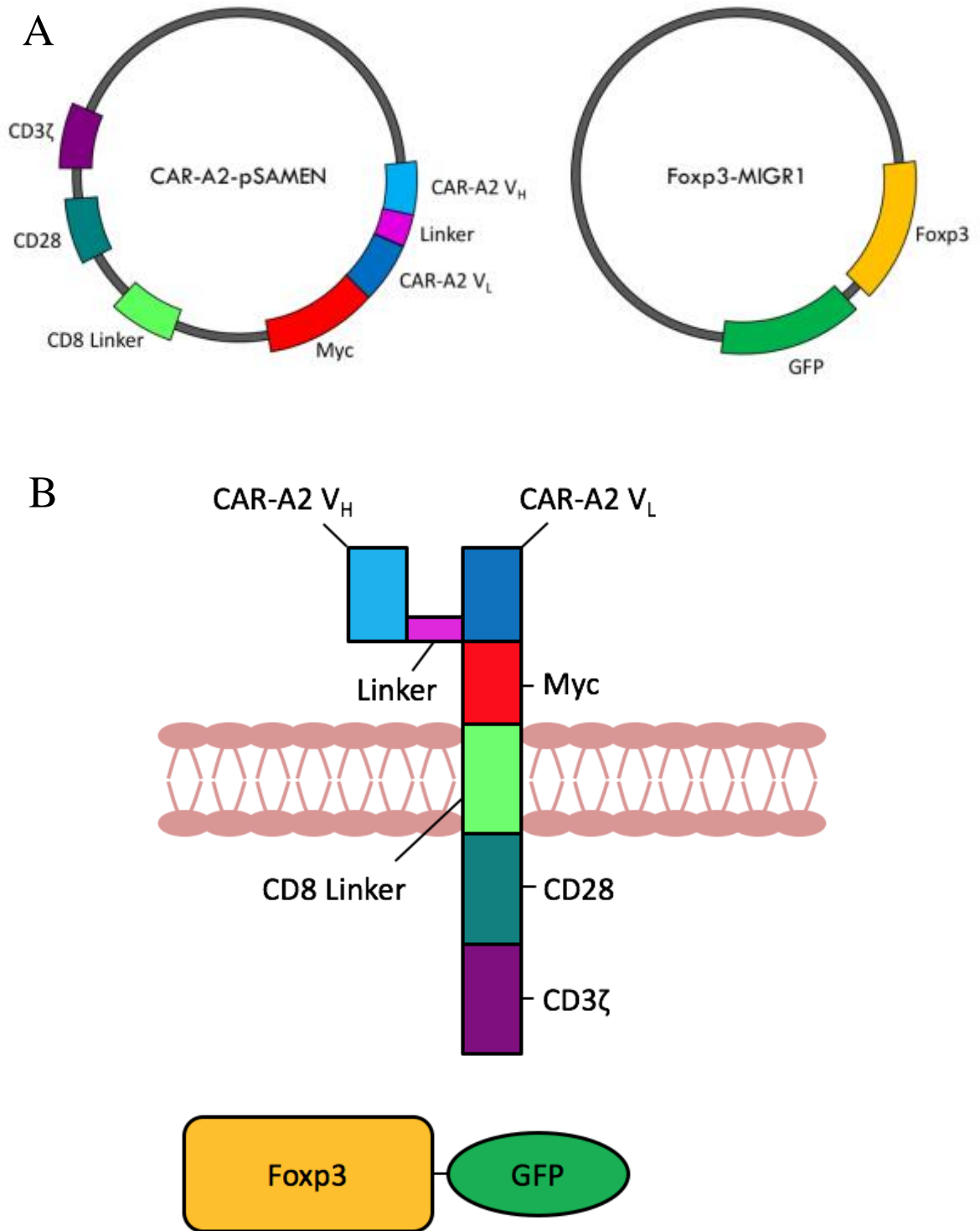
Figure 3.2. Schematic of CAR-A2-pSamen and Foxp3-MIGR1 plasmids and their gene products. The pSAMEN backbone was obtained from Kershaw, et al. (Kershaw *et al.* 2006) and Foxp3-MIGR1 was obtained from Hori, et al. (Hori, Nomura & Sakaguchi 2003). A) Schematic of plasmids used in the study. B) Schematic of plasmid products in transduced cells.

Figure 3.1 shows the sequence of the CAR-A2 plasmid that we used throughout the project. The scFv sequences were derived from Watkins et al. and can be located with GenBank accession numbers AF163303 and AF163308 (Watkins *et al.* 2000). Representations of the plasmids and their products are shown in figure 3.2. CAR-A2 is a transmembrane receptor with an extracellular domain for antigen interactions and an intracellular domain for signal transduction, while Foxp3 is a transcription factor protein.

3.1.3   PCR Confirmation of CAR-A2 and Foxp3 Plasmids
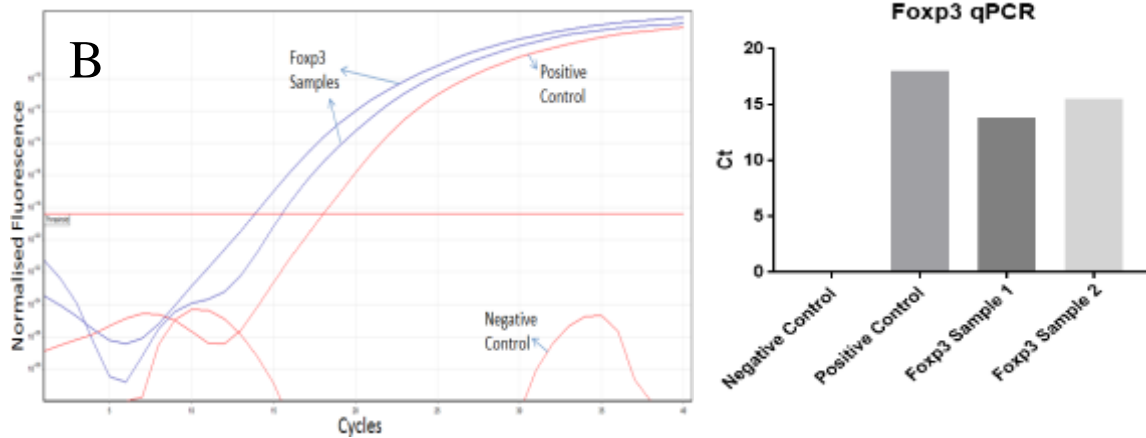
Figure 3.3. Confirmation of plasmids by PCR. A) Confirmation of CAR-A2 plasmid by PCR. Two colonies were selected from two different plates of CAR-A2-transformed *Escherichia coli* bacteria and were lysed by heating them up to 100ºC for five minutes. The lysates were then tested for the presence of the CAR-A2 plasmid by PCR. B) Confirmation of Foxp3-MIGR1 plasmid by qPCR. Amplified Foxp3-MIGR1 plasmids were confirmed to be the right plasmids by qPCR. qPCR was performed by Jevin Karunia.

Referring to the primer sequences in Appendix 2 – Anti-HLA-A2 Variable Chain Primer Sequence, the size of the CAR-A2 heavy chain gene between the forward and reverse primers is 316 bp, which aligns with the observed bands from PCR (figure 3.3A), confirming their presence in the two colonies of CAR-A2-transformed *E. coli* bacteria. These colonies went on to be amplified to obtain larger amounts of CAR-A2 plasmid. The Foxp3 plasmids that were amplified were also tested by qPCR using TaqMan primers from Dr. Yuan Min Wang (figure 3.3B). The rise in fluorescence compared to the negative control and parallel to the positive control confirms the presence of Foxp3 in the MIGR1 vector backbone.

3.2 Calcium Phosphate Transfection of EcoPack™ 293 Cells

3.2.1 Poor Transfection and Viability of 293 Cells
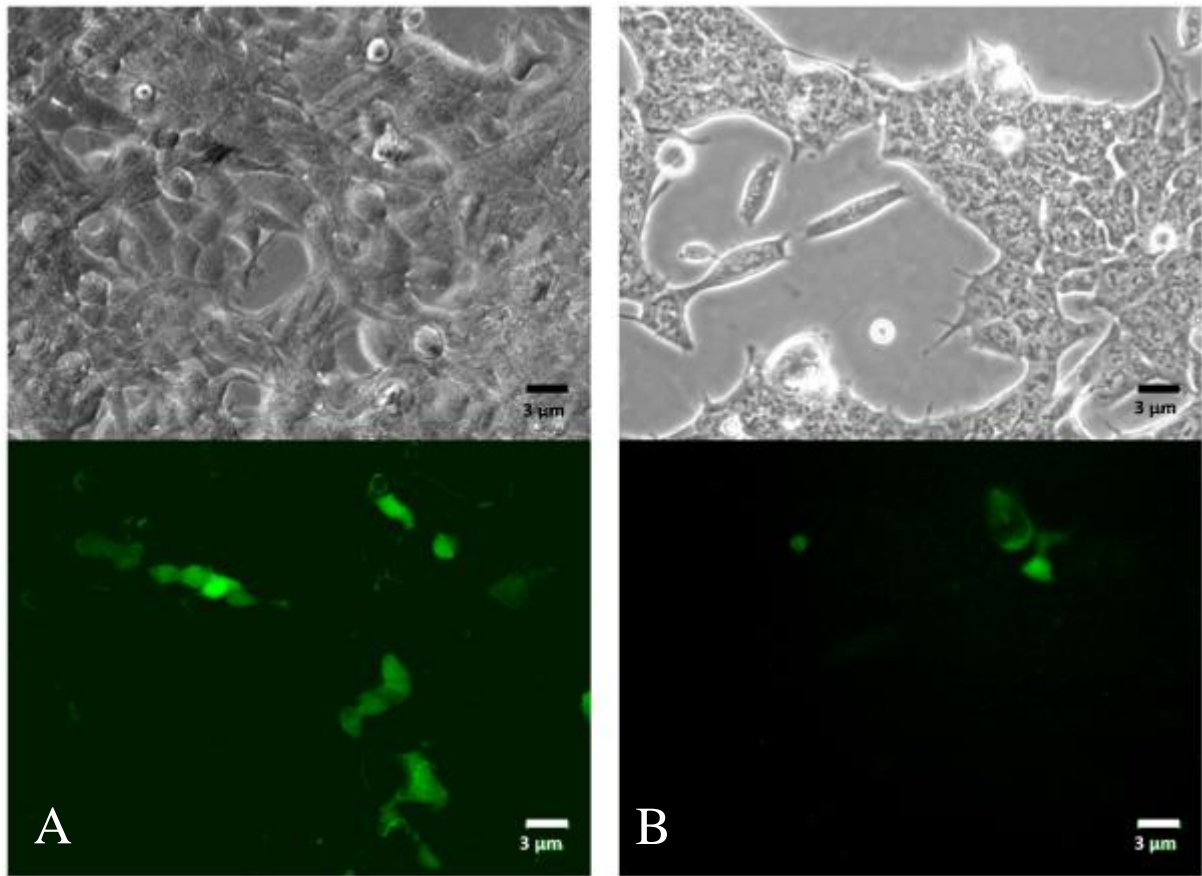
Figure 3.4. Healthy and unhealthy EcoPack™ 293 cells. A) Healthy 293 cells co-transfected with CAR-A2 and Foxp3 from 21/04/2017. B) Unhealthy 293 cells co-transfected with CAR-A2 and Foxp3 from 08/06/2017.

Following plasmid amplification, the two plasmids were used to transfect EcoPack™ 293 cells to produce retroviruses of the CAR-A2 and Foxp3 plasmids for T-cell transduction. Some hurdles in producing high retroviral titres for T-cell transduction occurred in 293 cell culture and transfection. 293 cells went from healthy (figure 3.4A) to unhealthy (figure 3.4B), which could have occurred due to infrequent passaging and overgrowth, characterised by the lack of visible cell borders and markedly reduced transfection

efficiency (figure 3.4B). With the help of Dr. Samantha Ginn, the 293 cell culture protocol was modified to promote healthier growth and better transfection efficiency.

3.2.2 Optimisation of Calcium Phosphate Transfection Protocol
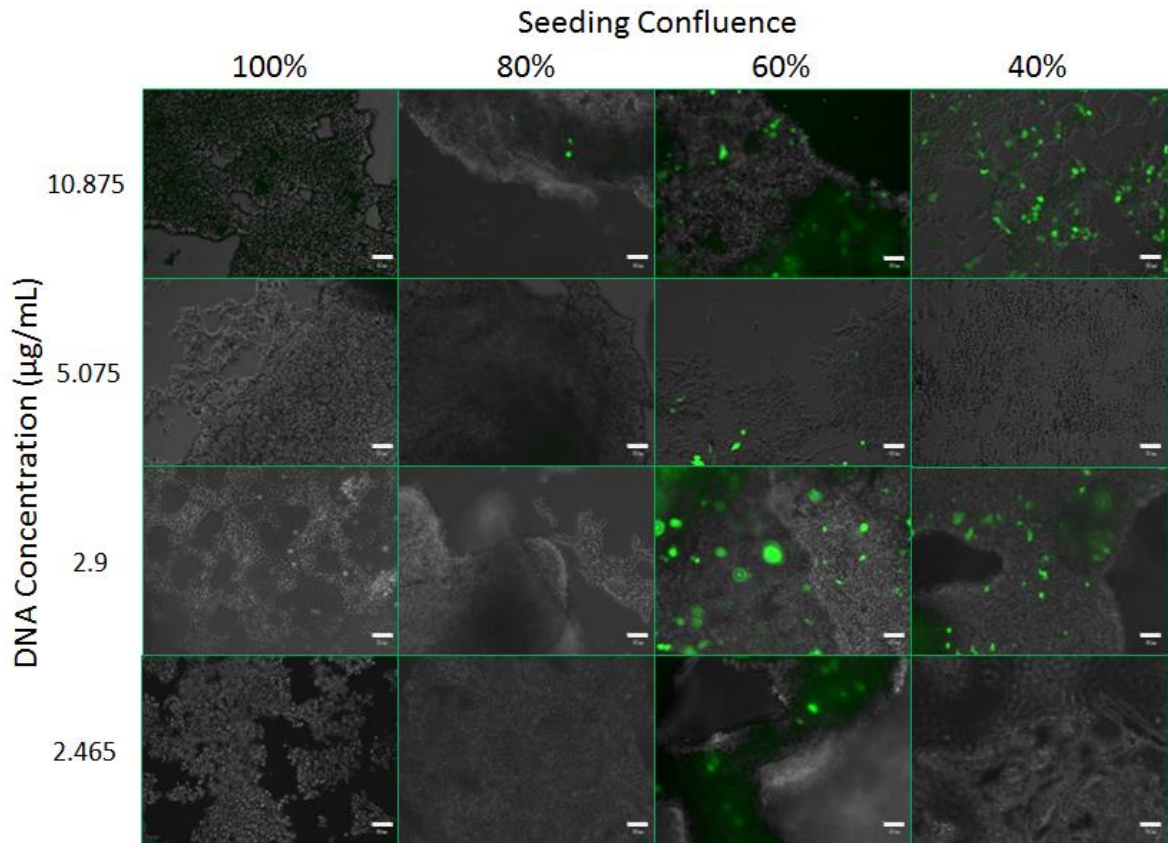
Figure 3.5. Optimisation of EcoPack™ 293 cell transfection. 293 cells were transfected with MIGR1 at varying amounts of DNA at different seeding confluences. Their media was replaced the next morning and they were imaged 2 days after media change. Scale bars represent 3 μm in length. Brightfield images of 293 cells were merged with green-fluorescence using ImageJ. This experiment was performed together with Jevin Karunia.

Optimisation and protocol modifications were also done for the transfection step. Calcium phosphate transfection efficiency is affected by cell confluence at the time of transfection and by the amount of DNA used. Poor transfection efficiency has debilitated the progress of this project significantly. In order to increase transfection efficiency, varying 293 seeding confluence (estimated by dilution from cells that had 100% confluence, 1 day

before transfection) and DNA concentrations were tested to optimise the transfection step of the project. From figure 3.5, even though all cells were visibly sloughed-off, 2.9 µg/mL of DNA and a seeding confluence of 60% was the optimal combination. With the advice of Dr. Samantha Ginn, the transfection protocol was modified to allow 2 days for 293 cells to grow to 40-60% confluence before transfection and minimise the sloughing off of cells.
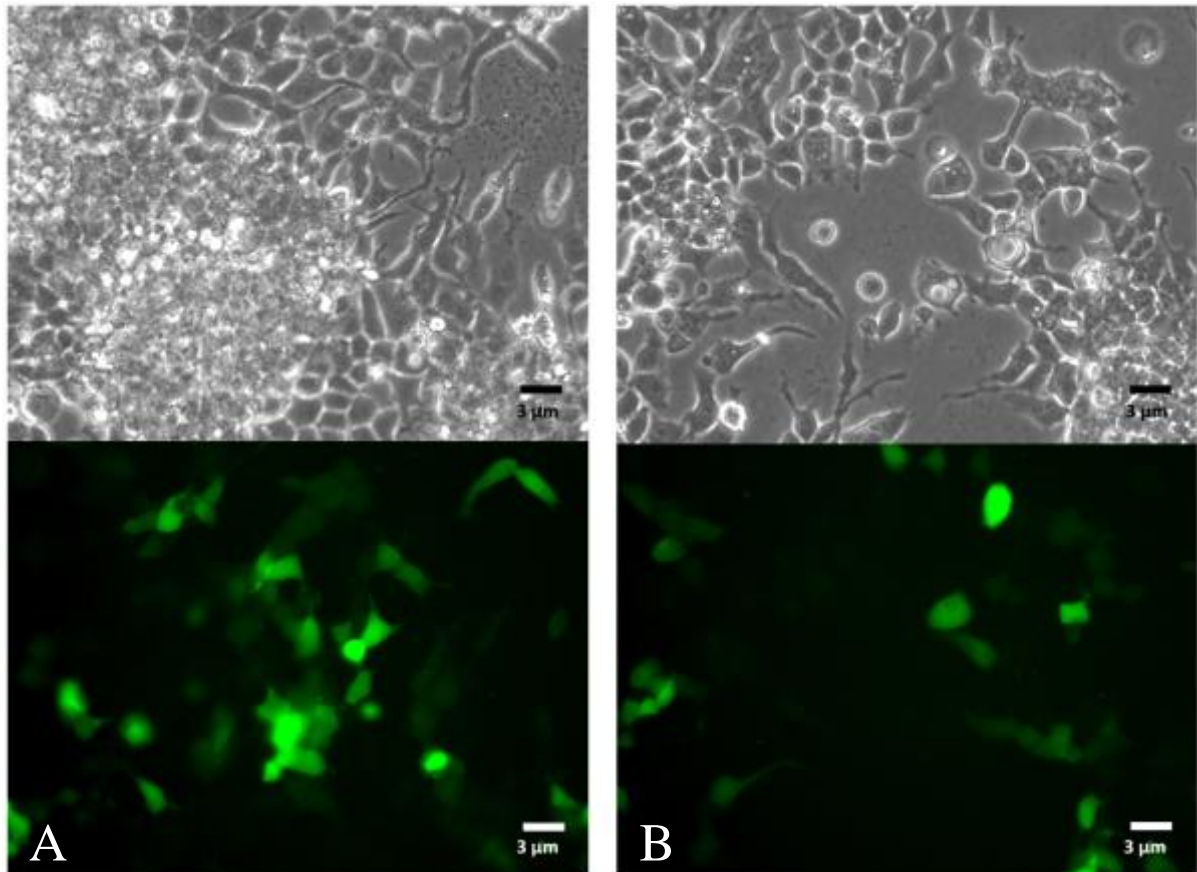
Figure 3.6. GFP expression of transfected 293 cells post-optimisation. 293 cells were imaged with a fluorescence microscope for GFP expression 3 days post-transfection. A) CAR-A2 and Foxp3 co-transfected 293 cells. B) CAR-HER2 and Foxp3 co-transfected 293 cells.

The optimised protocols were applied to subsequent 293 cell experiments. 293 cells were visibly healthier (more obvious cell borders and more GFP expression) (figure 3.6) after recovering earlier passages from the liquid nitrogen archive. There were also less

sloughing-off observed following transfection, and an increase in transfection efficiency
was obtained.

3.2.3   Measuring 293 Cell Transfection Efficiency
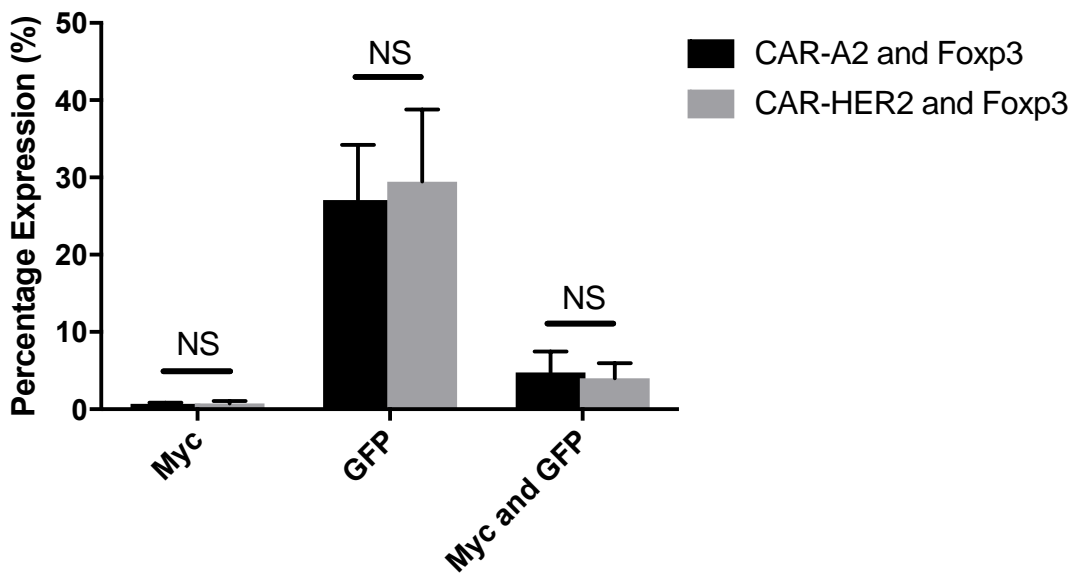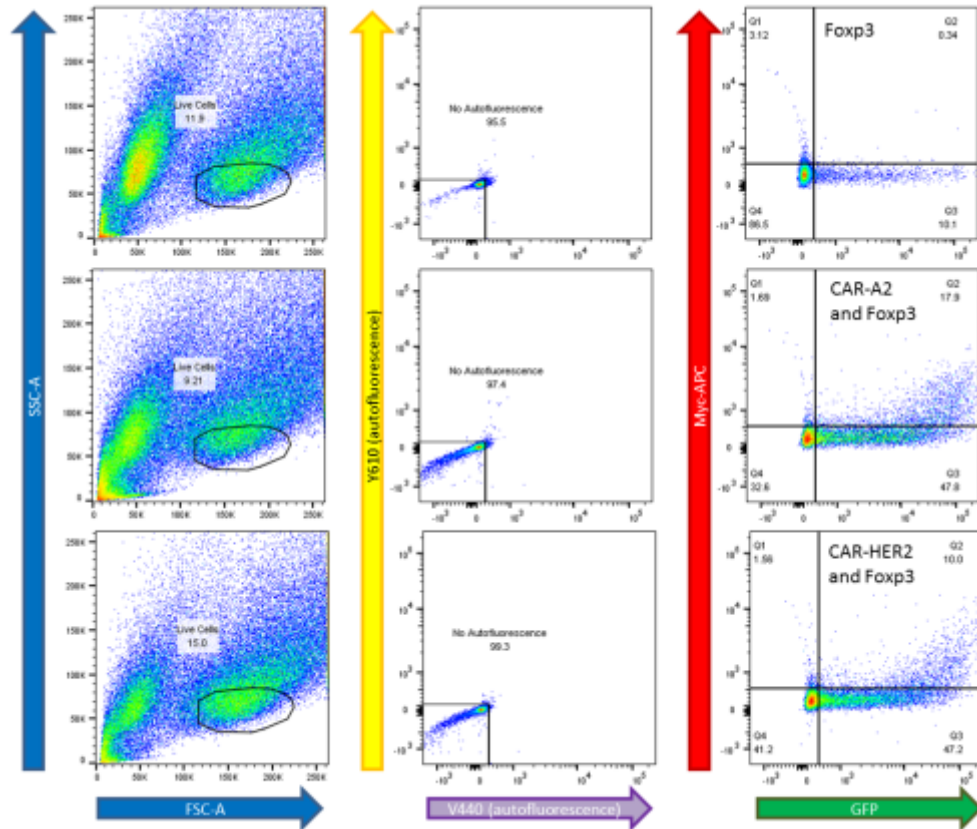
Figure 3.7. EcoPack™ 293 cell transfection efficiency. 293 cell transfection efficiency was measured by flow cytometry. Cells were recovered from liquid nitrogen, trypsinised, and then stained for Myc before being analysed using a flow cytometer. The means and standard error of mean (SEM) of CAR-A2/Foxp3 (n=5) and CAR-HER2/Foxp3 (n=4) co-transfected 293 cells were plotted together and statistically tested by ANOVA. NS = non-significant.

Transfection efficiency of the 293 cells was then quantitatively measured by flow cytometry (figure 3.7). In both groups of co-transfected 293 cells, GFP expression was most prominent, while Myc (CAR-A2 or HER2) and double expression was markedly poorer.
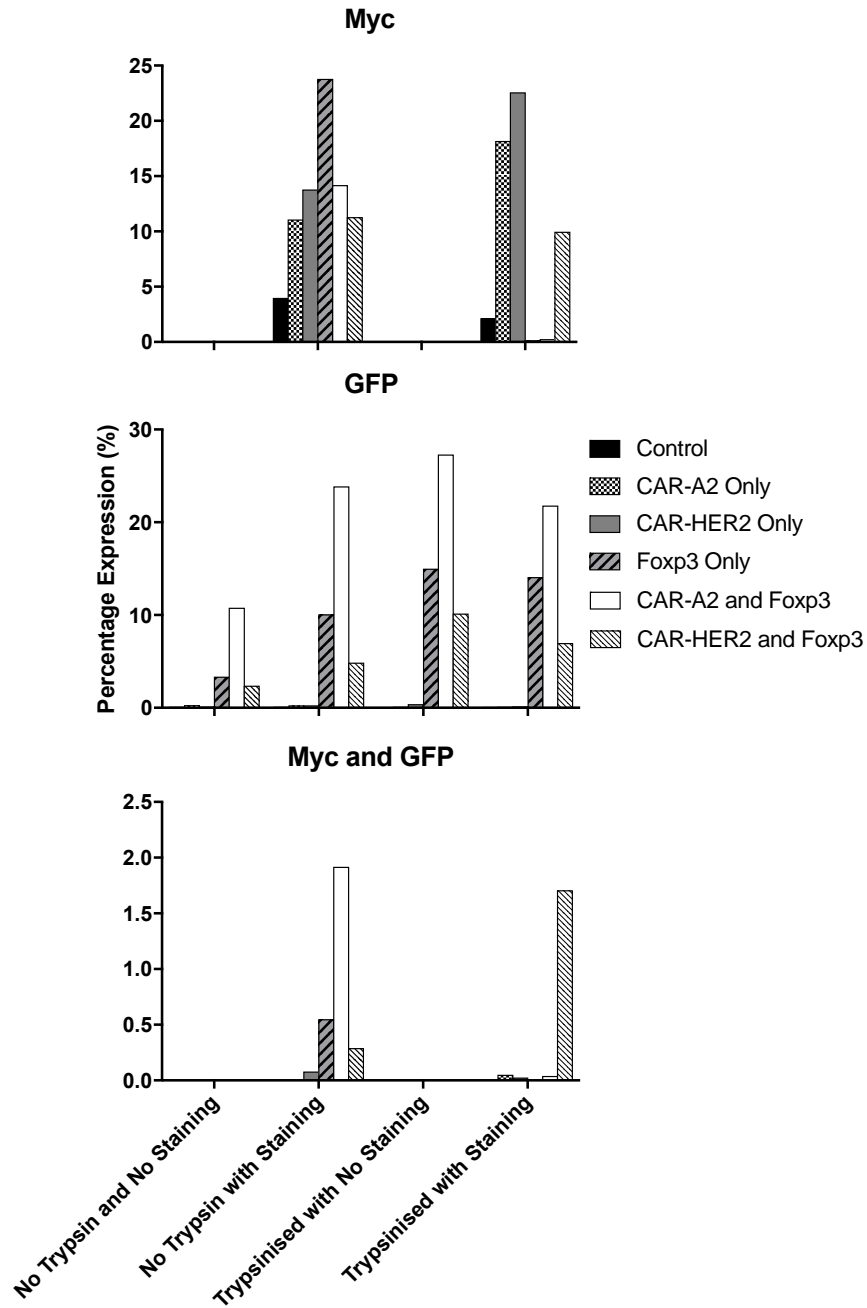
Figure 3.8. Testing the influence of trypsinisation on flow analysis of transfection efficiency. The effect of 5 minutes of trypsinisation on the Myc-expression of single and co-transfected 293 cells was tested. Trypsinised and non-trypsinised cells were stained for Myc and analysed using a flow cytometer.

Trypsinisation of cells can cause the cleavage of surface receptors. In order to determine whether trypsinisation has affected Myc-expression analysis, single and co-transfected 293 cells treated with trypsin were compared against cells without treatment (figure 3.8). The

expression of Myc after trypsinisation was particularly high for CAR-A2 and CAR-HER2 single-transfection groups, suggesting that trypsinisation does not affect surface Myc-expression. Lack of trypsinisation also caused a false positive in the Foxp3 single-transfection group for surface Myc-expression, most likely as a result of endogenous expression of Myc detected by the antibodies due to dead cells flowing through.

3.3 CD4+ T-cell Transduction and Functional Assessment

3.3.1 CD4+ T-cell Transduction
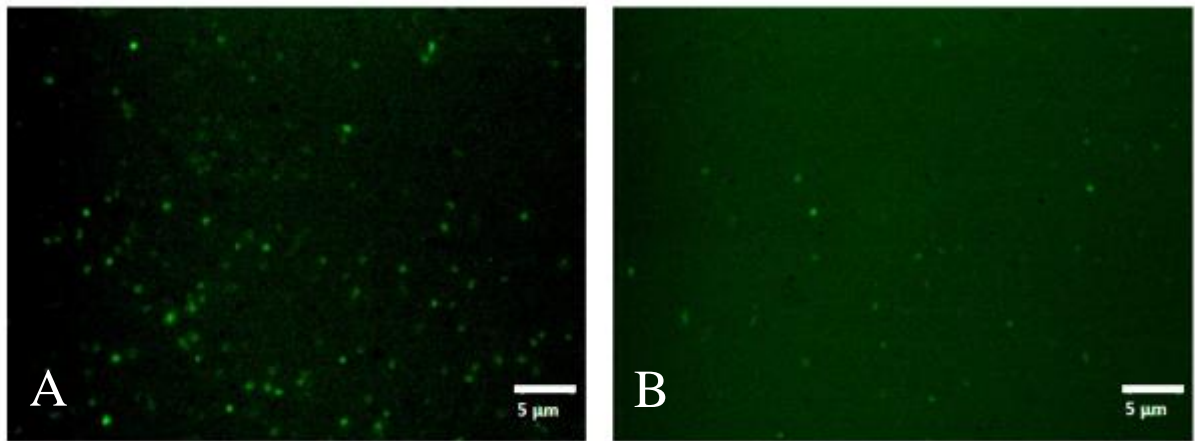
Figure 3.9. GFP expression of transduced CD4+ T-cells. Transduced T-cells were imaged with a fluorescence microscope for GFP expression 3 days post-transduction. A) CAR-A2 and Foxp3 co-transduced CD4+ T-cells. B) CAR-HER2 and Foxp3 co-transduced CD4+ T-cells.
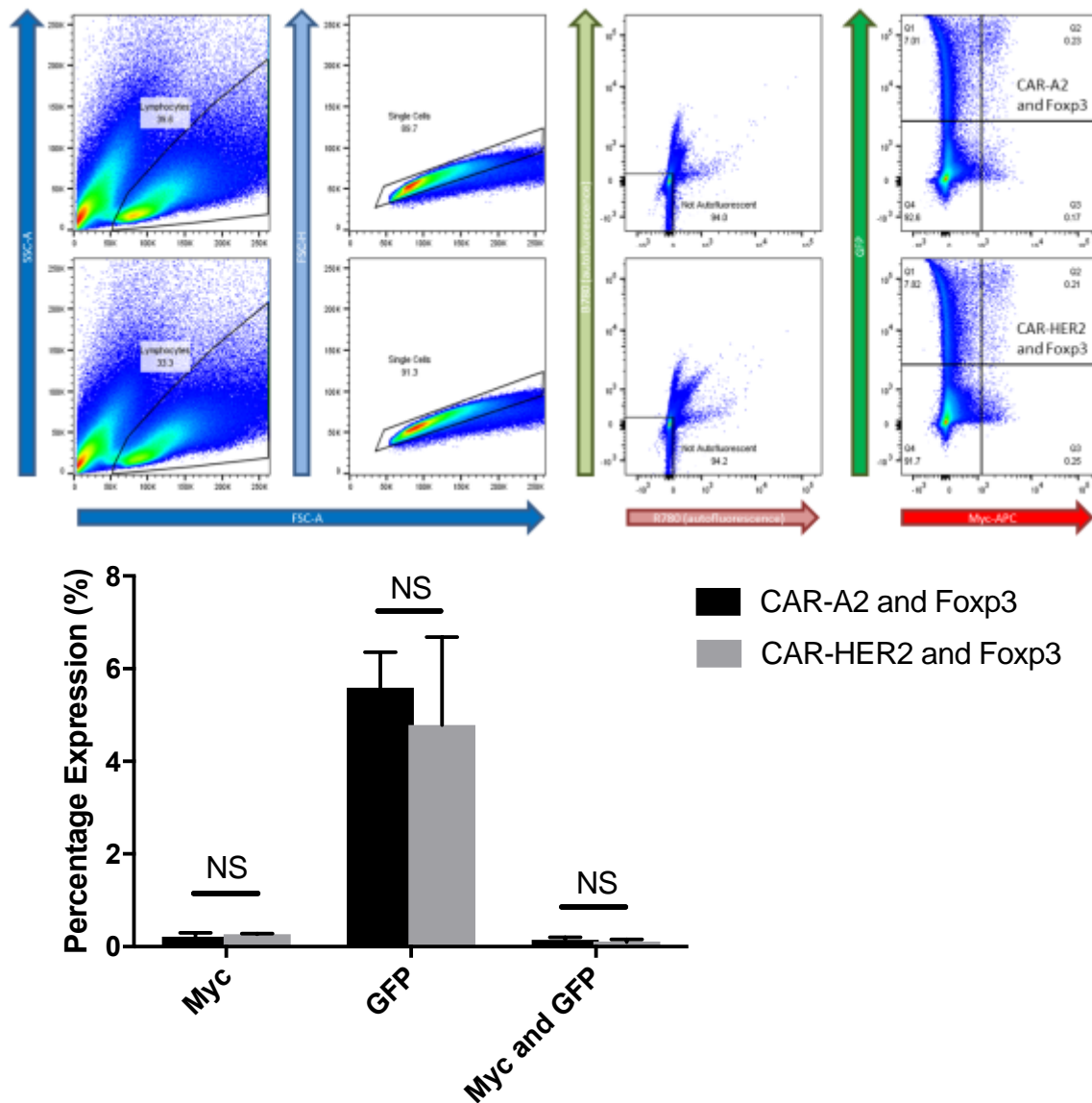
Figure 3.10. CD4+ T-cell transduction efficiency. Transduced T-cells were stained for Myc and were sorted using a flow cytometer. The means and standard error of mean (SEM) of CAR-A2/Foxp3 (n=4) and CAR-HER2/Foxp3 (n=3) co-transfected CD4+ T-cells were plotted together and statistically tested by ANOVA. NS = non-significant.

In the next stage of the project, retroviral supernatant obtained from 293 cells were used to transduce murine CD4+ T-cells. Following the trend observed in the transfection of 293 cells, transduced CD4+ T-cells expressed substantially higher GFP expression as opposed to Myc (figures 3.9 and 3.10). CAR-A2 or HER2+ T-cells and Tregs were obtained in low numbers as a result, limiting the number of reactions that can be executed for their

functional assays. However, this experiment shows that CAR-A2^{+} Tregs have been successfully generated.

3.3.2 Mixed Lymphocyte Reaction of Transduced CD4^{+} T-cells
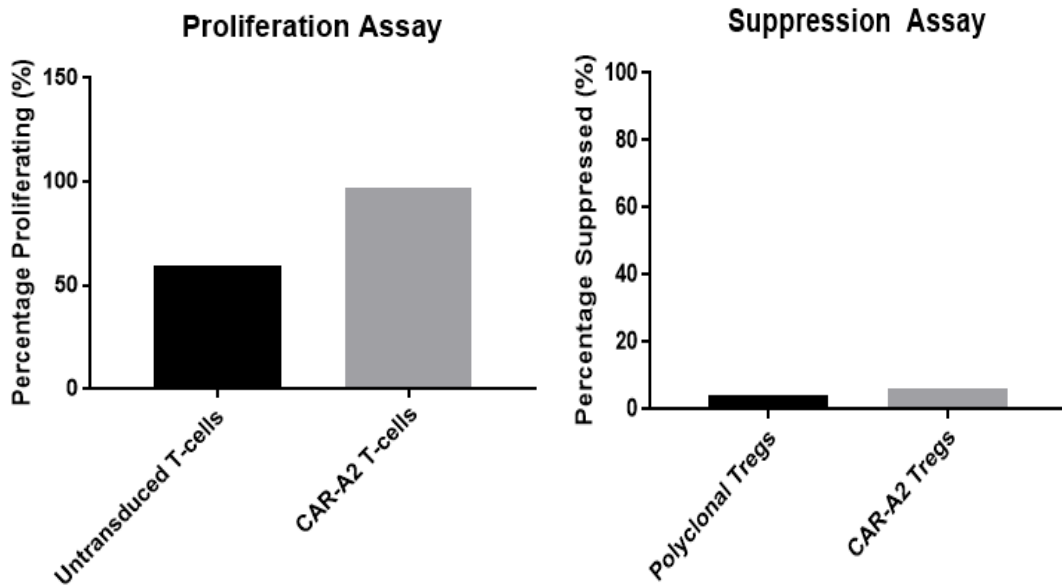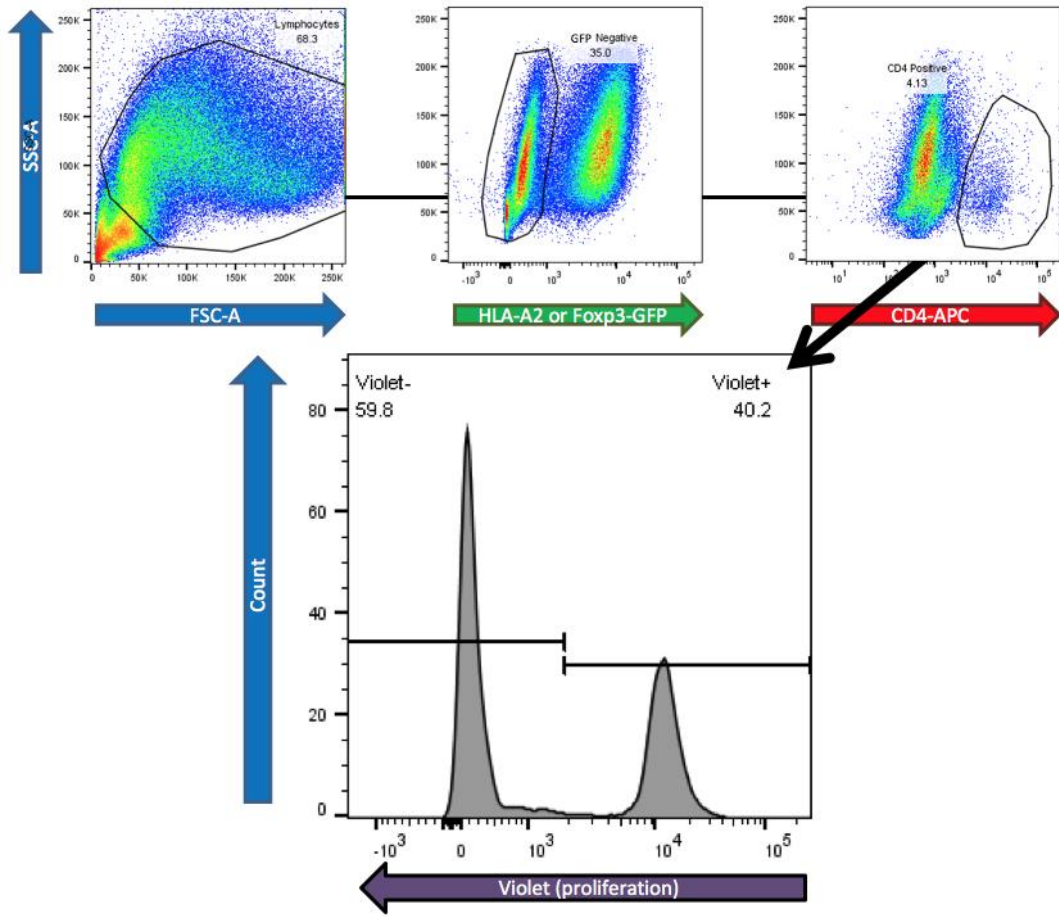
Figure 3.11. Preliminary MLR assay of transduced T-cells. Transduced and Violet-stained T-cells were stimulated with A2-K562 cells for 5 days. The cells were then stained with anti-CD4-APC and analysed using a flow cytometer. The proportion of Violet- cells from the proliferation assay containing Control Teffs (n=1) and CAR-A2$^+$ Teffs (n=1), and from the suppression assay containing polyclonal Tregs (n=1) and CAR-A2$^+$ Tregs (n=1) were plotted together.

Transduced T-cells were then functionally assessed through MLR. Figure 3.11 depicts the preliminary MLR of transduced T-cells, where transduced T-cells were cultured with live HLA-A2-expressing K562 cells (not irradiated). In the proliferation assay, Control CD4$^+$ Teffs did not proliferate as much as CAR-A2$^+$ Teffs when stimulated with A2-K562 cells. This shows that the presence of the HLA-A2 antigen activated the proliferation of CAR-A2$^+$ T-cells. Interestingly, about 50% of Control T-cells were proliferating as well, even though K562 cells do not express any MHC molecules.

In the suppression assay, CAR-A2$^+$ Teffs were cultured with A2-K562 cells together with polyclonal Tregs or CAR-A2$^+$ Tregs. The CAR-A2$^+$ responders were equally suppressed by both polyclonal Tregs and CAR-A2$^+$ Tregs. This may have occurred because the proliferative responses of the CAR-A2$^+$ responders when activated by the HLA-A2 antigen were too strong to be suppressed by both polyclonal Tregs or CAR-A2$^+$ Tregs when cultured at a 1:1 ratio.
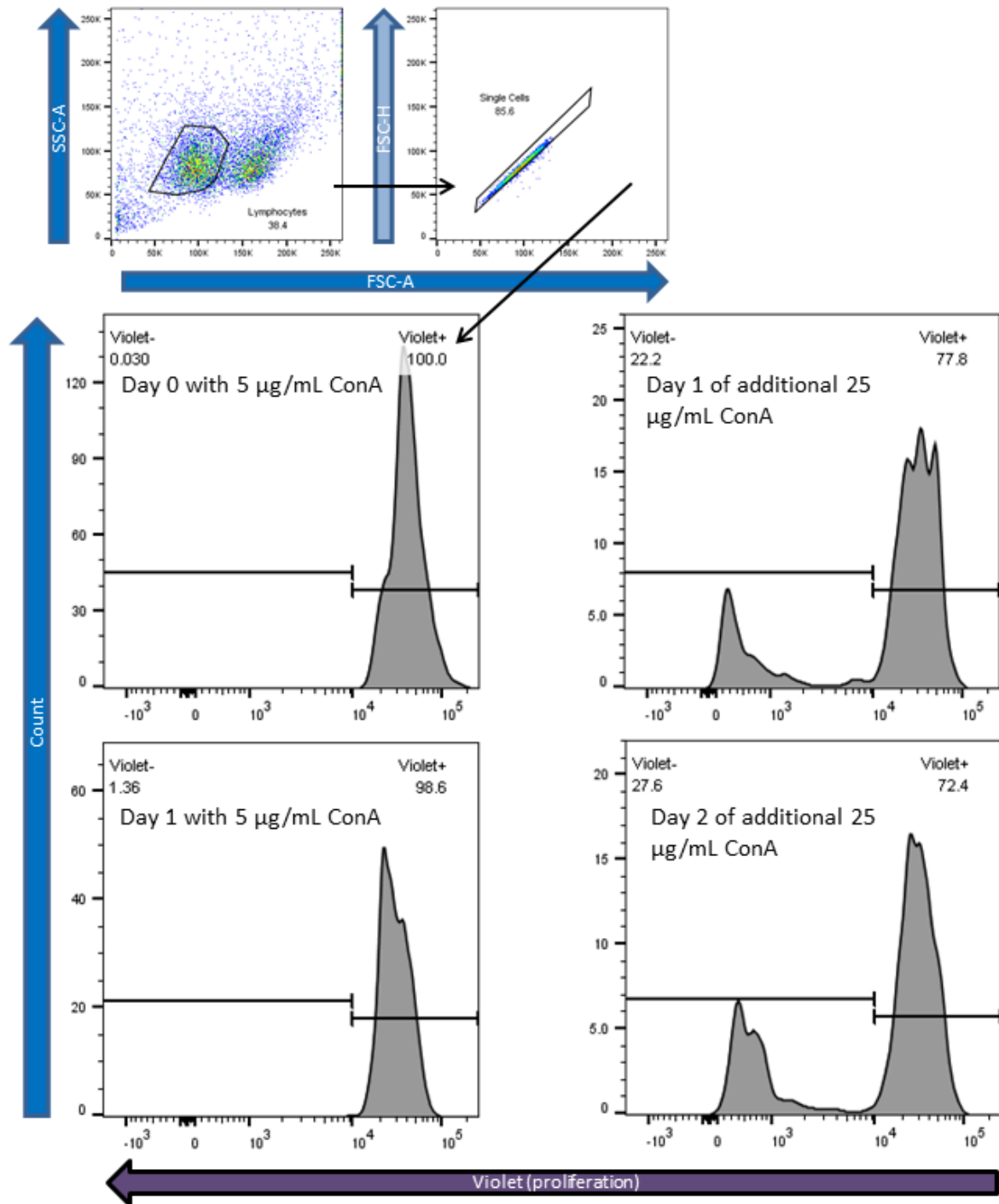
Figure 3.12. Optimisation of CellTrace™ Violet dye. Whole splenocytes were stained with CellTrace Violet and stimulated with 5 μg/mL ConA over 4 days. On day 2, stimulated cells were given an additional 25 μg/mL ConA. Cells were analysed using a flow cytometer daily.

Subsequently, more MLRs were performed with modifications in the protocol in order to include control stimulator cells (using B6 and A2Kb splenocytes). However, poor staining

of Violet prevented proper analysis of the functional changes in the CD4$^+$ T-cells that were transduced. This happened due to an error of quenching of the Violet dye with serum (present in complete T-cell media) unknowingly before using the dye solution to stain T-cells, rendering two MLRs unusable. In the optimisation step shown in figure 3.12, PBS was used as a medium for Violet staining, yielding 100% staining of target splenocytes. The splenocytes were then stimulated with 5 µg/mL Concanavalin A (ConA) with no observed proliferation until day 1. An additional dose of 25 µg/mL ConA was given on day 1 to promote stimulation, and their proliferation was successfully observed and traced for 2 days. PBS was then used to stain responder cells in subsequent MLRs.
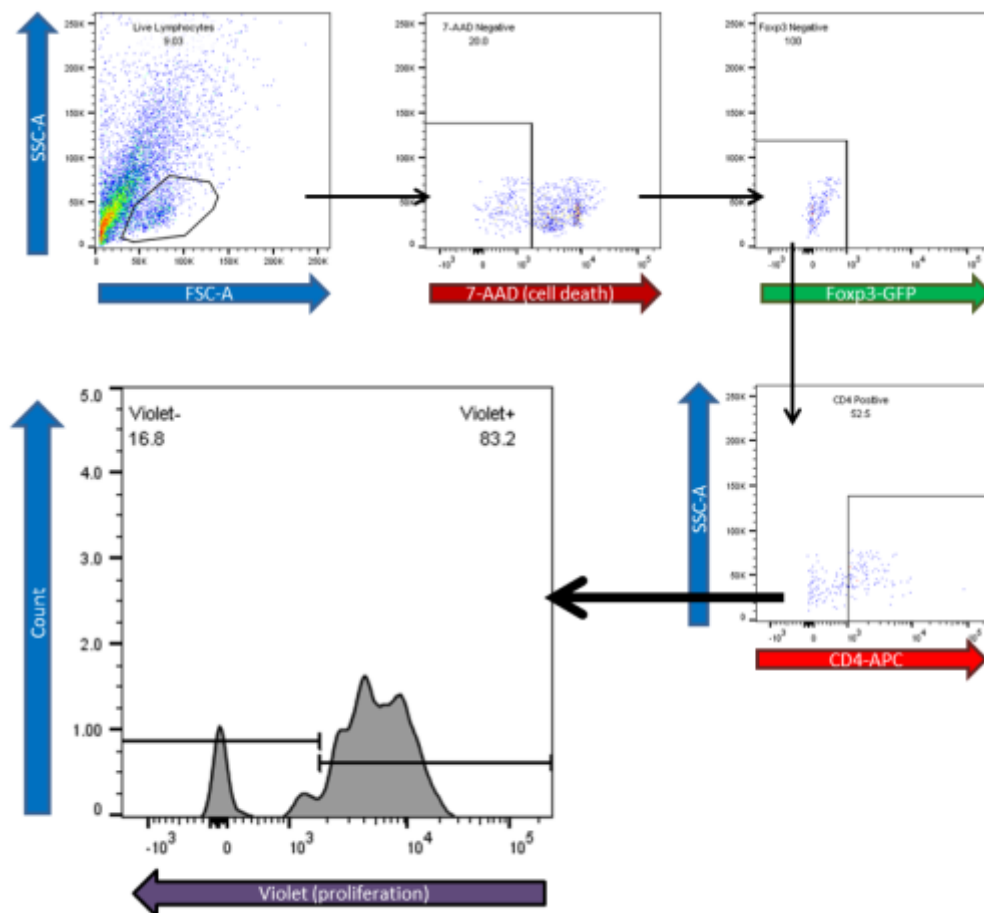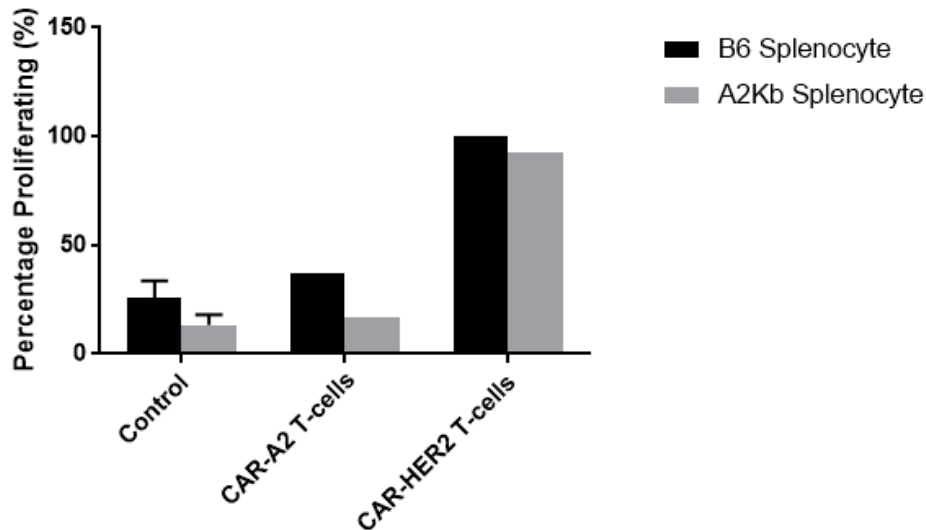
Figure 3.13. Proliferation assay of transduced T-cells with auto/allogeneic splenocytes. All cells in the MLR were stained with anti-CD4-APC and 7-AAD as a live/dead cell marker, and then analysed using a flow cytometer. The means and the SEM of the proportion of Violet- cells in the Control (n=3), CAR-A2 (n=1), and CAR-HER2 (n=1) groups were plotted together. Black bars denote T-cells stimulated with B6 splenocytes and grey bars denote stimulation with A2Kb splenocytes.

Following optimisation of Violet staining, transduced T-cells were cultured with either irradiated B6 or A2Kb splenocytes (figure 3.13). Live responder Control CD4$^+$ Teffs appeared to have similar responses as CAR-A2$^+$ CD4$^+$ Teffs when stimulated with either B6 or A2Kb splenocytes, which was unexpected. The poor proliferation response observed in CAR-A2$^+$ CD4$^+$ Teffs when stimulated with A2Kb splenocytes could have occurred due to a combination of poor T-cell maintenance before the MLR and a lower HLA-A2 molecule density in the A2Kb splenocytes compared to A2-K562 cells. CAR-HER2$^+$ CD4$^+$ Teffs had much greater proportion of proliferating cells compared to both control and CAR-A2$^+$ Teffs. Further repeats are required to confirm this observation.
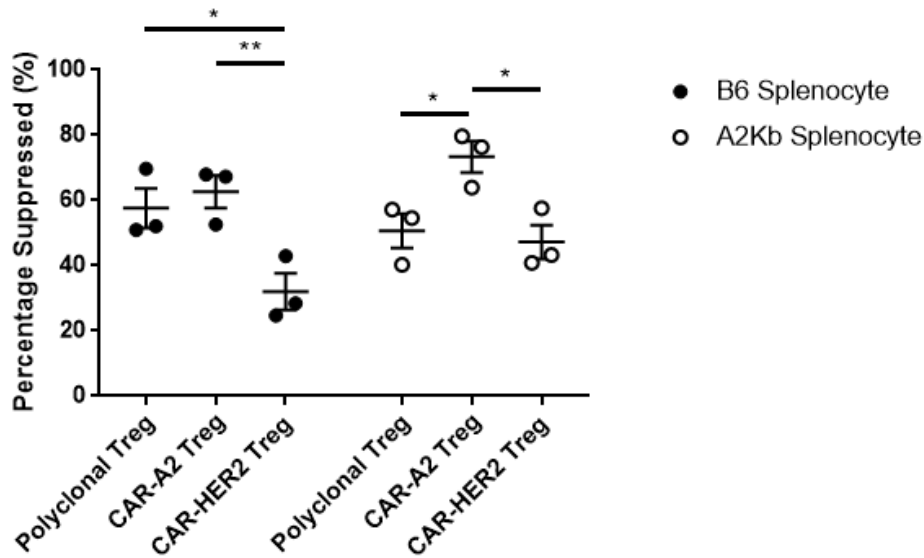
Figure 3.14. Suppression assay of transduced T-cells with auto/allogeneic splenocytes. All cells in the MLR were stained with anti-CD4-APC and 7-AAD as a live/dead cell marker, and then analysed using a flow cytometer. The means and the SEM of the proportion of Violet- cells in the polyclonal (n=3), CAR-A2 (n=3), and CAR-HER2 (n=3) Treg groups were plotted together and statistically tested by ANOVA. Black dots denote T-cells stimulated with B6 splenocytes and white dots denote stimulation with A2Kb splenocytes. NS = non-significant, * = $p<0.05$, and ** = $p<0.01$.

In the suppression assay (figure 3.14), Control CD4+ Teffs were cultured with either irradiated B6 or A2Kb splenocytes, together with either polyclonal, CAR-A2+, or CAR-HER2+ Tregs. When untransduced T-cells (responders) were stimulated with B6 splenocytes, polyclonal and CAR-A2+ Tregs suppressed responder proliferation similarly, while CAR-HER2+ Tregs had the lowest suppression. When responders were stimulated with A2Kb splenocytes, CAR-A2+ Tregs suppressed responder proliferation significantly better compared to polyclonal or CAR-HER2+ Tregs. CAR-A2+ Tregs suppresses allogeneic responses mediated by HLA-A2 better than polyclonal Tregs or CAR-HER2+ Tregs.

Chapter 4   Discussion

4.1 Alloantigen-Specific Proliferative Response of CAR-A2$^+$ Teffs

In the current study, a CAR-HER2 construct that has been effectively utilised to redirect cytolytic T-cells against HER2$^+$ tumour cells (John *et al.* 2013) was modified to alter its specificity by replacing the anti-HER2 scFv gene with an anti-HLA-A2 scFv gene. The resulting CAR-A2 construct was successfully expressed in both transfected EcoPack™ 293 cells (figure 3.7) and in transduced CD4$^+$ T-cells (figure 3.10). The CAR-A2$^+$ T-cells (both Teffs and Tregs) were then used for functional studies by MLR.

The first MLR (figure 3.11) gave us a preliminary result showing that CAR-A2$^+$ Teffs proliferate better than Control Teffs when stimulated by the HLA-A2 antigen. This is consistent with the antigen-specific proliferation response observed in CAR-A2$^+$ Tregs by both Levings (2016) and Noyan (2017). There were, however, several limitations to this *in vitro* experiment design. It was performed by culturing CAR-A2$^-$ and CAR-A2$^+$ CD4$^+$ Teffs together with live A2-K562 cells (not irradiated). K562 cells are a cell line that were derived from a patient with chronic myeloid leukaemia (CML) that are deficient of MHC molecules. They are transfection competent (Esendagli *et al.* 2009), are commonly used as a model for hematopoietic cell transduction studies (Ling *et al.* 2016), and can be used to create artificial antigen-presenting cells (AAPCs) as they express molecules that promote T-cell and APC binding (Suhoski *et al.* 2007). K562 cells also have the capacity to stimulate γ,δ receptors found in $\gamma\delta$ T-cells (Di Fabrizio *et al.* 1991). When CAR-A2$^-$ (Control) and CAR-A2$^+$ CD4$^+$ Teffs were cultured together with live A2-K562 cells, a high proportion of T-cells proliferated from both groups. Since the A2-K562 stimulators were not irradiated, they have proliferated together with the T-cells. Allogeneic responses

may have activated Control Teffs, which can potentially release blast cell (K562 cells are myeloid blast cell lines) activating cytokines such as IFN-γ and TNF-α (Bruserud *et al.* 1993; Munker & Andreeff 1996; Ngai *et al.* 2007) causing K562 proliferation. This makes it challenging to isolate the Teff population in the MLR during flow cytometric analysis, as the T-cell population is not distinct. While GFP-expressing cells (A2-K562) can be gated out, and $CD4^+$ T-cells can be selected for, no live-dead stain was incorporated. The cells that were analysed for their proliferation may have also included dead cells, which would have displayed as Violet-negative. In subsequent MLRs, the proliferation assay protocol was then modified for several reasons:

1. A control ($HLA-A2^-$) stimulator was required to assess antigen-specificity.
2. Irradiation of stimulator cells was required to prevent stimulator proliferation.
3. A live-dead stain was required to avoid confounding variables in proliferation analysis.

Irradiated $HLA-A2^-$ and $HLA-A2^+$ splenocytes (with the same B6 genetic background as the T-cells used) were then used as stimulators, and 7-AAD was incorporated as a live-dead stain for the Teffs.

In the following MLR, when Control Teffs were stimulated with either B6 or A2Kb splenocytes, the proportion of proliferating cells were similar to that of $CAR-A2^+$ Teffs (figure 3.13). This was unusual because $CAR-A2^+$ Teffs are expected to proliferate at a much higher rate when stimulated with A2Kb splenocytes. This observation occurred in contrast to what was seen in the first MLR, and is not consistent with other studies regarding CAR-mediated stimulation.

The failure of CAR-A2 Teffs to respond to the HLA-A2 antigen could have resulted due to several reasons, with the first being the difference in the density of the HLA-A2 antigen

expressed in the stimulator cells. HLA-A2-K562 cells were originally used in the preliminary MLR because 100% of the cells express the HLA-A2 antigen. In comparison to these cells, A2Kb splenocytes do not express the HLA-A2 antigen in all of the lymphocytes (Appendix 5 – HLA-A2 Antigen Expression in A2Kb Lymphocytes).

Another reason for the observed unresponsiveness of the CAR-A2$^+$ Teffs to the HLA-A2 antigen may be the lack of stimulation to maintain CAR expression. CAR-A2$^+$ and CAR-HER2$^+$ Teffs were obtained in low numbers and cultured at low density (~130,000 cells/mL) for 5 days before the MLR. Cell numbers remained constant (little or no proliferation) as no additional stimulus was given. The lack of activating stimuli could have allowed time for the CAR-receptors to decay in each of the cells, as they have finite half-lives (Roybal *et al.* 2016). The 5-day period of rest (no stimulation) was given to Control and CAR-A2$^+$ Teffs as they were given anti-CD3 and anti-CD28 antibodies prior to transduction, which functions to elicit a strong TCR response and co-stimulation (Li & Kurlander 2010). This allows for cells to rest before from their actively proliferating state before allogeneic restimulation in order to accurately assess only the extent of CAR-mediated proliferation, however, this step may be counter-productive if the CAR receptors degrade in these cells.

Interestingly, CAR-HER2$^+$ Teffs had much higher proportion of proliferating cells compared to Control and CAR-A2$^+$ Teffs whether stimulated with B6 or A2Kb splenocytes in the proliferation assay (figure 3.13). It is unlikely that CAR-HER2 had cross-reactivity with antigens in B6 splenocytes (as it would have activated the suppression of CAR-HER2$^+$ Tregs, figure 3.14), but the responses observed could have come about due to poor Violet staining of CAR-HER2$^+$ Teffs, presenting themselves as Violet negative. As CAR-HER2$^+$ Teffs were obtained in very low numbers (~ 15,000 cells) and a small volume of 14

μL of Violet dye was used, it is possible that the small cell pellet could have been poorly stained due to operator error.

In the future, CAR-expression on the T-cell responders should be assessed at least once before commencing the MLR, and optimisation to be done to determine the best duration of T-cell resting before re-stimulation using the splenocytes.

4.2 CAR-A2$^+$ Treg Suppression is Specific to HLA-A2 Antigen Stimulation

In the first MLR suppression assay (figure 3.11), no differences in suppression were observed between CAR-A2$^+$ Tregs and polyclonal Tregs. CAR-A2$^+$ Tregs were expected to have better suppression than polyclonal Tregs in the presence of the HLA-A2 antigen, as CAR-mediated activation of Tregs should exhibit an activated suppressive phenotype (Edinger 2016). This happened in contrast to the observations made in Levings (2016) and Noyan's (2017) studies, which could have resulted due to several reasons:

1. Tregs have lost their suppressive function before commencing the MLR.
2. The proliferation of CAR-A2$^+$ CD4$^+$ T-cells were too strong in the presence of the HLA-A2 antigen.

One of the limitations of this suppression model using CAR-A2$^+$ Teffs as responders was that it was not the most clinically relevant model. In a transplant patient, not 100% of the host T-cells will specifically be against the HLA-A2 antigen. As a result, the protocol was modified for subsequent suppression assays. Instead of using CAR-A2$^+$ Teffs, CAR-A2$^-$ CD4$^+$ Teffs (Control Teffs) were used as responders, and the stimulators were replaced to either be irradiated B6 or A2Kb splenocytes.

When control responders were stimulated with B6 splenocytes (figure 3.14), both polyclonal and CAR-A2$^+$ Tregs showed similar suppression capacity. The differences occurred when control responders were stimulated with A2Kb splenocytes. CAR-A2$^+$

Tregs had significantly higher suppression compared to polyclonal Tregs and CAR-HER2$^+$ Tregs, showing an antigen-specific suppression capacity of CAR-A2$^+$ Tregs. This was consistent to Levings (2016) and Noyan's (2017) findings, showing that CAR-A2$^+$ Treg are alloantigen specific and inhibit the proliferation of Teffs when activated. Levings showed alloantigen specificity of CAR-A2$^+$ Tregs through the suppression of GVHD in a xenogeneic PBMC transplant model (HLA-A2$^+$ PBMCs), while Noyan used a HLA-A2$^+$ skin graft mouse model to show antigen-specificity and suppressive capacity of their CAR-A2$^+$ Tregs. Their studies were different in that they used tissue expressing the whole HLA-A2 antigen, compared to the chimeric HLA-A2 molecules expressed in A2Kb mice used in the current study which were derived from a combination of human HLA-A2 and mouse H2Kb molecules. This difference adds to the existing knowledge in that it shows that epitopes of the HLA-A2 antigen can still trigger CAR-A2$^+$ Treg-mediated suppression. Whether or not binding affinity of the antigen to the CAR correlates with suppression strength is still unknown, but can be part of the future direction in understanding CAR-Treg physiology.

This antigen-specific activation occurred in contrast to the proliferation assay, which was possibly due to the different treatment of transduced cells post-sorting. All Foxp3$^+$ cells were expanded for 5 days with anti-CD3 and CD28 beads, possibly allowing for CARs to be more stably expressed. This can only be confirmed by flow cytometry before commencing the MLR.

4.3 Future Direction: Towards Clinical Application of CAR-A2$^+$ Tregs

In the current study, it has been shown that CAR-A2$^+$ Tregs suppress allogeneic stimulation of CD4$^+$ Teffs better than polyclonal Tregs in an *in vitro* model. Adding on to Levings' GVHD model (2016) and Noyan's skin graft model (2017), the next knowledge gap to explore is the performance of CAR-A2$^+$ Tregs in a kidney transplant model. Similar

to Levings and Noyan, NOD-SCID IL2Rγ-null (NSG) mice can be used as the host, and A2Kb mice can be used as kidney donors. This will bring CAR-A2$^+$ Tregs closer to being applied in clinical settings.

In the current study, CAR-A2$^+$ Tregs are shown to be better at suppressing Teff proliferation than polyclonal Tregs when stimulated with the HLA-A2 antigen. Clinically, this is a significant step forward to alloantigen-specific suppression of immune responses. Single antigen mismatches can possibly be overcome with CAR Tregs against the mismatched MHC (MacDonald *et al.* 2016), allowing for more freedom in donor selection. While the findings are exciting, there are still many aspects of CAR-Treg physiology that are not completely understood.

Foxp3$^+$ Tregs generally suppress by cell-cell contact, secretion of inhibitory cytokines, and competition for growth factors. Membrane-bound TGF-β, CTLA-4, granzyme B, and LAG3 (Sojka, Huang & Fowell 2008) have been shown to contribute to suppression through cell-cell contact, and suppressive cytokines such as TGF-β, IL-10, and the more recently discovered IL-35 are also necessary for Treg mediated suppression (Bettini & Vignali 2009; Collison *et al.* 2007). To date, there are no extensive molecular characterisation studies on CAR Tregs. Characterising the cytokine profile and surface molecule expression of CAR Tregs will be necessary to understand their suppression mechanisms. Genomic analysis at a single cell level will be helpful in correlating the level of Foxp3 expression and T-cell subtype with CAR-A2$^+$ Treg phenotype.

The safety of CAR-A2$^+$ Tregs is also a concern, with the first being the toxicities related to the use of retroviral vectors for CAR expression. The two main safety concerns for using retroviral vectors are the risk of insertional mutagenesis and the production of replication-competent viruses (Yi, Jong Noh & Hee Lee 2011). To minimise the risk of insertional

mutagenesis, altering the viral proteins and the studying the changes in the patterns of insertion using next-generation DNA sequencing is necessary. It can also ultimately lead to selection of integration sites in the host chromosome. Alterations in the viral proteins can also eliminate recombination to avoid producing replication-competent viruses. Another solution is to use different methods for genomic editing of T-cells. The CRISPR/Cas9 method, for example, can be used to target specific integration sites in the genome (Lee, JS *et al.* 2015).

Another safety concern of CAR-A2$^+$ Tregs is the stability of the Treg phenotype. Cell lineage tracking of Foxp3$^+$ Tregs show that with stimulation using certain inflammatory cytokines (Yang *et al.* 2008), when adoptively transferred to T-cell deficient mice (Duarte *et al.* 2009), or simply overtime in healthy mice (Zhou *et al.* 2009), some phenotypically plastic Foxp3$^+$ Tregs can downregulate in Foxp3 expression and lose their Treg phenotype. Moreover, they can also gain an effector phenotype resembling helper or memory Teffs (Duarte *et al.* 2009; Zhou *et al.* 2009). Retroviral transduction of Foxp3 hopes to promote the stable expression of the Treg phenotype, but whether this can change with inflammatory stimuli following adoptive transfer to immunocompetent hosts still needs to be investigated.

4.4 Overcoming Hurdles Encountered with EcoPack™ 293 Cells

4.4.1 Phenotypic Changes Due to Irregular Subculturing

With diligent subculturing, EcoPack 293 cells can be easily maintained as hosts for vector expression and retroviral packaging. Between the months of May to July, the 293 cells used have gradually changed in morphology and a reduction in cell viability and transfection efficiency was observed (figure 3.4), halting further progress of the project. This occurred due to the overgrowth of the cell line between late April and early May,

leading to phenotypically altered cells that were gradually selected for with each subculture. Typically, it is recommended that cells are subcultured at 80-90% confluence (Ooi *et al.* 2016; Phelan 1998; Phipps *et al.* 2007) to avoid contact inhibition (which results in a longer recovery time if these cells are frozen) and a decrease in mitotic index when cells become over-confluent. As such, the subculture frequency of our 293 cells was modified from every 4-5 days to every 2-3 days.

4.4.2   The Problem with Plasmid Co-Transfection of EcoPack™ 293 Cells

Co-transfection of 293 cells did not result in equal expression of CAR-A2 and Foxp3 (figure 3.7). Foxp3-GFP expression was consistently the highest compared to CAR-A2-Myc and double expression. In optimal circumstances, when the same amount of each plasmid is used for transfection, the overall expression of both plasmids should be roughly equal, even with variations in the expression of each plasmid in each cell. Optimal circumstances for equivalent plasmid expression in a co-transfection system include:

- Similar plasmid promoter sequence
- Similar plasmid size
- Similar half-life of plasmid products

Both the CAR-A2 and Foxp3 plasmids have similar nucleotide sequences upstream of their transgenes, however both plasmids differ in about 900 bp, and the exact half-lives of their products are unknown. Depending on the expression system, whether it is by mRNA transfection or by retroviral integration of DNA, CARs can have very different half-lives. mRNA-mediated CAR expression lasts as long as the mRNA remains intact (Kenderian *et al.* 2014), but when it is integrated into DNA, CAR expression can continue as long as the cell remains capable of transcribing the gene. A CAR gene integrated into the DNA in a T-cell can have a half-life of up 8 hours when the CAR-synthesising stimulus is restrained (Roybal *et al.* 2016). The Foxp3 protein is found to be less stable. When Foxp3 is

transduced into T-cells, its half-life is 2 hours when protein translation is inhibited (Morawski *et al.* 2013). Figure 3.8 suggests that protein half-life is not necessarily the differentiating factor in the discrepant expression of Myc and GFP. 293 cells that were only transfected with CAR plasmids expressed high levels of Myc, which was not observed in CAR-A2/HER2 and Foxp3 co-transfected cells. The Myc expression level was also similar to the expression of GFP in Foxp3-transfected cells. This suggests that the translation efficiency of the transgenes from both CAR and Foxp3 plasmids were not the major differentiating factor, but the preferential uptake of Foxp3 plasmids over CAR plasmids could be the underlying cause.

Calcium phosphate transfection is generally thought to be unaffected by plasmid size (Guo *et al.* 2017; Kim & Eberwine 2010), however it may be different in the case where two plasmids are used. This transfection method creates calcium-phosphate-DNA (CP-DNA) particles of varying sizes (Cao *et al.* 2011). In a system where more than one plasmid is used, the ratio of each plasmid within each CP-DNA particle may impact the ratio of DNA effectively endocytosed into each cell, causing a difference in transgene expression. As the CAR plasmid is about 1 kb larger than the Foxp3 plasmid, it is likely that the difference in plasmid ration in the CP-DNA particles affected the expression of both plasmids in the co-transfected 293 cells. In order to avoid the plasmid ratio problem in each CP-DNA particle, single transfections of CAR plasmids should be performed instead of co-transfection in the future.

4.5 T-cell Transduction: Improving Methods for the Future

4.5.1   c-Myc as a Reporter Gene for CAR Expression

c-Myc is an endogenously expressed-transcription factor found in all tissues in the body, but higher expression is observed in highly proliferative tissues (Pubmed gene ID: 4609).

Through association with the MAX protein, c-Myc functions to promote cell cycle progression, which is linked with inhibiting cell differentiation and promoting sensitivity to apoptosis (Pelengaris, Khan & Gerard 2002). In our expression system, surface c-Myc expression is coupled with the CAR genes, similar to Levings' (2016) construct, allowing them to be ectopically expressed on the cell surface to be detected by anti-Myc antibodies. Typically, having a protein endogenously present in our target cells as an expression marker, as opposed to using other markers such as CD19 (not expressed in T-cells) or LNGFR (low-affinity nerve growth factor receptor, used in Noyan's construct (2017)), may result in false positives. However, as permeabilisation was not performed on transduced T-cells prior to sorting, positive c-Myc expression can be inferred to be surface expression with enough confidence.

An alternative to using c-Myc is to use the HLA-A2 antigen itself (or HER2 antigen) to isolate cells expressing CARs. This can be achieved using tetramers conjugated with magnetic beads or fluorescent tags (Wang, X-C *et al.* 2013). A tetramer system using Streptavidin and Biotin can also help with dissociating the antigens from the CARs following sorting (IBA 2017). Tetramer-based methods of isolation can improve confidence when sorting for CAR-expressing T-cells compared to using c-Myc as a reporter.

4.5.2 Increasing the Yield of CAR-A2$^+$ Teffs

One of the challenges in CAR research is in generating high numbers of T-cells that express the CAR gene. Typically, both Teffs and Tregs can be polyclonally expanded following transduction using anti-CD3 and anti-CD28 antibodies to obtain higher numbers of CAR-expressing cells (Trickett & Kwan 2003; Veerapathran *et al.* 2011). In our study, only transduced Tregs were expanded post-sorting, while transduced Teffs were rested for 5 days. Due to the lack of post-sorting expansion of Teffs, there were limited numbers of

CAR-A2$^+$ Teffs that were usable for re-stimulation in the MLR, and replicates could not be done within the limited time of the study.

The proliferation assay (figure 3.13) was used to assess the functions and antigen-specificity of CAR-A2. To increase CAR-Teff numbers, Teffs can be expanded by TCR stimulation using anti-CD3 and CD28 post-sorting (Liu, L *et al.* 2016), and then rested before re-stimulation using A2Kb splenocytes. Alternatively, CAR-expressing T-cells can also be expanded by culturing them with genetically modified K562 cells as AAPCs which can be altered to express an array of co-stimulatory molecules (Wang, X & Rivière 2016), or with A2-K562 cells to stimulate proliferation via the CAR receptors. Expanding CAR-A2$^+$ Teff numbers will allow for more repeats to confirm the functionality of the CAR-A2 molecule.

Chapter 5   Conclusion

CAR-A2$^+$ Tregs have been shown by MLR with A2Kb splenocytes to be more potent than polyclonal Tregs in suppressing allogeneic stimulation of CD4$^+$ Tregs. An *in vivo* study using a kidney transplant model will be the next step, and extensive molecular characterisation and phenotypical studies of CAR-A2$^+$ Tregs will be required to determine their safety and efficacy to further advance them for clinical therapy. Modifications to the techniques used can also be done in terms of 293 cell transfection and post-sorting Teff expansion to improve the yield of CAR-A2$^+$ Teffs and Tregs for further studies.

Appendix

Appendix 1 – Primer Sequence for the A2Kb Mouse Chimeric MHC Molecule

A2Kb mice express a chimeric MHC molecule made up of the α1 and α2 domains of HLA-A2.1 and the α3 to the cytosolic domain of H-2Kb. Primers were designed based on the sequences of these three domains.

α1 of HLA-A2:
GGCTCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCAG
TGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAGCCGCGGGC
GCCGTGGATAGAGCAGGAGGGTCCGGAGTATTGGGACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGACT
CACCGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAGAGCGAGGCC

	Sequence (5'->3')	Template Strand	Length	Start	Stop	Tm	GC%	Self-Complementarity	Self 3' Complementarity
Forward Primer	TTCTTCACATCCGTGTCCCG	Plus	20	22	41	60.04	55.00	3.00	3.00
Reverse Primer	CGCTCTGGTTGTAGTAGCCG	Minus	20	265	246	60.53	60.00	3.00	2.00
Product Length	244								

α2 of HLA-A2:
GGTTCTCACACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCGGGTACCACC
AGTACGCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCGCTCTTGGACCGCGGCGGACATGGC
AGCTCAGACCACCAAGCACAAGTGGGAGGCGGCCCATGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCACG
TGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGAGACGCTGCAGCGCACG

	Sequence (5'->3')	Template Strand	Length	Start	Stop	Tm	GC%	Self-Complementarity	Self 3' Complementarity
Forward Primer	GGTTCTCACACCGTCCAGAG	Plus	20	1	20	60.04	60.00	3.00	1.00
Reverse Primer	TCTCCTTCCCGTTCTCCAGG	Minus	20	262	243	60.61	60.00	3.00	3.00
Product Length	262								

α3 to Cytosolic Domain of H-2Kb:

TGGGAGCCTCCTCC**ATCCACTGTCTCCAACATGGC**GACCGTTGCTGTTCTGGTTGTCCTTGGAGCTGCAATAG
TCACTGGAGCTGTGGTGGCTTTTGTGATGAAGATGAGAAGGAGAAACACAGGTGGAAAAGGAGGGGACTATGC
TCTGGCTCCAGGCTCCCAGACCTCTGATCTGTCTCTCCCAGATT**GTAAAGTGATGGTTCATGACCCTC**ATTCT
CTAGCGTGA

	Sequence (5'->3')	Template Strand	Length	Start	Stop	Tm	GC%	Self-Complementarity	Self 3' Complementarity
Forward Primer	ATCCACTGTCTCCAACATGGC	Plus	21	15	35	60.34	52.38	4.00	2.00
Reverse Primer	GAGGGTCATGAACCATCACTTTAC	Minus	24	214	191	59.36	45.83	8.00	1.00
Product Length	200								

The sequences highlighted in yellow are the target sequences of their respective primers.

Appendix 2 – Anti-HLA-A2 Variable Chain Primer Sequence

Anti-HLA-A2 scFv:

CAG**GTGCAGCTGGTGCAGTCT**GGGGGAGGCGTGGTCCAGCCTGGGGGGTCCCTGAGAGTCTCCTGTGCAGCGT
CTGGAGTCACCCTCAGTGATTATGGCATGCATTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGC
TTTTTATACGGAATGATGGAAGTGATAAATATTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGAC
AACTCCGAGAAAACAGTGTCTCTGCAAATGAGCAGTCTCAGAGCTGAAGACACGGCTGTGTATTACTGTGCGA
AAAATGG**CGAATCTGGGCCTTTGGACT**ACTGGTACCTCGATCTCTGGGGCCGTGGCACCCTGGTCACCGTGTC
G

	Sequence (5'->3')	Template Strand	Length	Start	Stop	Tm	GC%	Self-Complementarity	Self 3' Complementarity
Forward Primer	GTGCAGCTGGTGCAGTCT	Plus	18	4	21	60.28	61.11	7.00	3.00
Reverse Primer	AGTCCAAAGGCCCAGATTCG	Minus	20	319	300	60.04	55.00	4.00	2.00
Product Length	316								

These primer sequences were made using reference to the scFv sequence for HLA-A2 by
doing a BLAST search. The sequences highlighted in yellow are the target sequences of

the primers. They were also sent to Applied Biosciences to create a custom TaqMan™ gene probe for quantitative PCR.

Appendix 3 – Plasmid Construct Sequences

Appendix 3.1 – pSAMEN Plasmid Sequence

Below is the sequence of the pSAMEN plasmid (6365 bp) backbone obtained from A/Prof. Michael Kershaw of The University of Melbourne.

```
GAATTAATTCATACCAGATCACCGAAAACTGTCCTCCAAATGTGTCCCCCTCACACTCCCA
AATTCGCGGGCTTCTGCTCTTAGACCACTCTACCCTATTCCCCACACTCACCGGAGCCAAA
GCCGCGGCCCTTCCGTTTCTTTGCTTTTGAAAGACCCCACCCGTAGGTGGCAAGCTAGCTT
AAGTAACGCCACTTTGCAAGGCATGGAAAAATACATAACTGAGAATAGGAAAGTTCAGATC
AAGGTCAGGAACAAAGAAACAGCTGAATACCAAACAGGATATCTGTGGTAAGCGGTTCCTG
CCCCGGCTCAGGGCCAAGAACAGATGAGACAGCTGAGTGATGGGCCAAACAGGATATCTGT
GGTAAGCAGTTCCTGCCCCGGCTCGGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGC
CCTCAGCAGTTTCTAGTGAATCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAAATGA
CCCTGTACCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCGCGCGCTTCCG
CTCTCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGCGCGCCAGTCTTCCGATAGA
CTGCGTCGCCCGGGTACCCGTATTCCCAATAAAGCCTCTTGCTGTTTGCATCCGAATCGTG
GTCTCGCTGTTCCTTGGGAGGGTCTCCTCTGAGTGATTGACTACCCACGACGGGGGTCTTT
CATTTGGGGGCTCGTCCGGGATTTGGAGACCCCTGCCCAGGGACCACCGACCCACCACCGG
GAGGTAAGCTGGCCAGCAACCTATCTGTGTCTGTCCGATTGTCTAGTGTCTATGTTTGATG
TTATGCGCCTGCGTCTGTACTAGTTAGCTAACTAGCTCTGTATCTGGCGGACCCGTGGTGG
AACTGACGAGTTCTGAACACCCGGCCGCAACCCAGGGAGACGTCCCAGGGACTTTGGGGGC
CGTTTTTGTGGCCCGACCTGAGGAAGGGAGTCGATGTGGAATCCGACCCCGTCAGGATATG
TGGTTCTGGTAGGAGACGAGAACCTAAAACAGTTCCCGCCTCCGTCTGAATTTTTGCTTTC
GGTTTGGAACCGAAGCCGCGCGTCTTGTCTGCTGCAGCATCGTTCTGTGTTGTCTCTGTCT
GACTGTGTTTCTGTATTTGTCTGAAAATTAGGGCCAGACTGTTACCACTCCCTTAAGTTTG
ACCTTAGGTCACTGGAAAGATGTCGAGCGGATCGCTCACAACCAGTCGGTAGATGTCAAGA
AGAGACGTTGGGTTACCTTCTGCTCTGCAGAATGGCCAACCTTTAACGTCGGATGGCCGCG
AGACGGCACCTTTAACCGAGACCTCATCACCCAGGTTAAGATCAAGGTCTTTTCACCTGGC
CCGCATGGACACCCAGACCAGGTCCCCTACATCGTGACCTGGGAAGCCTTGGCTTTTGACC
CCCCTCCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTCCGCCTCCTCTTCCTCCATCCGC
CCCGTCTCTCCCCCTTGAACCTCCTCGTTCGACCCCGCCTCGATCCTCCCTTTATCCAGCC
CTCACTCCTTCTCTAGGCGCCGGAATTCCGGCCGTGACAAGAGTTACTAACAGCCCCTCTC
TCCAAGCTCACTTACAGGCTCTCTACTTAGTCCAGCACGAAGTCTGGAGACCTCTGGCGGC
AGCCTACCAAGAACAACTGGACCGACCGGTGGTACCTCACCCTTACCGAGTCGGCGACACA
GTGTGGGTCCGCCGACACCAGACTAAGAACCTAGAACCTCGCTGGAAAGGACCTTACACAG
TCCTGCAGACCACCCCCACCGCCCTCAAAGTAGACGGCATCGCAGCTTGGATACACGCCGC
CCACGTGAAGGCTGCCGACCCCGGGGGTGGACCATCTCTAGACTGACGCGGCCGCTACGTA
GTCGACGGATCCCTCGAGGATCAATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTG
GCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATT
GCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCT
AGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAG
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TTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAA
CCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCA
AAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGC
TCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGG
ATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTC
TAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATAATACCATGATTG
AACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGA
CTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGG
CGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGG
CAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGT
CACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCA
TCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATA
CGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACG
TACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTC
GCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCG
TGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATT
CATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGT
GATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCG
CCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGG
ACTCTGGGGATCCGATAAAATAAAAGATTTTATTTAGTCTCCAGAAAAAGGGGGGAATGAA
AGACCCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGAAA
AATACATAACTGAGAATAGAGAAGTTCAGATCAAGGTCAGGAACAGATGGAACAGCTGAAT
ATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGA
TGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCA
GGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAGAACCATC
AGATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAA
TCAGTTCGCTTCTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCA
CAACCCCTCACTCGGGGCGCCAGTCCTCCGATTGACTGAGTCGCCCGGGTACCCGTGTATC
CAATAAACCCTCTTGCAGTTGCATCCGACTTGTGGTCTCGCTGTTCCTTGGGAGGGTCTCC
TCTGAGTGATTGACTACCCGTCAGCGGGGGTCTTTCATTTGGGGGCTCGTCCGGGATCGGG
AGACCCCTGCCCAGGGACCACCGACCCACCACCGGGAGGTAAGCTGGCTGCCTCGCGCGTT
TCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGT
CGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGC
GGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGC
GTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT
CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCAC
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CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACA
AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTT
TCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTG
TCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCA
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CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCG
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AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGC
TCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACC
ACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT
CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACG
TTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAA
AAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT

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GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTG
ACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCA
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GAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG
TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATT
GCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCC
AACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGG
TCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCA
CTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT
CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAAC
ACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCT
TCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTC
GTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAAC
AGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATA
CTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA
TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGT
GCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATC
ACGAGGCCCTTTCGTCTTCAA
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Dark green highlighted region is the site replaced by the CAR-A2 scFv gene.

Grey highlighted regions are the sequences of the pSAMEN backbone.

Appendix 3.2 – MIGR1 Plasmid Sequence

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TGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCC
CCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGG
CCACGATGCGTCCGGCGTAGAGGCGATTAGTCCAATTTGTTAAAGACAGGATATCAGTGGT
CCAGGCTCTAGTTTTGACTCAACAATATCACCAGCTGAAGCCTATAGAGTACGAGCCATAG
ATAAAATAAAAGATTTTATTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCCACCTGTA
GGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGAAAATACATAACTGAGA
ATAGAGAAGTTCAGATCAAGGTTAGGAACAGAGAGACAGCAGAATATGGGCCAAACAGGAT
ATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGG
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TTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGCGCGCCAGTCCTCCG
ATAGACTGCGTCGCCCGGGTACCCGTATTCCCAATAAAGCCTCTTGCTGTTTGCATCCGAA
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TCTTTCATTTGGAGGTTCCACCGAGATTTGGAGACCCCTGCCCAGGGACCACCGACCCCCC
CGCCGGGAGGTAAGCTGGCCAGCGGTCGTTTCGTGTCTGTCTCTGTCTTTGTGCGTGTTTG
TGCCGGCATCTAATGTTTGCGCCTGCGTCTGTACTAGTTAGCTAACTAGCTCTGTATCTGG
CGGACCCGTGGTGGAACTGACGAGTTCTGAACACCCGGCCGCAACCCTGGGAGACGTCCCA
GGGACTTTGGGGGCCGTTTTTGTGGCCCGACCTGAGGAAGGGAGTCGATGTGGAATCCGAC
CCCGTCAGGATATGTGGTTCTGGTAGGAGACGAGAACCTAAAACAGTTCCCGCCTCCGTCT
GAATTTTTGCTTTCGGTTTGGAACCGAAGCCGCGCGTCTTGTCTGCTGCAGCGCTGCAGCA
TCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTGAAAATTAGGGCCAGAC
TGTTACCACTCCCTTAAGTTTGACCTTAGGTCACTGGAAAGATGTCGAGCGGATCGCTCAC
AACCAGTCGGTAGATGTCAAGAAGAGACGTTGGGTTACCTTCTGCTCTGCAGAATGGCCAA
CCTTTAACGTCGGATGGCCGCGAGACGGCACCTTTAACCGAGACCTCATCACCCAGGTTAA
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GATCAAGGTCTTTTCACCTGGCCCGCATGGACACCCAGACCAGGTCCCCTACATCGTGACC
TGGGAAGCCTTGGCTTTTGACCCCCCTCCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTC
CGCCTCCTCTTCCTCCATCCGCCCCGTCTCTCCCCCTTGAACCTCCTCGTTCGACCCCGCC
TCGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGGCGCCGGAATT`AGATCTCTCGAG`
`GTTAACGAATTC`CGCCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCG
G`TG`TGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCC
CGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAG
GAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACA
AACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTC
TGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACG
TTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGG
GCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACA
TGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACG
TGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACAACC`ATGGTGAGCAAGGGCGAGG`
`AGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAA`
`GTTCAGCGTGTCTGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTC`
`ATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACG`
`GCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGC`
`CATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAG`
`ACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA`
`TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCA`
`CAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGC`
`CACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCG`
`GCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA`
`AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATC`
`ACTCTCGGCATGGACGAGCTGTACAAGTAA`TGAATTAATTAAGAATTATCAAGCTTATCGA
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TGAATCCACATACTCCAATACTCCTGAAATAGTTCATTATGGACAGCGCAGAAGAGCTGG
GGAGAATTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAA
AGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACT
GCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCG
GGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT
CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCAC
AGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAAC
CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACA
AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTT
TCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTG
TCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGA
CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCG
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AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGC
TCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACC
ACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGAT
CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACG
TTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAA
AAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTG
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ATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCG
GAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG
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GCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCC
AACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGG
TCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCA
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CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAAT
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CTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA
TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGT
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TCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGG
CGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATT
GTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACC
GCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGC
CTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTA
ACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCGCAAGGAATGGTGCATGCAA
GGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCC
```

Blue highlighted region shows a deletion of G between TG after position 1771.

Green highlighted region shows the GFP sequence.

Appendix 4 – CAR-A2-pSAMEN Sequence

The CAR-A2-pSAMEN plasmid is 7934 base pairs long.

```
GAATTAATTCATACCAGATCACCGAAAACTGTCCTCCAAATGTGTCCCCCTCACACTCCCA
AATTCGCGGGCTTCTGCTCTTAGACCACTCTACCCTATTCCCCACACTCACCGGAGCCAAA
GCCGCGGCCCTTCCGTTTCTTTGCTTTTGAAAGACCCCACCCGTAGGTGGCAAGCTAGCTT
AAGTAACGCCACTTTGCAAGGCATGGAAAATACATAACTGAGAATAGGAAAGTTCAGATC
AAGGTCAGGAACAAAGAAACAGCTGAATACCAAACAGGATATCTGTGGTAAGCGGTTCCTG
CCCCGGCTCAGGGCCAAGAACAGATGAGACAGCTGAGTGATGGGCCAAACAGGATATCTGT
GGTAAGCAGTTCCTGCCCCGGCTCGGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGC
CCTCAGCAGTTTCTAGTGAATCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAAATGA
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CCCTGTACCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCGCGCGCTTCCG
CTCTCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGCGCGCCAGTCTTCCGATAGA
CTGCGTCGCCCGGGTACCCGTATTCCCAATAAAGCCTCTTGCTGTTTGCATCCGAATCGTG
GTCTCGCTGTTCCTTGGGAGGGTCTCCTCTGAGTGATTGACTACCCACGACGGGGGTCTTT
CATTTGGGGGCTCGTCCGGGATTTGGAGACCCCTGCCCAGGGACCACCGACCCACCACCGG
GAGGTAAGCTGGCCAGCAACTtATCTGTGTCTGTCCGATTGTCTAGTGTCTATGTTTGATG
TTATGCGCCTGCGTCTGTACTAGTTAGCTAACTAGCTCTGTATCTGGCGGACCCGTGGTGG
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TGGTTCTGGTAGGAGACGAGAACCTAAAACAGTTCCCGCCTCCGTCTGAATTTTTGCTTTC
GGTTTGGAACCGAAGCCGCGCGTCTTGTCTGCTGCAGCATCGTTCTGTGTTGTCTCTGTCT
GACTGTGTTTCTGTATTTGTCTGAAAATTAGGGCCAGACTGTTACCACTCCCTTAAGTTTG
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TCCTGCAGACCACCCCCACCGCCCTCAAAGTAGACGGCATCGCAGCTTGGATACACGCCGC
CCACGTGAAGGCTGCCGACCCCGGGGGTGGACCATCCTCTAGACTGACGCGGCCGCTACGT
ACCATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGT
CTAGACAGGTGCAGCTGGTGCAGTCTGGGGGAGGCGTGGTCCAGCCTGGGGGGTCCCTGAG
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GAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTAC
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GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGT
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCC
TCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTC
GGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCG
GTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCAC
TGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGG
CCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTA
CCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGG
TTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTG

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ATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCA
TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATC
AATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA
CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA
TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCC
ACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA
AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAG
TAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGT
GTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTT
ACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCA
GAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTAC
TGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGA
GAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGC
CACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTC
AAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCT
TCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCG
CAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATA
TTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAG
AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAG
AAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCT
TCAA
```

Yellow highlighted region is the insertion site of the anti-HLA-A2 scFv genes (adopted from Kershaw's CAR-HER2-pSAMEN plasmid).

Turquoise highlighted region is the anti-HLA-A2 scFv heavy chain sequence.

Blue highlighted region is the anti-HLA-A2 scFv light chain sequence.

Pink highlighted region is the linker sequence.
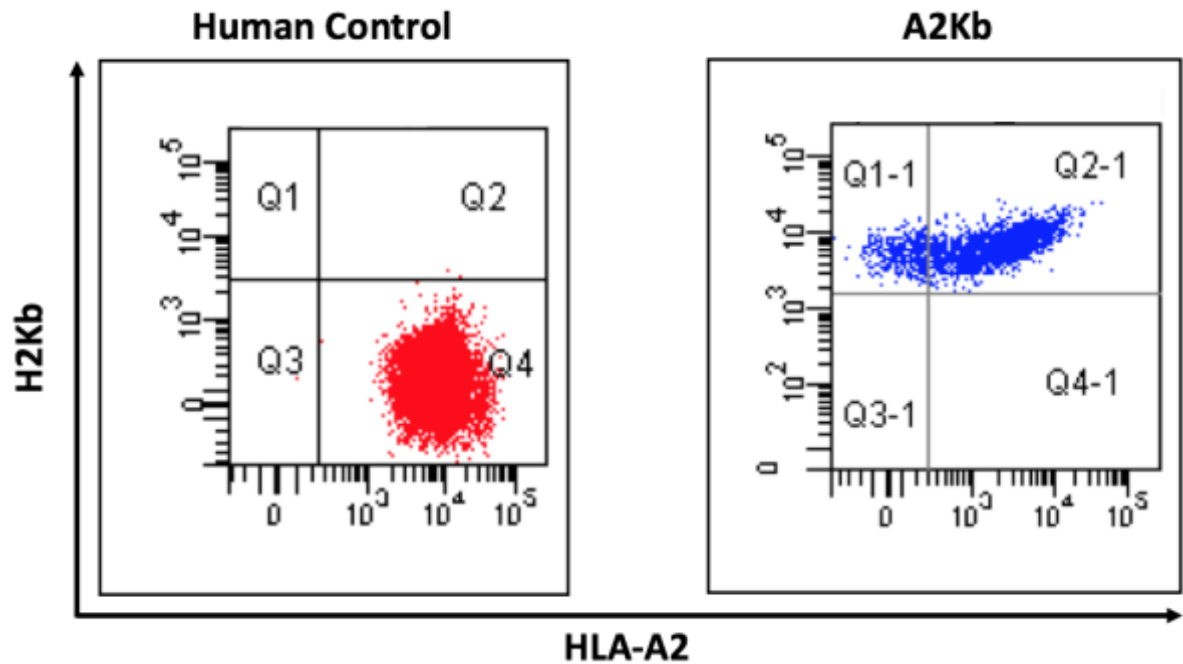
Red highlighted region is the c-Myc marker.

Bright green highlighted region is the CD8 linker sequence.

Teal highlighted region is the CD28 sequence.

Violet highlighted region is the CD247 (CD3ζ) sequence.

Grey highlighted regions are the sequences of the pSAMEN backbone.

Appendix 5 – HLA-A2 Antigen Expression in A2Kb Lymphocytes

This figure shows the extent of HLA-A2 antigen expression on A2Kb lymphocytes compared to human control lymphocytes. This data was obtained from Dr. Min Hu.

Appendix 6 – LabArchives DOI

The laboratory diary and all data obtained in this project can be accessed through LabArchives using the following DOI: dx.doi.org/10.6070/H4CJ8BXN.

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