Investigation of the Cell Biology of Human Regulatory T Cells in the Context of Transplantation

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Thesis submitted for DPhil in Surgical Sciences in Trinity Term 2016

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Abstract

Regulatory T cells (Tregs), lymphocytes that suppress immunological reactions, are of great interest for our comprehension of basic immunology and as a therapeutic agent to treat immune-mediated pathologies. Understanding the physiology of these cells will help to inform clinical strategies targeting Tregs.

In order to study the homing of human Tregs, we utilised genetic engineering to drive expression of fluorescent protein in human Tregs, permitting *in vivo* cell tracking. We optimised a protocol for lentivirus-mediated transduction of human Tregs during *in vitro* expansion, to generate high yields of stably-engineered cells. After infusing labelled cells into a humanised mouse model of skin allotransplantation, we detected human Tregs within a human skin graft by PCR and visualised Tregs moving in the graft, in a live mouse, by two-photon microscopy.

Through reverse genetic analyses, we explored molecular mechanisms that allow Tregs to respond adaptively to environmental cues. Neuropilin-1 (NRP1), a transmembrane co-receptor, has been implicated in the function of mouse Tregs. Tregs transduced with shRNA to knock down *NRP1* were severely impaired in their capacity to suppress cell proliferation *in vitro* and to prolong allograft survival in a humanised mouse model. qRT-PCR analysis revealed that transcription the gene encoding the anti-inflammatory cytokine IL-10, and the autophagy-associated genes *BECN1, COPS4* and *MAP1LC3B*, was significantly diminished in NRP1-deficient Tregs. We concluded that in human Tregs, NRP1 is necessary for suppressive function, most likely via regulation of NRP1-dependent regulation of cytokine production and metabolism.

Having identified a molecular target via which Treg function might be potentiated, we explored methods to target such molecules for cell therapy applications. Tregs engineered to over-express IL-10, but not NRP1, exerted significantly enhanced suppression of cell proliferation *in vitro*. Thus, relatively straightforward genetic engineering, compatible with generation of therapeutic cell yields, could be exploited to improve the efficacy of Treg cellular therapy.

Acknowledgements

During my graduate studies, I have been incredibly fortunate to have been supported by many colleagues and associates. My deepest gratitude must be reserved for my supervisor Joanna Hester. Joanna taught me almost everything I know about research and laboratory practice, and has done so with inexhaustible patience and kindness. On every occasion that I have encountered a problem that I believed to be insoluble, a discussion with Joanna has solved it. This is not exaggeration or flattery; Joanna really is an exceptional supervisor and colleague. I am also thankful to my supervisor Kathryn Wood, firstly for employing me as a completely inexperienced, insecure new graduate into her lab, and then encouraging me to take on challenges and build my confidence.

Other TRIG members have all played a part in this project. Before I embarked on my DPhil, I had the benefit of witnessing all stages of the PhD process, not always pretty, and yet the model set by previous students has been an inspiration. Meanwhile, the PostDocs and Research Assistants have been generous with their time, expertise, advice, encouragement and empathy. Among my colleagues, who all deserve thanks from me, some have made particular contributions that must be acknowledged here. Fadi Issa not only taught me most of the animal procedures but also procured the skin for the humanised mouse transplantation model. Fadi, and the other "Team Dominoes" members, Masaaki Zaitsu and Sushma Shankar, also deserve recognition for the team effort that made our skin-grafting factory feasible. Renee Robb guided me expertly through the two-photon microscopy and can be credited with the innovation that allowed us to image the skin non-invasively. Dr Stephen Juvet instructed me in the live cell imaging protocol and also earned my gratitude for being an enduring source of wisdom and reassurance. I am grateful to Monica Dalton for dealing so efficiently with all of the administration and practicalities that confound us scientists. Liz Wallin has been a perpetual source of moral support and camaraderie. I am also indebted to Liz for getting me out of the lab just enough to thrive within it.

Aside from colleagues in our lab, Dr Christoffer Lagerholm provided invaluable training and technical support for the microscopy experiments. The microarrays were performed as a service by Dilair Baban and colleagues at the, High Throughput Genomics group of the Wellcome Trust Centre for Human Genetics, University of Oxford. Rudd Van Stiphout very generously rescued me from a potential quagmire of microarray data by performing the bioinformatics analysis. The departmental facilities that we use have been managed brilliantly by Gareth Plant and Jakub Kupinski. I am also very grateful to staff in the Department of

Plastic and Reconstructive Surgery at the John Radcliffe Hospital, who procured the human tissue samples used in our mouse experiments. The animal house staff at BMS JR have been most supportive in ensuring the welfare of our mice.

This project was funded by a British Heart Foundation studentship and supported by funding from the European Society for Organ Transplantation, Kidney Research UK and the Wellcome Trust.

Finally, I owe a huge debt of gratitude to my parents, who raised me to enjoy, rather than endure, learning and academic challenges, without imposing any pressure to succeed and excel. My sister, the first Dr Milward, demonstrated beautifully how the task should be accomplished: efficiently, with enthusiasm and without drama.

Abbreviations

Abbreviation	Full
7AAD	7-Aminoactinomycin D
Ab	Antibody
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog 1; Protein Kinase B (PKB)
AMP	Adenosine Monophosphate
AnnV	Annexin V
APC	Antigen Presenting Cell or allophycocyanin
ATP	Adenosine Triphosphate
ATP5H	Mitochondrial ATP synthase subunit d
ATRA	All-Trans Retinoic Acid
BCL-2	B Cell Lymphoma protein 2
BCL-XL	BCL-like 1 isoform 1
BECN1	beclin-1
BIM	Bis(indolyl)maleimide
Breg	Regulatory B Lymphocyte
CAG	Cytomegalovirus Actin Globin
cAMP	Cyclic Adenosine Monophosphate
CAR	Chimaeric Antigen Receptor
Cas9	CRISPR-Associated protein 9
CCL	Chemokine (ligand)
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester

CLA	Cutaneous Lymphocyte Antigen
COPS4	Constitutive Photomorphogenesis 9 (COP9) Signalosome complex subunit 4
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cSMAC	Central Supramolecular Activation Cluster
CTLA-4	Cytotoxic T Lypmphocyte – Associated 4
cTreg	Central regulatory T cell
CXCR/CCR	Chemokine Receptor
Cy7	Cyanine 7
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
ECD	Energy Coupled Dye = R Phycoerythrin-Texas Red-X
EDTA	Ethylenediaminetetraacetic Acid
eGFP	Enhanced Green Fluorescent Protein
eTreg	Effector regulatory T cell
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase-1, CD39
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GF	Growth Factor
GFP	Green Fluorescent Protein
HGF	Hepatic Growth Factor
HLA	Human Leukocyte Antigen

HLA-DR	Human Leukocyte Antigen - Antigen D - related
HS	Human serum
i.p.	Intra-peritoneal(ly)
i.v.	Intra-venous(ly)
ICOS	Inducible T-cell COStimulator; CD278
IFN	Interferon
IKZF	Ikaros family Zing Finger
IKZF4	Ikaros Zing Finger 4, EOS
IL	Interleukin
IL-10	Interleukin-10
iTreg	*in vitro*-induced Treg
KD	Knock-down
LAG	Lymphocyte Activation Gene
LGALS1	Galectin-1 (gene/transcript)
LV	Lentivirus
MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B
MCL-1	Myeloid Leukemia Cell differentiation protein
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
moDC	Monocyte-Derived Dendritic Cell
Mreg	Regulatory Macrophage
mTOR	Mammalian Target of Rapamycin
N	Sample size (number of donors/mice)
NRP1	Neuropilin-1
NT / non-Td	Non-transduced

OE	Over-expressing/Over-expression
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic Cell
PDGF	Platelet-Derived Growth Factor
PD-L1	Programmed Death Ligand 1; CD274
PE	Phycoerythrin
PerCP	Peridinin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pSMAC	Peripheral Supramolecular Activation Cluster
PTEN	Phosphatase and Tensin Homologue
pTreg	Peripherally-derived Treg
qPCR	Quantitative Polymerase Chain Reaction
RAG/Rag	Recombination Activating Gene (protein/gene)
RNA	Ribonucleic Acid
ROR	RAR-related Orphan Receptor
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-Time Polymerase Chain Reactio
SD	Standard Deviation
SEM	Standard Error of the Mean
SEMA	Semaphorin
TALEN	Transcription Activator-Like Effector Nuclease
T-bet/*TBX21*	T-box 21 transcription factor
Td	Transduced

Teff	Effector T Cell
TGF	Transforming Growth Factor
Th	Helper T cell
TNF	Tumour Necrosis Factor
tolDC	Tolerogenic Dendritic Cell
TRAIL	Tumour Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand
Treg	Regulatory T cells
tTreg	Thymus-derived Treg
TU	Transforming Units
VEGF	Vascular Endothelial Growth Factor
VPD	Violet Proliferation Dye
WNT	Wingless-related Integration site
α-	Anti-

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Chapter 1: Introduction

1.1 A brief history of Transplant Immunology

Until further progress in regenerative medicine and bionic technologies permits restoration of failing tissues, transplantation is the most viable solution to end-stage organ dysfunction[1]. With advances in surgical expertise and technologies for transplantation, the greatest impediment to successful allogeneic transplantation (transplantation from one individual to another of the same species) is immunological rejection of the graft, whereby the recipient's immune system attacks the donor tissue. Before the advent of immunosuppressive treatments in the 1970s, success in clinical transplantation was rare.

Disregarding anecdotal reports dating to the fourth century CE[2], the first case of clinical allogeneic transplantation to be published in a scientific journal was an allogeneic skin graft performed in 1869. Throughout the late nineteenth and early twentieth century, cases of allogeneic transplantation remained relatively rare and almost universally unsuccessful in the long-term. Whilst destruction of grafted tissue was observed, it was generally attributed to surgical failure or poor tissue viability. Notably, the first tissue successfully transplanted between humans, reported in 1906[3], was a cornea, which is recognised today as an immunologically privileged tissue[4]. Not until the 1940s, when plastic surgery became commonplace in the treatment of military casualties, was destruction of grafted tissue recognised as an immunological phenomenon and the field of Transplant Immunology born.

Pioneering studies by Peter Medawar and colleagues shed the first light upon the immunological basis for graft rejection. Medawar observed that, in a patient who received both autologous and allogeneic skin grafts for burns injuries, only the allogeneic grafts were rapidly lost. A second attempt to graft skin from the same donor onto this patient resulted in accelerated loss of the grafts. Noting astutely that this accelerated secondary response was reminiscent of the secondary immune response following re-exposure to a pathogen, Medawar investigated the phenomenon further in an animal model, revealing that rabbits rejected skin grafts derived from a different strain with kinetics proportional to the mass of the graft. Furthermore, a second graft was rejected with substantially greater rapidity than the first graft only if the second graft was derived from the same donor as the first graft[5,6]

The description of transplant immunity bred a natural interest in methods by which this immunity might be overcome. In 1941, Frank Macfarlane Burnet proposed a theory of immunological tolerance founded upon the concept that a distinction, at the cellular and molecular level, between "self" versus "non-self" dictates antibody formation[7]. Meanwhile, the concept of *acquired* immunological tolerance had been observed experimentally in dizygotic twin calves, between whom skin grafts were tolerated. This phenomenon was attributed to the occurrence of mixed chimaerism among the blood cells of these calves, resulting from transfer of precursor blood cells between fraternal twins, via venous anastomoses *in utero*[8]. These findings inspired Medawar with the notion that immunological tolerance could be induced by prior exposure of a recipient to

foreign cells during embryogenesis and prompted his team to investigate the mechanisms underlying this tolerance. The resulting seminal report by Billingham, Brent and Medawar (1953) experimentally demonstrated the phenomenon termed "actively acquired immunological tolerance"[9]. Billingham et al observed that mice engrafted with cells from a second strain during foetal development, exhibited delayed rejection of skin grafts same strain as adults, when compared to mice that had received those cells as neonates or those that had received no cells. This tolerance was specific to the strain from the initial cell inoculation was derived, as skin grafts from a third strain were rejected with normal kinetics in these foetally- inoculated mice.

Whilst Medawar had identified graft rejection as an immunological phenomenon, theorising that allogeneic cells could be recognised as "non-self" by the immune system, the mechanism underlying this immune recognition within one species could not be understood until the field of immunogenetics developed. The existence of genetic determinants of histocompatibility between individuals of the same species (now known as "alloantigens"), and the concept of immune recognition of alloantigens as a physiological mechanism for immune rejection, had been explored theoretically in early scientific literature. Peter Gorer had identified histocompatibility antigens, including "Antigen II", now known as H-2, in 1936[10]. The observation that different animal strains were differentially susceptible to adoptively-transferred tumour cells, with tumours being destroyed in strains other than the strain from which the tumour derived, was not novel. However, the heritability of resistance to tumours had commonly been attributed

to non-Mendelian inheritance of some factor that could destroy malignant tissue whilst sparing healthy tissue. Loeb was the first to postulate that differential survival of transplanted tissue, depending upon the donor and recipient strains, was not restricted to tumours. Instead, resistance might be determined by the allogeneic nature of the transferred tissue ("individuality differentials"), rather than its malignant characteristics. Gorer recognised that the destruction of allogeneic tumours was analogous to the effects of antigenic variation (differential expression of blood group antigens) between erythrocytes from different blood groups. In his own words, Gorer proposed that "the genes determining susceptibility to tumour transplantation might be identical to those determining antigenic differences". In 1938, Gorer provided unequivocal experimental evidence for the immunogenicity of alloantigens by demonstrating the production of antibodies with affinity for "Antigen II"[10], after transplantation of tumours from an Antigen II-expressing mouse strain into an Antigen II-negative strain[11,12]. Later, a predominantly cellular mechanism for transplant rejection was demonstrated in a study by Mitchison, in which immunity to a transplanted tumour was conferred in previously tumour-susceptible mice after adoptive transfer of lymph nodes, but not serum or peritoneal exudate, from mice that were resistant to tumour growth[13].

George Snell and George Higgins subsequently characterised the critical genetic locus that was sufficient to cause rejection of tumour cells from a donor that differed genetically from the recipient only at this locus, the Major Histocompatibility Complex (MHC)[14] (or Human Leukocyte Antigen (HLA) in

humans) (discussed in more detail in sections 1.2.1 & 1.2.2). Proteins encoded by the MHC, we now know, play central roles in adaptive immunity mediated by T lymphocytes. In particular, the MHC class I and class II proteins provide a mechanism by which foreign peptides can stimulate T cell responses that target specifically cells that express those peptides. Indeed, these peptides can only be recognised by T cells when presented in a complex with a MHC protein. As discussed below, certain properties of MHC proteins, and the T cell antigen receptors (TCRs) that recognise them, explain why these molecules are particularly immunogenic in the context of allogeneic transplantation.

The concept of active, dominant mechanisms of tolerance was disclosed by later animal studies. Operational tolerance (defined as long-term graft survival in the absence of immunosuppression) towards a cardiac allograft was induced in rats using cyclosporine immunosuppression. When adoptively transferred into a naïve irradiated recipient, T cells from these rats were capable of conferring tolerance to an allograft from the same donor strain as the primary graft (but not to a third party graft)[15]. Subsequent investigations revealed that this immune suppression was conferred by a population of $CD4^+CD25^+$ T cells, termed "suppressor T cells" or "regulatory T cells" (Tregs) (described in detail in section 1.4)[16].

Since these pioneering studies, transplantation has proved to be an invaluable model system for investigating immunological processes[17–20].

1.2 Overview of Transplant Immunology

1.2.1 Immunological Tolerance

Vertebrates, including humans, bear an adaptive immune system that recognises foreign molecules (antigens) that are not normally present in the host organism. By default, the developing adaptive immune system will generate huge numbers of lymphocytes (adaptive immune cells), each recognising a unique set of antigens. Collectively, these cells are reactive against an almost unlimited range of putative antigens. Whilst the resulting immune responses provide critical protection from pathogens and malignant cells, the energetic costs and collateral tissue damage associated with inflammatory reactions must be minimised. The immune system must be able to discriminate between molecular signatures of potentially harmful cells or particles and antigens derived from the host and non-harmful biological material in their environment (including their microbiome and diet).

Within the adaptive immune system, the distinction between "self" and "non-self" occurs principally by virtue of tolerising mechanisms acting in the central lymphoid organs during development. These mechanisms purge the immune system of cells that react to auto-antigens (or "self-antigens", molecules expressed by the host), leaving only those reactive against antigens that are not encountered in the host. T cell antigen receptors ("T Cell Receptors" or TCRs) can only bind peptides that are bound to MHC (Major Histocompatibility Complex) glycoprotein complexes on the surface of antigen presenting cells (APCs). In order to induce signalling downstream of the TCR, the TCR must bind residues in both

the peptide and in the peptide-binding region of the MHC. Therefore, in order to provide protective recognition of non-self antigens, a T cell must express a TCR that recognises self-MHC. In the thymus, naïve T cell precursors undergo a process known as positive selection, whereby only those cells with TCRs that are able to bind self-MHC on cortical thymic epithelial cells receive signals from these APCs that are permissive to survival. Any T cell with low or no affinity for self-MHC (accounting for over 90 percent of the precursor cells) undergoes programmed cell death and is thus excluded from the mature T cell pool.

The T cells that survive positive selection all exhibit some degree of self-restriction. Hence, in order to avoid autoimmune reactivity, it is critical to eliminate T cells that can be activated above the threshold for inducing effector function by ligation of self-MHC bound to peptides from benign sources. In a process known as "clonal deletion", or "negative selection", thymocytes bearing TCRs with high affinity for autoantigens, presented on thymic epithelial cells or dendritic cells, are induced to undergo apoptosis[21].

Meanwhile, in the bone marrow, developing B cells with high affinity for autoantigen are subject to clonal deletion (apoptosis) or receptor editing (whereby genetic rearrangement at the BCR locus creates a BCR with altered antigen specificity). Low valence soluble antigens encountered in the bone marrow during development can induce B cell anergy, whereby the cell expresses little IgM and becomes unresponsive to antigen[22]. By these mechanisms, lymphocytes are "educated" to be unresponsive to molecules expressed by the host organism[23,24]. This is the basis of central tolerance.

Whilst central tolerance mechanisms are effective, they cannot account for every autoantigen and every antigen receptor specificity. Some cells will inevitably escape into the periphery with some degree of autoreactivity. A second layer of protection is conferred by peripheral tolerance mechanisms that restrain undesirable immune activity in non-lymphoid tissues. Some peripheral antigens are never encountered by T cells at high enough concentrations to induce an immune response, by virtue of their anatomical location being inaccessible to lymphocytes, and are ignored. In the absence of inflammation, expression of costimulatory molecules (discussed in Section 1.2.3.2) is not induced in APCs. TCR stimulation without costimulation induces anergy, rather than activation, in the T cells[21]. Without T cell help, B cells recognising multivalent autoantigen in the periphery undergo apoptosis, whilst those stimulated by soluble autoantigen in the absence of T cell help become anergic. In addition to these recessive mechanisms of peripheral tolerance, the immune system is subject to active, dominant regulation by regulatory cells, including regulatory T cells (discussed in detail in Section 1.4). These regulatory cells restrict the magnitude and duration of immune responses in order to prevent autoimmune reactions and to maintain immune homeostasis[23].

1.2.2 The Allogeneic Response

1.2.2.1 The physiological basis of the allogeneic response

The protection of host tissue conferred by central tolerance does not extend to antigens expressed by conspecific, but genetically disparate, organ donors. As a consequence of small differences in the genetic code between individuals of the

same species, one individual will express proteins that are functionally indistinguishable from those of another individual but differ slightly at the amino acid sequence level. These apparently insignificant differences in protein primary structure are detected by lymphocytes, due to the high specificity with which lymphocyte antigen receptors bind peptides. Thus, the molecular determinants of allorecognition by the adaptive immune system, known as alloantigens, comprise any variants of polymorphic peptides expressed by the donor that are not present in the recipient, due to the genetic disparity between the two individuals.

The MHC locus is highly polymorphic, displaying an unusually high level of allelic variation between individuals of a species. Considered in an ecological context, it is likely that any individual will be susceptible to a particular subset of pathogens, due to constraints upon the breadth of T cell specificities for different pathogens that can be generated in one genome. Under these circumstances, there is a strong selective advantage in harbouring a repertoire of immune specificities that differs from that of one's neighbours, including those specificities that are lacking in one's neighbours. Over evolutionary history, natural selection has favoured unprecedented polymorphism at the MHC locus because this genetic polymorphism manifests as diversity in immune profiles within a population.

As the most polymorphic locus in the genome, the Major Histocompatibility Complex Locus (the Human Leukocyte Antigen (HLA) locus in humans) is the source of potently immunogenic alloantigens. Additionally, MHC is expressed at high density on the cell surface, where these antigens can be bound by

antibodies, stimulating a humoral response, and by B cell receptors, permitting presentation of these antigens by B cells. Furthermore, MHC antigens are expressed broadly (even ubiquitously, in the case of class I MHC proteins), so grafts from any tissue will contain a high load of antigen. In relation to T cell activation, MHC proteins are uniquely immunogenic as a consequence of selection for TCRs with affinity for MHC during negative thymic selection, combined with cross-reactivity among the resulting TCR repertoire.

It is estimated that up to ten percent of an individual's T cell repertoire can respond to alloantigen[25,26]. The risk of rejection escalates with each "mis-match" between donor and recipient and so can be minimised by matching recipients to donors with the fewest genetic disparities at the MHC locus[27,28].

Alloantigens other than the MHC proteins (collectively called minor histocompatibility antigens, mHAgs) can be recognised as foreign peptides bound to either self-MHC or donor-MHC, or as B cell epitopes. mHAgs can derive from any locus in the genome that varies in sequence between individuals. Even fully HLA-matched grafts harbour mHAgs capable of stimulating graft rejection[29,30].

Whilst clinicians endeavour to match the genetic background of donor and recipient as closely as possible, an allograft inevitably expresses antigens to which the recipient immune system has not been tolerised. Peripheral tolerance mechanisms are not adapted to deal with such a high load of novel antigen, in an inflammatory context, which, in nature, would signal a rampant infection or malignancy. Consequently, without treatment to control the immune response, allogeneic grafts are inevitably subject to immunological rejection that will

eventually destroy the donor tissue and damage recipient tissue exposed to the inflammation.

1.2.2.2 Origins of the alloresponse

Briefly, before discussing the proximal mechanisms of graft rejection, it is informative to consider the ultimate causation (i.e. the evolutionary context) of both allorecognition and tolerance to allogeneic tissue. According to the revered Evolutionary Biologist Theodosius Dobzhansky, "nothing in Biology makes sense except in the light of evolution"[31]. As discussed above (section 1.2.2.1), the alloreactivity of the adaptive immune system is a by-product of the drive for genetic diversity in the MHC repertoire and the selection for MHC-restricted, yet cross-reactive, TCRs. With the exception of pregnancy, long-term and invasive exposure to allogeneic tissue is a purely artificial phenomenon and has, therefore, not been subject to neither positive nor negative natural selection.

When considering the evolutionary rationale for innate allorecognition, the observation of rejection of conspecific cells in several species of invertebrates (which lack adaptive immune systems) is particularly informative[32]. It is speculated that, in invertebrate colonial species, innate allorecognition helps to protect the organism from parasitism and to deter one colony from growing in close proximity to other colonies with which it would compete for resources. Thus, in contrast to adaptive allorecognition mechanisms, which are largely an unfortunate by-product of anti-pathogen and anti-cancer immunity, it appears that the innate mechanisms of allorecognition were favoured through evolution expressly for the outcome of immunity against conspecific cells. The conservation

of these mechanisms in mammals, where they appear to be redundant, is more difficult to explain but might be attributed to the benefits of excluding invasive foetal cells from maternal tissue[33].

Conversely, pregnancy also presents a context in which evolution of mechanisms that favour tolerance to alloantigen might be favoured by natural selection. During pregnancy, the mother's immune system is exposed to hemi-allogeneic cells of the foetus. Foetal cells bear antigens encoded by paternally-inherited genes to which the maternal lymphocytes have not been tolerised. Multiple mechanisms have been invoked to account for materno-foetal tolerance[34,35], which may also account for the numerous reported cases of spontaneous graft acceptance after withdrawal of immunosuppression. It has even been suggested that sustained tolerance to non-inherited maternal antigens, to which a foetus is exposed *in utero*, might confer upon female offspring a reproductive advantage, if those antigens are borne by their own foetus in a future pregnancy[36].

1.2.3 Mechanisms of Graft Rejection

1.2.3.1 Innate recognition of non-self and altered self and innate mechanisms of graft rejection

Grafted tissue is subject to several waves of immunological responses, beginning even before transplantation and enduring for the life of the graft, employing different stimuli and different mediators[37]. The early phases of the immune response, before and immediately after transplantation, are mediated by the innate immune system (first that of the donor, then that of the recipient). Even prior to retrieval from the donor, brain death and cardiac death in deceased

donors compromise the physiology of the donor tissue, in a manner that stimulates the innate immune response to tissue damage. Cells undergoing physiological stress express molecules, referred to as Damage-Associated Molecular Patters (DAMPs), that are detected by cells of the innate immune system via dedicated receptors. Recognition of DAMPs stimulates the immune system to clear damaged or dysregulated tissue, as well as any pathogens that might have initiated the damage, and to facilitate tissue repair. In transplantation, stimulation of the innate system by DAMPs promotes graft rejection[38]. Tissue retrieval inevitably induces further tissue damage, which propagates cascades of inflammatory reactions. Whilst organ preservation and preconditioning strategies are improving, any time spent outside the body results in a deterioration in cell viability that further exacerbates this inflammatory response. The sterile inflammation initiated by the surgical procedure, including ischaemia-reperfusion injury accompanying the perfusion of the graft, create an inflammatory environment within and surrounding the transplant[39].

This proinflammatory milieu not only exposes the graft to a fresh assault by the recipient's innate immune response but also, in the case of an allogeneic graft, determines the nature of the adaptive immune response that follows[40]. Broadly, proinflammatory cytokines (especially interleukins, such as IL-6, class I interferons, chemokines and Tumour Necrosis Factors (TNF)) promote activation, cell cycle and migration of immune cells[40]. The complement system, a proteolytic cascade in the blood plasma that can be activated by DAMPs or by antibody, generates molecular mediators that augment both innate and adaptive immunity.

Complement 3b and 4b (C3b and C4b) bind to the surface of target cells and recruit phagocytes expressing complement receptors, which phagocytose target cells. In addition to acting as chemoattractive stimuli for various immune cells, C3a, C4a and C5a induce vasodilation that is permissive to migration of leukocytes into the damages tissue. Meanwhile, other complement factors assemble as a membrane attack complex to lyse target cells[41].

An increasing appreciation of the major role that the innate immune system plays in initiating graft rejection has grown from greater understanding of the molecular and cellular mediators of graft rejection[42–44]. These innate inflammatory responses can be induced non-specifically, without any detection of foreign antigen. However, an emerging paradigm asserts that the innate immune system is capable of discriminating between self and conspecific non-self[33,45,46]. Studies have shown that inflammatory reactions, including infiltration by IL-12-secreting DCs, are induced at a greater magnitude by allogeneic transplants than by syngeneic transplants, even in mice that lack lymphocytes[47,48]. Monocytes[47,48], macrophages[49] and NK cells[49,50] have been identified as innate cells capable mediating innate allorecognition but the exact identity of the molecular determinants of innate allorecognition in mammals remain a mystery at present.

1.2.3.2 Recognition of alloantigen by the adaptive immune system

Within hours of transplantation, the recipient's adaptive immune response is mobilised. Upon exposure to alloantigen, lymphocytes are stimulated to proliferate and instigate destruction of the allogeneic cells of the graft.

Alloantigen-dependent activation of T cells requires three signals (see **Figure 1.1**)[51]. Binding of the TCR of a T cell to a MHC-peptide complex in which either peptide or MHC (or both) is donor-derived, resulting in intracellular signalling via the TCR-CD3 complex, provides the first signal required for priming of alloantigen-reactive T cells (i.e. any T cells in the existing repertoire capable of recognising donor-derived antigens). The second signal necessary for T cell activation by alloantigen is engagement of costimulatory ligands, expressed on APCs, by costimulatory receptors on the T cell[52]. Cytokines also confer chemical signals that are essential for T cell activation. The resulting recruitment of signalling and adhesion molecules into an immunological synapse initiates a program of gene expression that permits full T cell activation.

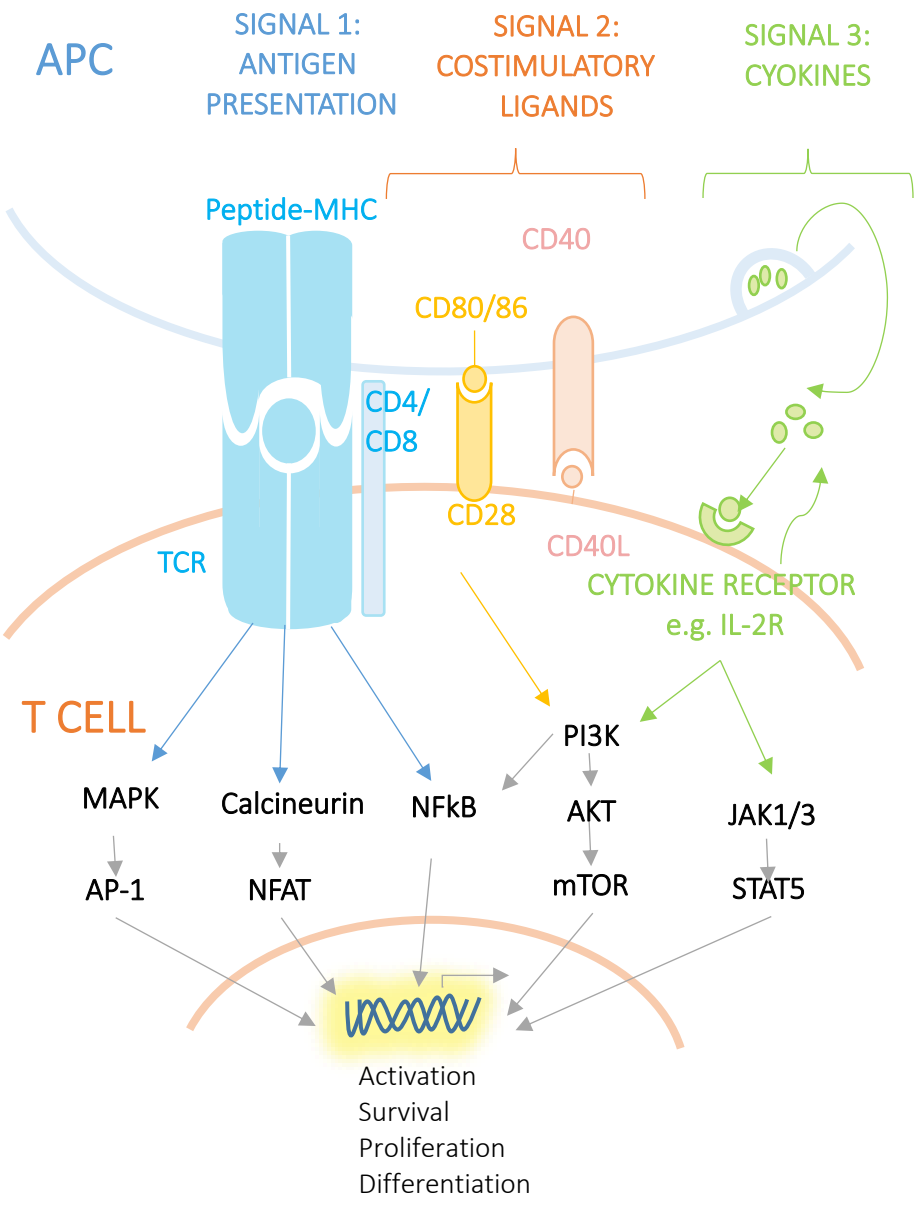

Figure 1.1. Three-Signal Model of T Cell Activation

Antigen-dependent T Cell activation requires three biochemical signals. The first event in T cell activation is engagement of the TCR by its cognate peptide-MHC complex. The stability of the TCR:MHC interaction and the intracellular signalling events propagated by TCR stimulation are also dependent upon the interaction of a co-receptor (CD4 or CD8) with the MHC. These interactions initiate signalling pathway that ultimately activate the transcription factors, NFkB, AP-1 and NFAT, responsible for orchestrating the transcriptional programs necessary for T cell activation. Further intracellular signalling, to stimulate T cell activation and survival, is induced downstream of costimulatory receptors or ligands on the surface of the T cell when these molecules engage with their binding partners expressed on the surface of the APC. One of the consequences of these signalling events is elevated secretion of mitogenic cytokines, especially IL-2, which stimulate proliferation in an autocrine and a paracrine manner,

Recognition of alloantigen by recipient T cells can occur via three mechanisms: direct, indirect and semi-direct allorecognition[53,54]. Donor APCs can migrate from the transplanted tissue into recipient lymphoid organs[55]. Additionally, donor vascular endothelium can present antigen. Intact donor MHC expressed on donor APCs, bound to any peptide, can be recognised by recipient T cells via the direct pathway. This recognition of non-self-MHC is thought to occur, in spite of positive selection of T cells with high affinity for self-MHC, as result from cross-reactivity or degeneracy of self-MHC-restricted TCRs, expressed by T cells that have survived positive selection in the thymus[56]. Cross-reactivity of TCRs with multiple MHC variants is an inherent property of the preselection TCR repertoire. Since there is no evolutionary impetus for selection against reactivity towards non-self-MHC, and no mechanism for imposing negative selection against donor MHC-reactive T cell precursors in the thymus, these TCRs persist in the post-selection pool.

The indirect pathway involves uptake, processing and presentation of donor-derived peptides by recipient APCs, via the exogenous antigen processing pathway. Donor antigen is then presented on recipient MHC class II to self MHC-restricted recipient $CD4^+$ T cells. mHAgs stimulate alloreactive immunity through this indirect pathway.

Exosome-mediated or trogocytic transfer of plasma membrane containing intact donor MHC class I or class II from donor cells to recipient APCs can allow semi-direct recognition of donor MHC-peptide, presented by recipient APCs to recipient $CD4^+$ or $CD8^+$ T cells with direct allospecificty[57]. The direct pathway of

alloantigen presentation is thought to be more potent, due to relatively high frequency of recipient T cells that are reactive to donor MHC, and therefore dominates during the initiation phase of rejection, early after transplantation. However, because passenger professional APCs (expressing donor MHCII) persist for only a relatively short time, the contribution of direct pathway must be superseded by the indirect and semi-direct pathways over time[58].

Among the many costimulatory ligand-receptor interactions identified in T cell activation, the B7 and TNF families dominate. The engagement of CD28 (expressed constitutively on T cells) with CD80 and CD86 (induced on activated APCs) induces signals in the T cell that induce cell cycle and IL-2 protein, whilst inhibiting activation-induced cell death. Costimulatory signals are also induced by binding of CD154 (CD40 ligand, CD40L), OX40[59] and ICOS on the T cell with CD40, OX40L and ICOSL, respectively, expressed on the APCs. Counteracting these costimulatory signals, coinhibitory molecules can confer unresponsiveness or anergy upon T cells. In particular, CD152 (Cytotoxic T Lymphocyte-Associated protein 4, CTLA-4) competitively inhibits CD28-mediated costimulatory signalling by binding CD80 and CD86 with much greater avidity than does CD28[60].

Secretion of cytokines, including IL-2, is upregulated in T cells as a consequence of transcriptional programs initiated by TCR-dependent signalling. These cytokines act in an autocrine and paracrine manner to further amplify the clonal expansion and differentiation of T cells, as well as activating other immune cells.

Various cytokines promote the activation of T cells that execute cell-mediated graft rejection. Certain cytokines will favour polarisation of this T cell response

toward a particular helper T cell (Th) subset[61]. For example, IL-12, derived principally from DCs, favours differentiation of Th1 cells. These Th1 cells promote macrophage- and $CD8^+$ T cell responses that classically mediate cell-mediated killing and clearance of infected or malignant cells. Meanwhile, IL-4 favours differentiation of Th2 cells that support B cells, eosinophils and mast cells and promote humoral (antibody-based) immunity towards multicellular parasites. Conversely, the presence of anti-inflammatory cytokines such as IL-10, IL-35 and TGFβ can induce unresponsiveness in T cells or differentiation of regulatory T cells (Tregs), promoting tolerance towards the allograft[62]. Ultimately, the outcome of T cell allorecognition, activation or anergy (promoting graft rejection or tolerance, respectively), is determined by the balance between stimulatory and inhibitory signals.

The antigen receptors of B cells, the B cell receptor (BCR), binds intact (unprocessed) antigen in its native conformation, in a MHC-independent manner. B cell activation is augmented by cytokines, derived from leukocytes and damaged tissue, and by complement[63]. B cells are not MHC-restricted and so do not exhibit the same predisposition to allorecognition as T cells. Nonetheless, BCRs are not tolerised to alloantigen and so any individual's BCR repertoire will be reactive to epitopes of both non-self MHC and mHAgs. Since B cells require help from $CD4^+$ T cells that are reactive to the same cognate antigen, alloreactive B cells become anergised unless the corresponding T cell clone is active.

Natural Killer (NK) cells are capable of recognising allogeneic cells via a different mechanism, referred to as "missing self". NK cells express inhibitory receptors

that recognise self MHC class I specifically and become activated by donor cells in the absence of self-MHC[50,64]. Once activated, NK cells upregulated perforin and granzymes, mediating cytotoxic lysis of target cells.

1.2.3.3 The adaptive immune response to allogeneic tissue

Following activation of antigen-reactive lymphocytes, the mechanisms by which the adaptive immune system targets tissue are numerous and interconnected, in a manner that permits rapid escalation of response. Pro-inflammatory cytokines convey signals between different cell types of the adaptive and innate immune systems, in order to recruit the most appropriate cell functions for the nature of the immune stimulus. Ultimately, the destruction of donor tissue is conducted by cytotoxic T cells and NK cells (in the case of cell-mediated rejection) or by antibodies derived from B cells and plasma cell (in the case of humoral rejection)[65].

B cells expressing B cell receptors (BCRs) that bind alloantigens receive signals via the BCR to mature and secrete donor-reactive antibodies. Upon binding of alloantibodies to their cognate alloantigen on the surface of a donor cell, especially in the vascular endothelium, these antibodies fix complement and recruit various innate immune cells via Fc receptors and complement receptors. Thus target donor cells can be targetted for phagocytosis, antibody-mediated cellular cytotoxicity (by macrophages and NK cells) or for complement-mediated cytolysis (via formation of the membrane attack complex)[66]. In particular, hyperacute rejection of vascularised solid organ transplants is firmly established as an antibody-dependent process. Hyperacute rejection can commence

immediately upon perfusion of the organ in recipients with preformed donor-reactive antibody specificities. As soon as the donor organ is perfused with the recipient's blood, donor-reactive antibodies can bind antigens on the graft vascular endothelium and blood cells.

Antibody-mediated rejection has also been implicated in acute and chronic allograft dysfunction following the generation of *de novo* donor-specific antibodies[67]. However, the relative contribution of humoral versus cell-mediated mechanisms of graft rejection is the subject of some controversy[68]. There is associative evidence of B cell infiltration, antibody deposition and complement 4d in the allografts of patients undergoing rejection episodes, which supports a role for B cells and antibody in the aetiology of graft rejection[69,70]. Some studies have also demonstrated a causal link between antibodies and graft rejection[65]. For example, transfer of alloreactive antibodies into lymphocyte-deficient mice is sufficient to induce chronic allograft vasculopathy in allogeneic heart transplants. Conversely, some studies have found B cells to be dispensable for graft rejection[71]. In some cases, intragraft B cells have been found to be neutral or even protective with respect to allograft rejection.

In recipients who do not generate pre-formed alloantibodies, cell-mediated mechanisms are classically thought to dominate the allogeneic response[72]. T cells are the main protagonists of cell-mediated alloreactivity. Cytotoxic T cells secrete cytolytic enzymes granzymes A and B and perforin, and express death ligands that induce apoptosis in target cells bearing the corresponding death receptors. Helper T cells, meanwhile, propagate the immune response against the graft in

several ways[73]. Firstly, Th cells secrete cytokines and express surface ligands that recruit other leukocytes to the graft or associated lymphoid organs and potentiate the activity of other immune effector cells. Another important function of Th cells is to provide help to B cells, initiating the humoral response to the allograft. Th cells also help cytotoxic $CD8^+$ T cells.

As mentioned above, T cells with specificity for microbial antigens can cross-react with alloantigens and exert heterologous immunity towards graft tissue. Critically, the same phenomenon has been observed in memory T cells. In contrast to naïve T cells that are dependent upon antigen presentation by professional APCs within lymphoid organs[74], memory T cells can be activated in the periphery (including within the graft) by any APC and at a lower threshold of costimulation[75]. This property of memory cells, combined with their high proliferative potential, makes the memory response to the allograft dangerously vigorous and rapid. Furthermore, by virtue of their reduced requirements for costimulation, are more resistant to costimulation[76]. In fact, this memory response is becoming recognised as critical determinant of graft rejection and failure of tolerance induction in patients[77]. Unfortunately, the memory response is often not represented in animal models that have not been exposed to microbial stimuli, such as those against which a memory cell pool is generated in humans.

In addition to their role in antibody production, B cells contribute to cell-mediated acute and chronic allograft rejection. Highlighting the importance of antibody-independent B cell function in allograft rejection, in a mouse model of cardiac transplantation, chronic allograft vasculopathy was found to be dependent upon

B cells, whilst antibodies were dispensable[78]. Probably the most influential role that B cells perform in the allogeneic response is as antigen presenting cells[79]. B cells can provide all three signals required for T cells activation: peptide presented on MHC class I and II, costimulatory ligands (CD80/86, CD40, OX40L and ICOSL) and cytokines (IFNγ, TNFα, IL-6 and IL-17). In particular, it has been shown that B cells are required for the generation of alloreactive memory T cells that are thought to be potent mediators of graft rejection[80]. In addition to inducing T cell proliferation and effector functions, the cytokines secreted by B cells promote cell-mediated immunity by modulating the differentiation of T cell subsets and recruiting the functions of polymorphonuclear cells that stimulate other lymphocytes[63].

1.3 Clinical Strategies to Resist Graft Rejection

1.3.1 Pharmacological Immunosuppression for Graft Rejection

Various approaches have been adopted to counteract the mechanisms of rejection at every stage. Strategies to increase organ donor recruitment, coupled with technological advances in organ preservation, increase the likelihood of obtaining good quality organs that have been exposed to minimal damage prior to transplantation. Tissue typing, to determine the HLA haplotype of potential live donors and recipients, permits some degree of strategic matching to minimise the genetic disparity between donor and recipient. However, inevitably there will always be alloantigens present in the graft to induce the recipient's lymphocytes and so further medical interventions are required to overcome the adaptive immune response.

Currently, the standard medical intervention for graft rejection is pharmacological immunosuppression. Immunosuppressive drugs act by various mechanisms to impair the activity of immune effector cells, especially T and B lymphocytes which are thought to be the major protagonists of the allogeneic response. Most commonly, regimens for solid organ transplantation involve a prophylactic induction of strong immunosuppression for a short period after the transplant operation, followed by life-long maintenance immunosuppression. Supplementary immunosuppression can be administered in the event of rejection being detected.

Immunosuppressive drugs have been designed to intercept all stages of the immune response; from lymphocyte survival and proliferation in response to primary antigen exposure, through TCR stimulation and costimulation, to production of immune effector molecules (summarised in 0)[81]. The most potent (but also rather crude) approach involves depleting the recipient of leukocytes, which is normally performed as induction therapy, for a short period immediately after transplantation. Leukocyte depletion can be achieved using monoclonal depleting antibodies directed against antigens expressed specifically on leukocytes, such as anti-CD52, which targets these cells for both antibody-dependent and complement-dependent cytotoxicity[82]. Polyclonal anti-thymocyte globulins are also used for T cell depletion.

For maintenance immunosuppression, lymphodepleting drugs would be too harmful to use long term so pharmaceuticals are selected that inhibit immune cell activation, proliferation or recruitment, rather than cell survival. Agents that

interfere with the cell cycle will affect lymphocytes preferentially, due to the unusually high proliferative capacity of these cells, compared with other adult tissues. For example, purine or pyramidine analogues and alkylating agents disrupt DNA synthesis required for cell division.

To target lymphocyte activation, drugs can be used to block the TCR (for example, anti-CD3 monoclonal antibodies) or BCR directly. Alternatively, some drugs block costimulatory pathways[83,84] that are required for T cell activation. For example, CTLA-4-Ig (Cytotoxic T Lymphocyte-associated Antigen 4 -Immunoglobulin) binds CD80/86, in competition with CD28, without activating downstream costimulatory signalling.

Drugs can also be used to neutralise the molecular mediators of the immune response, or their receptors, in order to quench the self-propagating cascade of inflammatory responses mediated by cytokines. Synthesis of interleukin-2, a potent stimulator of T cells, can be blocked by calcineurin inhibitors[85]. Alternatively, sensing of IL-2 by T cells can be inhibited using antibodies that block the IL-2 receptor protein CD25[86].

Table 1: Classes of Immunosuppressive Drugs used in Transplantation

Class of immuno-suppression[81,87]	Examples	Target molecule/pathway	Effect on immune system	Adverse effects[88] (in addition to infection & malignancy)
CORTICOSTEROIDS (GLUCOCORTICOIDS)[89]	Prednisone Methylprednisolone	Nuclear translocation of glucocorticoid receptor → transcriptional activation/repression via Glucocorticoid Response Element in genes encoding cytokines and NFkB	Reduced pro-inflammatory cytokine secretion; increased anti-inflammatory cytokine secretion; impaired T cell	Hypertension; peptic ulcers; hypothalamic-pituitary-adrenal suppression; Insulin-resistance; osteonecrosis &

			development; reduced T cell proliferation; T cell apoptosis	osteoporosis; retinopathy; neurotoxicity[90]
CALCINEURIN INHIBITORS	Cyclosporin A/G Tacrolimus	Inhibit calcineurin phosphatase → NFAT pathway	Reduced T cell activation & cytokine secretion	Nephrotoxicity, neurotoxocity, *de novo* diabetes
ANTIMETABOLITES[91]	Azathioprine Mycophenolate mofetil	Inhibit synthesis of nucleotides for DNA synthesis, blocking cell cycle preferentially in lymphocytes	Reduced lymphocyte expansion	Gastrointestinal disturbance; genitourinary symptoms; anaemia, leukopeania; neutropeania; thrombocytopaenia; neurological symptoms (fatigue, insomnia, tinnitus)
MONOCLONAL ANTIBODIES[92]	Muromonab CD3 (OKT3); Alemtuzumab (CAMPATH-1H, anti-CD52); Rituximab (anti-CD20)	Bind cognate antigen expressed specifically by target cell population e.g. CD3 on T cells, CD52 on all leukocytes, CD20 on B cells	Depletion of immune cells bearing antigen	Cytokine Release Syndrome; leukopenia; coagulation disorders; aseptic meningitis/encephalopathy; hypersensitivity to mouse protein[93,94]
POLYCLONAL ANTIBODIES[95,96]	Antithymocyte globulin (ATG), usually from rabbit	Bind antigens expressed on surface of human T cells, (including receptors required for lymphocyte activation, recruitment & survival), blocking the function of these receptors, inducing apoptosis and targeting cells for C'-mediated lysis.	Depletion of T cells; reduced recruitment of lymphocytes; B cell depletion; lysis of DCs; sparing (possibly inducing) Tregs & NKT cells	Cytokine Release Syndrome; Post-Transplantation Lymphoproliferative Disorder
MTOR INHIBITORS[97,98]	Sirolimus (Rapamycin, Rapamune) Everolimus	Inhibits mammalian Target of Rapamycin Complex 1 (mTORC1), leading to reduced activity of AKT, which promotes the cell cycle through transcriptional regulation.	Inhibit proliferation of Teffs>Tregs	Stomatitis; anaemia; hyperglycaemia; Interstitial Lung Disease; fatigue; nausea; diarrhoea; impaired wound healing[99]

COSTIMULATION BLOCKADE[84]	Belatacept (CTLA-4-Ig); αCD28; ICOS antagonists	Bind and block costimulation receptors/ligands or downstream transducers of costiumulatory signalling.	Inhibit T cells activation in the presence of cognate antigen, inducing antigen-unresponsiveness	Risk of agonism of target e.g. massive cytokine storm induced by αCD28
CYTOKINE RECEPTOR ANTAGONISTS (e.g. IL-2R antagonists)	Daclizumab Basiliximab	Bind to and block cytokine receptor components e.g. CD25 (alpha chain of IL-2R), preventing pro-survival and -proliferation signalling	Inhibit T cell proliferation	

Since the advent of immunosuppressive chemotherapy for transplantation in the 1970s, the short-term outcome of transplantation has improved consistently, with UK graft survival rates at 1 year post-transplant reaching 97% for first adult kidney transplant from live donors in 2013[100]. Whilst the progressive reduction in acute graft rejection in transplant patients over history is undoubtedly a testament to the effectiveness of these drugs, little improvement has been observed in the rate of chronic allograft dysfunction (CAD). Furthermore, the benefits of these drugs are off-set by the severe side-effects associated with systemic immune suppression[88]. The impotent state of the immune system following immunosuppression leaves the patient vulnerable to infections and metastases that would be contained and destroyed by an intact immune system. Additionally, each drug conveys a suite of toxicities. The calcineurin inhibitor Tacrolimus, for example, is associated with greatly increased risk of hypertension, new onset diabetes and nephrotoxicity. Since the protection conferred by these drugs is maintained only for the duration of the treatment (rarely is tolerance to

a graft sustained after cessation of the therapy), a transplant recipient is obliged to submit to all of the sequelae of immunosuppression for their entire future life.

1.3.2 Tolerance Induction in Transplantation

For these reasons, focus has shifted from immunosuppression towards tolerance induction, with the goal of rendering the recipient's intact immune system unresponsive to alloantigen. It is thought that costimulatory blockade may contribute to tolerance induction, since, in theory, T cells recognising alloantigen in the absence of co-stimulation should be rendered anergic[101]. Induction of donor haematopoietic macrochimearism, whereby the recipient's blood is populated by donor blood cells after the infusion of bone marrow or haematopoietic stem cells, can cause apoptotic deletion of donor-reactive T cells, to create a more stable tolerant state. Several cases have been reported of patients receiving solid organ transplants following stem cell transplants from the same donor, who were successfully weaned off immunosuppression[102–104]. Intuitively, it may be supposed that dominant mechanisms of peripheral tolerance, such as those exerted by regulatory cell subsets, could be exploited for more potent tolerance induction.

1.3.3 Cellular Therapies for Transplantation

Ideally, any treatment for allograft rejection should suppress the immune response against the graft specifically, without systemic effects upon the immune system. The next generation of transplant therapeutics should be designed with minimal toxicity and maximal specificity for the graft. One promising avenue being explored as a solution to drug-associated morbidity is to complement or

replace pharmacological treatments with cellular therapies. Rather than using drugs to manipulate immune cells *in situ*, it might be preferable to modulate the number or quality of suppressive immune cells by infusing more regulatory cells into the patient[105].

The concept of cellular therapy for transplantation is appealing since cells are exquisitely adapted over the course of evolution to respond to their environment and to behave in a manner that is beneficial to their host organism. Furthermore, regulatory cells exert diverse pro-tolerogenic effects upon the immune system[106], rather than targeting one cell type or one biochemical pathway. Critically, tolerance induced by regulatory cells is thought to be self-perpetuating, such that a single treatment episode might be sufficient to sustain long-term therapeutic outcomes.

Whilst facultative regulatory capacity has been observed in a broad range of cell subsets (described in Table 1.7, below) this regulatory function is often context-dependent or possibly confined to a subset of these cells that is yet to be formally characterised. Many of these cell populations, including plasmacytoid DCs (pDCs)[107], NKT cells[108] and γδT cells[109], also exhibit effector function that would be detrimental to tolerance-inducing cellular therapies. Among the apparently "professional" regulatory cells, various populations are being explored as putative cell therapies for immune-mediated pathologies, including transplantation[105]. In addition to the published studies listed in **Table 2** (below), the ONE Study is a multi-centre clinical trial testing regulatory immune cell therapies in kidney transplantation, including classical regulatory T cells (Tregs), Tr1 cells, tolerogenic

DCs and regulatory macrophages (Mregs). Regulatory T cells, which have probably the strongest body of evidence to support their efficacy as a cell therapy product, are the focus of this thesis and shall be described in more detail below.

Table 2: Immune cells with regulatory properties

Cell population	Phenotype (human)	Principal regulatory mechanism(s)	Published clinical trials (refs)
Plasmacytoid DCs[107]	$CD4^+CD11c^{lo}MHCII^{lo}BDCA2^+ILT7^+CD68^+CD123^+CD303^+CD304^+ILT3^+$	Treg induction	
Immature myeloid DCs, inc. tolerogenic DC (TolDC)[110]	$CD11c^{lo}CD11b^{hi}MHCII^{lo}CD86^{lo}$	Low expression of costimulatory molecules → antigen presentation induced unresponsiveness in Teffs; IDO - & FasL- dependent activation-induced cell death in Teffs; Galectin-1-dependent Treg expansion; IDO-dependent Treg induction; HO-1-dependent inhibition of Teff proliferation	Phase I healthy volunteers[111,112]; diabetes[113]; RA[114,115]; Crohn's[116]; SOT (prospective)[110]
Myeloid-derived suppressor cells (MDSCs)[117]	$CD11b^+CD33^+CD34^+HLA\text{-}DR^{lo}$	Nitric oxide synthase- & arginase 1-dependent inhibition of Teff proliferation & cytokine production; IFNγ- & IL-10-dependent Treg induction; HO-1; promote regulatory macrophage differentiation	
Mesenchymal stromal cells (MSCs)[118]	$CD34^-CD45^-CD73^+CD90^+CD105^+HLA\text{-}DR^-$	TGFβ-, prostaglandin E2- and contact-dependent Treg induction & inhibition of DC maturation; inhibition of Ab production	RA[119]; SOT[120,121]
Regulatory macrophages[122]	?	IL-10; Inhibit cytokine production in Teffs; promote tissue repair	SOT[123,124]
NKT cells[108,125]	$Vα24\text{-}Jα18^+Vβ11^+$	IFNγ & IL-4; promote Treg expansion	

γδ T cells[109,126]	TCRVγ9Vδ2	TGFβ & IL-10; granzyme/perforin- and FasL-mediated cytolysis of Th2 and macrophages; inhibit proliferation of Teffs; CTLA-4 & PD-L1; induce senescence in Teffs; inhibit DC maturation;	
Regulatory T Cells (Tregs) ($CD4^+$)[127,128]	$CD4^+CD25^+CD127^{lo}FOXP3^+$	See section 1.4.4	diabetes[129,130]; cord blood transplantation[131]; HSCT[132,133]
$CD8^+$ Tregs[134–136]	Various, inc. $CD8^+CD25^+FOXP3^+$ $CD8^+CD28^-$	IFNγ → induction of IDO, IL-10 & TGFβ expression in other cells; IL-10 & TGFβ; CTLA-4; inhibition of DC-dependent Teff activation	
DN Tregs[137]	$CD3^+CD4^-CD8^-NK1.1^-$	CD95-mediated induction of apoptosis of Teffs & DCs; trogocytic uptake of alloantigen from DCs; downregulation of co-stimulatory ligands on DCs	
Tr1 cells[138,139]	$CD4^+CD49b^+LAG-3^+IL\text{-}10^+$	Predominantly IL-10. TGFβ; granzyme/perforin-mediated cytolysis of myeloid cells; CTLA-4-, LAG-3- & PD-1-mediated inhibition of DC-dependent Teff; metabolic disruption of Teffs via CD39 & CD73	HSCT[140]
Regulatory B cells (Bregs)[141–143]	$CD19^+CD20^+CD24^{hi}CD27^-CD38^{hi}IgD^{hi}IgM^{hi}$	IL-10; Treg induction & expansion; DC tolerisation; induction of unresponsiveness in Teffs	

HSCT = Haematopoietic Stem Cell Transplantation; SOT = Solid Organ Transplantation; RA = rheumatoid arthritis

1.4 Overview of Regulatory T Cells

1.4.1 Phenotype of Tregs

Regulatory T Cells are a subset of T lymphocytes whose function in normal physiology is to maintain immune homeostasis and suppress autoreactive

immune response. The necessity for Treg-mediated immune regulation is illustrated by the severe pathologies associated with defects or deficits in this cell subset. IPEX (Immune-Dysregulation Polyendocrinopathy Enteropathy X-Linked) patients bearing a genetic mutation in the canonical Treg-associated transcription factor, FOXP3 (Forkhead Box 3) (discussed in more detail below), suffer from systemic autoimmunity[144–146]. Meanwhile, defects in Treg function have been implicated in many autoimmune diseases such as type I diabetes and multiple schlerosis[147]. Conversely, elevated densities of $FOXP3^+$ T cells have been associated with a chronic infections[148] and metastases[149]. Given the critical role that Tregs evidently perform in regulating immunity, there has been great interest in characterising these cells; identifying markers by which this cell population can be recognised and understanding the development, function and diversity of cells within this subset.

Despite a plethora of molecules that have been championed as Tregs markers, no single molecule has yet been identified that uniquely distinguishes human Tregs from any other T cell subset. This paucity of markers greatly confounds the identification, isolation and manipulation of Tregs. The regulatory T cell subset first identified in mice were characterised as $CD4^+CD25^+$ [150,151]. In mice, expression of the transcription factor FOXP3 is restricted to T cells with regulatory function and thus defines the Treg subset. FOXP3 can act as a transcriptional activator or repressor and is known to instruct epigenetic modification of its target genes. In this capacity, FOXP3 is understood to be critical for establishing genome-wide transcriptional and epigenetic regulation of a Treg-specific gene

expression program that specifies Treg cell fate[152,153]. However, the practical application of this marker is limited by the intracellular localisation of the molecule, which prohibits access to this protein in viable cells without a reporter construct. Furthermore, several regulatory T cell subsets have been described that reside outside the $CD4^+FOXP3^+$ population, including the IL-10-producing Tr1 cells[139] and $CD8^+FOXP3^+$[134], Double Negative (DN) T cells[137] and TGFβ-producing Th3 cells. Even disregarding these non-$FOXP^{3+}$ regulatory cells, the identification of human $FOXP3^+$ Tregs is confounded by the promiscuity of FOXP3 expression amongst human lymphocytes. FOXP3 is strongly upregulated upon TCR stimulation in human $CD4^+$ and $CD8^+$ Teffs[154,155], which negates the use of this transcription factor as a marker of Tregs.

Instead, researchers must rely upon a Treg "signature" comprising cell surface molecules that are preferentially, though not exclusively, expressed by Tregs. Most notably, the IL-2 receptor α chain (CD25), Glucocorticoid-Induced Tumour Necrosis Factor (TNF) Receptor (GITR)[156] , Lymphocyte Activation Gene 3 (LAG-3), CD39[157,158] and Cytotoxic T Lymphocyte-associated Antigen-4 (CTLA-4, CD152)[151,159] are all enriched on the surface of Tregs but can also be transiently upregulated to equivalent densities by Teffs.

Transcriptional profiling can also aid the discrimination between Tregs and Teffs, at the population level, for compatible applications. Currently, the most reliable hallmark of Treg phenotype is an epigenetic signature, specifically demethylation of DNA in the Treg-Specific Demethylated Region (TSDR) of the *FOXP3* promoter[153,160–162] (see **Figure 1.2**). Since neither transcriptional nor epigenetic

level molecular characterisation is conducive to the isolation of live cells, Tregs for research or therapeutic applications must be selected upon the basis of cell surface markers, however imperfect these may be. The current consensus recommends that selection upon the phenotype $CD4^+CD25^+CD127^{lo}$[163–165], and arguably $CD45RA^{hi}$ [166], yields the most suppressive $CD4^+FOXP3^+$ Tregs from human peripheral blood.

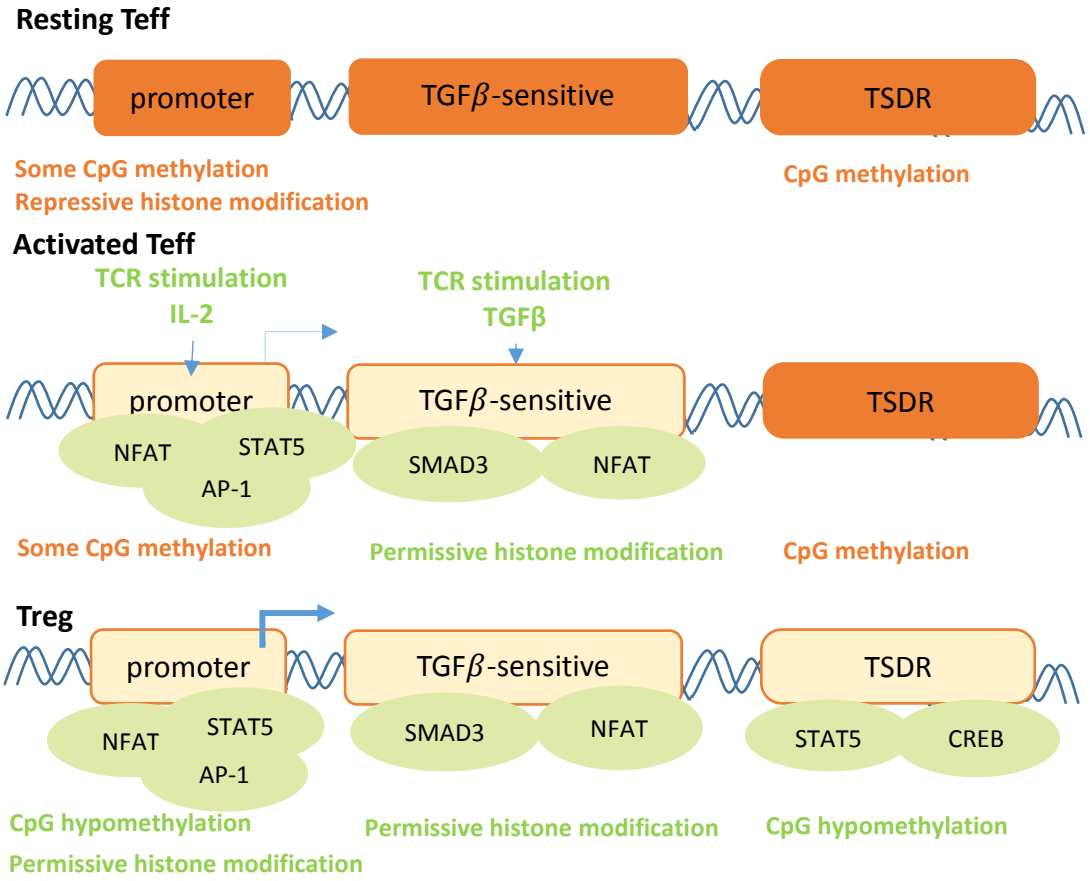

Figure 1.2 FOXP3 gene structure & regulation

The FOXP3 locus is subject to extensive epigenetic regulation, especially within three domains: the promoter, a TGFβ-sensitive enhancer and the Treg-Specific Demethyated Region (TSDR). In resting Teffs, the enhancer and TSDR are subject to inhibitory histone modifications and, in the case of the TSDR, DNA methylation. These epigenetic modifications create inaccessible higher-order chromatin structures that preclude binding of transcription factors and accessory proteins, rendering the gene poorly transcribed. The DNA methylation that regulates the TSDR is stable over cell generations and accounts for the stable basal repression of FOXP3 in Teffs. This stable repression can be overcome partially and transiently by permissive post-translational histone modifications within the TSDR and enhancer element. These modificaitons are transient, inducible and reversible, in response to environmental stimuli, such as cytokines. Permissive histone modifications include H3- & H4-acetylation and H3K4 methylation. H3K27 methylation is generally repressive. Upon TCR stimulation, in the presence of IL-2, the FOXP3 promoter undergoes epigenetic modifications that are permissive to transcription of the gene. TGFβ-dependent chromatin modifications permit access of transcriptional activators to the enhancer element. Together, these modification result in unstable expression of FOXP3 protein. In contrast, the stable FOXP3 protein expression observed in Treg cells results from constitutive CpG hypomethylation of the TSDR and is further propagated by permissive histone modifications within all three regulatory domains. NFAT = Nuclear Factor of Activated T cells; STAT = Signal Transducer and Activator of Transcription; AP-1 = Activator Protein 1; CREB = cyclic-AMP-responsive-element-binding protein; SMAD-3 = Mothers Against Decapentaplegic homolog 3.

Whilst FOXP3 is not infallible as a marker of Tregs, this transcription factor is undoubtedly responsible for orchestrating regulatory behaviour in the cells that express this protein constitutively. The transient upregulation of FOXP3 in activated Teffs is to insufficient to confer regulatory potential upon these cells[167], yet reduced proinflammatory activity, and even suppressive properties, have been reported in $CD4^+$ Teffs engineered to overexpress FOXP3[168,169] .

Broadly, the requirements for Treg development and function differ in several respects from those of Teffs. The activity of Teffs is antigen-specific, since Teffs require secondary exposure to their cognate antigen in order to induce their pro-inflammatory capacity. Whilst TCR stimulation is required for the development of Tregs, and for their initial activation, the suppression mediated by Tregs is TCR-independent and can be antigen-nonspecific. Consequently, Tregs exhibit a phenomenon termed "bystander suppression", whereby Tregs activated in response to one antigen are capable of suppressing Teffs with a different antigen-specificity, provided they are present in the same microenvironment. Some studies suggest that Tregs can suppress in the absence of the Treg cognate antigen[170]. Others claim that bystander suppression occurs only when the cognate antigens of the Treg and the Teff are presented by the same APC[171] .

Compared to $CD4^+$ Teffs, Tregs differ in their response to cytokines. Tregs are more sensitive to IL-2 than Teffs, since Tregs express higher constitutive levels of CD25, and more sensitive to antigen. Conversely, Tregs express lower levels of the IL-7 receptor CD127, rendering Tregs less sensitive to IL-7. Despite the pro-apoptotic properties of FOXP3, Tregs are also more resistant to apoptosis than

Teffs, partly due to the higher expression of anti-apoptotic protein MCL-1 in Tregs[172,173]. The metabolic profiles of Tregs and Teffs differ too, with Tregs exhibiting a greater dependence upon fatty acid oxidation, as opposed to the glycolytic pathway favoured by Teffs and various myeloid cells[174]. As a consequence of the disparity between Treg and Teff cell biology, Tregs occupy a distinct homeostatic niche, as demonstrated by the selective expansion of adoptively-transferred Tregs in Treg-depleted mice[175] .

1.4.2 Development of Tregs

In common with all T lymphocytes, the development of Tregs in the thymus begins from a naïve thymocyte precursor. Tregs can be classified into two categories according to their developmental history: those that acquired their Treg phenotype within the thymus ("thymic-derived Tregs" or "tTregs") and those that emerged from the thymus as Teff and were later induced to differentiate into Tregs in the periphery ("peripheral Tregs" or "pTregs"). (See **Figure 1.3**)

Thymic differentiation of Tregs is dependent upon engagement of the TCR by self peptide-MHCII complex. Unlike Teffs bearing TCRs that recognise autoantigen, autoreactive Tregs survive clonal deletion[176,177]. Certain disparities between Tregs and Teffs may account for the resistance of Tregs to clonal selection[178]. Tregs are stimulated by much lower affinity TCR:peptide-MHC interactions than Teffs, permitting survival of Tregs with an affinity for their cognate antigen that is below the threshold required for Teffs to survive positive selection[179]. Additionally, the biochemistry of Tregs is inherently pre-disposed to resist apoptosis more than that of Teffs[172], which may spare Tregs from clonal deletion. With their TCR

repertoire enriched for low-abundance autoantigens[180], tTregs are adapted for maintaining steady-state tolerance to self-antigens in order to prevent autoimmunity.

Meanwhile, the differentiation of Teffs into Tregs in the periphery is induced by TCR stimulation with exogenous antigen under tolerogenic conditions (with anti-inflammatory cytokines, especially TGFβ dominating over pro-inflammatory cytokines, and with APCs expressing inhibitory co-receptor ligands and low levels of costimulatory molecules)[181]. The prevailing model asserts that abundant exogenous antigens derived from commensal organisms and from innocuous environmental matter, including food antigens, stimulate this differentiation. The resulting pTregs confer tolerance to those harmless non-self antigens in the periphery, thus conferring protection from hypersensitivity reactions.

Once Tregs have progressed to secondary lymphoid organs, stimulation via the TCR and CD28 propagates upregulation of the transcription factors IRF4 and Blimp-1[182]. These transcription factors orchestrate further differentiation from the so-called central Treg ("cTreg") phenotype to an effector Tregs ("eTreg") phenotype. cTregs are resilient but quiescent cells, which localise to the T cell zones (in a CCR7- and CD62L-dependent manner), where they appear to exert control over immune cell priming. eTregs express much lower levels of CD62L, permitting more migration out of lymphoid tissues. eTregs are characterised by greatly elevated expression of activation markers including the IL-2 receptor α chain CD25, associated with a reduced dependence upon IL-2 that permits these cells to proliferate prodigiously in niches where paracrine IL-2 is limited. As such,

eTregs are better adapted to function in the marginal zones and B cells follicles of secondary lymphoid organs and at peripheral sites of inflammation, where the density of Teffs, and hence of paracrine IL-2, is relatively low. Indeed, it is to precisely these IL-2-poor anatomical sites that eTregs home, by virtue of the distinct repertoire of chemokine receptors and adhesion molecules expressed by these cells[183,184]. Overall, cTregs exert greater control over priming of effector cells in secondary lymphoid tissue to enforce tolerance to autoantigens, whilst effector Tregs are adapted to control ongoing inflammation in peripheral sites, to enforce immune homeostasis and tolerance to innocuous foreign antigens[185,186].

In common with eTreg activation, the generation of pTregs is most likely a reaction to immune perturbation, requiring more than basal regulation by Tregs to re-assert tolerance or immune homeostasis. pTreg differentiation in the periphery can be stimulated by some factors acting on T cells directly, such as the vitamin D metabolite calcitriol, extracellular adenosine (a vitamin A metabolite)[187] and certain bacterial short chain fatty acids derived from the gut microbiota[188–190]. Other environmental cues exert tolerising effects upon dendritic cells. Tolerogenic DCs subsequently promote pTreg differentiation via various soluble factors. For example, tryptophan metabolites generated by DC-derived IDO act via the Aryl-Hydrocarbon Receptor in T cells to favour polarisation towards a Treg phenotype[191]. Meanwhile, DCs expressing integrin αvβ8 convert latent TGFβ into its active form, which drives local differentiation of Teffs into pTregs[192].

Further differentiation of Tregs into a memory subset (mTregs) may account for the emerging phenomenon of regulatory memory[193]. Analogous to memory Teffs

and plasma cells, which persist after resolution of inflammation and rapidly initiate an inflammatory response upon secondary exposure to their cognate antigen, mTregs have been identified that can persist in peripheral tissues in the absence of cognate antigen and suppress these secondary immune responses. Intuitively, regulatory memory must be regarded as an adaptation to intermittently-encountered antigens, such as foetus- or semen-derived alloantigen, neo-autoantigens (such as pregnancy-associated antigens) and antigens derived from innocuous microbes that occasionally breach mucosal barriers. This specialised function is reflected in the enrichment of mTregs at mucosal barriers, in skin[194], and during pregnancy[195].

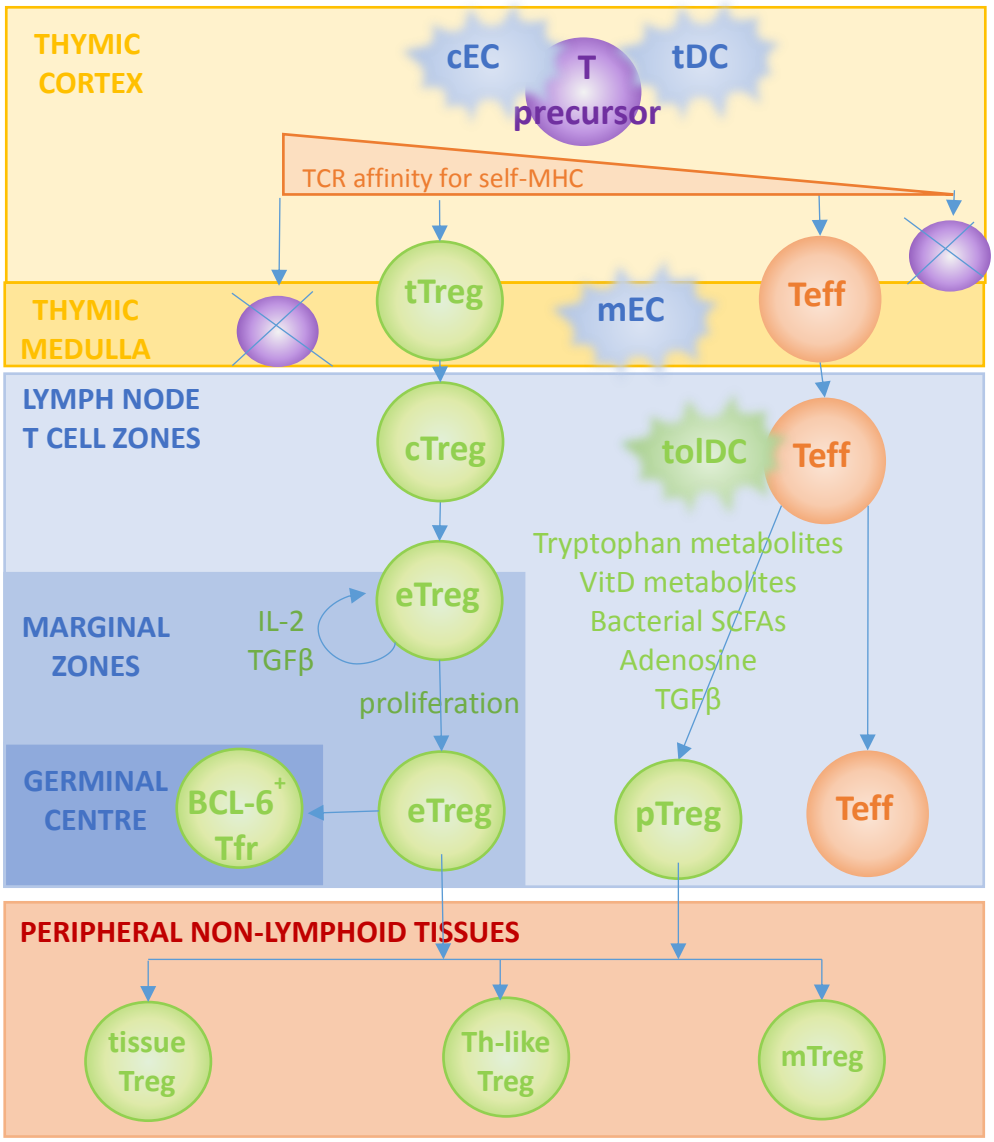

Figure 1.3 Development of Tregs

As with all T cells, Tregs derive from lymphoid progenitors in the bone marrow that migrate into the thymus as immature thmyocytes. These T cell precursors undergo TCR gene rearrangement and positive selection in the thymic cortex and negative selection primarily in the thymic medulla. The specification of Tregs can be imprinted in the thymus, during thymocyte development. Treg cell fate appears to be induced in thmyocytes bearing TCRs with moderately high affinity for self antigen, lower than the threshold required for negative selection. Thymic Tregs migrate to secondary lymphoid organs, where these cells differentiate into central Tregs (cTregs). cTregs can undergo further maturation to become highly proliferative effector Tregs (eTregs) in response to IL-2 and IL-15. Alternatively, a regulatory phenotype can be induced in Teffs within secondary lymphoid organs, yielding peripheral Tregs (pTregs). Differential expression of chemokine receptors and adhesion molecules directs migration of eTregs or pTregs to lymphoid compartments or non-lymphoid tissues, where these cells adopt tissue-specific phenotypes and functions

1.4.3 Treg Diversity and Specialisation

It is becoming increasingly evident that Tregs, and their Teff precursors, are highly responsive to local environmental factors; not only cytokines but also metabolites of both lymphoid and non-lymphoid cells and of commensal microorganisms. The resulting phenotypic and functional heterogeneity within the Treg population (summarised in Table 1.8, below) has added a further layer of complexity to the study of Treg biology.

Within secondary lymphoid organs, eTregs have been observed to undergo further functional diversification, mirroring that of Th, into functional subsets that orchestrate a suite of immune mechanisms most appropriate to the immunological challenge[196]. Specifically, Tregs can upregulate the canonical T helper 1 (Th1) transcription factor T-bet and express the chemokine receptor CXCR3 that directs migration of these cells to sites of Th1-mediated inflammation[197,198]. Likewise, Tregs expressing the Th2-associated transcription factor IRF4 home to sites of Th2 activity in a CCR4-dependent manner[199,200]. Th17 cells are shadowed by a Treg subset expressing the transcription factor STAT3 and the chemokine receptor CCR6[201]. Finally, BCL-6-expressing Tregs home to germinal centres where they antagonise T follicular helper (Tfh) cells.

Some regard phenotypic plasticity of Tregs with concern, questioning whether the expression of Th-associated molecular signatures by Tregs is indicative of loss of regulatory phenotype in these cells. The prospect that Tregs might acquire a pro-inflammatory phenotype under the influence of a proinflammatory milieu is

naturally daunting in the context of Treg-based therapeutics. Reassuringly, several studies have demonstrated that the regulatory function of Tregs is not compromised following differentiation into Th-like subsets [196,202,203]. Whilst the plasticity of Treg phenotype may be accountable for some controversy and confusion within the field of Treg biology, this functional diversity may prove a valuable substrate for finer resolution modulation of the immune system, by exploiting the refined functions of each cell subset differentially.

Treg suppressive function is not confined to secondary lymphoid organs but rather Tregs also exert immune regulation within non-lymphoid peripheral tissues. A large proportion of eTregs appear not only to migrate to peripheral sites but also to reside therein, by virtue of chemokine receptors and adhesion molecules that direct these cells to particular tissues or inflamed sites. Tissue-resident Tregs comprise phenotypically and functionally distinct subsets, expressing different transcription factors, different cell surface markers and distinct TCR specificities compared with lymphoid-resident counterparts (see Table 1.8.) Thus, tissue -infiltrating and tissue-resident Tregs are adapted for their microenvironment and for the nature of the immune milieu to which they are exposed[186].

The idiosyncratic transcriptomes exhibited by tissue-resident Tregs reflect the expression of the molecules required for migration to, and retention in, those specific peripheral tissues and for the specialised function exerted by Tregs within those tissues. For example, infiltration into the visceral adipose tissue is achieved by a subset of Tregs expressing adipose-homing molecules GATA3 and CD103[204–

[206]. The upregulation of these homing molecules is dependent upon expression of nuclear receptor Peroxisome Proliferator-Activated Receptor γ (PPARγ), a transcription factor that also regulated differentiation in adipocytes[207]. Meanwhile, Tregs can reside in the skin by virtue of higher expression of skin-homing chemokine receptors, such as CCR4, and adhesion molecules, including Cutaneous Lymphocyte Antigen (CLA)[194,199,208–210]. Tissue-resident Treg subsets may also perform roles beyond immune regulation *per se*, by promoting tissue-support function, such as muscle regeneration[211]. Notably, the study of tissue-resident Treg subset has, so far, been restricted mostly to animal models. Since this is not a phenomenon that is easily modelled *in vitro*, evidence for tissue Tregs in humans is currently sparse and, necessarily, mostly associative or indirect[212].

Table 3: Regulatory T cell subsets

Treg subset		Cytokine receptors & adhesion molecules	Transcription factors	Tissue tropism	Enriched Cytokines	Principle functions
Th-like Treg subsets[196]						
	Th1-like [197,198,213]	CXCR3	T-bet		IFNγ IL-10	Regulating Th1-dominated immune reactions (intracellular microbes, autoimmunity)
	Th2-like [200]	CCR4	GATA-3 IRF4		IL-4	Regulating Th2-dominated immune reactions (parasites, hypersensitivity)
	Th17-like [202,214]	CCR6 CCR4	RORγt		IL-17 IL-10	Regulating Th17-dominated immune reactions (extracellular microbes)
	Th22-like	CCR6 CCR4 CCR10 CLA				Regulating Th22-dominated immune reactions (epidermal immunity & remodelling)[215,216]
	Tfr [217–220]		BCL-6	Germinal centres		Regulating germinal centre response
Tissue-homing/tissue-resident Tregs[186,221]						
	Skin [199,208–210,222]	CCR4 E-selectin CLA		Skin		Tolerance to autoAgs
	Gut [188,190,223]	CCR9 α4β7		Gut		Tolerance to commensal & food Ag
	Muscle [224,225]			Muscle	IL-10	Resolution of inflammation Tissue repair
	Adipose [204–207,212,226]	St2 (IL-33R)	GATA-3 PPARγ IRF4 BATF	Adipose	1L-10	Resolution of inflammation Maintaining metabolic homeostasis, including response to hypothermia
Memory Treg						
	Memory Tregs [222,227–229]			Skin, Mucosal tissues	IL-17	Tolerance to autoAg & intermittently -encountered foreign Ags, especially in skin & muscosae

1.4.4 Mechanisms of Treg-Mediated Immune Suppression

The mechanisms by which Tregs exert their suppressive influence on the immune system are numerous and varied[106,230,231] . The suppressive mechanisms employed by Tregs can modulate diverse cellular processes and target multiple cell subsets; some directed at Teffs, others preferentially influencing APCs. Early *in vitro* studies revealed both contact-dependent and contact-independent Treg-mediated suppression of cell proliferation, differentiation and cytokine secretion (see **Figure 1.4**).

Contact-dependent mechanisms of Treg-mediated suppression include engagement of inhibitory co-receptors expressed on the surface of Tregs with ligands on Teffs or APCs. Probably the most well-defined of these co-inhibitory receptors is CTLA-4, which binds CD80 (B7.1) and CD86 (B7.2). Binding of CTLA-4 to CD80/86 on dendritic cells appears to transmit intracellular signals that tolerise these APCs via multiple mechanisms: impairing maturation, inducing generation of immunoregulatory molecules, including indolamine 2,3-dioxygenase (IDO) and downregulating expression of CD80/86. Not only does the ligation of CD80/CD86 by CTLA-4 transmit inhibitory intracellular signals but CTLA-4 also physically competes with the co-activator CD28 for interaction with CD80/CD86, effectively blocking co-stimulation[232,233]. Similarly, Lymphocyte-activation gene 3 (LAG3) expressed on Tregs inhibits DC maturation upon ligation of MHC class II[234].

The array of anti-inflammatory soluble factors secreted by Tregs, including IL-10, TGFβ and IL-35, contributes to a potent suppressive milieu that develops within the microenvironment in which they reside[62]. Treg-derived IL-10 has been

implicated in contact-independent suppression of cell proliferation and protection from various immunopathologies, including colitis[235]. Not only do Tregs secret anti-inflammatory cytokines themselves, they also induce generation of these cytokines by APCs. Likewise, chemokines secreted by Tregs[236], and by dendritic cells under the influence of Tregs[237], also modulate the behaviour of neighbouring effector cells.

Conversely, Tregs actively deplete the proinflammatory cytokine IL-2 from their microenvironment, due to the high density of IL-2 receptor on the surface of Tregs. Teffs deprived of IL-2 as a mitogen and activation stimulus are impaired in their proliferation and function[238–240].

Tregs also instigate metabolic disruption of Teff cells through depletion of extracellular ATP. The ectoenzymes CD39 and CD73, expressed preferentially by Tregs, convert ATP into AMP plus ADP and metabolise AMP into adenosine, respectively[241,242]. Since Teffs are dependent upon extracellular ATP for their survival, whilst adenosine suppresses Teff cells, the adenosine-rich environment surrounding Tregs becomes less conducive to Teff cells. Conversely, due to differential expression of different variants of adenosine receptor, the impact of adenosine upon Tregs is favourable to their suppressive function. Engagement of the P1A2 receptor on Tregs by adenosine results in a fluctuation in cAMP, which when delivered from the cytosol of Tregs into Teffs via gap junctions, inhibits proliferation and IL-2 secretion in Teffs[243,244] . Meanwhile, Treg-mediated contact-dependent suppression has been shown to require membrane-bound TGFβ[245].

Tregs are also capable of inducing apoptotic cell death in Teffs directly by secretion of cytolytic enzymes granzyme A, granzyme B[246] and perforin[247]. Expression of Fas ligand on the surface of Tregs has also been shown to induce Fas-dependent apoptosis in Teffs[248,249].

The exact suppressive mechanisms employed by Tregs are most likely context-dependent, being determined by the tissue microenvironment and the inflammatory milieu. The relative dominance of different regulatory mechanisms is determined by several factors: the differentiation status and anatomical location of the Tregs, the nature of the immune reaction, the type and frequency of activated effector cells and the site of inflammation. For example, within the same patients the suppression mediated by tumour-infiltrating Tregs was found to be dependent upon IL-10 and TGFβ, whilst Tregs isolated from the peripheral blood were found to secrete negligible quantities of either cytokine[250].

According to a three-tier model of Treg function proposed by Tang and Bluestone[106], different suppressive mechanisms are employed by Tregs to exert immune tolerance in three phases: homeostatic control, damage control and infectious tolerance. In the steady state, maintenance of immune tolerance by Tregs seems to be most dependent upon TGFβ and CTLA-4, which act directly upon neighbouring cells in lymphoid organs, whilst other regulatory mediators are dispensable. In the context of ongoing inflammation, activated Tregs employ more diverse medium-range, rapidly mobilised anti-inflammatory mediators, including IL-10, IDO, HO-1 and adenosine. Cytolytic mechanisms, the sledge-hammer of Treg-mediated suppression, are most dominant in the context of

persistent inflammation, associated with chronic activation of Tregs. Finally, the tolerogenic milieu established by Tregs is maintained through several positive-feedback mechanisms even after resolution of inflammation. Notably, several of the Treg-induced mediators, such as IL-10[251,252], TGFβ[181,253], IDO[191] and adenosine[187,242], not only impede effector cells but also enhance Treg function directly or promote pTregs induction via their tolerising effect upon DCs.

Beyond their function in regulating the immune system, emerging evidence suggests that Tregs also promote tissue homeostasis and repair in parenchymal tissues, via interaction with non-lymphoid cells[211]. For example, Tregs infiltrating damaged muscle in dystrophic mice were found to express the myogenic growth factor amphiregulin and to contribute to muscle regeneration[224]. Such non-immunological functions of Tregs may serve to further expedite resolution of inflammatory tissue damage. Since damaged tissue is a source of inflammatory mediators and potentially immunogenic neo-antigens, tissue homeostasis is also conducive to tolerance.

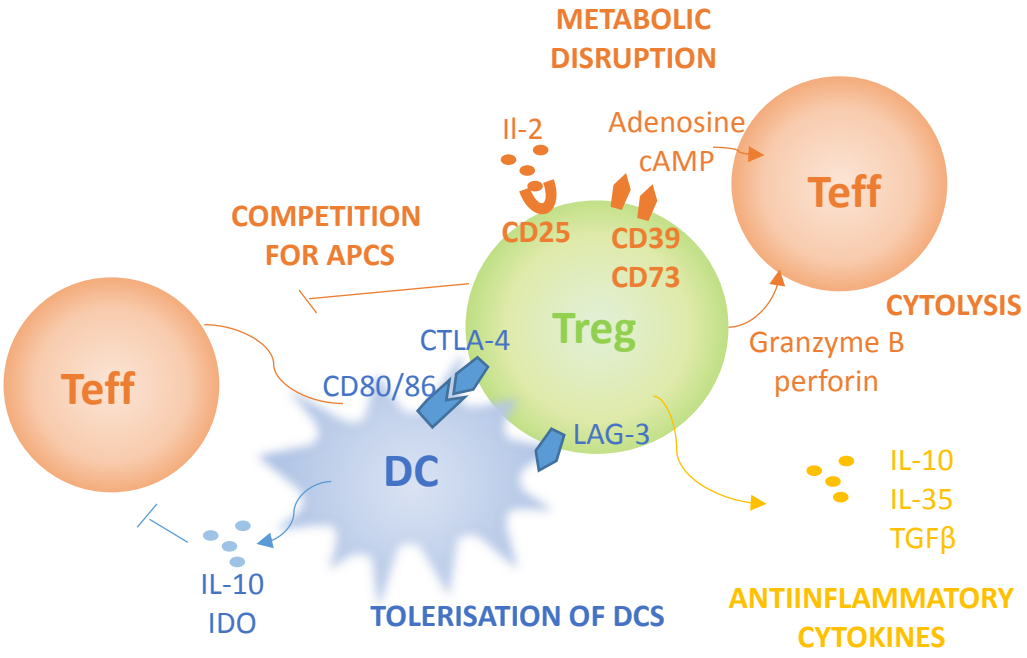

Figure 1.4 Mechanisms of Treg-mediated Immune Suppression
Tregs can exert suppressive effects upon Teffs directly, in a contact-dependent manner. For example, Tregs can induce cytolysis of Teffs via delivery of granzymes or cAMP. Adenosine, secreted abundantly by Tregs, disrupts Teff metabolism. Tregs regulate diverse immune cells via secretion of anti-inflammatory soluble factors, especially IL10, IL-35 and TGFβ. Tregs can compete with Teffs for activation stimuli, including interactions with DCs and the mitogenic cytokine IL-2. Additionally, Tregs interact with DCs in a manner that renders these APCs tolerogenic, i.e. capable of inducing unresponsiveness (as opposed to activation) in Teffs. For example, CTLA-4, expressed by Tregs binds to CD80 and CD86 costimulatory molecules expressed by DCs. The interaction of CTLA-4 with CD80/86 not only inhibits DC maturation but also antagonises the binding of CD80 and CD86 to CD28 on Teffs, an interaction that would otherwise promote antigen-dependent Teff activation. Immature or tolerogenic DCs secrete anti-inflammatory factors, including IL-10 and IDO, that favour induction of anergy or activation-induced cell death in Teffs upon antigenic stimulation.

1.4.5 Clinical Strategies to Augment Treg Function

Just as pathogens exploit Tregs to protect themselves from the endogenous immune response, so researchers have long recognised the therapeutic potential of manipulating Tregs as a means of modulating the immune system.

Pharmacological agents can be used to promote or impede the expansion or function of endogenous Tregs. Rapamycin, for example, has been favoured as an immunosuppressive drug in transplantation because it selectively abrogates proliferation of Teffs, whilst sparing Tregs, thus increasing the effective ratio of Tregs to Teff cells[254,255]. Nonetheless, the anti-proliferative effects of Rapamycin can have adverse effects upon the parenchyma and may prove detrimental to tissue repair in the context of transplantation.

Recently, Transplant Pharmacology has focussed upon molecules that have a much more selective effect upon Tregs. Ligation of CD45 via an anti-CD45RB antibody has been shown to induce expansion of antigen-specific Tregs within secondary lymphoid organs, by promoting the integrin-dependent interactions between Tregs and DCs[256]. Crucially, Teffs appear to be resistant to the pro-proliferative effects of anti-CD45RB. Meanwhile, IL-2/anti-IL-2 complexes have been reported to augment the expansion and function of Tregs within non-lymphoid tissues, reducing disease severity in various models of immunopathology[256–258]. Whilst some studies claim that Teffs were not affected by IL-2 compex therapy, it has been reported that, depending upon the clone of antibody used, IL-2/anti-IL-2 complexes can induce expansion and maturation of $CD8^+$ Teffs[259,260]. Similarly, low dose IL-2 therapy is currently undergoing phase I/II

clinical trials for the treatment of type 1 diabetes and graft-versus-host disease[261,262]. Intuitively, administration of exogenous IL-2 evokes a major concern: inadvertent activation of $CD8^+$ T cells and NK cells, which are also stimulated by IL-2. Indeed, these concerns were realised in a recent clinical trial of IL-2 for T1D[263,264]. For conditions where enhanced Treg function is desired, a promising alternative approach is Treg cellular therapy.

1.4.6 Application of Tregs as a Cellular Therapy

Whilst pharmaceutical immunosuppressive therapies have evolved through several generations since their genesis in the 1970s, the morbidity associated with broad, global immunosuppression are not trivial (see Section 1.3.1 & 0)[88].

Cell therapies are being heralded as the next generation of therapeutics for immune-mediated pathologies, including transplant rejection[127,128,265,266]. In theory, cell-based therapeutic agents should confer fewer side effects than pharmacological agents. Over the course of evolution, cells have undergone adaptation and selection to provide finely-tuned and "intelligent" regulation of the immune system. Cells are able to sense and respond to their environment and adapt their behaviour to provide the most appropriate activities for their context.

Infusion of *ex vivo*-expanded cells has several advantages over manipulation of a native cell population. The molecular characteristics and biochemical profiles of many regulatory cell types are not sufficiently well characterised to design effective drugs to target these cells specifically. Regulatory cells are so closely related to their effector counterparts that targeting one or other subset differentially is extremely challenging. Any attempt to promote the survival or

activity of a regulatory cell subset *in situ* will most likely provide some degree of positive stimulus to an effector cell subset. Extracting and culturing the regulatory cells *ex vivo* provides an opportunity to manipulate these cells in isolation. Furthermore, the resulting cell product can undergo further selection before infusion, to isolate only those cells that exhibit favourable properties for the treatment. For example, researchers have explored the benefits of selecting alloantigen-reactive Tregs, by stimulating the cells *in vitro* with donor-derived APCs or APCs pulsed with donor peptides[267,268]. In theory, Tregs expanded in the presence of alloantigen should preferentially regulate immune responses triggered by alloantigen, without impairing responses against unrelated antigens.

A parallel approach exploits culture supplements, such as TGFβ[181], all-trans retinoic acid (ATRA)[269], interferon-γ[270] or cilostimide[271], to generate Tregs from $CD4^+$ Teffs. This approach would be useful in clinical scenarios where a patient's endogenous Tregs are inadequate in number or function to be used as progenitors for cells therapy.

Alternatively, gene transfer has been used to force expression of transgenic TCRs of known specificity in Tregs[272–274]. Genetic engineering strategies, including gene transfer, gene silencing and genome editing, might also be employed to create "designer" cell therapy products to fit a particular specification (see Section 1.5.3).

One of the major limitations of immune cell therapy is that immunogenicity of allogeneic cells restricts clinicians to using autologous cells. The process of ex vivo expansion imposes a lag between retrieval of the progenitor cells and

administration of the product. Hence, the progenitor cells must be retrieved several weeks in advance of the treatment starting. For cadaveric donor transplant procedures, the date of transplantation cannot be predicted in advance and it would be too costly to prepare cell products for potential recipients prospectively, without having secured a donor. Immediately after transplantation, it would be too late to retrieve cells from which to generate a cell therapy product, as recipients are treated with induction immunosuppression that generally ablates those cells. Considering other applications of immune cell therapy, patients suffering from certain autoimmune pathologies have inherent deficits in their regulatory immune cell populations that make retrieval of a sufficient quantity and quality of cells very challenging[275,276].

1.5 Aims of this Thesis

A. To optimise a protocol for generating high yields of genetically engineered primary human Tregs, which is ideally compatible with *in vitro* expansion of these cells (Chapter 3)

B. To track adoptively-transferred, fluorescent protein-expressing human Tregs *in vivo* in order to measure the longevity and homing behaviour of these cells in a humanised mouse model. (Chapter 3)

C. To determine whether the protein Neuropilin-1 (NRP1) constitutes a marker of, or contributes to the suppressive function of, human Tregs. (Chapter 4)

D. Should NRP1 prove to contribute to human Treg function, to identify molecular mechanisms by which NRP1 influences Treg physiology. (Chapter 4)

E. To explore the feasibility of driving expression of immunomodulatory molecules in human Tregs to enhance their suppressive potency, with particular focus on NRP1 and interleukin-10 (IL-10). (Chapter 5)

1.5.1 Background to aims A and B: Genetic approaches to study the cell biology of cellular therapeutic agents

Many advances in the understanding of mouse immunology have been achieved with the aid of transgenic models. Genetic labelling of cells permits dissection of the physiology and cellular biology of immune cells. Meanwhile, molecular-scale analyses can be aided by manipulation of protein expression. Certain aspects of human immunology are not perfectly reflected in mouse models. In particular, human Tregs appear to differ in several fundamental properties from their mouse counterparts. Therefore, it would be desirable to apply genetic engineering methodologies to the study of human immunology, which can be achieved by transfection of cells *in vitro* using viral vectors.

In Chapter 3, we optimised a method for generating stably genetically-engineered Tregs, whilst preserving the suppressive function of these cells. Building upon a method for lentivirus-mediated transduction of T Cell Receptors into human Tregs that has been published previously by Brusko et al[277], we identified transduction conditions that generated consistently high yields of transduced Tregs. We developed a 16-day protocol for concurrent expansion of, and gene

transfer into, FACS-sorted Tregs. Finally, we explored some of the applications of genetic manipulation of Tregs: transcriptional silencing of genes implicated in Treg suppressive function and tracking of fluorescently-labelled Tregs *in vivo* by flow cytometry, polymerase chain reaction (PCR) and *intravital* microscopy.

1.5.2 Background to Aims C and D: Studying the role of Neuropilin-1 in human Tregs by reverse genetics

The suppressive mechanisms of Tregs must be regulated according to the inflammatory context. Finer comprehension of the mechanisms by which Tregs respond to their environment would greatly benefit our understanding of basic immunology and our ability to translate this knowledge into therapeutic strategies. Genetic analyses could be employed to investigate environmental cues that regulate Treg activity and the molecular pathways by which Tregs transduce salient contextual signals.

Neuropilin-1 (NRP1) is transmembrane glycoprotein that acts as a co-receptor for plexins and growth factor receptors that bind ligands of the Semaphorin and growth factor families (including VEGF, HGF, PDGF and TGFβ[278,279]. First characterised as a mediator of neural and vascular development[280,281], NRP1 has been implicated more recently in immune system function[282–284]. Within the immune system, NRP1 is expressed by T cells and antigen presenting cells and, moreover, is required for immunological synapse formation in mice[285–287].

Interest in this transmembrane glycoprotein as a marker of Tregs arose from studies showing that, in mice, NRP1 is preferentially expressed by Tregs, compared with Teffs[286,288], or by thymus-derived Tregs specifically[289,290].

Moreover, NRP1 has been proposed to promote the suppressive capacity of Tregs, either extrinsically, through modulation of APCs, or intrinsically, via NRP1-dependent signalling that induces molecular mechanisms of suppression[288291]. Whilst it has been reported that NRP1 is not preferentially expressed in human peripheral blood Tregs[292], it has not been established whether suppressive function is influenced by NRP1 in human cells.

In chapter 4, we demonstrate that, in spite of the minimal expression of NRP1 by human Tregs, the immune suppression mediated these cells is dependent upon Treg-intrinsic NRP1 expression. NRP1 expression by human Tregs was found to be dispensable for cell survival, proliferation and activation. However, gene expression analyses revealed that expression of three key mediators of autophagy was dependent upon NRP1 expression. Additionally, synthesis of various cytokines was moderately perturbed in NRP1 deficient cells, suggesting some role for NRP1 in maintaining Treg phenotype.

1.5.3 Background to Aim E: Genetic engineering of cell therapy products

The necessity of culturing cells *ex vivo* prior to infusion for cell therapy applications provides an ideal window of opportunity for genetic manipulation of cells *in vitro,* in order to enhance the function or safety profile of the cells. Genetic engineering could be used to confer upon the cells a greater specificity or bespoke functionality. Likewise, safety switches could be introduced to disable cells in order to reverse or down-regulate the immune regulation exerted by the cells[293]. Finally, in conditions where the patient's own Tregs are functionally defective, a clinician may elect to use cells from an allogeneic or xenogeneic

source that have been modified to be less immunogenic[294]. Alternatively, if the defect can be attributed to a particular genetic locus, the function of autologous cell might be restored by *ex vivo* genetic engineering.

In chapter 5, we explored the potential to enhance Treg function by forcing expression of two immunoregulatory molecules, IL-10 and NRP1, in expanded human Tregs. One might expect that driving expression of these proteins in cells that already express them would not add to their function. However, the expression of these molecules in native Tregs is dictated by the cell's environment via mechanisms that were tuned to operate for the purpose of maintaining immune homeostasis. Native Tregs are adapted to permit physiological inflammation and only impede immune responses that are interpreted as pathological. However, Tregs applied for cellular therapy need to perform above and beyond their native function if they are required to overcome the rather unnatural scenario of a fully allogeneic tissue placed at a site that is not immunologically privileged. Tregs expressing these molecules at constitutively high levels might overcome the restrictions upon Treg activity conferred by endogenous homeostatic mechanisms, permitting these cells to function with maximal potency. Therefore, we hypothesised that constitutive overexpression of IL-10 or NRP1 in human Tregs would enhance the suppressive capacity of these cells.

Chapter 2: Methods

2.1 Cell isolation and cell culture

2.1.1 Isolation of PBMCs by density gradient centrifugation

Human PBMCs were isolated from the blood of healthy donors (provided by the National Health Service Blood and Transplant, Oxford, U.K) using LSM 1077 (GE Healthcare, Chicago, IL, USA) for density gradient centrifugation at 2200rpm, at room temperature, for 30mins. Buffy coats were collected and washed with PBS. Erythrocytes were lysed by incubating the PBMCs for 5mins in PharmLyse lysing buffer (BD biosciences, Franklin Lakes, NJ, USA).

2.1.2 Isolation of human Tregs by FACS

Isolation of $CD25^+$ cells from PBMCs was performed by incubating PBMCs with CD25 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15mins at 4°C, washing and applying the cell suspension to Miltenyi LS column (Miltenyi) on a MACS (Magnetic Cell Separation) magnet (Mitenyi), according to the manufacturer's protocol. $CD4^+CD25^+CD127^{lo}$ cells were FACS-sorted, from $CD25^+$-enriched PBMCs, using a BD FACSAria instrument, after staining with αCD4-ECD (Beckmann Coulter, Brea, CA, USA) αCD25-PE-Cy7 (BD Biosciences) αCD127 – PE (BD Biosciences). Meanwhile, from the $CD25^-$ fraction of cells, $CD4^+$ Teffs were isolated using CD4 microbeads (Miltenyi), according to the manufacturer's protocol. The remaining PBMCs were cryopreserved before use. For all *in vitro* cell culture and assays, leukocytes were cultured in complete medium, composed of RPMI medium supplemented with L-glutamine (Sigma-Aldrich, St. Louis, MO,

USA), 100U/mL penicillin and 10mg/mL streptomycin (Sigma-Aldrich) and 10% human AB serum (Seralab, Haywards Heath, U.K) (heat-inactivated for 20 minutes at 55˚C).

2.1.3 in vitro Expansion of human Tregs and Teffs

After isolation, T cells were cultured for sixteen days in complete medium. During the first fourteen days, culture medium was supplemented with 1000U/mL (for Tregs) or 200U/mL (for Teffs) recombinant human IL-2 (Novartis Pharmaceuticals UK Ltd, Surrey, UK). Tregs were stimulated with αCD3αCD28 T cell activator beads (ThermoFisher Scientific) on day 0 of culture, at a ratio of 3 beads: 1 cell, and on day 7, with 1 bead: 1 cell. Teffs were stimulated on both day 0 and day 7 with 1 bead: 1 cell. Cultures were split, and medium replaced, as required. Tregs and Teffs were rested, without beads, in the presence of 200U/mL or 100U/mL IL-2, respectively, for the final two days.

2.1.4 Generation of human monocyte-derived dendritic cells

$CD14^+$ monocytes were isolated from PBMCs using MACS $CD14^+$ microbeads and LS (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol. $CD14^+$ mononuclear cells were incubated for five days at $5x10^5$ cells/mL medium in the presence of 1000U/mL IL-4 (Peprotech, Rocky Hill, NJ, USA) and 50ng/mL GM-CSF (Peprotech)

2.1.5 Cryopreservation and thawing of human leukocytes

Cells were resuspended in freezing medium (45% RPMI, 45% Foetal Calf Serum (FCS), 10% DMSO) at 50-200$x10^6$ cell/mL for PBMCs or 2-25$x10^6$/mL for T cells.

Cells were frozen in 1.5mL cryovials in freezing boxes at -80°C, before being transferred to -160° for long-term storage.

Frozen vials of cells were transferred to -80°C for one hour before thawing at 37°C. Thawed cell suspensions were immediately washed in 10mL RPMI, added dropwise.

2.2 Flow cytometric analyses

2.2.1 Labelling of cell surface antigens with fluorophore-conjugated antibodies

Cells, washed with FACS buffer or PBS and plated onto 96-well v-bottomed plates at 5-20x10^4 cells per well, were incubated with 0.15µL per sample of each fluorophore-conjugated antibody, at 4°-8°C, in the dark. Antibody was washed from the cells with FACS buffer after 30mins. All washes were performed by adding PBS to each well and centrifuging samples at 1500rpm for 5mins at 4°C, then tipping off the supernatant. Samples from *in vivo* experiments were stained in 4mL FACS tubes, with 1µL of each antibody per sample. After washing, samples were fixed in a volume of at least 100µL 1% paraformaldehyde (PFA). Flow cytometry was performed using a BD FACSCanto II instrument (BD) and data were analysed using FACSDiva software (BD).

2.2.2 Antibodies

All antibodies for flow cytometry are reactive against human antigens, unless otherwise stated.

Table 2.7 Flow Cytometry Antibodies

antigen	fluorochrome	Species of origin	clone	supplier
CD127	PE	Mouse	hIL-7R-M21	BD
CD14	APC	Mouse	61D3	BD
CD14	APC-Cy7	mouse	MϕP9	BD
CD19	PE	Mouse	HIB19	BioLegend
CD25	PE Cy7	Mouse	M-A251	BD
CD25	APC	Mouse	M-A251	BD
CD3	PE	Mouse	HIT3a	BD
CD3	PE Cy7	Mouse	SK7	BD
CD3	eFlour450	Mouse	OKT3	eBioscience
CD4	ECD	Mouse	SFCI12T4D11	Beckmann Coulter
CD4	APC	Mouse	RPA-T4	BD
CD45	APC	Mouse	H130	eBioscience
CD69	APC Cy7	Mouse	FN50	BD
CD8	FITC	Mouse	SK1	eBioscience

CD8	PE	Mouse	HIT8a	BD
CD8	APC Cy7	Mouse	SK1	BD
CD80	FITC	Mouse	2D10.4	BD
CD83	PE Cy7	Mouse	HB15e	eBioscience
CD86	PE	Mouse	IT2.2	BD
FOXP3	FITC	Rat	PCH101	eBioscience
FOXP3	PE	Rat	PCH101	eBioscience
FOXP3	eFluor450	Rat	PCH101	eBioscience
HLA-DR	ECD	Mouse	Immu-357	Beckman Coulter
ICOS	FITC	Mouse	ISA-3	eBioscience
Ki67	eFluor 450	Mouse	20Raj1	eBioscience
Mouse CD45	PE	Rat	30F11	eBioscience
NRP1	APC	Mouse	12C2	BioLegend

2.2.3 Intracellular immunostaining

Following immunostaining of cell surface antigens (Section 2.2.1), cells were incubated for 45mins-16hours at 4°C in 100µL per well, or 500µL per tube,

fixation-permeabilisation buffer (eBioscience). Permeabilisation buffer was then added before centrifuging samples to remove the buffers. Samples were incubated for 10-20mins with 2µL per well, or 5µL per tube, 2% rat serum (eBioscience). Antibodies were added at 1-2µL per well and washed out after incubating for 40mins at 4°C in the dark, after which, samples were washed and fixed in 1% PFA (as for surface staining Section 2.2.1).

2.2.4 Cytokine bead array

Supernatants were collected from cell cultures, after 72hrs stimulation, unless otherwise stated, and stored at -80°C until use. Thawed supernatants were probed using a multiplex bead-based immunoassay. A LEGENDplex™ Human Th Cytokine Panel (BioLegend) was used to analyse Treg culture supernantants. Supernatants from suppression assays were analysed using LEGENDplex Human Inflammation Panel (BioLegend) Flow cytometric bead array kit.

2.2.5 Staining with cell viability dyes

Cell were incubated for 30mins at 4°C in the dark with 0.3µL per sample of each dye. 7-aminoactinomycin D (7AAD) (eBioscience) was stained concurrently with antibodies in all flow cytometry assays. Annexin V-APC staining solution (eBioscience) was also incorporated into antibody staining master mixes for cell viability assays. When staining for Annexin V, all washes were performed in Annexin V binding buffer (eBioscience) and cells were resuspended in binding buffer for acquisition on the flow cytometer, immediately after staining.

2.2.6 Staining with cell proliferation dyes

For staining with Violet Proliferation Dye (VPD) (BD), cells were washed in PBS then resuspended in PBS at $30x10^6$ cells/mL in PBS. Cells were incubated with 1µM VPD for 10mins at 37°C before being washed in PBS 5% FCS, followed by PBS. To stain with carboxyfluorescein succinimidyl ester (CFSE), cells were incubated in 1mL RMPI with 10µM CFSE for 10mins at 37°C, then washed with RPMI supplemented with 10% FCS.

2.3 lentivirus-mediated transduction of human Tregs

2.3.1 Lentiviral vectors

The VSV-G envelope plasmid, pMD2.G (Addgene plasmid #12259), lentiviral packaging plasmids, psPAX2 (Addgene #12260), pMDLg/pRRE (Addgene #12251) and pRSV-Rev (Addgene #12253) and eGFP transfer vector pWPXL (Addgene #12257) were a gift from Didier Trono. Plasmid maps and sequences for these vectors are available at www.addgene.org.

The NRP1 shRNA plasmid (TRC0000063525) was acquired from ThermoFisher (Waltham, Massachusetts, USA), whilst the pLKO.1 - TRC cloning vector was a gift from David Root (Addgene plasmid # 10878)[295].

Plasmids for IL-10 and NRP1 overexpression, and the control vector CAG-GFP, were designed for this study using VectorBuilder software and constructed by Ciagen Biosciences. Both CAG-IL10 and CAG-NRP1 vectors contained the ORF (*IL10* or *NRP1*), linked via P2A sequence to an *EGFP* ORF, driven by a CAG promoter (see plasmid maps and sequences for CAG-GFP [Appendix 1, Appendix

2] CAG-IL10 [Appendix 3, Appendix 4] and CAG-NRP-1 [Appendix 5, Appendix 6]. All transfer vectors also encoded ampicillin resistance proteins.

2.3.2 Preparation of plasmids

Plasmids were isolated from 100mL or 250mL of overnight cultures of E.coli using a Plasmid Maxi Prep kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Plasmid DNA was quantified using a NanoDrop instrument (ThermoScientific) and frozen in TE buffer (Qiagen) at -80˚C.

2.3.3 Generation of lentiviral vectors

293T cultures were maintained in DMEM supplemented with 10% FCS, 4mM L-Glutamine, 100U/mL penicillin and 10mg/mL Streptomycin. 293T cells were seeded at $1.6x10^6$ cells per $75cm^2$ tissue culture flask one day prior to transfection. Transfection mixtures, containing envelope, packaging and transfer plasmids, were prepared using Profection Mammalian Transfection System reagents (Promega, Fitchburg, WI, USA), according to the manufacturer's protocol. The IL-10- and NRP1-overexpression vectors (10µg per flask) were packaged using a third-generation lentiviral packaging system, utilising pMD2.G (3µg per flask), pRSV-Rev (2.5µg per flask), pMDLg/pRRE (5µg per flask). All other transfer vectors (6µg per flask) were packaged using a second generation system, utilising pMD2.G (2.4µg per flask) and psPAX2 (3.6µg per flask). Transfection mixture was added dropwise to each flask of 293T cells. Medium was replaced 10-16hrs after transfection and supernatants were harvested two to three times, at approximately 12hr intervals. Lentivirus-containing supernatants were cleared by centrifugation for 5mins at 1700rpm and filtered through 0.22µM filters.

Cleared supernatants were concentrated by centrifugation for 3 hours at 18000rpm, 4˚C and resuspended in culture medium at 100x concentration. Concentrated supernatants were stored at 4˚C, in the dark, for up to three days.

2.3.4 Transduction of human Tregs and selection of transduced cells

Transduction of human Tregs or Teffs was performed concurrently with the expansion protocol outlined above (Section 2.1.3), by adding concentrated lentivirus (50µL of 100x viral concentrate per well of a 96-well plate, containing 200µL medium and 5-10x10^5 cells, unless otherwise stated) to freshly isolated T cells on day 0 of the expansion. Cells transduced with shRNA or the TRC pLKO.1 control vector were selected by adding puromycin (Millipore, Billerica, MA, USA) to cultures at 1µg/mL from day 7 until day 12, and then at 2µg/mL on day 13-14. Cells transduced with vectors encoding fluorescent proteins were selected by FACS at least 10 days post-transduction.

2.3.5 Titration of lentiviral vector by flow cytometry

293T cells were seeded at 10^5 cells per well of a 24-well plate one day prior to addition of concentrated lentivirus stock at varying concentrations. Cells from one well were enumerated manually at the time of lentivirus addition (day 1). 72hrs after addition of the virus, cells were fixed in 1% PFA and the proportion of GFP^+ cells was quantified by flow cytometry. The concentration of a viral stock was calculated by the following formula:

Concentration (TU/mL) = # cells day 1 x fraction GFP^+ cells day 4 / volume viral supernatant (mL)

2.4 *in vitro* cell culture assays

2.4.1 NRP1 Expression timecourse

Freshly isolated Tregs and Teffs, and *in vitro* expanded Tregs, were cultured in the presence of beads (1bead: 5cells) and IL-2 (250U/mL) for seven days. Aliquots of cells were stained with αNRP1 antibody (BioLegend, San Diego, CA, USA) on day 0, 2, 4 and 7 of culture and analysed by flow cytometry.

2.4.2 *in vitro* beads-stimulated Treg suppression assay

Responder PBMCs were stained with 10μM CFSE (Invitrogen) or 1uM VPD (BD) (Section 2.2.5) and cultured (1 x 10^5 per well of a 96 well plate) for 72hrs with Tregs, at ratios from 1 Treg: 1 responder to 1/64 Treg: 1 responder. T cells were stimulated with αCD3αCD28 T cell activator beads (invitrogen) at a 1 bead: 5 responder cells ratio. Assays were performed in triplicate, unless otherwise indicated. To quantify responder proliferation from CFSE/VPD dilution profiles, division indeces were calculated using the following formula[296]:

$$division\ index = \frac{\#\ \text{cell divisions}}{\#\ \text{precursor cells}} =$$

$$\frac{\left(\frac{\#\ \text{cells division}1}{2^1}\right)+2\left(\frac{\#\ cells\ division\ 2}{2^2}\right)+\cdots+n\left(\frac{\#\ cells\ division\ n}{2^n}\right)}{\left(\frac{\#\ \text{cells division}\ 0}{2^0}\right)+2\left(\frac{\#\ cells\ division\ 1}{2^1}\right)+\cdots+n\left(\frac{\#\ cells\ division\ n}{2^n}\right)}$$

Where "divisions" refer to peaks of the proliferation dye dilution profile, and n= the number of the division peak (corresponding to the number of cell cycles completed by cells in that peak), with the peak of highest intensity (corresponding to undivided cells) designated as "division 0".

2.4.3 *in vitro* mixed lymphocyte reaction

in vitro-expanded Tregs were cultured for 6 days with autologous responder PBMCs (1 x 10^5 cells per well of a 96 well plate), at ratios from 1 Treg:1 responder to 1/64 Tregs: 1 responder. Monocyte-derived dendritic cells (moDCs) (allogeneic to the responder cells and Tregs) were included as stimulators (1x10^4 per well). Six replicates were performed for each condition. Relative cell proliferation in each well was determined by incorporation of ^{3}H-Thymidine (Perkin Elmer), added for the last sixteen hours of culture, detected using a beta-plate reader. Percentage suppression of responder proliferation was calculated as:

Proliferation =100x ([cpmPBMC+stim – cpmPBMC+stim+Treg]/cpmPBMC+stim)

2.4.4 Flow cytometric analysis of *in vitro* T cell proliferation

Tregs or Teffs were stained with 1µM violet proliferation dye (BD) (Section 2.2.5) prior to being cultured (1x10^4 cells per well) for 72hrs with or without αCD3αCD28 beads (2x10^4 per well) before harvesting cells for flow cytometry to determine dye dilution. Assays were performed in triplicate. Division indeces were calculated as in 2.4.2

2.4.5 Flow cytometric analysis of *in vitro* T cell activation

After culturing for 72 hours with or without αCD3αCD28 beads (1x10^4 per well), Tregs or Teffs (5x10^4 cells per well) were stained with antibodies against activation markers: αICOS-FITC (BD), αCD25-PE-Cy7 and αCD69-APC-Cy7 (BD). Cell surface expression of these activation markers was determined by flow cytometry.

2.4.6 Flow cytometric analysis of *in vitro* apoptotic cell death

Tregs or $CD4^+$ Teffs ($5x10^4$ per well) were stimulated with or without αCD3αCD28 beads ($1x10^4$ per well) and IL-2 (200U/mL for Tregs or 100U/mL for Teffs) for 72hrs. After staining cells with 7AAD (eBioscience) and Annexin V-APC (eBioscience), $AnnV^+7AAD^-$ (early apoptotic) and $AnnV^+7AAD^+$ (late apoptotic) cells were enumerated by flow cytometry.

2.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.5.1 RNA isolation

Cells were cryopreserved as dry pellets at -80°C after washing in PBS. Total cellular RNA was isolated from cryopreserved cell pellets using a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) or Ambion mirVana™ miRNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturers' protocols. RNA that was not used immediately was stored in endotoxin-free water at -80°C for up to 12 months.

2.5.2 cDNA synthesis

cDNA was reverse transcribed from RNA using a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific), according to the manufacturer's protocol. cDNA that was not used immediately was stored at -80°C for up to 12 months.

2.5.3 qRT-PCR

Quantitative real-time PCRs were performed with Taqman probe mixes (ThermoFisher Scientific) and PerfeCTa® qPCR ToughMix® UNG Low ROX™ from Quanta Biosciences (ThermoFisher Scientific), using an Applied Biosystems

QuantStudio™ 7 Flex instrument (ThermoFisher Scientific). Each reaction was performed with 2µL cDNA: 18µL PCR master mix per well of a 384-well plate (ThermoFisher Scientific). *HPRT* mRNA was measured in all samples as an endogenous control. In all cases, the reporter dye was FAM, the quencher NFQ-MGB and the reference dye ROX. Results represented as $2^{-\Delta Ct}$ were calculated from mean ΔCt values (threshold cycle of each target relative to *HPRT* for the same sample) generated automatically by the QuantStudio 6 and 7 Flex™ 7 software (ThermoFisher Scientific).

2.6 Microarray

Total RNA was isolated from control and NRP1 KD samples of three cell types: expanded, rested Tregs (N=5 donors); expanded, rested Teffs (N=3) and expanded, stimulated Tregs (N=4 donors). Stimualted Tregs had been cultured for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). RNA was isolated using Qiagen's RNeasy kit (Qiagen) and hybridised to Illumina Human HT-12 arrays. The microarray was conducted by the Bioinformatics and Statistical Genetics Core, Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics. Analysis of differential gene expression between groups was performed, with pairing of samples from the same donor, using LIMMA[297], by Dr Ruud Van Stiphout.

2.7 Microscopy assay to measure duration of intercellular contacts between Tregs and DCs

Two wells of an 8-well microscopy chamber (Lab-Tek II, Nalge Nunc International, Roskilde, Denmark) were loaded with 10^5 CFSE-stained dendritic cells and 10^5

autologous *in vitro*-expanded human Tregs per well, in 200µL complete culture medium (2.1.1) with 20nM HEPES (Sigma). 10µg/mL of anti-human NRP1 blocking antibody (R&D Systems, Minneapolis, MA, USA) was added to one well and Sheep IgG (R&D Systems) control antibody to the second well. Cells were incubated at 37°C, 5%CO_2 for 90 mins prior to imaging. Live cell imaging to detect polarised light and CFSE (FITC channel) was performed using a DeltaVision Elite Imaging System (GE Healthcare, Chicago, IL, USA), inside a chamber at 37°C, 5%CO_2. Images were taken at 20s intervals for 40mins, at two points of interest in each well, alternating between wells at each time point. For each timelapse video, all Treg: DC contacts were analysed for ten DCs, selected on the basis of being relatively stationary and positioned at a moderate cell density. Only direct contacts between the plasma membranes of two cells were counted as contact events. Duration of each Treg: DC contact was calculated by recording the number of frames for which the cells remained in continuous contact. Interrupted contacts were counted as separate events. Cells that were judged to be passively trapped in close proximity were excluded from the analysis.

2.8 Humanised mouse model of skin allograft rejection

2.8.1 Mice

BALB/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed in the John Radcliffe Hospital Biomedical Services Unit under specific pathogen-free conditions. All protocols were conducted in accordance with the UK ASPA (Animals (Scientific Procedures) Act, 1986) and approved by Oxford University's Committee on Animal Care and Ethical Review.

2.8.2 Procurement of human tissue

Human tissue was obtained with informed consent from patients undergoing plastic surgery procedures at the Department of Plastic and Reconstructive Surgery, John Radcliffe Hospital, Oxford. Ethical approval was granted by the Oxfordshire Research Ethics Committee, study number 07/H0605/130. The outermost 1/100'' of the skin was isolated from underlying tissues using a dermatome (Zeiss, Jena, Germany). Patients diagnosed with inflammatory skin conditions, or receiving immunosuppressive medication, were excluded as skin donors. Skin was stored at 4°C in University of Wisconsin Solution for up to 12hrs prior to transplantation.

2.8.3 Skin grafting procedure

Under anaesthesia (Domitor/Ketamine), BALB/c $Rag2^{-/-}$ $c\gamma^{-/-}$ recipients, aged 8-12wks, were prepared for surgery by removing a $1cm^2$ square of skin from the shaven left flank. Surgical grafting $1cm^2$ of human skin into the incised site was performed using 8-0 polypropylene sutures (Ethicon, Somerville, NJ, USA). A small nick was made using microscissors in the centre of each graft to allow gas bubbles to escape. Skin grafts were covered with a patch of iodine-infused gauze (Syntagenix, Gatwick, U.K.). A 1x10cm strip of sterile, non-woven swab was rolled into a cylinder, placed over the graft, and secured with autoclave tape wound around the abdomen of the mouse, to apply light compression to the graft. After ten days, these bandages were removed under anaesthesia.

2.8.4 Adoptive transfer of human leukocytes

5 weeks after skin grafting, recipients received $5x10^6$ cryopreserved PBMC in pure RPMI via intra-peritoneal injection, with or without $2.5x10^6$ *in vitro*-expanded Treg. Tregs were autologous to the PBMCs. Both PBMCs and Tregs were allogeneic to the skin graft. Grafts were assessed at least three times per week for macroscopic markers of rejection. Mice were sacrificed after graft rejection or at day 100 post-transplantation (whichever occurred sooner).

2.8.5 Flow cytometric analysis of lymphocytes in secondary lymphoid organs and graft

Peripheral blood was harvested from the inferior vena cava, under anaesthesia, prior to euthanasia. Half of each skin graft was digested for 1hr in 1.6mg/mL collagenase D (Roche, Basel, Switzerland). Spleens were macerated, using a syringe plunger, through a 70µm nylon mesh. Erythrocytes were lysed in blood and spleen cell suspensions in 10mL PharmLyse buffer (BD) for 8mins, before washing in FACS buffer. Blood, skin and spleen cells were stained for analysis of cellular composition by flow cytometry using the following antibodies: CD3-PE (BD), CD4-ECD (Beckman Coulter), 7AAD (eBioscience), CD25-PE-Cy7 (BD), hCD45-APC (eBioscience), CD8-APC-Cy7 (BD) and mCD45-eFlour450 (eBioscience).

2.9 Detection of GFP-expressing graft infiltrating cells by PCR

2.9.1 Preparation of tissues

Skin grafts, dissected from skin graft recipient mice that had been reconstituted with human PBMCs and GFP^+ Tregs (Sections 2.8.3-2.8.4) between three and

seven weeks post-transplantation, were snap-frozen in OCT compound and stored at -80˚C. Skin samples were cut into fine shreds using sterile scalpels, after removal of the OCT compound.

2.9.2 Preparation of standards for computation of PCR detection threshold

Standards were prepared by spiking known numbers of eGFP-expressing Tregs, (from the same stocks as those used for the adoptive transfer into skin graft recipient mice) into aliquots of GFP^- PBMCs. These cell samples contained $5x10^5$ cells in total, with a five-fold serial dilution of GFP^+ Tregs from 10^5 to 1 cell. Cells were frozen as dry cell pellets prior to DNA isolation using the Red ExtraNAmp kit (Sigma), according to the manufacturers protocol for tissue samples.

2.9.3 DNA isolation

DNA was extracted from these skin samples (Section 2.9.1) and cell pellet standards (Section 2.9.2) using extraction, tissue preparation and neutralisation buffer from Sigma's Red Extract N Amp DNA isolation kit (Sigma-Aldrich), according the manufacturer's protocol for tail tips.

2.9.4 Polymerase Chain Reaction (PCR)

PCR primers were designed using OligoPerfect software (ThremoFisher Scienctific) to target sequences within the eGFP ORF of the pWPXL plasmid and had the following nucleotide sequences:

Forward primer for eGFP: AGTTCACCTTGATGCCGTTC
Reverse primer for eGFP: CACATGAAGCAGCACGACTT

PCR mixes were prepared using 0.5µL of DNA extract, 0.2µM of each primer and reagents from the HotStarTaq master mix kit (Qiagen), following the manufacturer's protocol. PCR was performed using a DNA Engine thermal cycler (Bio-Rad Laboratories), with an annealing temperature of 60°C, for 35 cycles.

2.9.5 Gel electrophoresis for detection of PCR products

Gels were prepared with 1.75% agarose in TBE 0.5x buffer, with 1xSYBR Safe reagent (ThremoFisher) to visualise bands. 8µL of each PCR reaction, with 2µL loading dye, was loaded per lane. DNA hyperladder IV (Bioline, Boston, MA, USA), containing oligonucteotides of 100-1000bp, at 100bp intervals, was loaded in two lanes. Electrophoresis was performed at 70V. Gels were imaged using a Gel Doc EZ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.10 *intravital* microscopy of fluorescently-labelled T cells in human skin grafts

2.10.1 Preparation of mice for imaging

BALB/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mice received human skin grafts (as in Section 2.8.3) and adoptively-transferred human PBMCs and GFP^{+} Tregs (as in Section 2.8.4). Human skin grafts were imaged, *in situ*, using a Zeiss 780 Upright instrument (Zeiss, Oberkocken, Germany), 5 weeks after injection of human cells into the graft recipients. Mice were anaesthetised using isofluorane during preparation and imaging. Imaging of mice was conducted using an apparatus comprising a pair of screws mounted on a base, onto which a metal plate, with a glass coverslip at the centre, could be attached. Under anaesthesia, mice were positioned between the two screws, cushioned by putty placed along the animal's dorsal side and head,

with the grafted flank uppermost. The skin of the flank was stretched over a block of putty next to the abdomen and secured with two sterile 26G syringe needles piercing either side of the graft. The metal plate was fixed directly over the flank of the mouse, with the graft visible through the coverslip, securely enough to flatten the skin without restricting blood circulation.

2.10.2 Instrument setup and imaging parameters

The tissue was excited at 860nm. Z-stack imaging was performed across 20 sections at intervals of 3µm up to a depth of 100µm, at 30 second intervals for 24 minutes. Images were corrected for autofluorescence and drift using Imaris software (Bitplane, Zurich, Switzerland).

2.11 Statistical analyses

All statistical analyses were performed using GraphPad Prism software. Wilcoxon Signed Rank tests were applied to data comparing numbers or percentages of cells or MFIs, pairing samples from the same donor. Unpaired data sets were analysed using Mann Whitney U tests. Data from suppression assays performed at varying Treg: responder ratios were analysed using a two-way ANOVA. Meanwhile, graft survival data were assessed using Log Rank tests.

Chapter 3: Optimisation and Application of Genetic Engineering in Human Tregs

3.1 Introduction

Research into Tregs has flourished over the past two decades. Nonetheless, greater comprehension of the mechanisms by which Tregs regulate other immune system components, and how these cells respond to their environment, would greatly benefit our understanding of basic immunology and our ability to translate this knowledge into therapeutic strategies.

Many advances in the understanding of mouse immunology have been achieved with the aid of transgenic models. Utilising a reverse genetics approach, the function of a particular protein in a cellular response can be probed using a model in which that molecule is genetically manipulated. A protein of interest may be over-expressed, knocked out or knocked down. Reporter constructs can be used to tag proteins, or promoters, enabling detection and localisation of protein expression. Stable labelling of cells with fluorescent protein has been invaluable for studying cell migration and cell fate, *in vivo*. Nowadays, fluorescently-labelled cells can also be detected by *intra-vital* microscopy to study cellular interactions *in situ* [298].

Certain aspects of human immunology are not perfectly reflected in mouse models and, in particular, human Tregs appear to differ in several fundamental properties from their mouse counterparts. For example, among mouse T

lymphocytes, expression of the transcription factor FOXP3 is ubiquitous in, and exclusive to, cells with a regulatory phenotype. In human T cells, FOXP3 can be expressed in Teffs upon activation[154]. Given the obvious impediments to transgenesis in humans, genetic engineering can be achieved by gene transfer into cells *in vitro.* Stable genetic modification can be accomplished via transduction of cells with a viral vector capable of integrating transgenes into the host cell genome. Among viral gene transfer vectors, replication-incompetent HIV-1 lentivirus exhibits the most favourable safety profile and is also known to be very efficient for transduction of mammalian lymphocytes[299–303]. In contrast to other retroviral vehicles, lentiviruses can traverse the nuclear envelope in its target cell, permitting integration in non-dividing cells.

In this chapter, we sought to optimise a method for generating high yields of stably genetically-engineered Tregs, whilst preserving the suppressive function of these cells. Building upon a method for lentivirus-mediated transduction of T Cell Receptors into human Tregs that has been published previously by Brusko et al[277], we identified transduction conditions that generated consistently high yields of transduced Tregs. We propose an optimised 16-day protocol for concurrent expansion of, and gene transfer into, FACS-isolated human Tregs. Finally, we demonstrated some of the applications of genetic manipulation of Tregs: transcriptional silencing of genes implicated in Treg suppressive function and tracking of fluorescently-labelled Tregs *in vivo* by flow cytometry, polymerase chain reaction (PCR) and *intravital* microscopy.

3.2 Objectives and Hypotheses

Objective 1: To optimise a protocol for generating high yields of genetically engineered primary human Tregs that is ideally compatible with *in vitro* expansion of these cells.

Hypothesis 1: Lentivirus-mediated transduction of human Tregs during *in vitro* expansion will yield sufficient numbers of stably-transduced cells (>1.5 x 10^7 cells per culture) to be used in adoptive transfer experiments *in vivo*.

Objective 2: To track adoptively-transferred, fluorescent protein-expressing human Tregs *in vivo* in order to measure the longevity and homing behaviour of these cells in a humanised mouse model.

Hypothesis 2: Human Tregs engineered to express enhanced Green Fluorescent Protein (eGFP) will be detectable in human and mouse tissues (by PCR, flow cytometry or two-photon microscopy) after adoptive transfer into a humanised mouse model.

3.3 Results

3.3.1 Optimal transduction efficiency in Tregs depends upon strength of TCR stimulation, lentiviral titre and duration of pre-activation of cells

Many informative discoveries in mouse immunology have been achieved with the aid of genetic engineering to label cells with a detectable marker, in order to track the cells *in vivo*. In order to emulate these studies, we wished to optimise a protocol for genetically engineering human Tregs to express a fluorescent marker, enhanced eGFP (eGFP) constitutively. Fluorescent markers such as eGFP are particularly amenable to *in vivo* cell tracking because they can be detected via

a range of accessible methodologies, including fluorescence microscopy and flow cytometry. For the same reason, this was also a pragmatic choice of transgene to use for optimisation and validation of the gene transfer protocol. Lentiviral vectors were chosen because these vectors have been identified as arguably the most effective for stable gene transfer into lymphocytes.

Since many experimental and clinical applications demand large numbers of cells and stable transgene expression, we sought to formulate a protocol for optimal transduction in human Tregs that would maximise the yield of stably modified cells. Whilst lentivirus can enter the nucleus via nuclear pores, independently of cell division, genomic integration is favoured by activation and cell cycling[300]. Hence, we decided to co-opt an existing protocol for *in vitro* expansion of human Tregs developed previously in our lab[304], since this protocol was designed to drive rapid and sustained cell proliferation [Figure 3.1].

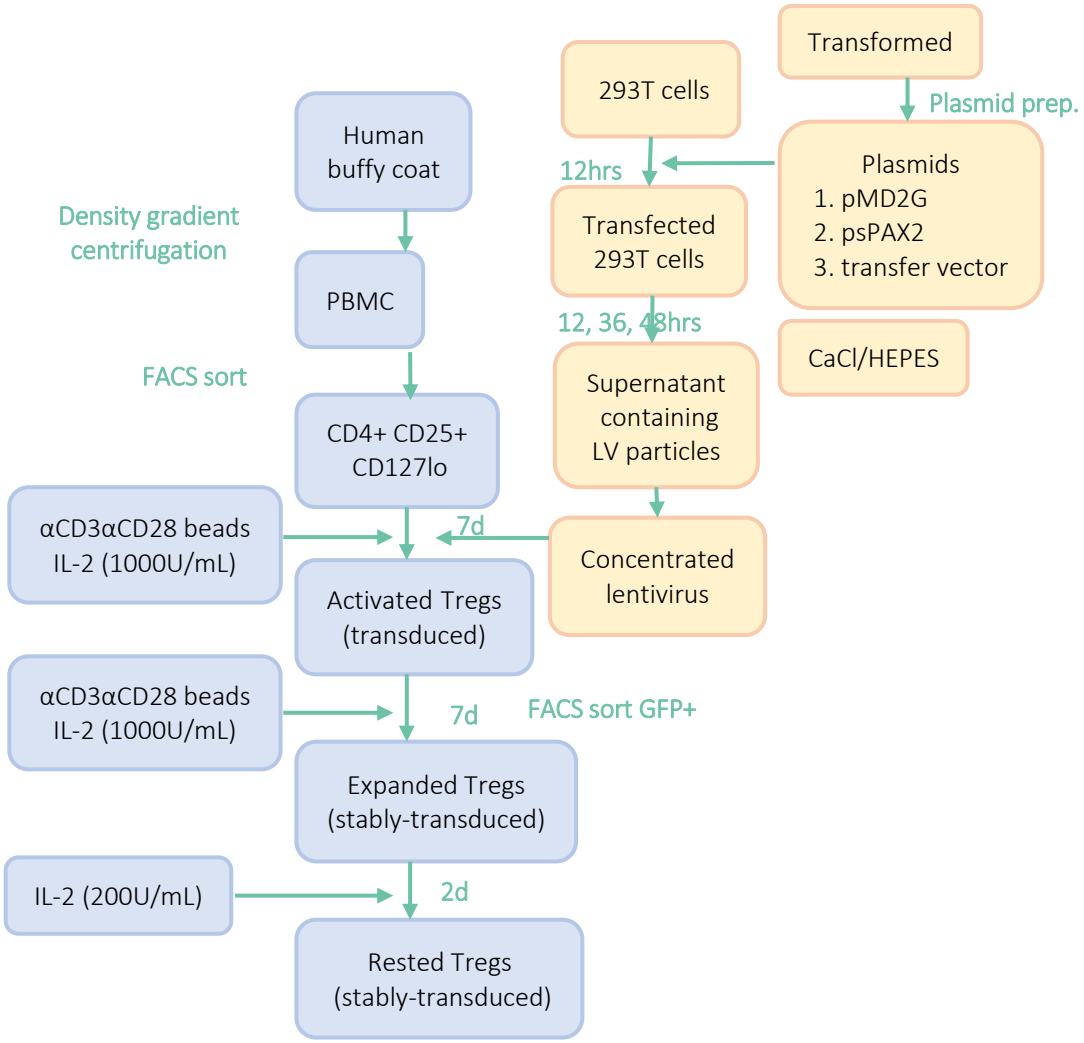

Figure 3.1 Protocol for transduction of human Tregs

Human PBMCs were isolated from buffy coats from healthy donors. $CD4^{+}CD25^{+}CD127^{lo}$ Tregs were isolated from these PBMCs by Fluorescence-Activated Cell Sorting (FACS) and stimulated with αCD3αCD28 beads (at a 3 beads: 1 cell ratio, unless otherwise stated) and recombinant human IL-2 (1000U/mL). On the day of isolation (unless otherwise stated), concentrated supernatant from lentivirus-producing 293T cells was added directly to the Treg cultures. One week after Treg isolation, the Treg cultures were re-stimulated with αCD3αCD28 beads (1 bead per cell) and cultured for a further week in the presence of IL-2 (1000U/mL). Finally, the stimulatory beads were removed from these Treg cultures, which were rested for two days in the presence of 200U/mL IL-2. Throughout culture, cell cultures were divided and medium replenished as required. Lentivirus-producing 293T cells were generated by transfecting 293T cells with plasmids encoding lentiviral envelope components, lentiviral packaging proteins and the transgene of interest (see plasmid maps, Appendix 1). Lentivirus was concentrated by centrifugation of supernatants collected from these transfected cells.

The effect of stimulation intensity on the yield of transduced Tregs was assessed by transducing Tregs stimulated with different ratios of stimulatory αCD3αCD28-coated beads. Treg cultures stimulated with the highest bead: cell ratio (3 beads: 1 cell) yielded numbers of $eGFP^+$ cells at least as great as lower ratios tested (1 bead: 1 cell and 1 bead: 5 cells) [Figure 3.2a].

It may be intuitive that a viral titre should correlate positively with transduction efficiency. Nonetheless, the increased transduction efficiency expected from higher viral titres might be negated by adverse effects of viral toxicity. Three viral titres were tested: 0.05TU/mL ("low"), 0.5TU/mL ("medium") and 5TU/mL ("high"). The highest viral titre yielded the greatest number of stably-transduced Tregs [Figure 3.2b]. In the existing literature, authors often advise that the lentivirus should be added to cells that have been pre-activated[277], inducing a highly proliferative state that promotes viral integration rapidly after viral entry into the cell. However, higher yields of transduced cells were obtained when lentivirus was added to target cells on the same day as the cells were stimulated, compared with adding virus to cells that had been pre-activated for three days [Figure 3.2c].

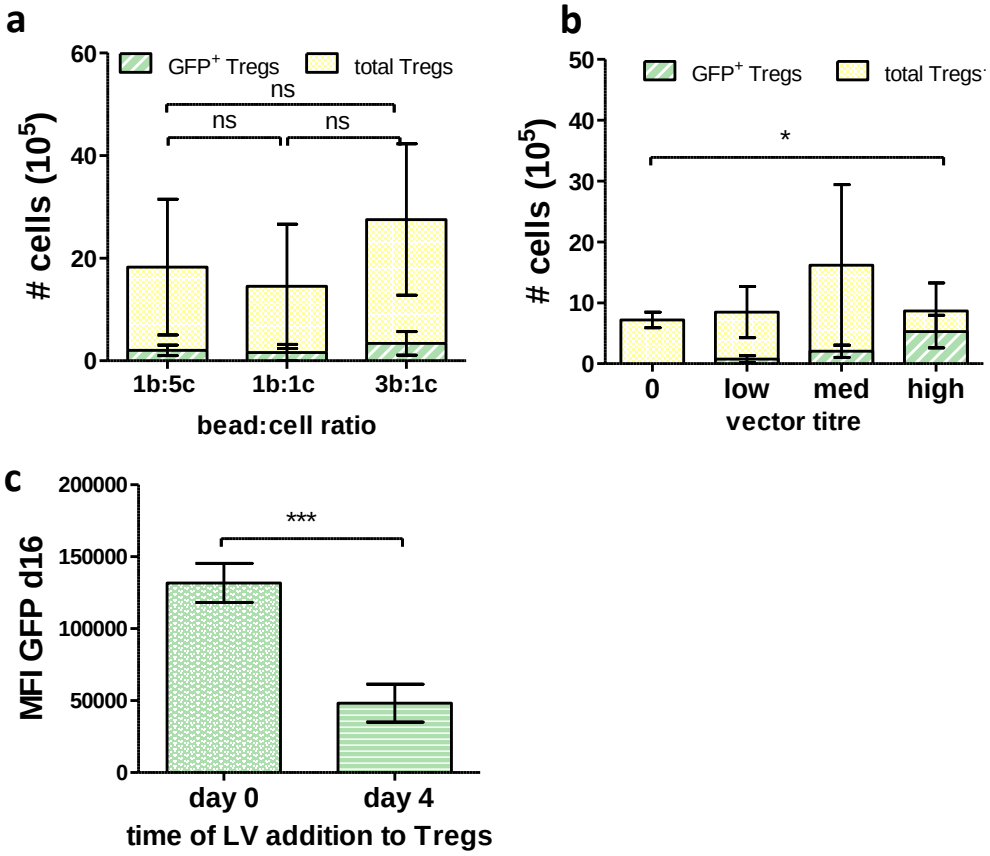

Figure 3.2 Effect of transduction conditions on transduction efficiency

$CD4^+CD25^+CD127^{lo}$ Tregs isolated from human peripheral blood were stimulated with αCD3αCD28-coated beads and IL-2, in the presence of lentiviral vector containing the eGFP transgene, under various conditions, as follows. (a) Tregs were stimulated at one of three bead: cell ratios (either 1 bead: 5 cells, 1 bead: 1 cell or 3 beads: 1 cell). Lentiviral vector (0.5TU/mL) containing the eGFP transgene was added to all cultures on day 3 after stimulation. Bars indicate mean+/-SEM of 3 cell donors. Statistical significance was assessed using Kruskal-Wallis Tests with Dunn's post-test for multiple comparisons. (b) Tregs were stimulated with αCD3αCD28-coated beads (at a ratio of 1 beads: 5 cells). On day 3, one of three different titres of lentiviral vector were added to each culture: either 0.05TU/mL ("low"), 0.5TU/mL("medium") or 5TU/mL ("high"). Bars indicate mean +/-SEM for 3 cell donors). Statistical significance was assessed using Kruskal-Wallis Tests with Dunn's post-test for multiple comparisons ($p<0.05$, unless indicated otherwise). (c) After stimulation with 3 beads: 1 cell, lentivirus was added to Treg cultures either on the day of cell isolation ("day 0") or 3 days after isolation ("day 4"). On day 14 after stimulation, cells counted manually and by flow cytometry to determine the percentage and absolute number of GFP^+ cells in each culture. Total number of live cells in each condition is plotted in yellow and number of GFP^+ cells in each culture is superimposed in green. Bars indicate mean +/- SD for 2 donors. Statistical significance was determined using Fisher's Exact Test: $p<0.001$.

3.3.2 Stable transgene expression in human Tregs can be achieved by lentivirus-mediated transduction

When relying upon a transgenic marker for long-term assays, it is critical to confirm stable expression of the transgene, such that the cells will not revert to a wild-type phenotype during the course of the experiment. Stable transgene expression requires genomic integration and permissive epigenetic regulation. Cultures containing greater than 85% eGFP$^+$ Tregs were obtained at the end of a 16-day transduction protocol. Throughout a further 54 days of *in vitro* cell culture, the frequency of eGFP$^+$ Tregs detected by flow cytometry remained constant, confirming that transgene expression persisting at day 16 post-transduction was sustained long term [Figure 3.3]. Furthermore, the stable frequency of transduced cells confirms that cells expressing transgene were not subject to negative selection when placed in competition with wild-type cells. These results provide confidence that the modified phenotype of transduced Tregs should be consistent, at the level of the cell population, over the course of a long-term experiment.

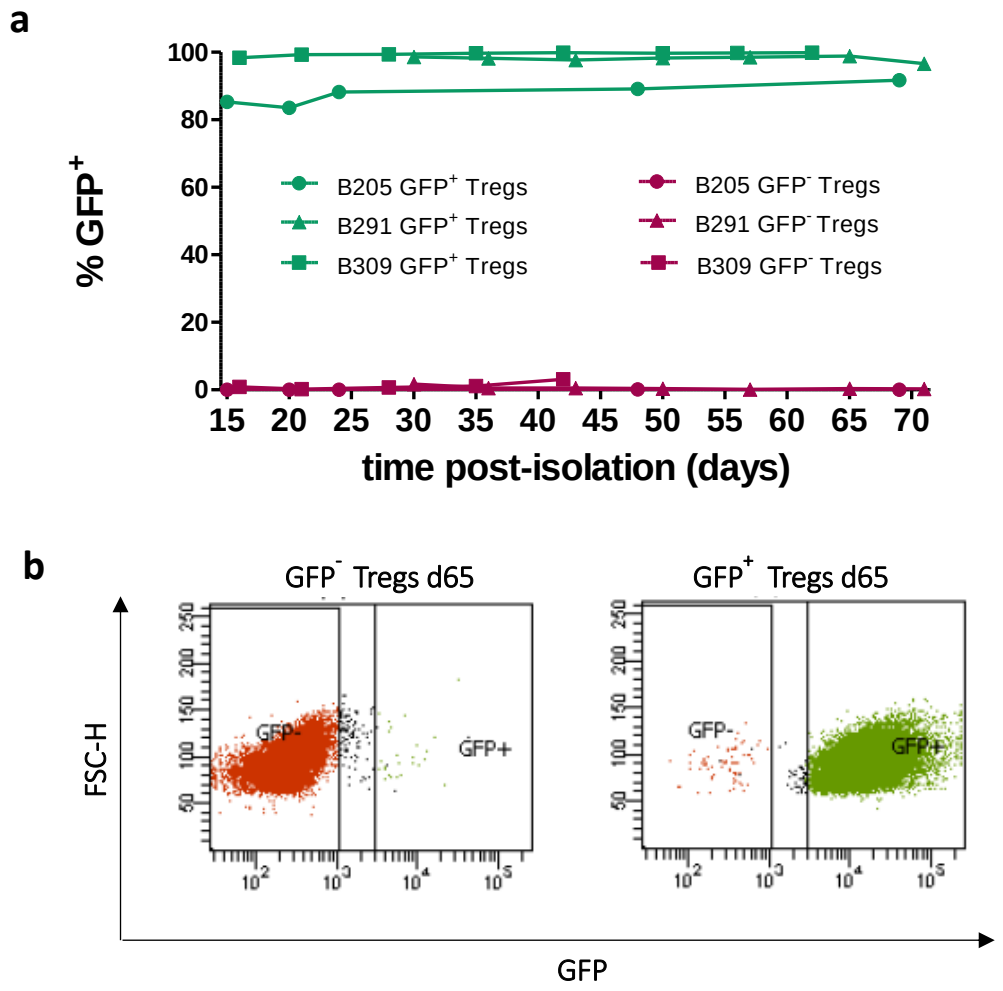

Figure 3.3: Stability of lentivirus-mediated transduction in human Tregs

Following expansion and lentiviral transduction with eGFP, $CD4^{+}CD25^{+}CD127^{lo}$ Tregs were sorted into GFP^{-} and GFP^{+} fractions. GFP^{-} and GFP^{+} Tregs were cultured for a further 8 weeks in the presence of 200U/mL recombinant IL-2 and αCD3αCD28-coated beads (1 bead: 5 cells). Cells were restimulated every 2 weeks with 1 bead: 1 cell. Cell cultures were divided and culture medium replenished as required. (a) GFP^{+} cells were enumerated periodically in aliquots from these cell cultures by flow cytometry. N=3 cell donors. (b) Representative FACS plots depicting GFP expression in GFP^{-} and GFP^{+} sorted Treg cultures on day 65 post-isolation.

3.3.3 Treg physiology is not impaired by lentivirus-mediated transduction

Previous studies have cautioned that the presence of virus within a cell, disruption of genomic integrity during viral integration, or the expression of transgene products (particularly those of xenogeneic origin) could all result in alterations to the fundamental biology of the target cell. Should transduction or transgene expression modify the biology of target cells, this might confound experimental assays or make the outcomes of clinical applications unpredictable. In order to ascertain whether this protocol affects Treg cell biology, particularly with respect to cell viability and activation, $eGFP^+$ Tregs were stimulated *in vitro*, alongside $eGFP^-$ cells (from the same culture as $eGFP^+$ cells but not expressing eGFP by day 14 post-isolation). After three days' stimulation *in vitro*, the frequency of apoptotic cells (determined by staining with Annexin V and 7AAD) did not differ significantly between $eGFP^+$ and $eGFP^-$ Tregs [Figure 3.4a]. In the same assay, the proliferation of $eGFP^+$ Tregs, as determined by dilution of a proliferation dye, was not significantly different from that of $eGFP^-$ Tregs [**Figure 3.4b**]. Finally, no significant difference was observed between $eGFP^+$ and $eGFP^-$ Tregs with respect to the cell surface density of two Treg activation markers, CD25 [**Figure 3.4c**] and CD69 [**Figure 3.4d**]. Although not statistically significant, there was a trend towards higher proliferation and lower survival among $eGFP^+$ Tregs, compared to $eGFP^-$ Tregs. For this reason, caution should be exercised in conducting *in vitro* assays that rely upon comparison of transduced versus non- transduced cells, as there appear to be slight but fundamental alterations in their biology that might affect sensitive assays.

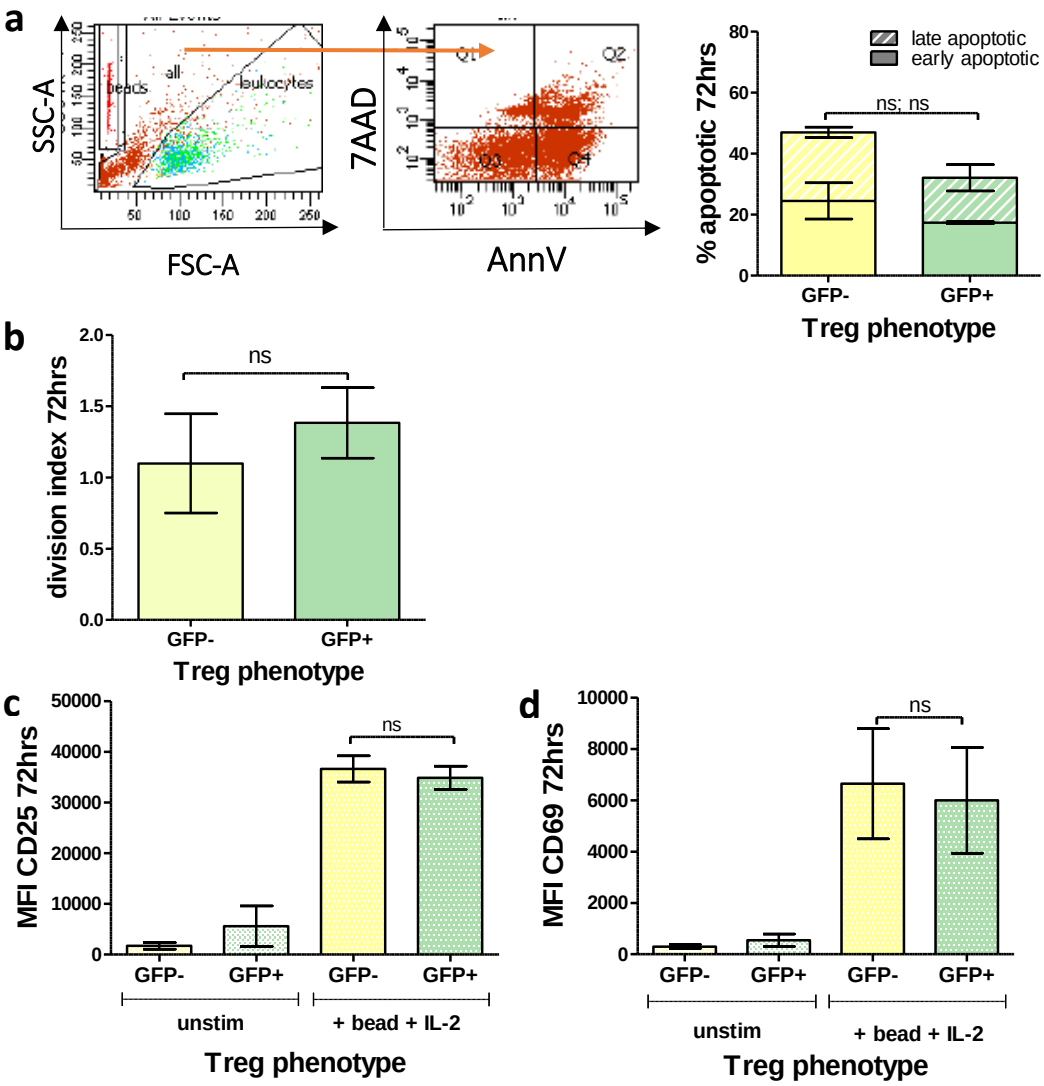

Figure 3.4 Survival, proliferation and activation of transduced Tregs in culture

GFP^+ and GFP^- Tregs were sorted from cultures transduced with the vector pWPXL according to the protocol in Figure 3.1. These Tregs were stained with violet proliferation dye and cultured with or without αCD3αCD28-coated beads (1bead: 5 cells) and 200U/mL IL-2. On day 3 after stimulation, cells were stained with vital dyes 7AAD and Annexin V for analysis by flow cytometry. (a) Cells undergoing early apoptotic cell death ($7AAD^-AnnexinV^+$) and late apoptotic cells ($7AAD^+AnnexinV^+$) were enumerated. (b) Dilution of violet dye by Tregs was determined by flow cytometry and used to calculate a division index. (c) Cell surface expression of activation markers CD25 and CD69 was quantified by flow cytometry. N=3 blood donors. Statistical significance was assessed using a Wilcoxon matched-pairs signed rank tests: (a) p=0.500, 0.250; (b) p=0.500; (c) p=0.250 ; (d) p=0.250.

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3.3.4 Treg suppressive function is not compromised by lentivirus-mediated transduced with a xenogeneic transgene

Ultimately, we are most concerned with whether suppressive function is modified in transduced Tregs. When cultured with autologous PBMCs, stimulated with αCD3αCD28 beads, the suppression of PBMC proliferation in the presence of $eGFP^+$ Tregs was not significantly different from the suppression mediated by $eGFP^-$ Tregs [Figure 3.5]. This result was consistent between $CD4^+$ [Figure 3.5a,b] and $CD8^+$ [Figure 3.5c,d] responder T cells. We conclude that human Tregs genetically engineered *in vitro* using lentiviral vector are not defective in their suppressive function and can, therefore, be used for functional assays.

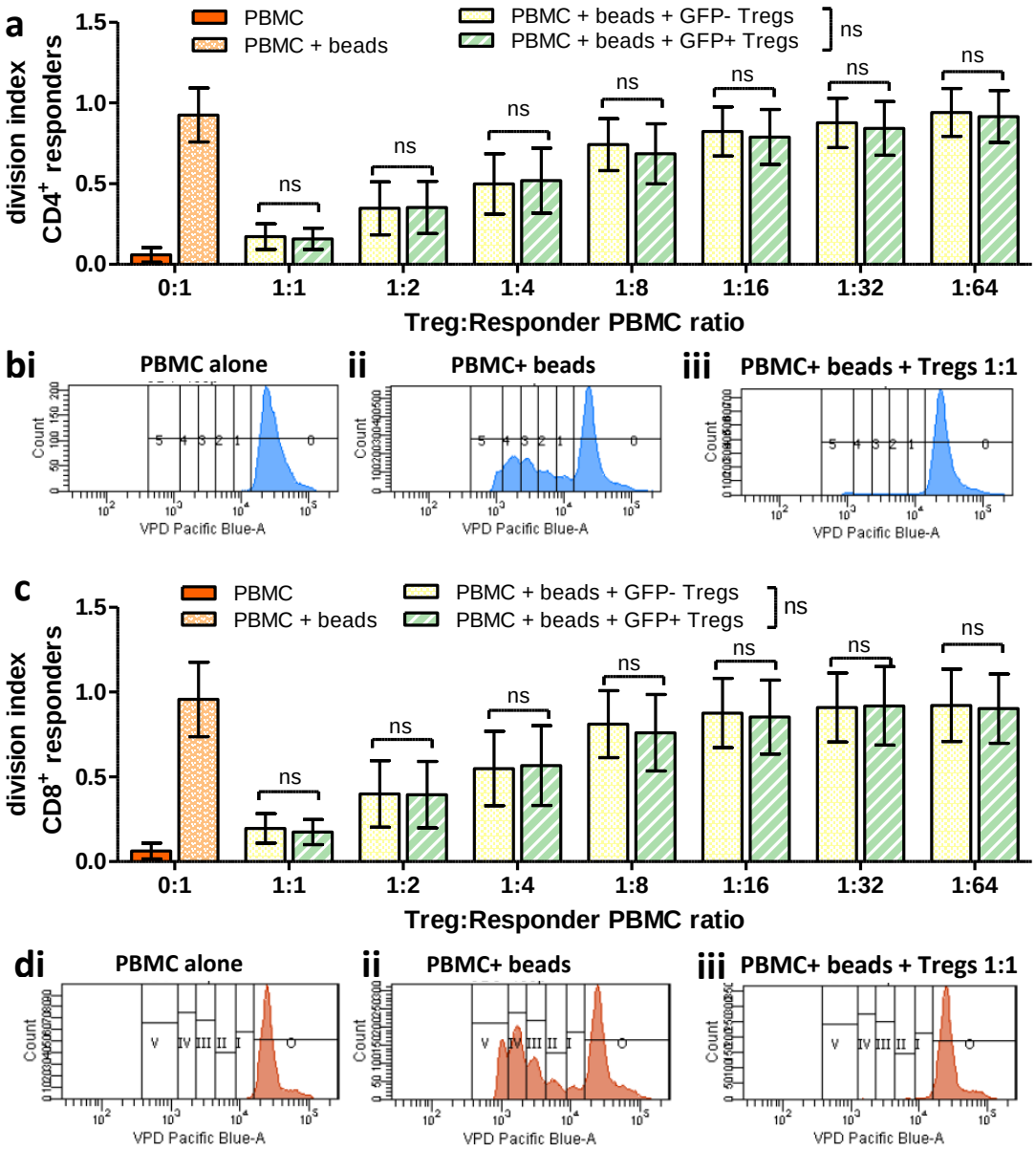


Figure 3.5 Effect of transduction upon the suppressive function of Tregs

PBMCs were stained with a proliferation dye and cultured for 72 hours with αCD3αCD28-coated beads (1 bead: 5 cells) in the presence or absence of Tregs at various Treg: responder PBMC ratios. (a,c) Dilution of the proliferation dye, indicating cell division, was measured by flow cytometry and used to calculate division indeces, which are plotted for (a) $CD4^+$ and (c) $CD8^+$ responder cells, as a mean and SEM of four cell donors. (b,d) FACS histograms from one representative donor depict proliferation dye dilution by (b) $CD4^+$ and (d) $CD8^+$ responder cells, under three conditions: (i) unstimulated, (ii) with beads, (iii) with beads and Tregs. Statistical significance was analysed by a two-way ANOVA with Bonferroni post-test: (a) p=0.149; (c) p=0.480.

3.3.5 Transduction with fluorescent protein permits detection and imaging of human Tregs *in vivo*

For cells that are being investigated as an adoptive cellular therapy, one particularly appealing application of fluorescently-labelled cells is *in vivo* tracking. We investigated briefly three techniques for tracing fluorescently-labelled cells *in vivo:* flow cytometry, PCR and intravital two-photon microscopy of live tissues, *in situ*.

For these experiments, we adoptively transferred eGFP$^+$ human Tregs into immunodeficient mice that had been surgically grafted with human skin, derived from a different donor to the cells. Blood and spleen, extracted from these mice five weeks after adoptive transfer of the cells, were macerated and stained for markers of human leukocytes. Whilst we detected substantial infiltration of human leukocytes within the blood and spleen, eGFP$^+$ cells were not detectable in meaningful numbers in these tissues by flow cytometry. eGFP$^+$ cells constituted less than 1% of the human CD4$^+$ cells in the spleen [**Figure 3.6a**] and less than 0.4% of the total human leukocytes in the spleen [**Figure 3.6b**], corresponding to a maximum of approximately 800 cells in this organ [**Figure 3.6c**]. Meanwhile, a negligible proportion of human cells in the blood (<0.03%) were eGFP$^+$. Considering that we had confirmed that cells from the same culture as those used in this experiment retained eGFP expression for longer than the duration of this experiment [**Figure 3.3**], the absence of detectable eGFP$^+$ cells cannot be attributed to loss of eGFP expression. Alternatively, human Tregs may not localise

to these particular tissues in this mouse model. We discounted this explanation because we know from previous experience that FOXP3^{+} human cells can be detected in these tissue in this model[305,306]. Whether these cells are the original Treg population that was infused or *de novo* induced Tregs, we cannot distinguish. In either case, we know that human cells with a Treg phenotype can reside in the blood and spleen of these humanised mice. The remaining explanation is that the method of detection, flow cytometry, is inadequate to detect eGFP or that that preparation of samples or flow cytometry compromises the detectability of eGFP in these samples.

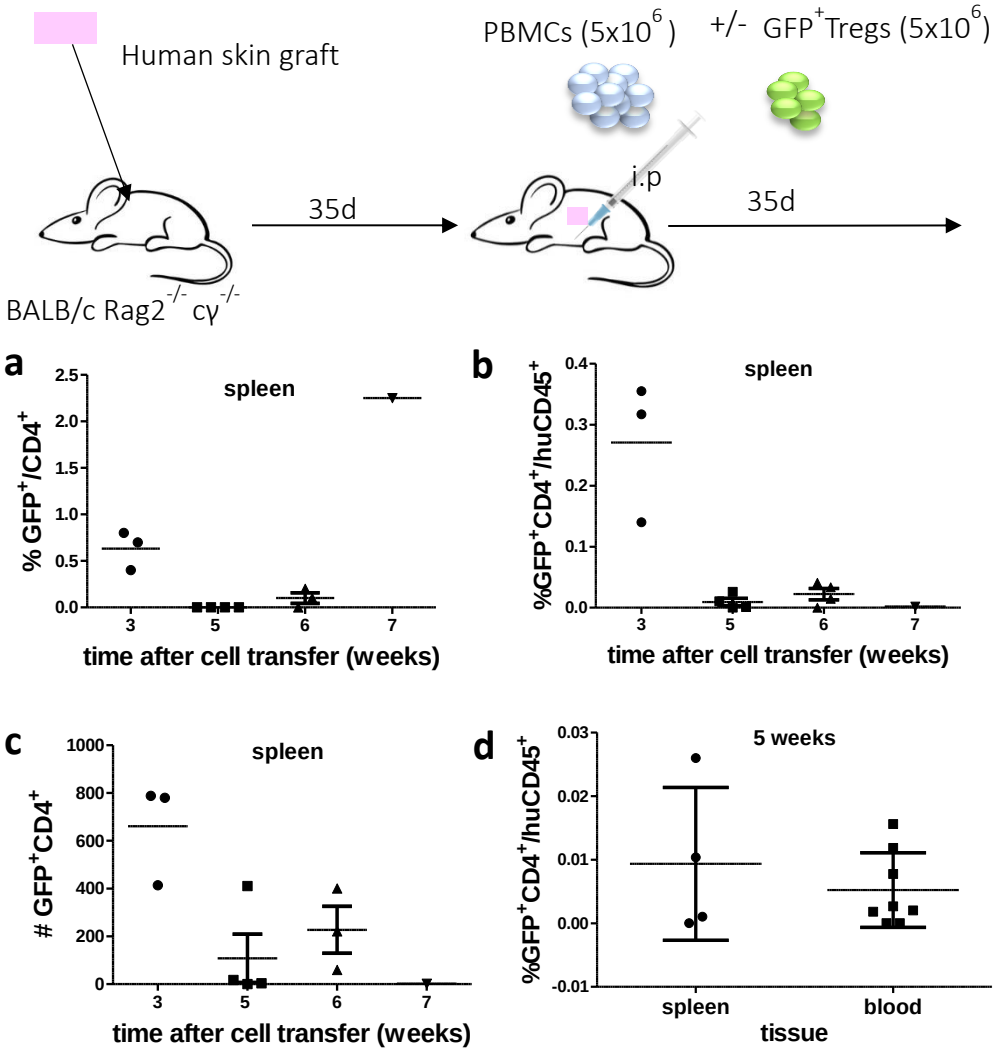


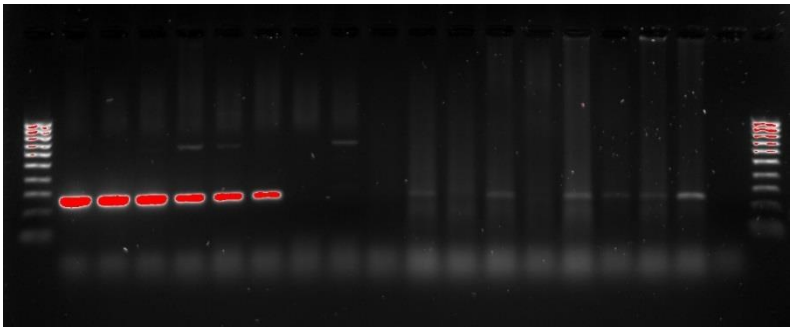
Figure 3.6 Attempted detection of GFP$^+$ Tregs within lymphoid tissues by flow cytometry

Human skin (1cm^2 split thickness) was surgically grafted onto the flank of BALB/c Rag2$^{-/-}$γc$^{-/-}$ mice. GFP$^+$ human Tregs (5x10^6 per mouse) were injected into skin graft recipients 35 days after transplantation, along with autologous PBMCs (5x10^6 per mouse). (a) GFP$^+$ cells detected by flow cytometry in spleen, at 3-7 weeks after cell transfer, are plotted as a percentage of CD4$^+$ cells and as a percentage of human CD45$^+$ leukocytes. (c) Number of GFP$^+$CD4$^+$ cells enumerated in spleens by flow cytometry are plotted over time. (d) Percentage of GFP$^+$ CD4$^+$ cells among total human leukocytes is plotted for spleen and blood at 5 weeks. Data for each mouse are as depicted as individual points, with bars indicating mean +/- SD.

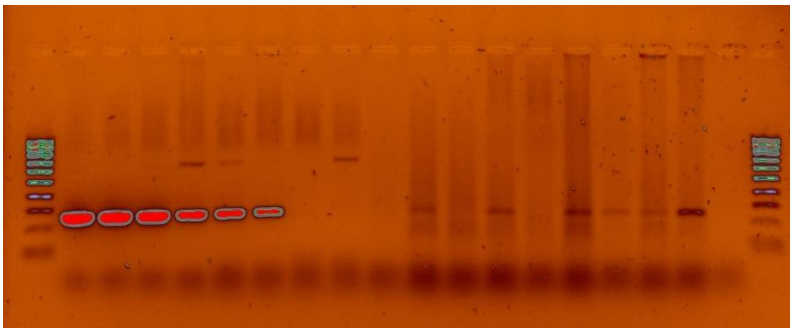
3.3.6 Detection of eGFP within skin grafts from humanised mice treated with eGFP-expressing human Tregs

In order to ascertain whether the eGFP$^+$ Tregs that had been infused into mice with human skin grafts had infiltrated the graft, we performed PCR on DNA extracts isolated from these skin grafts between three and seven weeks after cell transfer. Since the genomes of neither the mouse nor the human PBMCs contain eGFP, we used primers that bound within the eGFP ORF. Using standards comprising known numbers of eGFP$^+$ Tregs from the same culture as those used for this *in vivo* experiment, we confirmed that this PCR protocol could detect eGFP in samples containing as few as 8 eGFP$^+$ Tregs [**Figure 3.7**]. We tested DNA extracts from human eight skin grafts derived from mice that had received eGFP$^+$ Tregs. The degree of reconstitution with human PBMCs, quantified by flow cytometry, was variable between these graft recipients. Of these eight skin grafts, eGFP was detected in 6 grafts [**Figure 3.7a,b**].

a



b



c

Lane	Sample ID	# GFP⁺
1	Ladder 100-1000bp	-
2	Standard C	20000
3	Standard D	4000
4	Standard E	800
5	Standard F	160
6	Standard G	32
7	Standard H	8
8	Standard I	1
9	Standard J	0
10	Skin D39 m3 (no GFP⁺ cells)	0
11	Skin D55 m5	?
12	Skin D55 m6	?
13	Skin D55 m7	?
14	Skin D55 m9	?
15	Skin D55 m12	?
16	Skin D55 m13	?
17	Skin D55 m14	?
18	Skin D55 m15	?
19	NTC	0
20	Ladder 100-1000bp	-

Figure 3.7 Detection of GFP-expressing Tregs in human skin allografts by PCR

Human skin grafts and infusion of PBMC with GFP^+ Tregs were performed as in Figure 3.. 35 days after injection of cells, skin grafts were cryopreserved. Genomic DNA isolated from each skin graft was used as the substrate for PCR with primers complementary to portions of the eGFP ORF. As standards, DNA was isolated from known numbers of GFP^+ Tregs from the same cell culture as those infused into the mice. After PCR amplification, PCR products were subjected to gel electrophoresis. Bands of DNA corresponding to ~300bp amplification products indicate the presence of eGFP in the sample. The same gel is pictured in (a) greyscale and (b) pseudo-colour, for better print reproduction. (c) Content of lanes is tabulated by lane number (with increasing numerical order from left to right of the gel).

3.3.7 Visualisation of human eGFP-expressing Tregs within a skin allograft in situ by *intravital* two-photon microscopy

Whilst flow cytometry and PCR can be used to detect labelled cells *in vivo*, both methods provide only a snapshot and require culling of the animals. One method that can be used for longitudinal analyses is *intravital* microscopy, whereby imaging of fluorescently labelled cells can be conducted in living animals, at serial time points if necessary. Using two-photon microscopy, we imaged human skin grafts, *in situ*, on the flank of a humanised mouse that had received adoptively-transferred human eGFP$^+$ Tregs five weeks prior to imaging. Crucially, we used a non-invasive set-up, whereby the skin of the mouse's flank was flexed out over a piece of insulating putty, making the graft accessible to the imaging apparatus without making any incision in the skin. Time-lapse z-stack images revealed that eGFP$^+$ Tregs could be detected in the graft using this two-photon microscopy set-up [Figure 3.8]. Furthermore, the cells that we could see were exhibiting roaming behaviour typical of healthy, active lymphocytes.

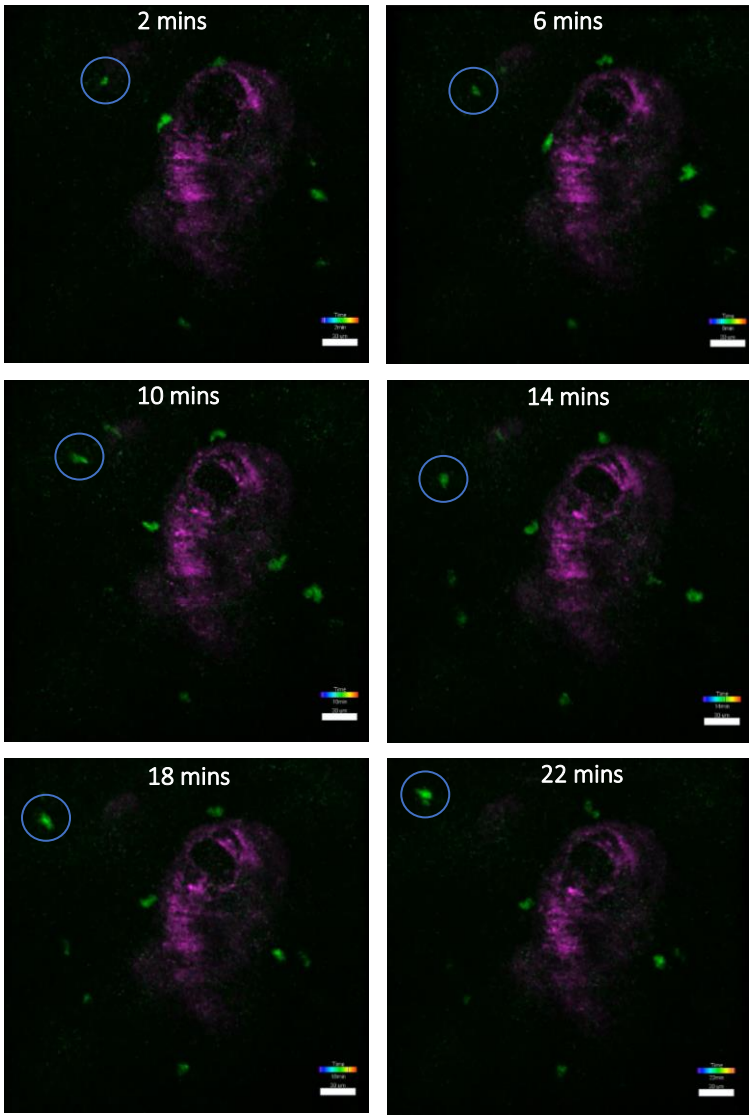


Figure 3.8 Visualisation of GFP-expressing human Tregs within a human skin graft

Human skin grafts and infusion of PBMCs and GFP$^+$ Tregs was performed as in **Figure 3.6** 35 days after injection of cells, skin grafts were imaged *in situ* by two-photon intra-vital microscopy. Images were taken at 5 frames per second, across a 60µm z-stack, up to a depth of approximately 100µm depth, for a total of 24mins. Signal from eGFP is depicted in green, whilst magenta indicates second harmonic signal from collagen. Still images captured at 4min intervals in the time-lapse video are presented to illustrate the dynamic behaviour of the cells. Scale bar indicates a 30µm distance. White text indicates time elapsed since commencing imaging. Blue circles track one cell to illustrate movement.

3.3.8 Lentivirus-mediated transduction of shRNA into human Tregs facilitates transcriptional silencing of genes for functional analyses

Another experimental application of genetic engineering is reverse genetics, blocking the expression of a protein in order to deduce the function of that molecule. We investigated the feasibility of utilising our protocol for transduction of human Tregs to knock down two genes that have been implicated in the function of mouse Tregs, in order to ascertain the contribution of their products to human Treg-mediated suppression. Tumour Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (*TRAIL*) is a cytokine that induces apoptosis in target cells via ligation of the death receptors DR4 and DR5. Galectin-1 (encoded by *LGALS1*) is a beta-galactosidase-binding protein that can induce apoptotic signalling in T cells and modulate integrin-dependent inter-cellular adhesion. Galectin-1 has been shown to exert anti-inflammatory effects upon the immune system via inhibition of leukocyte recruitment and modulation of DC phenotype[307–309]. shRNA directed against each of these genes was introduced into primary human Tregs, and $CD4^{+}CD25^{-}$ Teffs, using the protocol for lentivirus-mediated transduction outlined in Figure 3.1. By the end of the protocol, which involved selection for puromycin resistance conferred by transduction, efficient silencing of target gene transcription was achieved for *LGALS1* [Figure 3.9a] and *TRAIL* [Figure 3.9b].

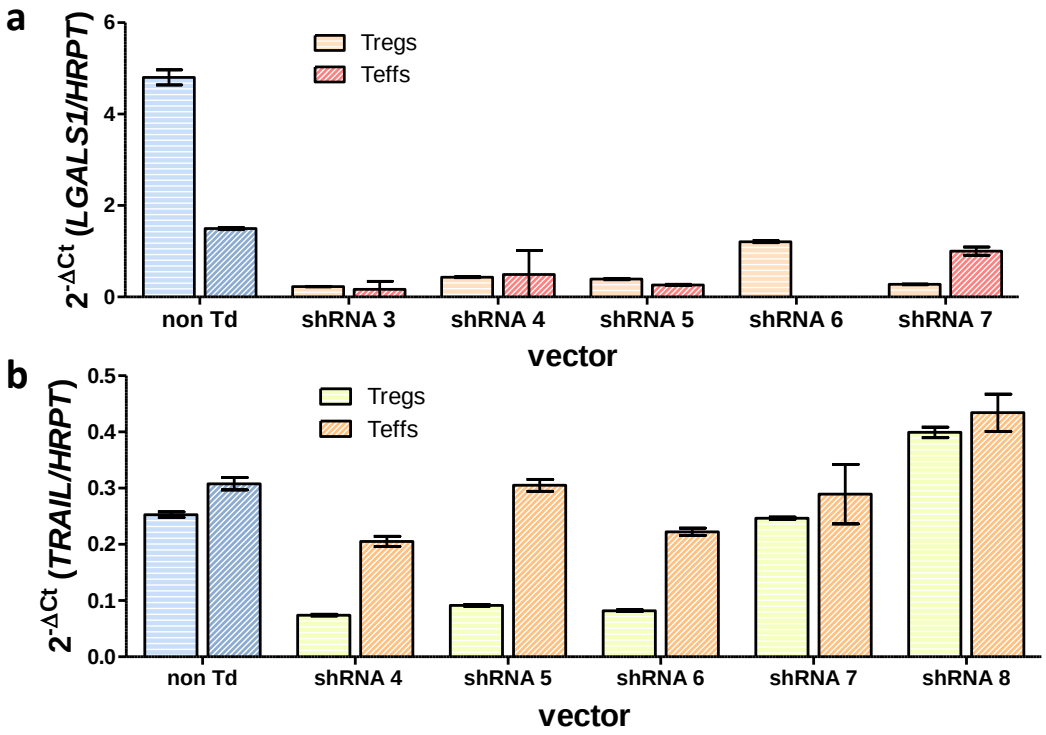


Figure 3.9 Efficiency of shRNA-mediated knock-down of *LGALS1* and *TRAIL* in human Tregs

During *in vitro* expansion, Tregs and $CD4^+CD25^-$ Teffs were either transduced with one of five shRNAs against (a) LGALS1 or (b) TRAIL, or not transduced ("non Td"). qRT-PCR was performed on cDNA from these cultures using probes for HPRT and (a) LGALS1 or (b) TRAIL. mRNA expression of each shRNA target, relative to *HPRT*, is depicted as a mean +/-SD for two replicates from one donor.

3.3.9 Genetic knockdown reveals that TRAIL is required, whilst Galectin-1 is dispensable, in human Tregs for optimal Treg-mediated suppression of cell proliferation

In order to determine whether TRAIL or Galectin-1 expression in human Tregs is required for Treg-mediated suppression, we performed an *in vitro* assay for suppression of PBMC proliferation. In the presence of *LGALS1* KD Tregs, PBMC proliferation was comparable to that observed in the presence of control Tregs, indicating that Galectin-1 expressed by Tregs is dispensable for Treg-mediated suppression [Figure 3.10a]. Meanwhile, suppression of PBMC proliferation in the

presence of *TRAIL* KD Tregs was marginally but significantly less than the suppression observed in the presence of control Tregs [**Figure 3.10b**]. This result suggests that TRAIL expressed by human Tregs promotes optimal suppressive function in these cells. However, reduced suppression by TRAIL KD Tregs is only evident at lower Treg: responder ratios, suggesting that TRAIL does not contribute to a dominant mechanism of Treg-mediated suppression.

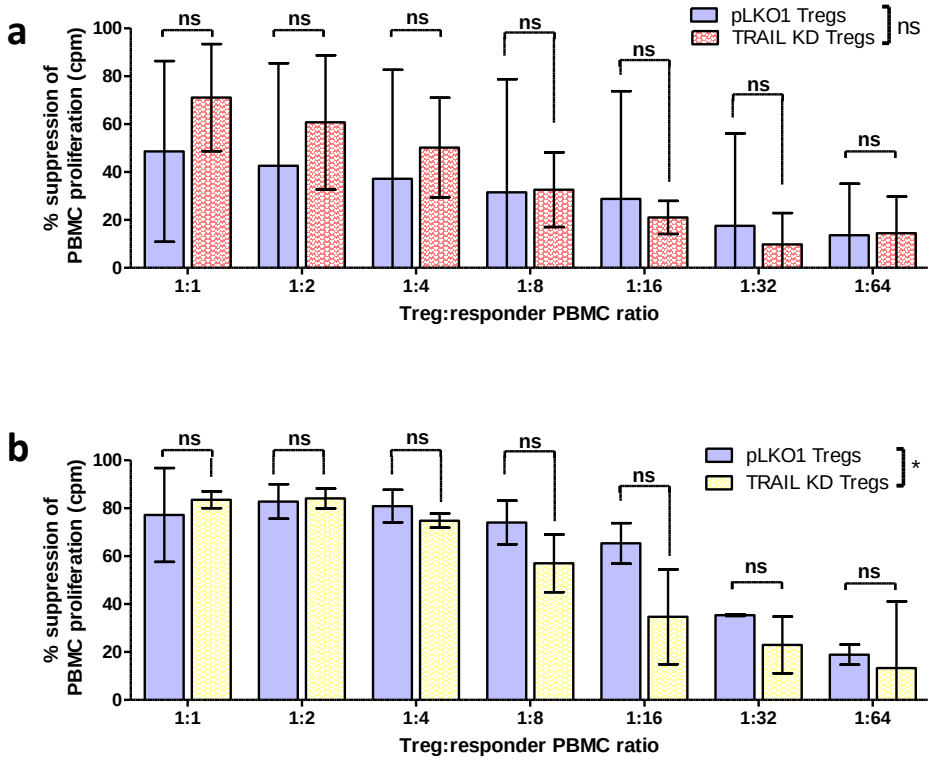


Figure 3.10 Suppression of PBMC proliferation by Galectin-1 KD and TRAIL KD Tregs

PBMCs (10^5 cells per well) stimulated with αCD3αCD28-coated beads ($2x10^4$ per well) were cultured for 6 days in the presence or absence of Tregs. Tritiated thymidine was added for the final 16 hours of co-culture and proliferation of responder cells was measured as uptake of tritium (counts per minute). Suppression of proliferation in the presence of Tregs was calculated as a percentage of proliferation of stimulated PBMCs in the absence of Tregs. (a) Suppression assay using Tregs transduced with either a control vector, pLKO.1, or with *LGALS1* shRNA. Bars indicate mean +/- SEM for three cell donors. (b) Suppression assay using Tregs transduced with either a control vector, pLKO.1, or with *TRAIL* shRNA. Bars indicate mean +/- SEM for two cell donors. Statistical significance was calculated using a two-way ANOVA with Bonferroni post-test: (a) p= 0.561; (b) p=0.0303.

3.4 Discussion

Therapeutic strategies harnessing the immune regulatory function of Tregs to inhibit graft rejection are the subject of much investigation. Optimal manipulation of these cells (either endogenous or adoptively-transferred) *in vivo* will depend upon an understanding of how these cells are regulated physiologically.

The study of mouse immunology has excelled through the exploitation of genetically engineered mouse models. For experiments in human immunology, a genetic model could be achieved by incorporating genetically engineered human immune cells into a humanised mouse model. The results presented here demonstrate that human peripheral blood Tregs are amenable to lentivirus-mediated transduction, with high transduction efficiency. Further investigation of the transduction conditions disclosed that activation of Tregs with a high ratio of stimulatory beads and exposure to relatively high viral titres generated optimal yields of transduced Tregs. Consistent with previous studies[277], it appears that strong stimulation via the TCR made Tregs more permissive to viral transduction. Yet it was not necessary for activation to precede addition of the virus. From these findings, we optimised a protocol for simultaneous *in vitro* transduction and expansion of human Tregs that reliably yielded sufficient engineered cells to be used in *in vitro* assays or in a humanised mouse model.

Whilst we had demonstrated the feasibility generating sufficient genetically engineered human Tregs for experimental applications, we felt that it was critical to examine the quality of these transduced cells. Both intuition and scientific evidence inform us that the process of transduction can have undesired effects

upon the target cell[310]. Firstly, the viral vector is bound to be immunogenic and has been shown to induce innate and adaptive immune mechanisms[311]. Genotoxicity resulting from insertional mutagenesis is a particular concern when using genome-integrating vectors such as retroviruses[312]. In fact, the regions of DNA in the vicinity of actively-transcribed protein-coding genes and regulatory elements are more likely to adopt an open chromatin structure that is permissible to retroviral integration[313,314]. Thus, retroviruses have a natural tropism for the very genes that are most active in their host cell. Furthermore, the products of the transgene being introduced into the cell may also be immunogenic[311], particularly if it is derived from a xenogeneic source. Given that eGFP is derived from a species of jellyfish, it would not have been surprising to find that this protein is not tolerated well by the target cell or by the immune system.

At the lentiviral titres tested (up to 5TU/cell), Treg biology was not substantially affected by lentiviral transduction. $eGFP^+$ Tregs exhibited marginally higher survival and proliferation rates than $eGFP^-$ Tregs, *in vitro,* yet these trends were not statistically significant. Furthermore, on average, the suppressive potency of transduced cell was not compromised by transduction of transgene expression. Notably, the mean trends obscure considerable heterogeneity between transduced cell cultures. It would advisable when using transduced human Tregs for experimental applications to replicate assays with Tregs from different transduction cultures. Thus, we conclude that lentivirus-mediated transduction of Tregs should be compatible with most experimental applications, provided that a vector control is used if transgene-positive cells are to be compared with

transgene-negative cells. Nonetheless, for each experiment, it is important to consider the specific effects of the transgene product on the cell. Our results are consistent with previous reports that, in spite of alterations in the expression of genes in the vicinity of integrated retroviruses in retrovirus-transduced T cells, the gross physiology of the cells was unaffected[313]. Furthermore, the authors of that study detected no evidence of clonal selection of transduced cells after infusion into patients.

For analysis of protein function, reverse genetics offers certain advantages over alternative approaches utilising blocking antibodies or small molecule inhibitors to neutralise the function of a molecule of interest. Stable genetic knockdown can be achieved in order to assess the impact of long-term deprivation of the protein of interest, without sustained treatment with a blocking agent. This is particularly useful if the target cells are to be adoptively transferred into an animal, where it may not be practicable to continue treating the cells *in vivo*. In this manner, the deficiency resulting from genetic knockdown can be restricted to the target cells, without exposing neighbouring cells in a co-culture or *in vivo*, after adoptive transfer of the target cells.

Adopting a reverse genetics approach, we used our Treg transduction method to investigate proteins that have been implicated in Treg function but have not yet been interrogated experimentally in primary human Tregs. Two candidate proteins identified in previous studies as contributing to mouse Treg immune regulatory mechanisms were selected for investigation: TRAIL and Galectin-1. TRAIL, a death receptor ligand, has been investigated mostly in tumour models,

in which it promotes the graft-versus-tumour activity of adoptively-transferred T cells. Serendipitously, it was observed that over-expressing TRAIL in T cells also reduced graft-versus-host by suppressing proliferation of alloreactive T cells[315]. Tregs have been shown to induce apoptosis in $CD4^+$ Teffs through a Fas-independent, TRAIL-dependent mechanism[249]. Galectin-1, a glycan receptor, has been shown to inhibit immune cell recruitment and induce a tolerogenic phenotype in DCs[307–309]. By transducing human Tregs with short hairpin RNA (shRNA) directed against the transcript of each gene of interest, we were able to silence expression of these candidate proteins efficiently. *In vitro* suppression assays revealed no significant impairment of suppressive function in Tregs deficient in Galectin-1. In fact, in two out of three assays, Galectin-1 KD Tregs suppressed PBMC proliferation more potently than control Tregs. TRAIL deficient Treg, in contrast were impaired in their ability to suppress PBMC proliferation, relative to control Tregs. Thus, we concluded that TRAIL contributes to Treg-mediated suppression, though other mechanisms of Treg-mediated suppression can compensate for a deficiency in TRAIL, whilst Galectin-1 is dispensable for the suppressive mechanisms that dominate in these *in vitro* assays.

The transduction of Tregs provided a relatively labour-efficient method for conducting a small-scale screen of candidate proteins. For larger-scale screens, a number of emerging technologies, particularly in the field of genome editing, would permit a much more efficient, high-throughput methodology. Nonetheless, for labs without expertise in genomics and bioinformatics, interested in validating a small number of target proteins in-house, we feel that

the protocol described herein is more appropriate. Crucially, genetic silencing provides a cleaner analysis of protein function than exposure of cells to reagents that block or stimulate the function of the protein. Genetic silencing or overexpression of the protein permits dissection of cell-intrinsic versus cell-extrinsic functions of the protein. Furthermore, long-term effects of deprivation of that protein can be determined without repeated application of the reagent.

Due to constraints on time and resources, we did not assess the function of TRAIL KD and Galectin-1 KD Tregs in an *in vivo* model. However, this genetic knockdown approach proved to be invaluable for an analogous study outlined in Chapter 4 of this thesis, in which we explore the function of one molecule, neuropilin-1, in human Tregs.

Beyond molecular studies, the methodology of Treg genetic engineering might be adopted for a variety of experimental applications. One application that is particularly pertinent to our research interests is *in vivo* tracking of adoptively-transferred cells. The successful detection of adoptively-transferred $eGFP^+$ Tregs in allogeneic skin grafts provides evidence that these cells do persist *in vivo* for at least five weeks and do retain their eGFP expression for this duration. In the future, we would consider expanding this experiment by performing PCR to detect GPF in various tissues at different time points after cell infusion. In this way, we could examine the kinetics of Treg infiltration or trafficking into various tissues, and their persistence within these tissues. Other cell types could also be tracked in this manner.

Considering that Tregs persisted and retained eGFP expression in skin for the duration of the experiment, and that other human $CD4^+$ T cells are abundant in the lymphoid organs of these mice, our failure to detect $eGFP^+$ Tregs in the blood and spleen of these animals is most likely attributable to limitations of the methodology. It appears that either our method of preparing tissues for flow cytometry or the flow cytometric analysis is not conducive to detection of eGFP-expressing cells present at low frequency. With optimisation, we hope that flow cytometry could be utilised in the future. Whilst PCR was sufficiently sensitive to detect the eGFP-expressing cells in animal tissues, this method merely discriminates presence versus absence of the cells. Flow cytometry would enable phenotypic analysis and absolute enumeration of the cells tracked by eGFP expression. This approach could be used for simple cell fate analyses, to account for phenotypic plasticity among T cells subsets that can be a confounding factor in experiments on Tregs.

Previous data from our lab has demonstrated that human $FOXP3^+$ cells are detectable in allografts from mice that have received adoptively-transferred human Tregs at 100 days post-cell infusion[305,306]. In the future, we envisage utilising this cell tracking method to determine whether these $FOXP3^+$ cells were the *in vitro*-expanded Tregs that we infused or whether these were derived from the PBMCs with which these mice were reconstituted, by *de novo* Treg induction. Additionally, it would be useful to compare the phenotype of adoptively-transferred Tregs before and after infusion. We would predict that the phenotype of these cells is altered after exposure to the inflammatory environment of an

allograft response. Furthermore, where phenotypic heterogeneity within this cell population is associated with different *in vivo* behaviours, such as homing to different tissues, it may be possible to deduce causality between molecular phenotype and behaviour.

The use of conditional reporter constructs might further facilitate cell tracking experiments. For example, in mouse studies, the combination of a eGFP-cre fusion protein driven by directly the FOXP3 promoter and RFP driven by a cre-activated constitutive, ubiquitous promoter has been used to discriminate between current and historical FOXP3 expression in T cells[316,317]. This distinction was critical for defining Treg phenotypic plasticity in both steady-state and inflammatory environments, by identifying populations of "exTregs" and Teff populations that express FOXP3 transiently.

We have demonstrated as a proof-of-principle that human Tregs engineered to express fluorescent proteins can be imaged intravitally in a humanised mouse model. Intravital imaging of immune cells in skin has been reported previously using explants or by inserting a plate beneath the skin during imaging. Both of these invasive approaches are likely to fundamentally alter the physiology of the tissue being imaged, increasing the risk of artefacts. One group has developed a protocol for non-invasive imaging of skin graft in the mouse ear[318]. Whilst this approach is a refinement on previous invasive methods, grafting of human skin (which is usually procured as a very delicate split-thickness sample) onto mouse ears is technically challenging. Furthermore, compared to skin grafts situated on the animal's flank, which can be protected with bandaging, skin grafts on the ear

are at much greater risk of damage inflicted by the animals. Our method for non-invasive imaging of skin graft on the flank of mice could greatly benefit future intravital skin imaging experiments. It must be noted that we could not detect these cells in all skin grafts that we examined. In particular, we found that the thickening of the skin associated with immunological rejection of the graft greatly reduced the clarity of the images that could be obtained, to the extent that it precluded discrimination of $eGFP^+$ cells from background noise. The use of an alternative fluorescent marker might help to mitigate the problem of discriminating between eGFP and auto-fluorescent signal. Furthermore, the $eGFP^+$ cells appeared to present at low frequency and to be concentrated in particular localities within the graft, rather than being distributed throughout the tissue. In the future, labelling other structures, such as the vasculature, within the grafts might help us to identify whether these Tregs are associating with particular anatomical structure. With some refinement, this methodology could be used to explore the *in vivo* dynamic behaviour of human Tregs, where previously mouse experiments have served as a proxy.

Venturing into the future, the prospect of applying genetically-manipulated Tregs in the clinic is tantilising[319]. Cellular therapies are regarded as a promising approach to treat cancer and immune-mediated pathologies and are progressing well through clinical trials[127,266]. Already, genetic engineering and gene editing are being exploited to improve the efficacy and safety of putative cellular therapeutics[320]. The possibility of enhancing the function of human Tregs by

employing the genetic engineering protocol described here, to augment the expression of immunoregulatory molecules, is explored in Chapter 5 of this thesis.

Chapter 4: Investigating the Role of Neuropilin in Human Regulatory T Cells

4.1 Introduction

All leukocytes must be responsive to environmental cues that encode information about local and systemic physiology, including immune and metabolic status. In order to strike the optimal balance between immunity and tolerance, regulatory T cells must themselves be subject to regulation, as components of an orchestrated network. Proliferation, migration and secretion of suppressive cytokines are potentiated in response to potentially pathological inflammation, whilst being prohibited during physiological inflammation. Identifying the molecular signals that dictate Treg activity will further our understanding of basic Treg cell biology and help to inform the design of Treg-based cellular therapy.

First characterised as a mediator of neural and vascular development[280,281], neuropilin-1 (NRP1) is a transmembrane glycoprotein that has been implicated broadly in the regulation of immunity [282–284]. Neuropilin-1 associates with various receptors, including plexins and growth factor receptors. Ligands of these NRP1-receptor complexes include diverse members of the semaphorin and growth factor families (including Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor beta (TGFβ)) [278,279].

Within the immune system, NRP1 is expressed by T cells and dendritic cells and NRP1-associated ligands are expressed broadly by lymphocytes and APCs. Depending upon the cell type and the receptors with which this co-receptor interacts, NRP1 and its ligands have been associated with both activation[285] and inhibition[321,322] of immune cells. Additionally, NRP1 and semaphorins have been shown to regulate immune cell migration[323]

In addition to supporting receptor-ligand interactions, trans-cellular homophilic interactions of NRP1 appear to perform an adhesive function that stabilises intercellular adhesions between mouse T cells and DCs. In mice, Nrp1 has been shown to contribute to immunological synapse formation as a component of the peripheral Supramolecular Activation Complex (pSMAC)[286]. Subsequent T cell priming was dependent upon Nrp1, as demonstrated by inhibition of DC-induced T cell proliferation following pre-incubation of either T cells or DCs with anti-NRP1 blocking antibody [285].

Both semaphorins and growth factors are known to be exploited extensively in the immune system for intercellular signalling, both among lymphoid cells and between cells of the immune system and cells of non-lymphoid tissues[284,287,324,325]. Migration has been shown to be impaired in DCs that are deficient in SEMA3A or plexinA1[326]. Sema4A signalling via Tim-2 contributed to the pathogenesis of Experimental Autoimmune Encephalomyalitis (EAE) in mice, by promoting T cell activation[327]. Meanwhile, optimal activation of $CD8^+$ T cells, DCs and B cells is dependent upon signalling via Sema4D-plexinB2[328–330]. Conversely, semaphorins can exhibit anti-inflammatory functions. For example,

in one study, PBMCs downregulated secretion of certain pro-inflammatory cytokines upon exposure to exogenous semaphorin 3A (Sema3A) and administration of SEMA3A delayed the development of experimental collagen-induced arthritis[322]. TGFβ has well-established roles as an anti-inflammatory cytokine[331–333]. VEGF has been implicated in mechanisms of tumour immune evasion[334], including inhibition of DC maturation[335]. Driving neuronal expression of Hepatocyte Growth Factor promoted induction of tolerogenic DCs and Tregs and reduction of other immune cell infiltrates in a mouse model of EAE [336].

The biochemistry of NRP1-dependent intracellular signalling is not firmly established. Upon ligand binding, NRP1 can recruit PTEN (Phosphatase and Tensin Homologue) to the plasma membrane, resulting in inhibition of AKT phosphorylation and changes in expression of AKT-dependent transcripts. More extensive signal transduction associated with NRP1 probably derives from the receptors to which NRP1 is complexed. In the context of osteogenesis, semaphorin-dependent signalling downstream of plexins has been found to modulate the insulin-like growth factor[337] and WNT (Wingless-related Integration site)[338] signalling pathways. At the nexus of so many pertinent signalling pathways, NRP1 appears to be ideally placed to integrate complex immunological signals.

In relation to Tregs, interest in NRP1 arose from studies showing that, in mice, Nrp1 is preferentially expressed by Tregs, compared with Teffs[286,288], or by thymus-derived Tregs specifically[289,290]. Based upon these findings, Nrp1 is generally regarded as a reliable cell surface marker of Treg phenotype in mice.

Furthermore, several studies have demonstrated a functional role for Nrp1 in mouse Tregs[286,288,291,339]. Of particular interest in the context of transplant immunology, prolonged survival of cardiac allografts was observed in mice receiving adoptively-transferred $CD4^+CD25^-Nrp1^+$ T cells, whilst $Nrp1^-$ T cells failed to prolong graft survival[339].

Some studies found Nrp1 expression by $CD4^+CD25^-$ T cells to be sufficient for suppressive of PBMC proliferation[288,340] *in vitro* and of EAE pathogenesis in a mouse model[340]. In fact, the latter study found $CD4^+CD25^-Nrp1^+$ cells to be more suppressive than $CD4^+CD25^+$ cells.

The mechanisms by which Nrp1 promotes mouse Treg function appear to be manifold. One study demonstrated that DCs formed immunological synapses with Tregs at a greater frequency and for a longer duration than with Teffs, which could be attributed to the higher density of Nrp1 expressed on Tregs[286]. The authors speculate that this prolonged interaction with DCs confers upon Tregs a competitive advantage over Teffs, by permitting activation at a lower threshold of cognate antigen concentration. An important mechanism of Treg-mediated suppression, induction of a tolerogenic phenotype in DCs, relies upon physical interaction of Tregs with DCs. Thus, NRP1 may support both activation of Tregs by DCs, with competitive exclusion of Teffs from DCs, and modulation of DC function by Tregs.

As well as the adhesive function of NRP1, ligand-dependent intracellular signalling downstream of this co-receptor might also facilitate Treg function in a cell-intrinsic manner. In a mouse tumour model, the downregulation of AKT-mTOR

signalling upon Nrp1-Sema4a ligation correlated with acquisition of a transcriptional profile associated with greater Treg phenotypic stability. The ligation of Teff- or DC-derived Sema4a to Nrp1 on Tregs was shown to be necessary and sufficient to potentiate contact-independent mechanisms of Treg-mediated suppression[291]. Interestingly, the dependence of Tregs upon Nrp1 was found to be critical in an inflammatory context, namely within a tumour microenvironment, but dispensable for steady-state Treg homeostasis. This finding suggests that Nrp1 does indeed convey signals encoding immunological cues in order to regulate Treg activity adaptively.

Whilst it has been reported that NRP1 is not preferentially expressed in human peripheral blood Tregs[292], it has not been established whether suppressive function is influenced by NRP1 in human Tregs.

4.2 Objectives and Hypotheses

Objective 1: To determine whether the protein neuropilin-1 (NRP1) constitutes a marker of, or contributes to the suppressive function of, human Tregs.

Hypothesis 1: NRP1 deficient human Tregs will exhibit impaired suppression of cell proliferation and cytokine secretion *in vitro* and of graft rejection *in vivo*.

Objective 2: Should NRP1 prove to contribute to human Treg function, to identify NRP1-dependent molecular mechanisms by which NRP1 influences Treg physiology and potentiated Treg suppressive function.

Hypothesis 2: NRP1-deficient Tregs will exhibit altered gene expression and impaired physical interaction with antigen presenting cells.

4.3 Results

4.3.1 NRP1 is expressed transiently by human Tregs and CD4$^+$ Teffs upon TCR stimulation

Previous studies have reported that among mouse T cells, NRP1 is expressed preferentially, or even exclusively, by CD4$^+$CD25$^+$ Tregs. In order to assess the extent to which NRP1 is expressed by human Tregs, CD4$^+$CD25$^+$CD127^{lo} Tregs and CD4$^+$CD25$^-$ Teffs were isolated from human peripheral blood and stimulated with αCD3αCD28 beads and IL-2. In contrast to the data from studies of mouse Tregs, both the frequency and density of NRP1 expression, determined by flow cytometry, were lower among freshly-isolated human Tregs, compared with Teffs. Whilst mouse studies reported constitutive expression of NRP1 by mouse Tregs, the expression of NRP1 on human Tregs increased transiently, peaking at 48-72hours after TCR stimulation [**Figure 4.1a**]. NRP1 expression was also assessed in human Tregs that had been exposed to prolonged TCR stimulation during a 16-day *in vitro* expansion procedure. NRP1 expression was elevated in expanded Tregs, compared with freshly-isolated Tregs, but still fluctuated in response to current TCR stimulation [**Figure 4.1b-c**].

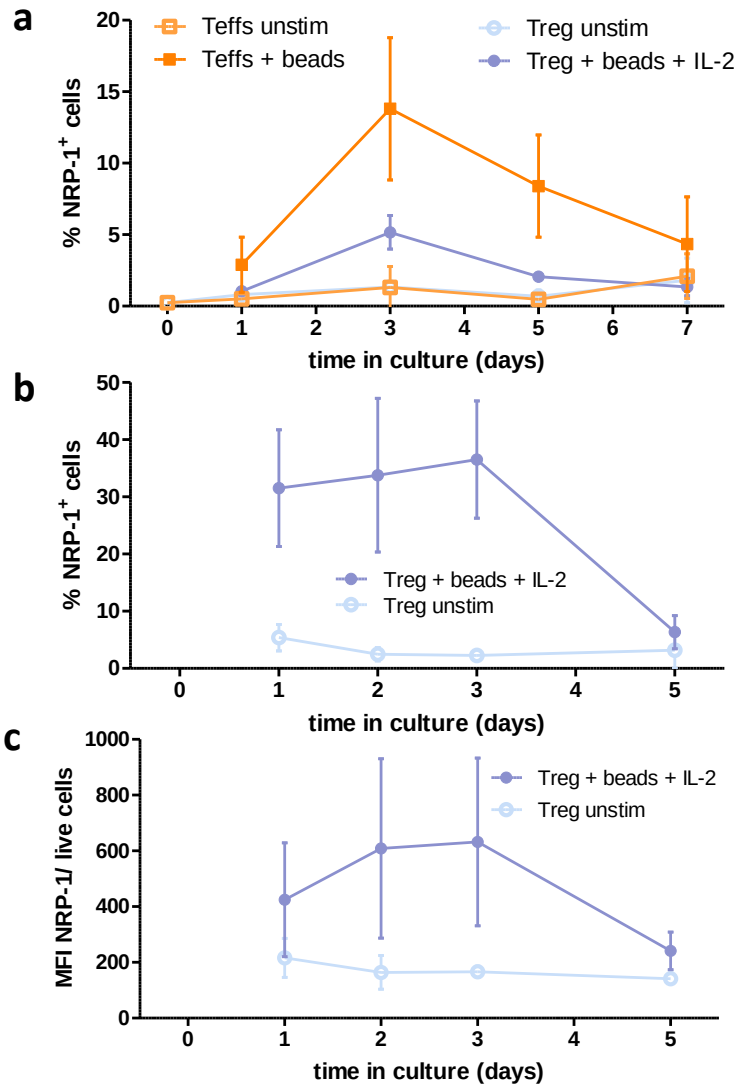


Figure 4.1 Expression of NRP1 at the surface of human Tregs and Teffs

(a) Directly after isolation, Tregs and Teffs from each donor were cultured for 7 days in the presence or absence of beads (1 bead: 5 cells) and IL-2 (200U/mL for Tregs and 100U/mL for Teffs). At each time point, cells were harvested for analysis of cell surface expression of NRP1 by flow cytometry. N=3 donors. (b) After cryopreservation, *in vitro*-expanded Tregs were cultured as described for (a). Percentage of $NRP1^+$ cells (a,b) and MFI of NRP1 (c) are plotted as mean +/- SD of three donors

Consistent with previous reports [292], the transitory nature of NRP1 expression by human Tregs suggests that NRP1 is not a marker of human peripheral blood Tregs and that $NRP1^+$ Tregs are not a stable population. Some *in vitro* assays could be performed in the presence of NRP1 blocking antibody, however the interpretation of these assays could be confounded if the effects of blocking NRP1 on responder T cells might negate the effects of blocking NRP1 on Tregs. We sought a new approach that would isolate any Treg-intrinsic requirement for NRP1. We discounted the alternative strategy of comparing FACS-sorted populations based upon their level of NRP1 protein expression on the basis of the instability of NRP1 expression in human Tregs. If sorted, these cells would not maintain their phenotype with respect to NRP1 expression for the duration of our experiments. Therefore, we examined the role of NRP1 in human Tregs by genetically silencing *NRP1* in these cells by lentivirus-mediated transduction with a construct encoding shRNA against *NRP1*. By the end of the 16-day expansion and transduction procedure, the density of NRP1 protein on the surface of NRP1 knockdown Tregs was reduced by 32.9-83.2%, relative to Tregs from the same donor transduced with a control vector [**Figure 4.2**].

a

	% NRP1+ cells		% reduction in frequency of NRP1+ cells	MFI of NRP1		% knockdown of NRP1 protein expression (MFI)
B263	10.0	2.5	75.0	190	100	47.4
B265	20.8	7.5	63.9	266	99	62.8
B266	15.9	1.8	83.0	249	141	43.4
B286	36.7	3.7	90.0	679	351	48.3
B292	13.7	4.8	65.0	164	110	32.9
B300	22.6	2.0	91.2	305	74	76.1
B301	29.2	4.1	86.0	369	62	83.2
B305	10.2	0.5	95.1	123	68	44.7

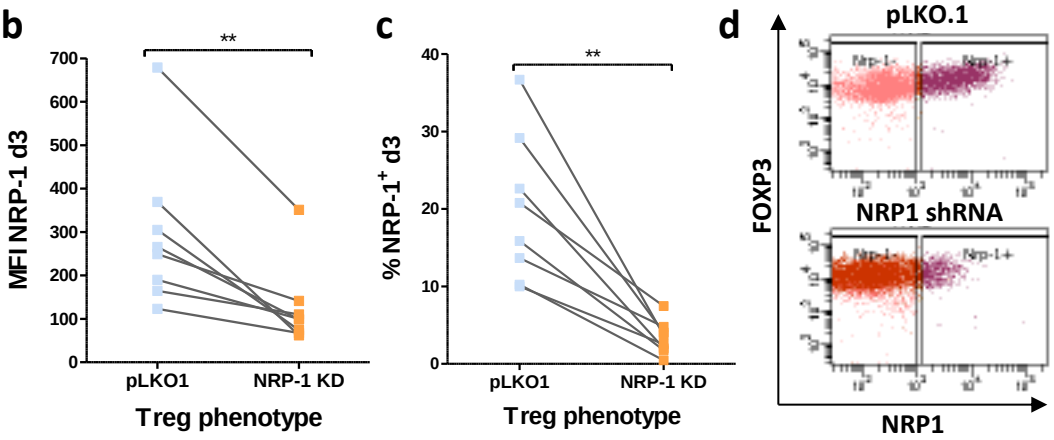


Figure 4.2 Knockdown Efficiency in human Tregs transduced with *NRP1* shRNA

$CD4^{+}CD25^{+}CD127^{lo}$ Tregs transduced with lentivirus containing plasmids encoding either *NRP1* shRNA or a control vector containing a stuffer insert. Transduced Tregs were stimulated for 72hrs with αCD3αCD28 beads and IL-2. Cell surface expression of NRP1, quantified by flow cytometry, is (a) tabulated and (b-c) plotted as (b) median fluorescence intensity and (c) frequency of cells expressing NRP1. Mean values for each donor are plotted separately, with lines adjoining cells from the same donor. Wilcoxon matched-pairs signed ranks tests were used for statistical analyses: (b) p=0.008; (c) p=0.008. (d) FACS plots from one representative donor are shown for NRP1 expression in (upper) control and (lower) NRP1 KD Tregs.

4.3.2 NRP1 expression by Tregs was required for Treg-mediated suppression of cell proliferation and pro-inflammatory cytokine secretion *in vitro*

In several studies, Nrp1 has been implicated in the suppressive function of mouse Tregs[286,291,339,341] yet the role of NRP1 in human Treg function has not been assessed experimentally. In order to determine whether NRP1 expression by Tregs is required for the function of these cells, NRP1-KD Tregs were compared with NRP1-sufficient Tregs in an *in vitro* suppression assay. The proliferation of PBMCs stimulated with allogeneic monocyte-derived dendritic cells was dramatically reduced in the presence of NRP1-sufficient Tregs, indicating Treg-mediated suppression of PBMC proliferation. In striking contrast, no suppression of PBMC proliferation was observed in the presence of NRP1-deficient Tregs [Figure 4.3].

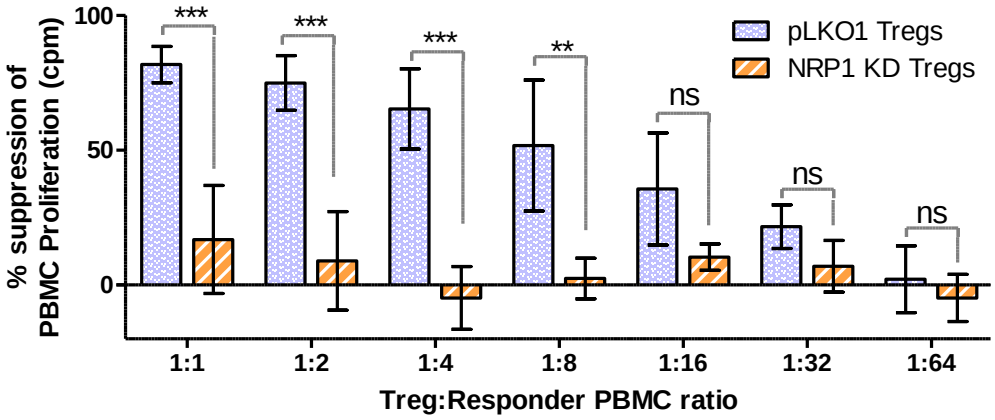


Figure 4.3 Suppression of PBMC proliferation by NRP1 KD Tregs *in vitro*

PBMCs (10^5 cells per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for six days in the presence or absence of Tregs, transduced with either control lentiviral vector, pLKO.1, or with *NRP1* shRNA. Tritiated thymidine was added for the final 16 hours of co-culture and proliferation of responder cells was measured as uptake of tritium (cpm). Bars indicate mean +/- SEM of three assays using different cell donors. Statistics were performed using a two-way ANOVA with Bonferroni post-tests ($p<0.0001$).

In addition to inhibiting T cell proliferation, Tregs are known to supress Th1, Th2 and, to a lesser extent Th17, cytokine production. In order to investigate the impact of Treg-intrinsic NRP1 on the ability of Tregs to affect cytokine production, we stimulated T cells polyclonally, in the presence or absence of Tregs. Secretion of proinflammatory cytokines IL-5 IL-6, IL-13 (and to a lesser extent IL17A and TNFα) by beads-stimulated PBMCs was also suppressed significantly less by NRP1 KD Tregs, than by control Tregs [**Figure 4.4b-f**]. Meanwhile, the concentration of anti-inflammatory IL-10 was elevated in co-cultures of PBMCs with NRP1-sufficient Tregs compared to PBMCs with NRP1-deficient Tregs [**Figure 4.4a**]. These data indicate a functional defect in human Tregs that are deprived of NRP1, suggesting that NRP1 is required for optimal Treg suppressive function.

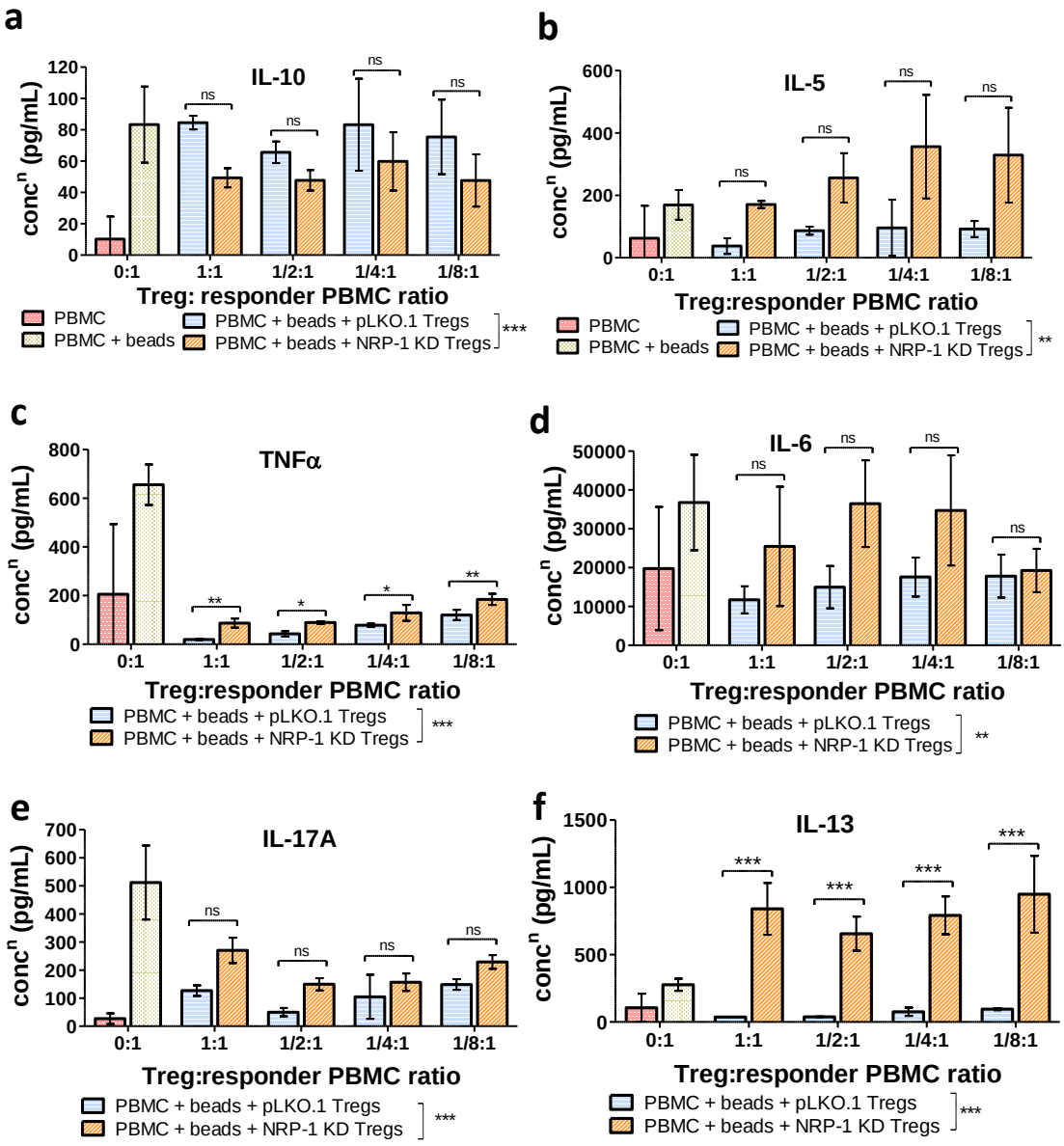
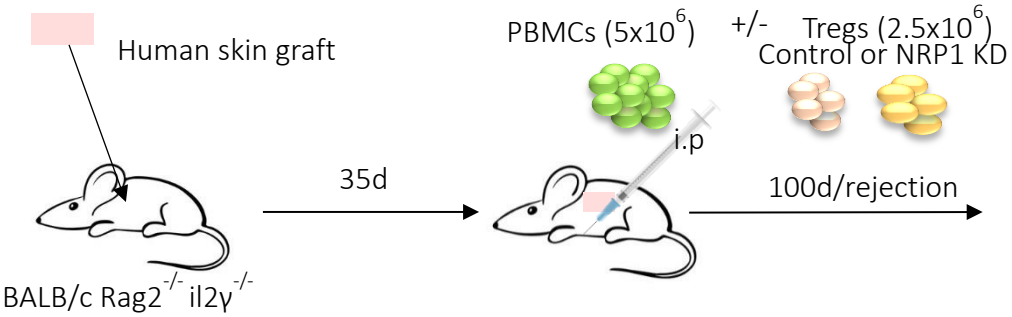


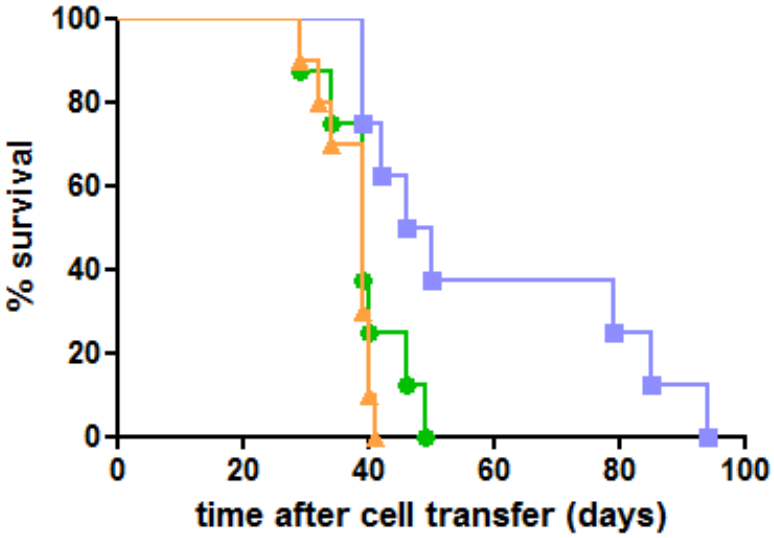
Figure 4.4 Cytokine secretion by PBMCs in the presence of NRP1 KD Tregs *in vitro*

PBMCs stimulated with αCD3αCD28-coated beads were cultured for 72hrs with or without either NRP1 KD or control Tregs. Supernatants from the co-culture assay were subjected to a 13-plex cytokine bead array. Of the 13 cytokines analysed, only those detected at substantial levels in all replicates are presented here. Each chart depicts representative data from one of two cell donors tested. Bars indicate mean +/- SD of at least 3 replicates. Statistical significance was determined by two-way ANOVA with Bonferroni post-tests.: (a) $p=0.0002$; (b) $p=0.0019$; (c) $p<0.0001$; (d) $p=0.0083$; (e) $p=0.0001$; (f) $p<0.0001$.

4.3.3 NRP1-deficiency impairs Treg-mediated suppression *in vivo*

In order to determine whether the functional dependence of human Tregs upon NRP1 is biologically significant *in vivo*, we assessed the ability of NRP1 KD Tregs to suppress immunological rejection of an allograft in a humanised mouse model of skin transplantation. Immunodeficient mice grafted with human skin and reconstituted with allogeneic human PBMCs rejected their grafts with a median survival time of 39days. Graft survival time was significantly prolonged if control Tregs were administered concurrently with the PBMCs. However, treatment with NRP1 KD Tregs from the same cell donor failed to prolong graft survival [**Figure 4.5**]. These data demonstrate that Treg-intrinsic NRP1 is required for Treg-mediated abrogation of allograft rejection *in vivo*.





Treatment	N	Median Survival Time (days)	
PBMC only	7	39	*
PBMC + control vector Tregs	8	48	** ns
PBMC + Nrp-1 KD Tregs	7	39	

Figure 4.5 Rejection kinetics of human skin allografts after adoptive transfer of NRP1 KD Tregs in a humanised mouse model

Split thickness human skin was grafted onto the flank of BALB/c Rag2$^{-/-}$Il2rγ$^{-/-}$ mice. Five weeks post-transplantation, each mouse received an intraperitoneal injection of PBMCs (5x10^6 per mouse) with or without Tregs (2.5x10^6 per mouse). Grafts were monitored for macroscopic signs of immunological rejection for 100 days after the transfer of cells. Percentage of grafts surviving (i.e. not rejected) is plotted over time for each treatment group and median survival time is tabulated below. Statistical significance was determined by a log-rank test: *p=0.038, **p=0.0013.

4.3.4 NRP1-deficiency does not increase susceptibility to apoptosis in human Tregs

One trivial explanation for the poor suppressive function of NRP1-deficient Tregs might be that NRP1-KD cells are inviable, undergoing cell death during the assay and thereby reducing the effective ratio of Tregs: responders. The lower cell yields that we observed after expansion of Tregs transduced with NRP1 shRNA, compared to cells transduced with a control vector, suggests that there may be some deficit in cell viability following silencing of NRP1 [**Figure 4.6**].

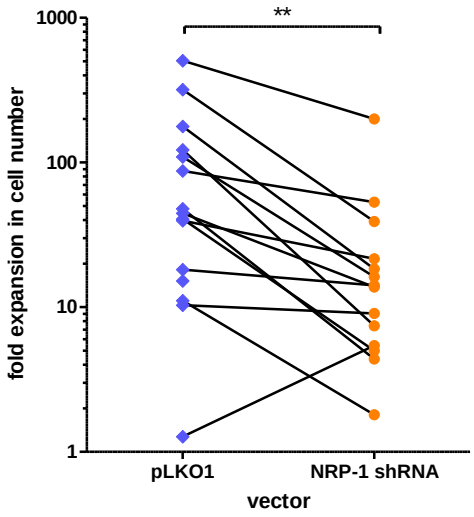


Figure 4.6 Cell yields from expansion of NRP1 KD Tregs

$CD4^{+}CD25^{+}CD127^{lo}$ Tregs transduced with lentivirus containing plasmids encoding either *NRP1* shRNA or a control vector (pLKO.1) containing a stuffer insert during a 16-day expansion protocol. The fold change in cell number from day 0 to day 16 (after selection for transduced cells with puromycin) is plotted for each donor, with lines adjoining cell cultures derived from the same donor. Statistical significance was determined by a Wilcoxon Matched-Pairs Sign Rank test, p=0.0003.

However, in a flow cytometric assay, the frequency of Tregs staining positive for cell viability dyes 7AAD and Annexin V was comparable between cultures of stimulated NRP1-deficent Tregs and NRP1-sufficient Tregs [Figure 4.7a-b]. Furthermore, the relative abundance of transcripts encoding anti-apoptotic proteins BCL-2 [Figure 4.7 c], BCL-XL [Figure 4.7d], MCL-1 [Figure 4.7e] and pro-apoptotic BIM [Figure 4.7f] was not significantly different between NRP1-deficient and control Tregs. These data suggest that after expansion, and within the time scale of our *in vitro* functional assays, at least, NRP1 silencing does not impair Treg survival. Therefore, we concluded that the functional defect of NRP1-deficient Tregs could not be attributed to greater susceptibility to apoptosis in these modified cells.

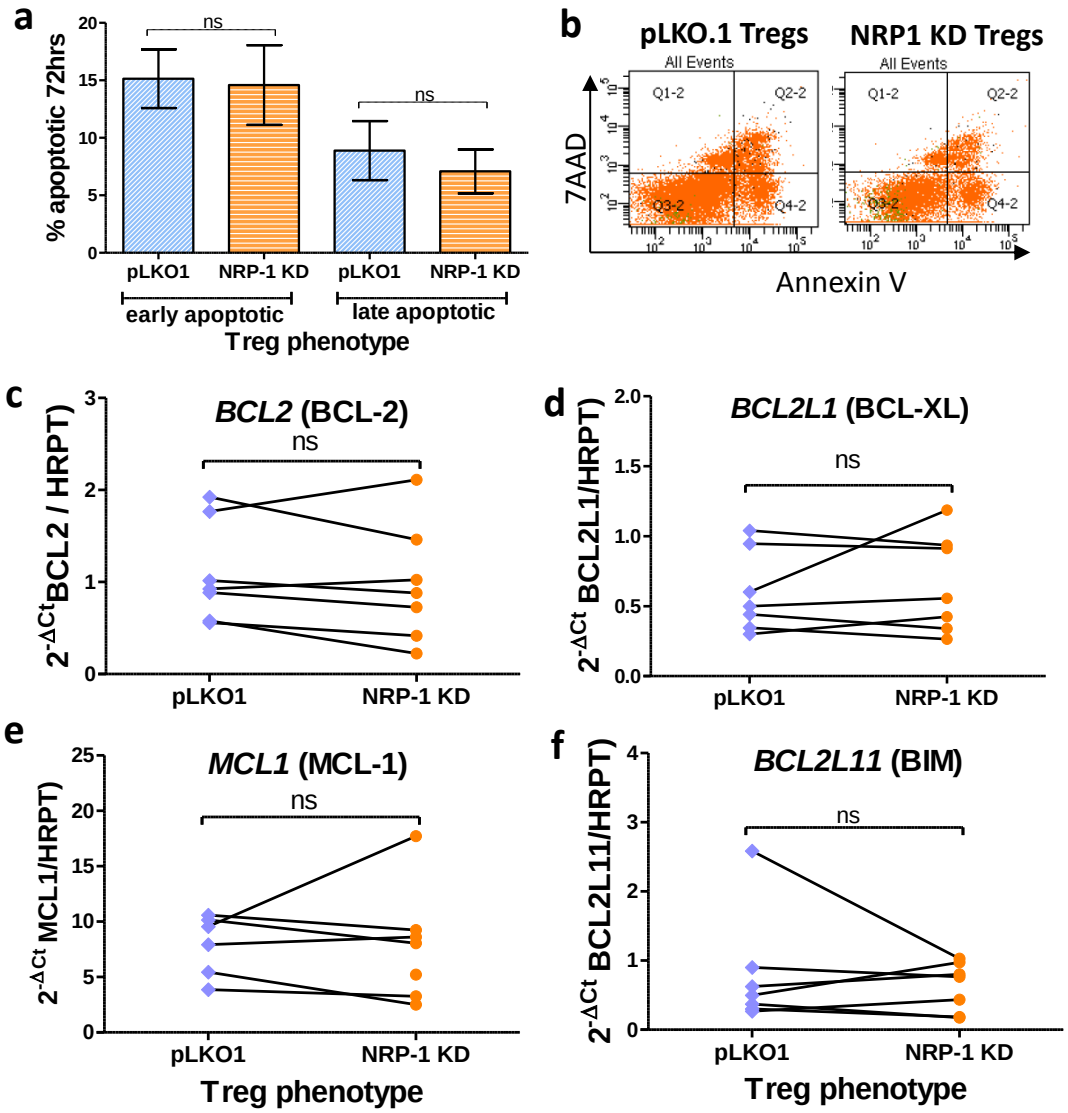


Figure 4.7 Viability of NRP1 KD Tregs *in vitro*

(a) NRP1 KD and control Tregs stimulated for 3 days with αCD3αCD28-coated beads (1bead :5cells) and IL-2 (200U/mL) were stained using cell viability dyes 7AAD and AnnexinV staining solution, which were was quantified by flow cytometry. Bars represent means +/- SEM for data derived from 5 donors, with 3 replicates per donor. FACS plots of 7AAD versus AnnexinV are shown for (left) control and (right) NRP1 KD Tregs from the same donor. (c-f) qRT-PCR was performed on cryopreserved cell pellets of NRP1 KD and control Tregs, using Taqman assays for anti-apoptotic factors(c) *BCL2*, (b) *BCL2L1* (BCL2-XL) and (c) *MCL1* and pro-apoptotic factor (d) *BCL2L11* (BIM). The mean of two replicates from each cell donor is plotted as a separate point, with lines joining data from the same cell donor. Statistical significance was assessed using Wilcoxon signed rank tests: (a) p=0.8703, p=0.6250; (c) p=0.2188 ; (d) p=0.9375 ; (e) p=0.6875 ; f) p=0.3125.

4.3.5 Proliferation and activation are not impaired in NRP1 deficient human Tregs *in vitro*

To illuminate more subtle changes in Treg viability, stimulated Tregs were also examined by flow cytometry for expression of activation markers and dilution of cell proliferation dyes. After three days' stimulation *in* vitro, dilution of a cell viability dye was not diminished in NRP1-deficient Tregs relative to control Tregs [Figure 4.8f], indicating that the proliferative potential of these cells is not reduced. The cell surface density of one Treg activation marker, ICOS (Inducible T Cell COStimulator), was marginally but significantly diminished at the protein level [Figure 4.8c], but not the mRNA level [Figure 4.8e], in NRP1-deficient Tregs, without a significant reduction in the proportion of ICOS-expressing cells [Figure 4.8d].

However, expression of another T cell activation marker, CD69, was significantly elevated in NRP1-deficient Tregs, compared with control Tregs, with respect to per-cell density and frequency of positive cells [Figure 4.8a-b]. We speculate that the reduction in ICOS expression may reflect a specific influence of NRP1 upon induction of ICOS protein expression, rather than a deficit in global activation. Therefore, we concluded that neither the proliferative nor the activation potential of Tregs is dependent upon NRP1 expression.

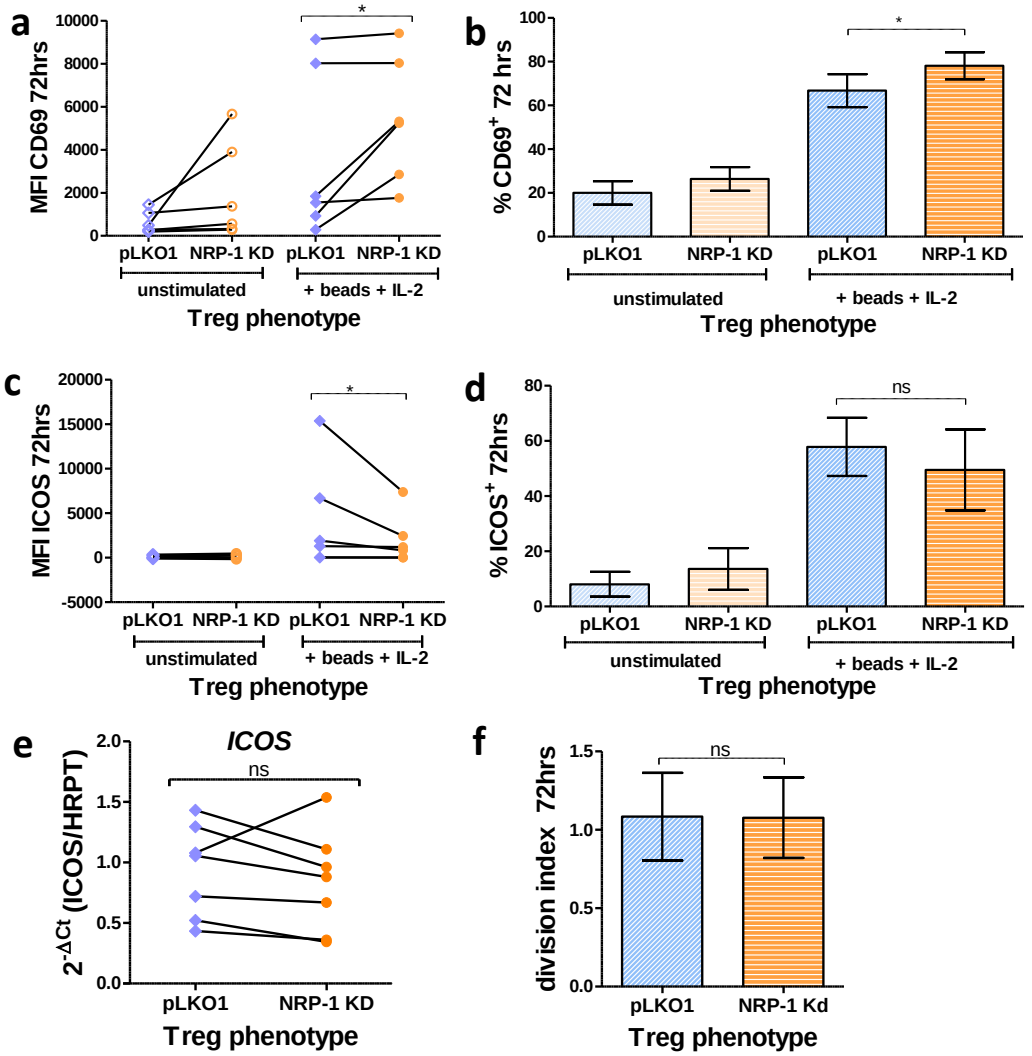


Figure 4.8 Activation and proliferation potential of NRP1 KD Tregs

NRP1 KD and control Tregs were stained with a proliferation dye and stimulated for 72hrs with αCD3αCD28 beads and IL-2 before staining cells with antibodies against Treg activation markers ICOS and CD69. Density (a, c) and frequency (b, d) of protein expression were quantified by flow cytometry. (e) Relative transcription of ICOS was measured by qRT-PCR using Taqman assays for *ICOS* (and *HPRT* as an endogenous control). (f) Division index was calculated from dilution of the proliferation dye, quantified by flow cytometry. Bars depict mean +/- SEM for 7 cell donors. Statistical significance was determined by Wilcoxon signed rank tests: (a) p=0.0313; (b) p=0.0355; (c) p=0.0313; (d) p=0.2188;(e) p=0.2969 ; (f) p=0.0625.

4.3.6 Intercellular interactions between human Tregs and Dendritic Cells are not dependent upon NRP1

In mice, intercellular adhesion mediated by homotypic interactions between Nrp1 expressed on mouse T cells and on dendritic cells was identified as an early event in immune synapse formation and subsequent DC-induced T cell proliferation [285]. To assess whether human Treg: DC interactions are dependent upon NRP1, we performed live cell imaging to quantify the frequency and duration of intercellular interactions between Tregs and monocyte-derived DCs (moDCs)[342], in the presence and absence of anti-NRP1 blocking antibody.

In contrast to the results published from an analogous experiment performed on mouse cells[286], blocking NRP1 had no effect upon the frequency or duration of contacts made between Tregs and DCs [**Figure 4.9**]. This result suggests that the contribution of NRP1 to human Treg function cannot be attributed to the adhesive properties of NRP1 mediating interaction between Tregs and DCs. Rather, it seems probable that NRP1 contributes to human Treg function via Treg-intrinsic NRP1-dependent intracellular signalling.

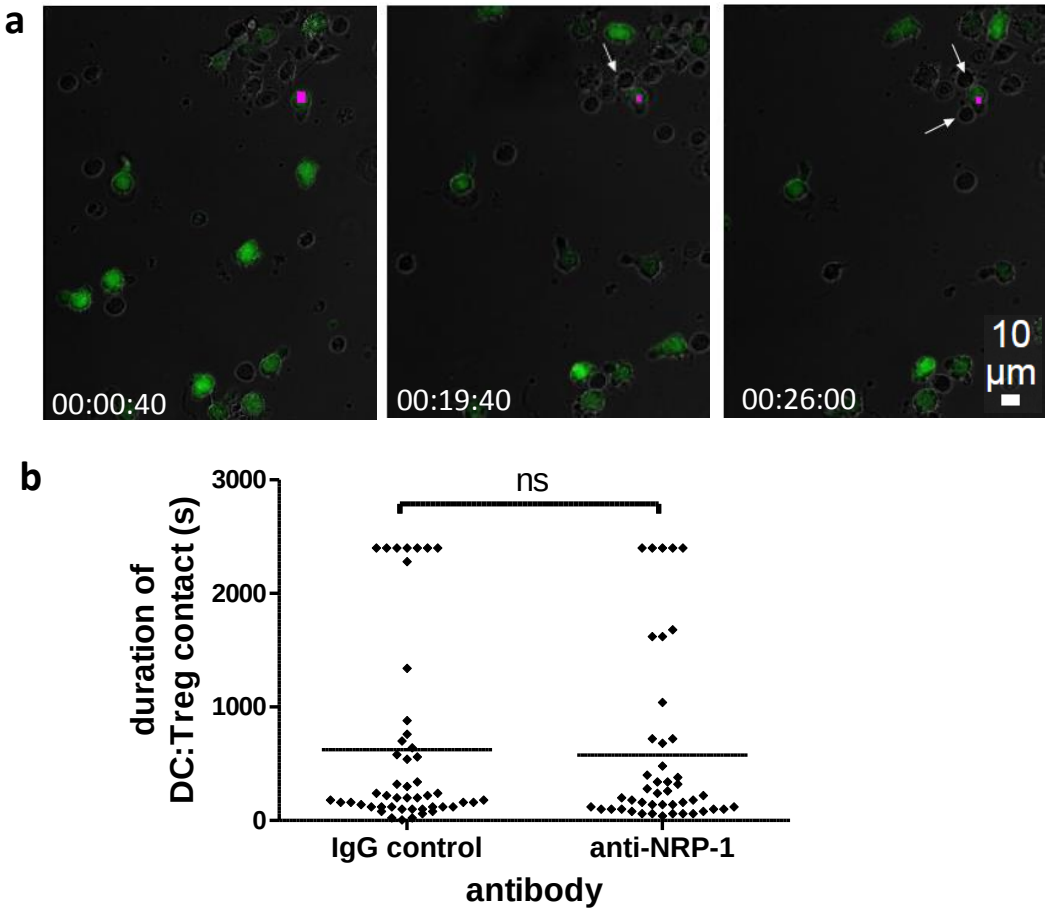


Figure 4.9 Effect of blocking NRP1 upon Treg:DC contact duration

Expanded human Tregs ($2x10^4$ per chamber) were incubated with CFSE-stained autologous moDCs ($2x10^4$ per chamber) in an 8-well microscopy chamber, in the presence of either anti-NRP1 blocking antibody or sheep IgG control antibody (10µg/mL). After 2hrs incubation at 37°C, 5%CO_2, live cell cultures were imaged by fluorescence microscopy. Images were acquired in the FITC and polarised light channels, every 20s for 40mins, at two positions for each culture. (a) representative frames from time-lapse video. One DC of interest is marked with a magenta square; Tregs in contact with the DC of interest are identified by white arrows. (b) The duration of physical contact between Tregs and DCs was measured manually for 13 DCs per condition. At least 45 intercellular contacts were measured for each position. Each Treg:DC contact is plotted individually, with bars indicating mean contact duration across all DCs. Statistical significance was calculated using a Student T-test (p=0.7661).

4.3.7 NRP1 expression is required to maintain the transcription of IL-10, but not TGFβ or CD39, by human Tregs

The ability of Tregs to suppress effector immune cells has been attributed to a plethora of molecular mechanisms, relying upon several immunomodulatory molecules. Among these, the anti-inflammatory cytokines IL-10 and TGFβ are believed to be critical to contact-independent Treg-mediated suppression[245]. The ectonuclease CD39 has also been implicated in the functionality of Tregs, by depleting extracellular ATP upon which effector cells depend for their function[242]. The functional deficit of NRP1-deficient Tregs might be explained if the expression of one of these molecules were found to be NRP1-dependent.

By qRT-PCR, we detected a significantly lower level of *IL10* mRNA expressed by NRP1 KD Tregs, relative to control Treg [Figure 4.10a]. No difference was detected between NRP1-deficient and control Tregs in the levels of transcript encoding the Treg-associated anti-inflammatory cytokine TGFβ (*TGFB1*) [Figure 4.10b]. Surprisingly, transcription of the ectonuclease CD39 (*ENTPD1*) was actually significantly elevated in NRP1 KD Tregs [Figure 4.10c]. The fact that NRP1 KD Tregs exhibit defective suppression, in spite of the presumed advantage conferred by higher ectonuclease expression, suggests that other Treg suppressive mechanisms must be impaired by loss of NRP1. Thus, we conclude that transcription of genes encoding IL-10, but not TGFβ or CD39, is dependent upon NRP1 in Tregs. Transcriptional regulation of NRP1-dependent transcriptional regulation of IL-10 could contribute to Treg suppressive function, although this does not exclude the possibility of NRP1-depenent post-transcriptional regulation.

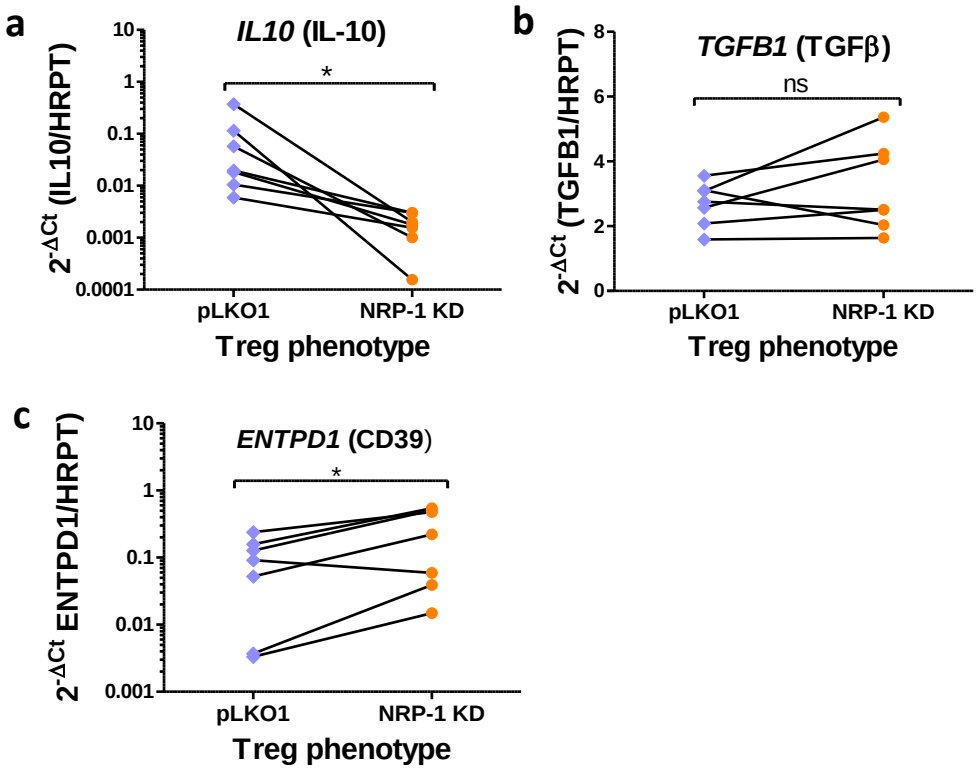


Figure 4.10 Expression of Treg immunomodulatory molecules by NRP1 KD Tregs
qRT-PCR was performed on cryopreserved cell pellets of NRP1 KD and control Tregs, using Taqman assays for immunomodulatory molecules *IL10* (encoding IL-10), *TGFB1* (encoding TGFβ) and *ENTPD1* (encoding CD39). Data from each cell donor is plotted as a separate point, with lines joining data from the same cell donor. Statistical significance was determined by Wilcoxon signed rank tests: (a) p=0.0156; (b) p=0.2696; (c) 0.0469.

4.3.8 The expression of Th- and Treg-associated transcription factors by human Tregs is not determined by the expression of NRP1

$CD4^+$ T lymphocytes are classically categorised into subsets according to their differential repertoires of ligands and cytokines, determined by expression of different transcription factors. In T helper 1 cells, for example, expression of IFNγ and CXCR3 is prescribed by the transcription factor T-BET. Meanwhile, the Th2 subset is defined by expression of the transcription factor GATA-3 and the Th17

subset by expression of RORγt. Expression of any Th-associated transcription factors in Tregs, which are defined by expression of FOXP3, can be regarded as an indicator of phenotypic instability, whereby Tregs acquire a more pro-inflammatory phenotype. No reduction in expression of FOXP3 mRNA [**Figure 4.11e**] or protein [**Figure 4.11g-h**] was observed in NRP1 KD Tregs. Although there was a discernible negative correlation between the expression of NRP1 protein and FOXP3 protein, this trend was not statistically significant [**Figure 4.11f**]. Counterintuitively, NRP1 KD Tregs transcribed significantly higher levels of IKZF4 mRNA (encoding the Treg-associated transcription factor EOS[343,344]) than control Tregs [**Figure 4.11**]. The mRNA levels Th-associated transcription factors, *GATA3* [**Figure 4.11b**] and *RORC* (encoding RORγt) [**Figure 4.11c**] were not significantly upregulated in NRP1 KD Tregs. Moreover, *TBX21* (encoding T-BET) [**Figure 4.11a**] was actually transcribed at lower levels in NRP1 KD Tregs than in control Tregs. Therefore, we concluded that, under homeostatic conditions, NRP1 deprivation does not compromise phenotypic stability of Tregs with respect to transcription factor expression. Nonetheless, the critical test of phenotypic stability is to ascertain whether the Tregs have the capacity to promote inflammation by producing pro-inflammatory mediators. Hence, we examined the secretion of cytokines by NRP1-deficient Tregs.

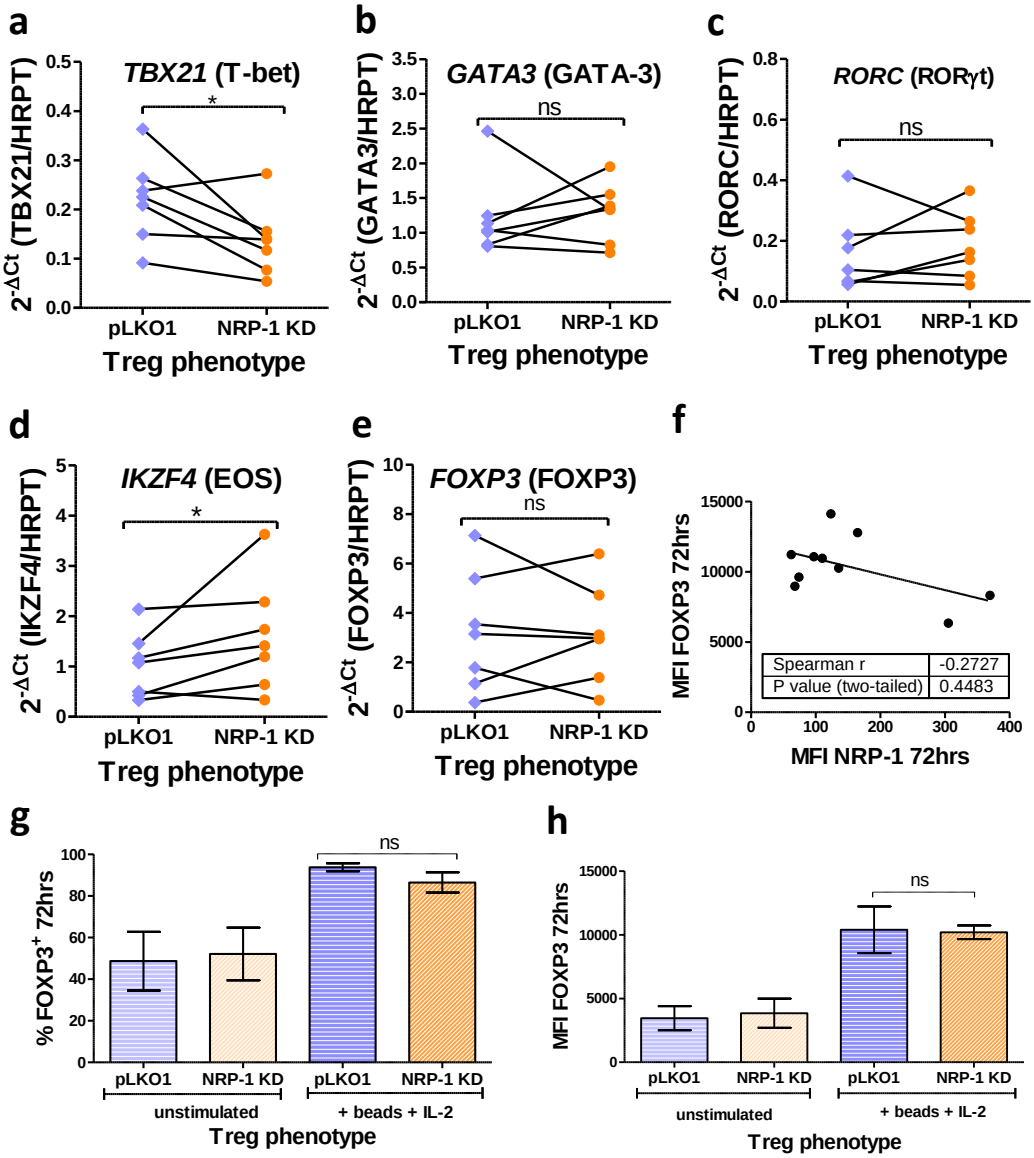


Figure 4.11 Expression of Treg- and Th-associated transcription factors by NRP1 KD Tregs

(a-e) qRT-PCR was performed on NRP1 KD and control Tregs, to quantify transcripts encoding (a) Th1-associated T-BET (*TBX21*), (b) Th2-associated GATA3 (*GATA3),* (c) Th17-associated RORγC (*RORC*), Treg transcription factors (d) EOS (*IKZF4*) and (e) FOXP3 (*FOXP3*). Data from each cell donor are plotted as a separate point, with lines joining data from the same donor. (f-h) Expression of FOXP3 protein was quantified by flow cytometry in NRP1 KD and control Tregs after 72hrs stimulation with αCD3αCD28-coated (1 bead: 5 cells) and IL-2 (200U/mL). (e) Density (MFI) of FOXP3 protein expression is plotted against density of NRP1 expression in the same samples. (g) Frequency and (h) density of FOXP3 protein expression are plotted as mean +/- SEM of 5 cell donors. Statistical were analysed by matched-pairs Wilcoxon Matched-Pairs Sign Rank tests: (a) p=0.0469; (b) p=0.5781; (c) p=0.5781; (d) p=0.469; (e) p=0.9375; (g) p=0.3125; (h) p=1.000.

4.3.9 NRP1 is not critically required to prevent pro-inflammatory cytokine secretion by human Tregs *in vitro*

Using a cytometric bead assay, the concentrations of thirteen T cell-derived cytokines were quantified in the supernatants of stimulated Treg cultures. In all four donors tested, the concentration of IL-10, an anti-inflammatory cytokine implicated in the mechanisms of Treg-mediated suppression, was consistently but not significantly diminished in cultures of NRP1-deficient Tregs, compared with control Tregs [**Figure 4.12g**]. This trend is corroborated by data from a qRT-PCR assay, which demonstrate that transcription of IL-10 is significantly diminished in NRP1 KD Tregs, relative to control Tregs [**Figure 4.10a**]. The concentration of none of the pro-inflammatory cytokines tested (IL-6, IL-5, IL-13, TNFα, IFNγ, or IL-17A) was significantly elevated in NRP1 KD Tregs [**Figure 4.12a-f** respectively]. Together, these results hint that the NRP1 expression by Tregs may influence anti-inflammatory cytokine production but, under steady-state conditions, is not critically required for the maintenance of phenotypic stability of Tregs with respect to cytokine secretion.

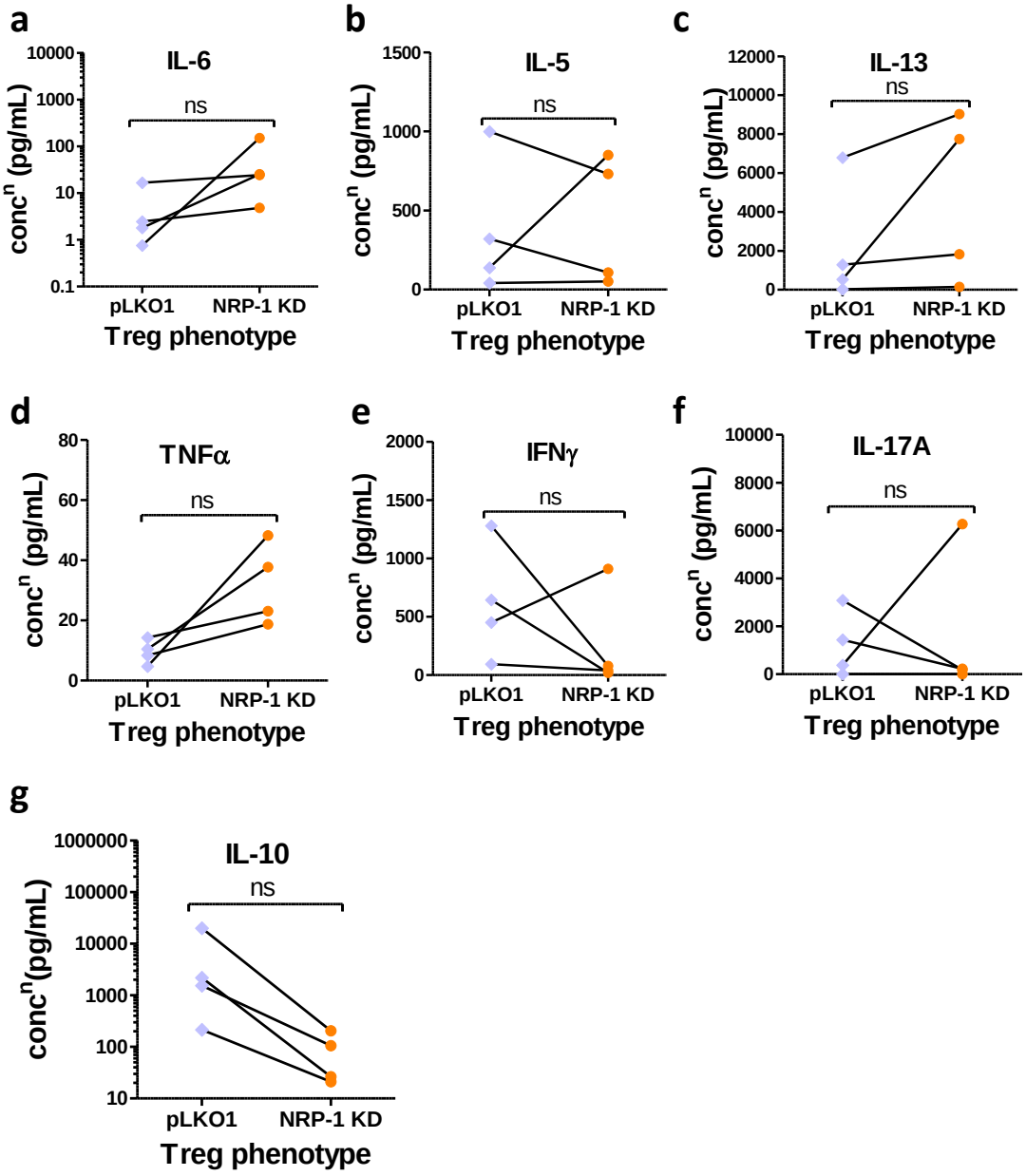


Figure 4.12 Cytokine secretion by NRP1 KD Tregs

A cytokine bead array assay was performed on supernatants from cultures of NRP1 KD or control Tregs stimulated for 72hrs with αCD3αCD28-coated (1 bead: 5 cells) and IL-2 (200U/mL). Concentration of cytokine, computed from median fluorescence intensities, is plotted for each cell donor, with lines joining data from the same cell donor. (a) IL-6, (b) IL-5, (c) IL-13, (d) TNFα, (e) IFNγ, (f) IL-17A, (g) IL-10. Statistical significance was assessed by matched-pairs Wilcoxon signed ranks tests: (a) p=0.1250; (b) p=1.000; (c) p=0.1250; (d) p=0.1250; (e) p=0.3750; (f) p=1.000; (g) p=0.1250.

4.3.10 Global gene expression in Tregs is modified by NRP1 expression

Finally, we sought to identify NRP1-dependent transcriptional signatures in human Tregs and Teffs by performing a microarray-based genome-wide transcriptional analysis on these cells. The microarray revealed significantly differential expression between unstimulated NRP1 KD and control Tregs at 205 loci. The majority of differentially expressed genes were down-regulated in NRP1-deficient cells, relative to control cells, in both unstimulated and stimulated Treg, as well as in Teffs [**Figure 4.13**]. Interestingly, a large proportion of differentially expressed loci were unique to Tregs, with a distinct set of loci identified in the comparison of NRP1 KD and control Teffs [**Figure 4.14**]. This suggests that Tregs and Teffs may utilise NRP1 to regulate different biochemical processes, perhaps contributing to the disparities in cell biology between these two populations.

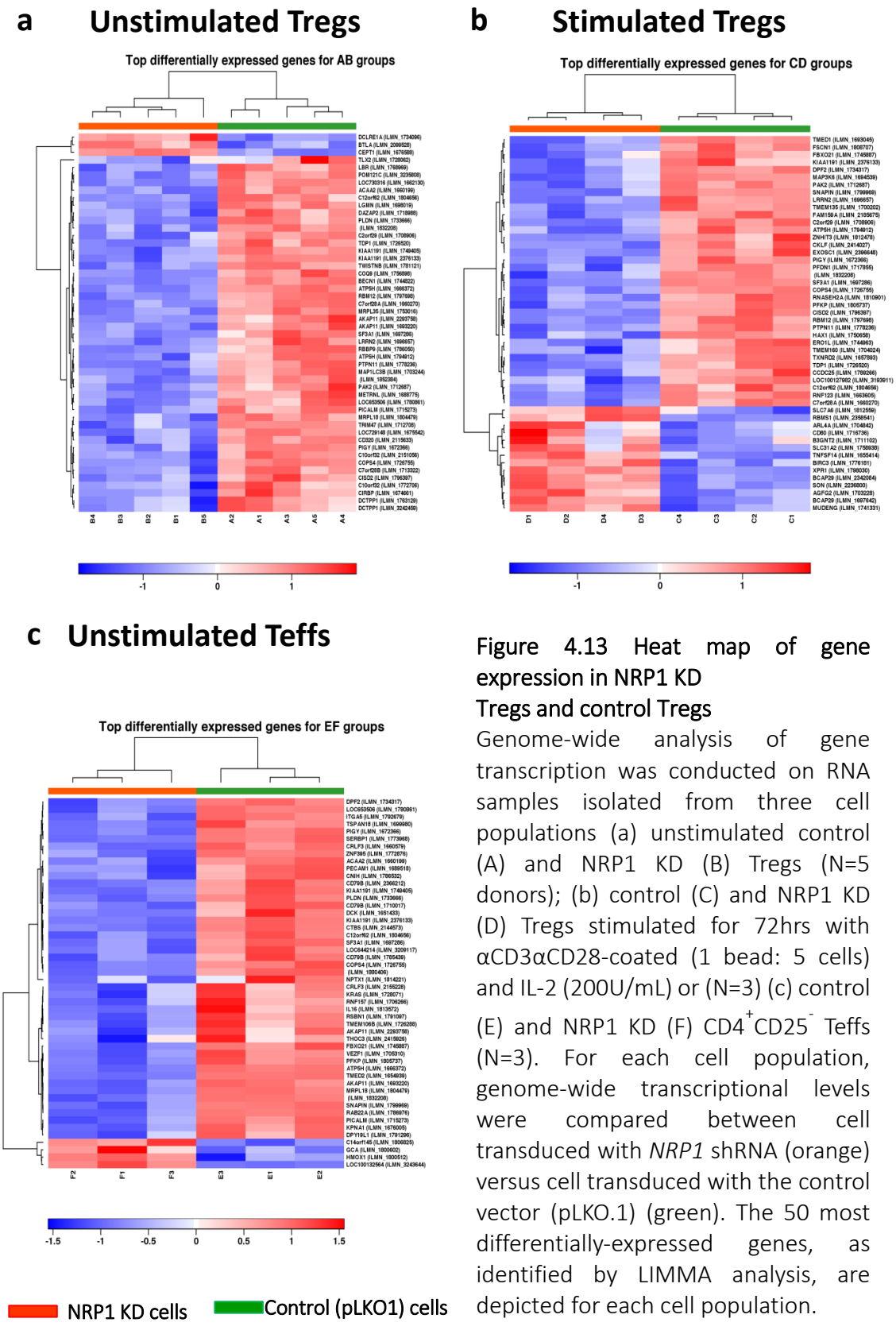


Figure 4.13 Heat map of gene expression in NRP1 KD Tregs and control Tregs

Genome-wide analysis of gene transcription was conducted on RNA samples isolated from three cell populations (a) unstimulated control (A) and NRP1 KD (B) Tregs (N=5 donors); (b) control (C) and NRP1 KD (D) Tregs stimulated for 72hrs with αCD3αCD28-coated (1 bead: 5 cells) and IL-2 (200U/mL) or (N=3) (c) control (E) and NRP1 KD (F) $CD4^{+}CD25^{-}$ Teffs (N=3). For each cell population, genome-wide transcriptional levels were compared between cell transduced with *NRP1* shRNA (orange) versus cell transduced with the control vector (pLKO.1) (green). The 50 most differentially-expressed genes, as identified by LIMMA analysis, are depicted for each cell population.

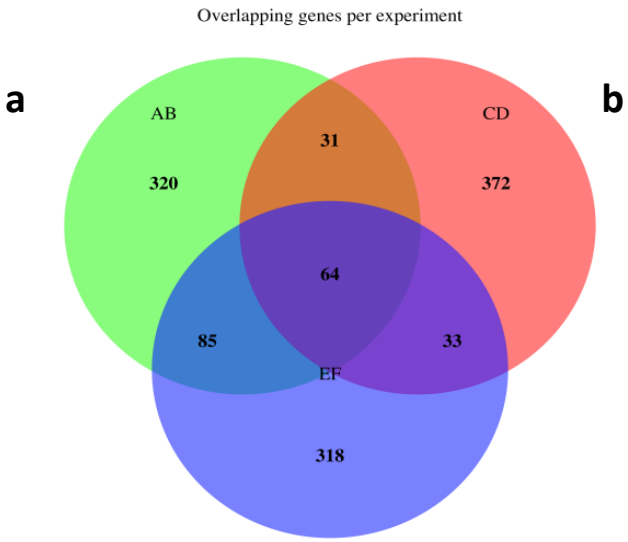


Figure 4.14 Distribution of genes differentially expressed between NRP1 KD and control cells in different cell populations

Genome-wide analysis of gene transcription was conducted on RNA samples isolated from three cell populations (a) control (A) and NRP1 KD (B) unstimulated Tregs (N=5); (b) control (C) versus NRP1 KD (D) Tregs stimulated for 72hrs with αCD3αCD28-coated (1 bead: 5 cells) and IL-2 (200U/mL) (N=3); or (c) control (E) versus NRP1 KD (F) $CD4^{+}CD25^{-}$ Teffs (N=3). For each cell population, transcriptional profiles were compared between cells transduced with NRP1 shRNA versus cells transduced with the control vector (pLKO.1). The Venn diagram illustrates proportions of genes, among the 500 genes identified as most differentially expressed between NRP1 KD and control cells, that are common between cell populations

In an unbiased manner, we selected the five genes (ATP5H, BECN1, COPS4, MAP1ILC3B and PTP11) for which the transcriptional level was most significantly disparate between NRP1 KD and control Tregs for validation and further analysis. ATP5H encodes a subunit of the mitochondrial ATP synthase[345]. *BECN1* encodes beclin-1, a component of the phosphatidylinositol-3 kinase (PI3K) complex that regulates autophagy and apoptosis[346,347]. COPS4 is a subunit of the COP9 (COnstitutive Photomorphogenesis 9) Signalosome (CSN), a protein complex that

regulates multiple signalling pathways as a protease component of the ubiquitin-proteosome pathway[348]. The CSN has also been implicated in autophagic protein degradation[349,350]. *MAP1ILC3B* (Microtubule-Associated Protein 1 Light Chain 3 Beta) encodes LC3B, a microtubule-associated protein that participates in autophagolysosome biogenesis and recruitment of substrates for autophagocytic degradation[351]. Tyrosine-protein phosphatase non-receptor type 11 (PTPN11), also called SRC homology protein 2 (SHP-2), encoded by *PTPN11*, transduces signals via diverse growth factor-, cytokine- and hormone-induced signalling pathways. SHP-2-dependent signalling pathways regulate various cellular processes, including cell cycle and metabolism[352]. qRT-PCR confirmed that all five genes were transcribed at significantly lower levels in NRP1 KD Tregs than in control Tregs [Figure 4.15]. Intriguingly, three out of these five genes (BECN1, COPS4 and MAP1ILC3B) are key components of the cellular machinery regulating autophagy. It is reasonable to speculate, therefore, that NRP1 regulates autophagy in human Tregs. A further inference, that Treg function is influenced by NRP1-dependent autophagy pathways, is particularly plausible considering recently-published findings that Tregs are more reliant upon autophagy than other T cells[353,354355,356].

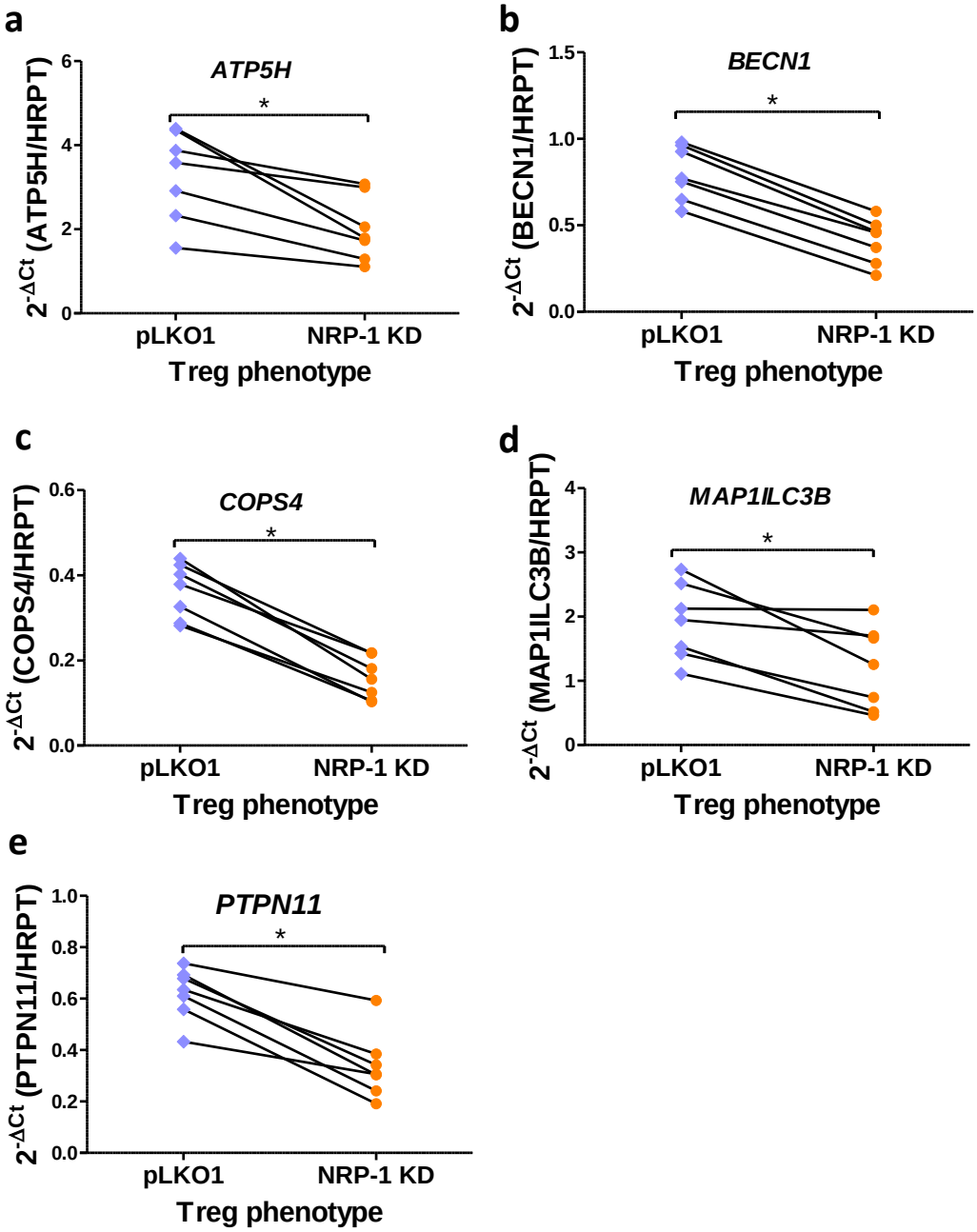


Figure 4.15 Validation of genes identified as differentially expressed between NRP1 KD Tregs and control Tregs in microarray

cDNA synthesised from total cellular RNA of NRP1 KD and control Tregs was subjected to qRT-PCR with Taqman assays for the following transcripts: (a) *ATP5H*, (b) *BECN1*, (c) *COPS4*, (d) *MAP1LC3B* and (e) *PTPN11*. Mean values from two replicates are plotted for each of 7 donors, with lines adjoining samples from the same donor. Statistical significance was determined by a Wilcoxon Signed Ranks tests: (a-e) p=0.156.

4.3.11 Not all NRP1-dependent cellular processes are restricted to Tregs

In order to determine whether the effects of NRP1 deficiency that we observed in Tregs are Treg-specific or general to $CD4^+$ T cells, we repeated certain *in vitro* assays on NRP1 KD $CD4^+CD25^-$ Teffs [**Figure 4.16**]. The proliferation of Teffs in response to TCR stimulation was comparable between NRP1 KD Teffs and control Teffs [**Figure 4.17**]. Nor did we observe a significant reduction in the level of FOXP3 protein [**Figure 4.19a**] or the frequency of $FOXP3^+$ cells [**Figure 4.19b**] among NRP1 KD Teffs, compared to control Tregs. In common with Tregs, neither the expression of CD69 [**Figure 4.18a,c**] nor CD25 [**Figure 4.18b,d**] upon TCR stimulation was diminished in NRP1 KD Teffs, compared with control Teffs. However, it is notable that there was a significant, strong positive correlation between NRP1 protein expression and the MFI of CD69 [**Figure 4.18e**] and CD25 [**Figure 4.12f**]. This correlation suggests that, contrary to our observations in Tregs, NRP1 is associated with activation status in Teffs.

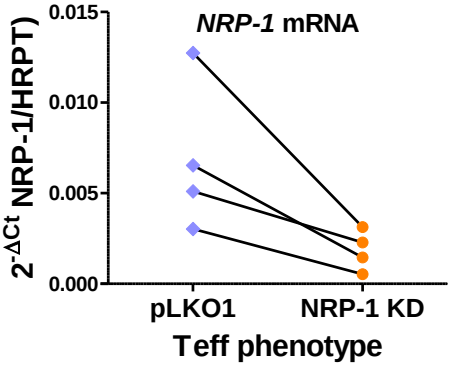
Interestingly, the transcription of the autophagy genes *BECN1* [**Figure 4.20b**], COPS4 [**Figure 4.20c**] and *MAP1LC3B* [**Figure 4.20d**], identified as significantly downregulated in NRP1 KD Tregs in the microarray, was reduced, though not significantly, in NRP1 KD Teffs relative to control Teffs. Two of the other transcripts that were most significantly downregulated in NRP1 KD Tregs, *ATP5H* [**Figure 4.20a**] and *PTPN11* [**Figure 4.20e**], were also expressed at not significantly lower levels in NRP1 KD Teffs relative to control Teffs. These results suggest that, whilst at a global level, the transcriptional targets of NRP1 differ between Tregs

and Teffs, these two cell subsets may share in common certain NRP1-dependent pathways.

a

	$2^{-\Delta Ct}$ (*NRP-1/HPRT*)		% knockdown of mRNA expression
B245	0.0051	0.0022	56.9
B273	0.0065	0.0015	76.9
B318	0.013	0.0031	76.2
B319	0.0030	0.00053	83.3

b



c

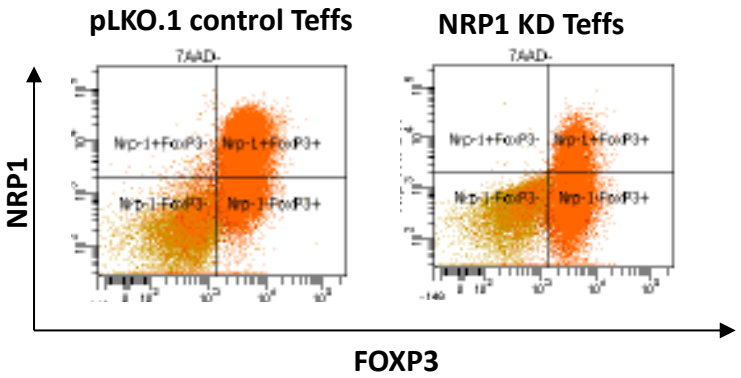


Figure 4.16 Knockdown Efficiency in human $CD4^{+}CD25^{-}$ Teffs transduced with NRP1 shRNA

$CD4^{+}CD25^{-}$ Teffs transduced with lentivirus containing plasmids encoding either NRP1 shRNA or a control vector containing a stuffer insert. Transduced Teffs were stimulated for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (100U/mL) before quantifying cell surface expression of NRP1 by flow cytometry. (a) qRT-PCR data for *NRP1* mRNA expression of Teffs are tabulated by cell donor and (b) plotted for each donor separately, with lines adjoining cells from the same donor. (c) FACS plots from one representative donor show cell surface NRP1 protein expression for (left) control and (right) NRP1 KD Tregs.

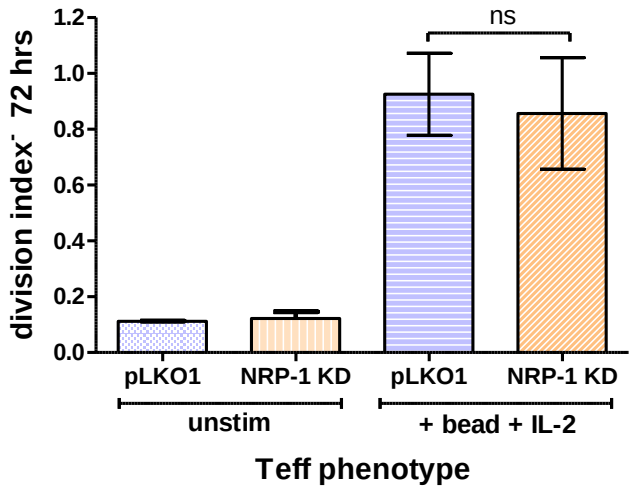
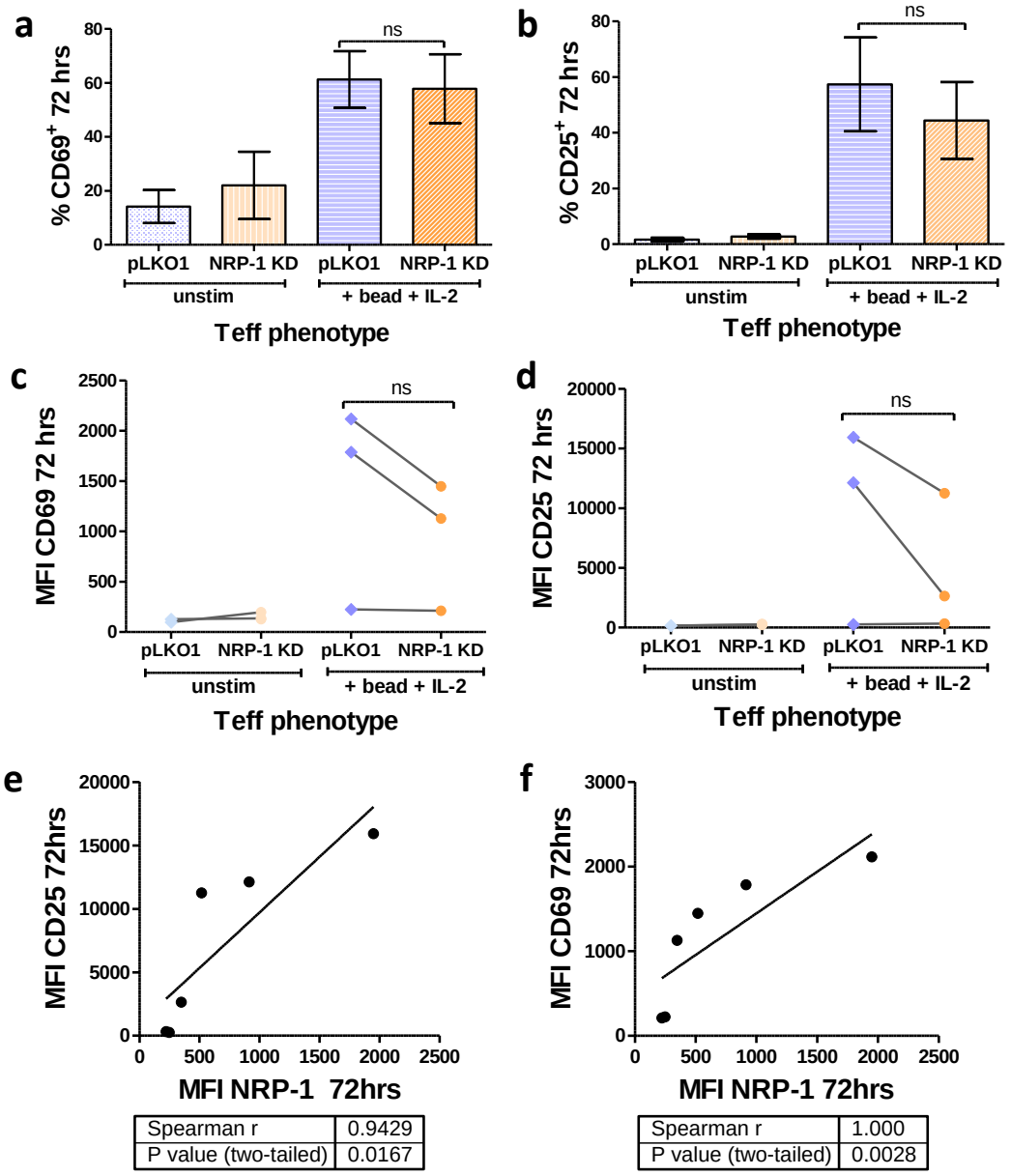


Figure 4.17 Proliferative potential of NRP1 Kd Teffs *in vitro*

NRP1 KD and control $CD4^{+}CD25^{-}$ Teffs were stained with cell proliferation dye and stimulated for 72 hours with beads (1 bead: 5 cells) and IL-2 (100U/mL). A division index was calculated from dilution of the proliferation dye, quantified by flow cytometry. Bars depict mean +/- SEM for 5 cell donors. Statistical significance was assessed using Wilcoxon Matched-Pairs Signed Rank tests: p=0.6250

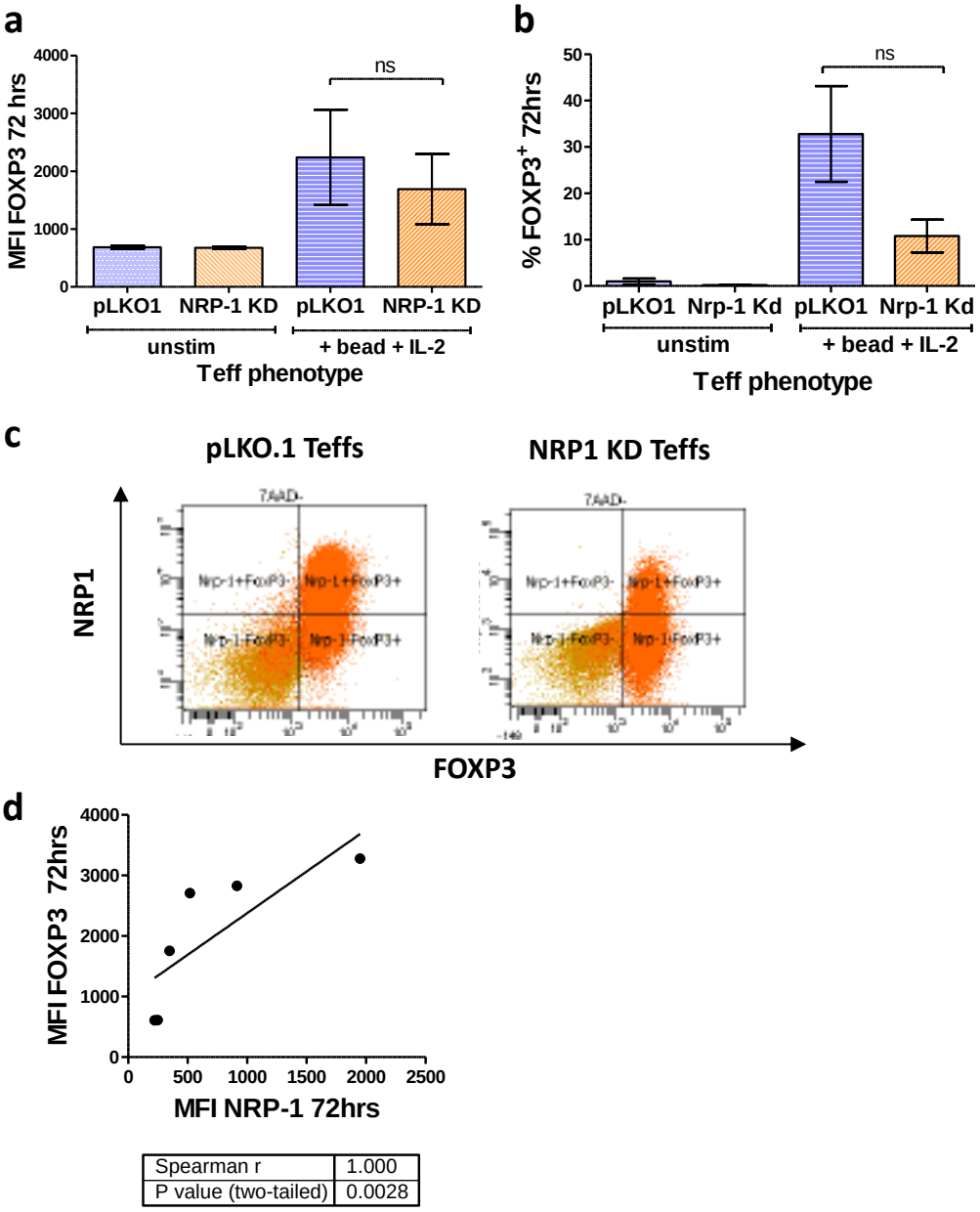


Spearman r	0.9429
P value (two-tailed)	0.0167

Spearman r	1.000
P value (two-tailed)	0.0028

Figure 4.18 Activation potential of NRP1 KD Teffs

NRP1 KD and control $CD4^+CD25^-$ Teffs were stimulated for 72hrs with αCD3αCD28-coated (1 bead: 5 cells) and IL-2 (100U/mL) before staining cells with antibodies against activation markers ICOS and CD69. (a, b) Frequency and (c, d) density of protein expression were quantified by flow cytometry. Bars depict mean and SEM for 7 cell donors. MFIs for each cell donor are plotted as separate points, with line joining data from the same donor. Statistical significance was determined by Wilcoxon signed rank tests: (a) p=0.3125 ; b) p=0.2500 ; (c) p=0.2500 ; (d) p=0.5000 . (e, f) Regression plots of CD69 and CD25 MFI against NRP1 MFI for all cells samples (control and NRP1 KD Teffs), with correlation assessed by Spearman's Rho tests.



Spearman r	1.000
P value (two-tailed)	0.0028

Figure 4.19 Expression of FOXP3 by NRP1 KD Teffs

(a) Density of FOXP3 protein expression and (b) frequency of $FOXP3^+$ cells in NRP1 KD and control $CD4^+CD25^-$ Teffs was quantified by flow cytometry. Means +/- SEM are depicted for 3 cell donors. Statistics were determined by Wilcoxon Matched-Pairs Signed Rank tests: (a) p= 0.2500; (b) p=0.2500. (c) FACS plots of FOXP3 against NRP1 are shown for (left) control and (right) NRP1 KD Tregs from the same donor. (d) MFI of FOXP3 protein is plotted against MFI of NRP1 in the same samples, with correlation analysis performed using Spearman's Rho test.

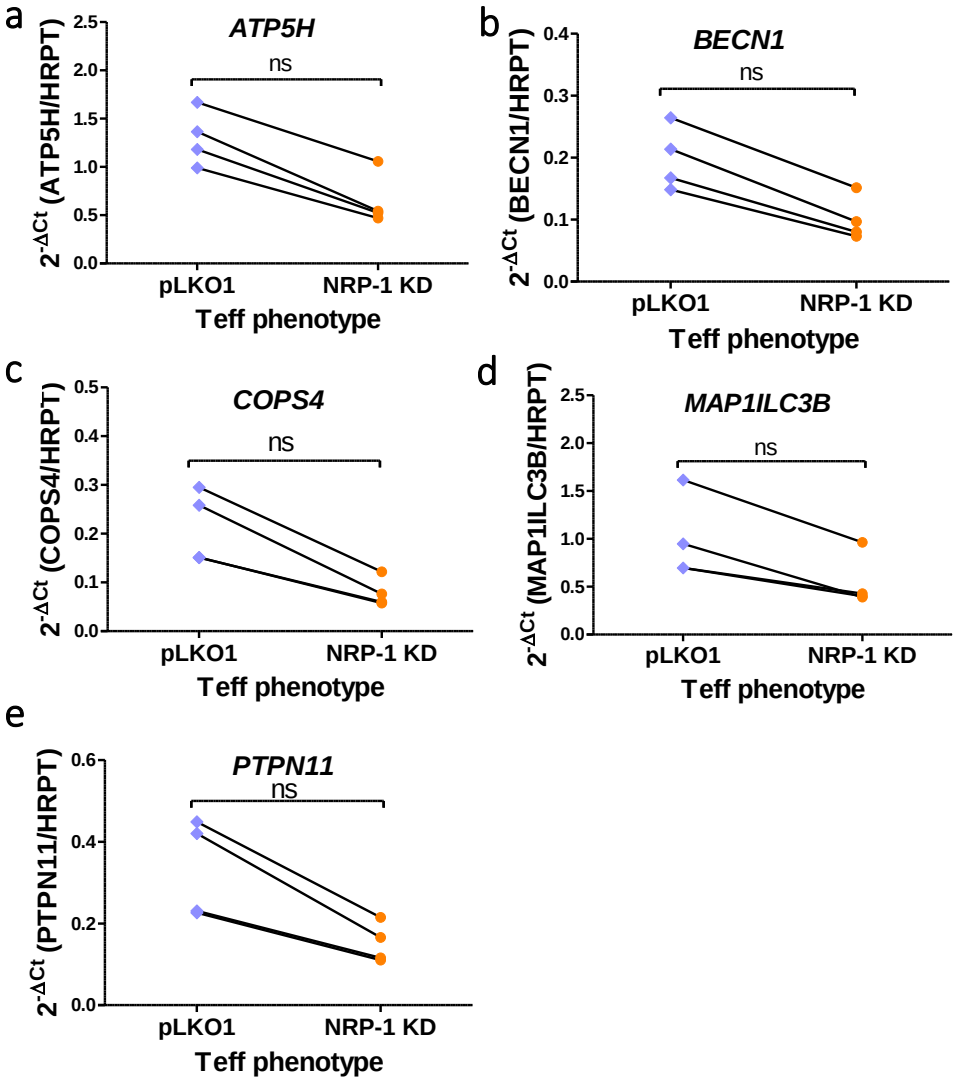


Figure 4.20 Expression by NRP1 KD Teffs of genes identified as differentially expressed between NRP1 KD and control Tregs

cDNA synthesised from total cellular RNA of NRP1 KD and control $CD4^{+}CD25^{-}$ Teffs was subjected to qRT-PCR with Taqman assays for the following transcripts: (a) *ATP5H*, (b) *BECN1*, (c) *COPS4*, (d) *MAP1LC3B* and (e) *PTPN11*. Each cell sample is plotted as a separate point, with lines adjoining samples from the same donor. Statistical significance was determined by a Wilcoxon Matched-Pairs Signed Rank test: (a-e) p=0.1250.

4.3.12 NRP1 expression is not sufficient to confer substantial suppressive function upon $CD4^+$ Teffs

The experiments outlined so far confirm that NRP1 is necessary for Treg-mediated suppression. In order to assess whether NRP1 is sufficient for Treg-mediated suppression, we transduced non-Treg $CD4^+CD25^-$ Teffs with a construct that drives expression of NRP1 and GFP, or with a control vector encoding GFP alone. At the end of the transduction procedure, we performed an *in vitro* suppression assay to measure the proliferation and expression of activation markers by PBMCs in the presence of NRP1 over-expressing Teffs. NRP1 over-expressing Teffs did not suppress PBMC proliferation significantly more than control Teffs [Figure 4.21a]. However, expression of CD25 [Figure 4.21b] and CD69 [Figure 4.21c] was suppressed to a significantly greater extend in the presence of NRP1-overexpressing Teffs, compared with control Teffs. These results suggest that the expression of NRP1 by Teffs does confer some immunoregulatory capacity upon these cells but is insufficient to confer the full suppressive capacity of Tregs.

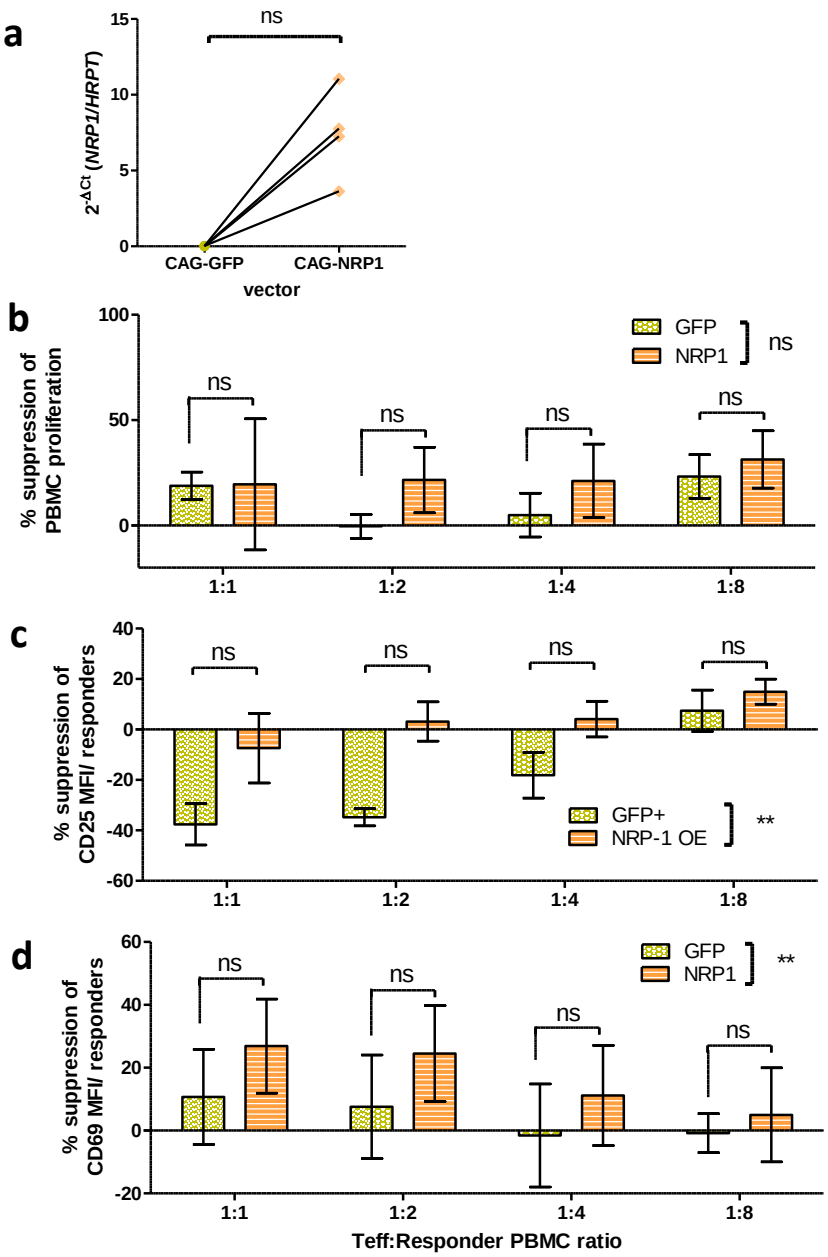


Figure 4.21 PBMC proliferation and activation in the presence of NRP1 over-expressing Teffs

(a) Tregs transduced with lentivirus encoding either *NRP1* and *EGFP* or a control vector encoding *EGFP*. Transduced Tregs were stimulated for 72hrs with beads and IL-2. Transcription of NRP-1, quantified by qRT-PCR, is plotted for each donor. P=0.125 (Wilcoxon matched pairs signed rank). (b-d) VPD-stained PBMCs (10^5 cells per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for 4 days in the presence or absence of $CD4^+CD25^-$ Teffs, transduced with either control lentiviral vector, CAG-GFP, or CAG-NRP1. Dilution of proliferation dye and median fluorescence intensity for CD25 and CD69 were measured by flow cytometry. Percentage suppression was calculated relative to DC-stimulated PBMC in the absence of Teffs. Bars indicate mean +/- SEM for three cell donors. Statistics are derived from a two-way ANOVA with Bonferroni post-tests: (b) p=0.3363; (c) p=0.0033; (d) p=0.0084

4.4 Discussion

Numerous studies have implicated Nrp1 in Treg function in mouse models[291,339,341,357] but far less has been reported relating to NRP1 in human lymphocytes. In mouse experiments, a genome-wide microarray identified Nrp1 as a transcript highly enriched in Tregs versus Teffs [288]. In the same study, Nrp1 expression was found to be constitutive in Tregs, not Teffs, and correlated with suppressive function of those Tregs. In contrast to the mouse data, and consistent with a previous report [292], we observed that the expression level of NRP1 protein was low among resting human peripheral blood Tregs, comparable to that of Teffs. Hence, we conclude that NRP1 is not a useful marker of Treg phenotype in humans, as it is in mice. NRP1 protein expression by Tregs increased marginally and transiently in response to *in vitro* stimulation [**Figure 4.1**] indicating that NRP1 expression by Tregs is not a stable phenotype delineating distinct $NRP1^-$ and $NRP1^+$ populations. Rather, we deduce that elevated NRP1 expression is a marker of activation in human $CD4^+$ T lymphocytes. Notably, expanded human Tregs expressed a higher density of NRP1 than freshly-isolated Tregs [**Figure 4.1b**], likely reflecting the higher global activation status of Tregs that have been exposed to long-term stimulation.

The minimal expression of NRP1 by human Tregs might suggest that NRP1 does not contribute substantially to the function of human Tregs. Rather than comparing Tregs sorted for high versus low levels of NRP1, which would not represent historical or future expression levels, we elected to manipulate NRP1 expression levels genetically. Introducing into primary human Tregs a shRNA

against the *NRP1* transcript successfully reduced NRP1 protein levels by a mean of 54.9% [**Figure 4.1**] Given the marginal expression of NRP1 in freshly-isolated Tregs, the impaired suppression mediated by NRP1 KD Tregs of T cell proliferation [**Figure 4.2**] and the suppression of pro-inflammatory cytokine secretion by PBMCs [**Figure 4.3**] was unexpected. Therefore, we validated these results *in vivo*, observing that adoptively transferred NRP1KD Tregs failed to prolong survival of the allograft in a humanised mouse model of skin allograft rejection, whilst graft survival was extended significantly in mice receiving the same number of control Tregs [**Figure 4.5**] Therefore, we concluded that Treg-intrinsic NRP1 is necessary for Treg-mediated suppression of allograft rejection *in vivo*.

Whilst NRP1 was necessary for Treg-mediated suppression, our experiments using NRP1-overexpressing Teffs demonstrated that NRP1 is not sufficient to recapitulate the suppressive capacity of Tregs [**Figure 4.21**]. Given the plethora of suppressive mechanisms employed by Tregs, it is unsurprising that expression of a single immunomodulatory molecule could not recapitulate the suppressive phenotype of Tregs. In fact, one might expect that, if NRP1 is required for generic T cell fitness, that NRP1-overexpressing Teffs would exhibit a more robust pro-inflammatory phenotype. The observation that activation marker expression on PBMCs is reduced in the presence of NRP1-overexpressing Teffs supports the theory that NRP1 is an inherently immunoregulatory molecule, rather than simply conferring fitness upon T cells that bear this molecule.

Since the mechanisms by which NRP1 influences Tregs in mice are varied and incompletely understood, we sought to understand more about the nature of

NRP1 activity in human Tregs. First eliminating more trivial explanations for the diminished function of NRP1-deficient Tregs, we demonstrated that the cell viability of NRP1-KD Tregs was not compromised, since cell survival and proliferation of NRP1 KD Tregs were equal to NRP1-sufficient Tregs. Therefore, it seemed plausible that NRP1 performed a more direct role in Treg function. Within the immune system, and in T lymphocytes specifically, NRP1 has been implicated in both cell-extrinsic functions (e.g. adhesive intercellular interactions)[285,357] cell-intrinsic functions (modifying cell behaviour, e.g. via regulation of gene expression)[282,291].

Considering the strong dependence of Treg-mediated suppression upon Treg activation, we assessed the effect of NRP1 signalling deprivation upon Treg activation *in vitro,* revealing that activation of Tregs is not globally impaired in Tregs deficient in NRP1 protein. Therefore, we conclude that the dependence of Treg function upon NRP1 cannot be attributed to regulation of Treg activation by NRP1 [**Figure 4.8**].

Given the reports from mouse studies that implicate NRP1 in the immunological synapse formation between T cells and DCs[286], we predicated that intercellular adhesion mediated by NRP1 may account for the functional deficit in NRP1 KD Tregs. However, blocking NRP1 with antibody had no effect upon the average duration of contact between human Tregs and DCs, suggesting that intercellular adhesion is not a dominant mechanism by which NRP1 contributes to the function of human Tregs [**Figure 4.9**]. Instead, it seemed probable that intracellular signalling downstream of NRP1 might have Treg-intrinsic effects that would

promote Treg suppressive function. Hence, we sought to survey cell-intrinsic events that might be modulated by NRP1.

One of the most crucial functional distinctions between Tregs and their Teff counterparts is the anti-inflammatory cytokine profile of Tregs, versus the pro-inflammatory cytokine repertoire of Teffs. Accumulating evidence of phenotypic plasticity among $CD4^+$ T cell subsets[358,359] , including Tregs[360], in recent years has led us to examine whether NRP1 expression in Tregs influences Treg lineage stability. Accordingly, we found that none of the Th-associated transcription factors was upregulated, nor were Treg-associated transcription factors diminished, in NRP-deficient Tregs [**Figure 4.11**]. Nonetheless, we demonstrated that, compared with control Tregs, the cytokine profile of NRP1 KD Tregs was mildly perturbed, with a non-significant trend towards enrichment of certain proinflammatory cytokines but also diminished secretion of other such cytokines. [**Figure 4.12**] The subset of cytokines that were elevated did not suggest polarisation towards a particular Th subset.

Whether the consequences of Treg reprogramming are physiologically adaptive or pathological probably depends upon the context. Some reports suggest that, by co-opting Th transcriptional programs, Tregs become adapted to target the Th subset whose behaviour they are emulating[196]. However, other studies identify a causal association between acquisition of Th-like phenotype in Tregs and exacerbated immune pathologies[198,361]. Whilst not statistically significant, the diminished levels of the anti-inflammatory cytokine IL-10 that we observed in NRP1 KD Treg cultures, in combination with the poorer control of pro-

inflammatory cytokine secretion by PBMCs by NRP1 KD Tregs, could plausibly contribute to a pro-inflammatory milieu that would promote inflammation and impede immune regulation. In our co-culture assays, secretion of Th2-associated cytokines (IL-5, IL-6 and IL-13) by PBMC was particularly poorly regulated by NRP1 KD Tregs [**Figure 4.4b, d, f**], suggesting that control of Th2 responses may depend upon NRP1 expression in Tregs.

The signalling events downstream of NRP1-associated receptors have not been fully characterised. The promiscuous nature of NRP1-receptor-ligand interactions likely generates a complex network of cellular processes that are influenced by NRP1-dependent intracellular signalling. In order to identify transcriptional targets of NRP1-dependent signalling, we compared global gene expression between NRP1 KD and control Tregs and Teffs. A microarray revealed a large number of genes whose transcription is NRP1 dependent in Tregs, and a distinct repertoire of genes whose transcription is NRP1-dependent in Teffs [**Figure 4.13, 4.14**].

Notably, among the five genes that were most significantly differentially expressed between NRP-deficient and control unstimulated Tregs were two, BECN1, COPS4 and MAP1LC3B, whose products are key mediators of autophagy. Autophagy is a catabolic process by which energy is released through the lysosomal degradation of cytosolic components, as an adaptive response to cellular stress and to recycle dysfunctional organelles and macromolecules. MAP1LC3B (LC3) is required for autophagolysosome biogenesis, whilst beclin-1 (encoded by BECN1) constitutes part of the phosphatidylinositol-3-kinase (PI3K)

complex that initiates autophagy[346]. COPS4 is a subunit of the COP9 (COnstitutive Photomorphogenesis 9) Signalosome (CSN), a protein complex that regulates multiple signalling pathways as a protease component of the ubiquitin-proteosome pathway[348]. Four recent publications have emphasised the importance of autophagy as part of a Treg- specific metabolic program that promotes survival and lineage stability of Tregs[353–356].

It is now well-established that utilisation of metabolic pathways differs between Tregs and Teffs[254,362–365]. Tregs rely more upon fatty acid oxidation, whilst glycolysis is favoured by Teffs, and exhibit higher activity of mTORC1 than Teffs (and hence, are more resistant to the cytostatic effects of Rapamycin). The influence of NRP1 upon autophagy might be considered in the context of existing reports of regulation of the PI3K-mTOR-AKT pathway by VEGFR and some semaphorin receptors[366]. Incidentally, an homologous co-receptor, NRP-2, has been reported to promote autophagy in tumour cells, upon ligation of VEGF-C, via negative regulation of mTORC1 activity[367]. We speculate that, via positive regulation of the autophagy pathway, NRP1 may promote metabolic homeostasis in Tregs.

Notably, beclin-1 also contains a BH3 domain that mediates interaction with anti-apoptotic, but not pro-apoptotic, members of the BCL-2 family, BCL-2, BCL-XL and MCL-1. In our analyses, none of these anti-apoptotic proteins was differentially transcribed between NRP1 KD and control Tregs but perhaps these proteins are subject to post-translational regulation via their interaction with beclin-1. Increasing evidence supports the notion that apoptosis and autophagy are

regulated in a mutually antagonistic manner and beclin-1 appears to be placed at a critical point of convergence between these two pathways[347]. Whilst we observed no alteration in susceptibility to apoptosis in NRP1-deficient Tregs, such alterations might be unmasked under nutrient-poor conditions. Integrating these lines of evidence, it is plausible to conjecture that NRP1 promotes the function of regulatory T cells by transducing salient environmental cues to regulate adaptive metabolic programming in these cells. By promoting expression of beclin-1 and thus modulating the activity of mTORC1, NRP1-dependent signalling could influence the balance between autophagy and apoptosis, and between different metabolic pathways, in response to ligands encoding nutritional and immunological cues.

Another function of NRP1 that we did not explore in our experiment, but which is likely to be pertinent to Treg function, is activation of latent TGFβ. Glinka et al demonstrated that this NRP-dependent TGFβ activity promoted Treg function in mice[368].

Characterisation of the differential outcomes of different NRP1-receptor-ligand interactions in Tregs will be valuable in designing methods of modulating Treg function via administration of NRP1 ligands. Several NRP1 binding partners, in particular TGFβ, VEGF-VEGFR[334,335,369], Sema3A- plexin A1[322,370], Sema4A-TIM-2[291,371,372] and Sema4D-CD72[287,328,373] have been shown to modulate lymphocyte and dendritic cell function in mice. In addition, the NRP1-independent effects of NRP1-associated receptors are already the subject of investigations into immune modulation[282,325] and should be considered with respect to Tregs. For example,

the TIM family of receptors has been implicated in the regulation of different Th subsets (Tim-1 in Th2 and Tim-3 in Th1) in mice[327,371]. Possibly NRP1 is one component of a broader network of immune regulatory mechanisms orchestrated by the semaphorin family.

We demonstrated that some effects of NRP1 deficiency in Tregs are recapitulated in Teffs. It is important to consider, if we are to regard NRP1 as a molecular target for therapeutic intervention, that dependency upon NRP1 *per se* may not be unique to Tregs. Strategies that target NRP1 may not affect Tregs and Teffs differentially. Thus, it will be crucial to dissect out the specific NRP1-dependent pathways that are Treg-restricted.

In conclusion, the results presented in this chapter demonstrate that the co-receptor NRP1 is required for the suppressive function of human regulatory T cells. Whilst NRP1 was found to be dispensable for human Treg survival, FOXP3 expression and activation, NRP1-dependent expression of the autophagy proteins LC3B, COPS4 and beclin-1 hints that metabolic regulation by NRP1 promotes optimal Treg cell function. Furthermore, the expression of a IL-10, a key mediator of immune regulation, by Tregs is dependent upon cell-intrinsic NRP1 expression. Tregs have been implicated broadly in the pathology of malignancies and chronic infections and have been exploited as therapeutic agents in immunopathological conditions. Therefore, the finding that NRP1 influences Treg function might inform future clinical strategies. NRP1 might constitute a molecular target via which endogenous Tregs could be modulated *in vivo* or adoptively-transferred Tregs could be manipulated in culture prior to infusion.

Chapter 5: Investigating Strategies to Enhance Human Treg Function during *in vitro* Expansion

5.1 Introduction

The period of *in vitro* expansion required to generate therapeutic doses of Tregs provides an opportunity to manipulate these cells prior to infusion, with the aim of improving their therapeutic potential *in vivo*. Already, it is known that the process of TCR-stimulation that is required to expand Tregs also, incidentally, improves the suppressive potency of expanded cells, compared with freshly-isolated cells[374]. Furthermore, several groups advocate the use of allogeneic stimulation to select for alloreactive Tregs, which confer stronger suppression on a per-cell basis[267]. The *in vitro* expansion procedure might also incorporate treatment with chemical agents to improve the purity of the final cell product or to promote the viability or suppressive function of the Tregs. Rapamycin, for example, is added as a supplement in Treg expansion cultures to favour the survival and proliferation of Tregs relative to Teff contaminants[254].

In vitro culture of Tregs also offers an opportunity for genetic manipulation aimed at enhancing the function or safety profile of the cells. Genetic engineering could be used to confer upon the cells a greater specificity or bespoke functionality. Likewise, safety switches could be introduced to disable cells in order to reverse or down-regulate the immune regulation exerted by the cells[293]. Finally, in

conditions where the patient's own Tregs are functionally defective, a clinician may elect to use cells from an allogeneic or xenogeneic source that have been modified to be less immunogenic[294]. Alternatively, if the defect can be attributed to a particular genetic locus, the function of autologous cells might be restored by gene therapy.

For example, in recent years, there has been much interest in generating T cells with defined antigen specificity by forcing expression of transgenic TCRs or chimaeric antigen receptors (CARs) into these cells. Transgenic TCRs are derived from natural TCRs that have been selected for their affinity for a salient disease-associated antigen. CARs are engineered constructs composed of variable, antigen-binding moieties of one molecule (most commonly the single chain variable fragment of a BCR) fused to the transmembrane domain of another receptor (classically, the CD3-zeta domain)[375,376]. By splicing the antigen-specificity of one receptor onto the intracellular signalling functionality of another, one can create a T cell in which MHC-independent, high affinity recognition of a chosen antigen is coupled to activation of the cell.

Currently, progress in the field of artificial antigen receptor technologies has mostly been directed at Teffs for cancer therapy but several research groups and companies are developing designer Tregs for application in immune-mediated pathologies[198,277,319,377]. Tregs have been engineered to express TCRs with specificity for alloantigen[272,378,379]. Expression of MHC class I-restricted TCRs in Tregs has been explored as a means of allowing these cells to be activated by endogenous antigens presented on the more ubiquitous class I MHC, as well as

MHC class II on professional APCs[171] The advent of genome editing technologies will no doubt expiate development of genetic strategies for enhancing cell therapy products in the future.

Synthetic biology can also be applied to modulate the *in vivo* behaviour of the cells after infusion. For example, transgenic receptors for artificial ligands can be coupled to the intracellular apparatus for lymphocyte activation, in order to place the activity of cell therapy products under the control of the user who administers the stimulus. More sophisticated transgenic antigen receptor designs employ inducible switch mechanisms, responding to a synthetic small molecule ligand, to exert an extra layer of user-controlled regulation upon cell activation[380]. Likewise, transgenic receptors, coupled to endodomains of chemokine receptors that modulate the migration of cells, have been used exert user control over the recruitment of engineered cells to a chosen anatomical site[381]. Other designs incorporate inhibitory receptors to transiently inactivate engineered T cells as a means of reducing on-target off-tumour activity[382]. Even more complex Boolean logic gates, whereby the activity of an engineered T cells is dictated by multiple molecular parameters, are being explored for refining the safety and efficacy of engineered cell therapy products[383–385].

Following our discovery that NRP1 is required for optimal suppressive function in Tregs, we were interested to determine whether NRP1, or its downstream targets, could be targeted to enhance the suppressive function of Tregs during *in vitro* expansion. If Treg suppressive function were found to be dependent upon a particular NRP1-associated ligand, this would provide an amenable molecular

target for enhancing Treg suppressive treatment during *in vitro* culture. In this chapter, we explored two methods of augmenting the activity of NRP1 in Tregs: exposure of Tregs to NRP1 ligands and genetic engineering of Tregs to induce over-expression of NRP1. Additionally, we selected a second target for genetic engineering, IL-10, as a molecule with established immunoregulatory properties[386], whose transcription is NRP1-dependent.

5.2 Objectives and Hypotheses

Objective 1: To explore the feasibility of driving expression of immunomodulatory molecules in human Tregs to enhance their suppressive potency, with particular focus on NRP1 and IL-10.

Hypothesis 1: Human Tregs engineered to overexpress NRP1 will exhibit more potent suppression of *in vitro* cell proliferation than wild-type Tregs.

Hypothesis 2: Human Tregs engineered to overexpress IL-10 will exhibit more potent suppression of *in vitro* cell proliferation than wild-type Tregs.

Hypothesis 3: Human Teffs engineered to overexpress IL-10 will exhibit some suppression of *in vitro* cell proliferation.

5.3 Results

5.3.1 Treg-mediated suppression of PBMC proliferation is not enhanced by increased availability of NRP1-associated ligands SEMA3A, SEMA4a or VEGF

From our results discussed in Chapter 4, we inferred that regulation of a NRP1-dependent transcriptional program was likely the dominant mechanism by which NRP1 potentiates human Treg function. Since NRP1-dependent cell-intrinsic

signalling is presumably initiated by engagement of extracellular ligand, we predicted that exposing Tregs to exogenous NRP1 ligands *in vitro* would enhance the suppressive function of these cells.

In order to test whether Treg function can be enhanced by increasing the availability of NRP1 ligands, we performed an *in vitro* assay for Treg-mediated suppression of PBMC proliferation, in the presence of three NRP1-associated soluble ligands: Vascular Endothelial Growth Factor (VEGF), semaphorin 3A (SEMA3A) and semaphorin 4A (SEMA4A). These ligands were selected on the basis of published evidence implicating these ligands in modulation of immune activity[327,334,369–371]. However, the suppression of PBMC proliferation in the presence of Tregs was not enhanced in the presence of any of these three ligands, at either moderate concentrations (250ng/mL) [**Figure 5.1a**] or high concentrations (1000ng/mL) [**Figure 5.1b**]. Therefore, we conclude that short-term incubation with VEGF, SEMA-3A SEMA4A is insufficient to enhance the suppressive function of human Tregs.

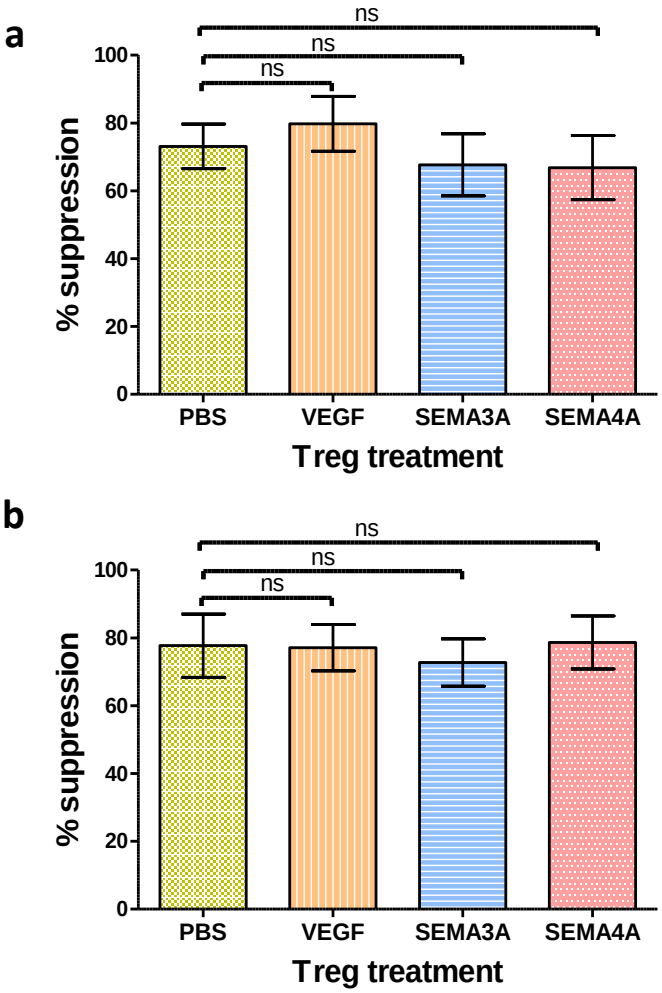


Figure 5.1 Suppression of PBMC proliferation by Tregs pre-incubated with NRP1 ligands *in vitro*

PBMCs (10^5 per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for 6 days in the presence or absence of Tregs (10^5 per well). NRP1 ligands SEMA3A, SEMA4A and VEGF were added at concentrations of (a) 250ng/mL and (b) 1000ng/mL or an equal volume of PBS. Tritiated thymidine was added for the final 16hours and proliferation of responder cells was measured as uptake of tritium (cpm). Percentage suppression was calculated relative to PBMCs with DCs and no Tregs. Bars indicate mean +/- SEM for 3 cell donors. Statistics were derived from Wilcoxon matched pairs signed rank test: (a) p= 0.7500, 0.2500 and 0.2500, for PBS vs VEGF, SEMA3A, SEMA 4A, respectively. (b) p= 1.000, 5.000 and 1.000 for PBS vs VEGF, SEMA3A, SEMA 4A, respectively.

5.3.2 Treg-mediated suppression of cell proliferation is not enhanced by over-expression of NRP1 in Tregs

In order to achieve durable expression NRP1, we transduced Tregs with a vector containing the *NRP1* ORF driven by a strong, constitutive promoter (CAG[1]). After sorting successfully transduced cells, marked by expression of GFP, by FACS, we measured the expression of cell surface NRP1 protein by flow cytometry. mRNA levels of NRP1 were elevated in cells transduced with the CAG-NRP1 construct. However, it must be noted that the increase in protein expression in CAG-NRP1-transduced Tregs was modest, ranging from 11.6-21.3% in the donors that were used for subsequent assay [Figure 5.2], despite successful transduction indicated by expression of GFP from the same promoter. Notably, also, the expression of NRP1 in resting Tregs was not altered significantly by introduction of the transgene.

[1] CAG is a gene expression construct composed of the cytomegalovirus (CMV) early enhancer element; a promoter comprising the first exon and the first intron of the chicken beta-actin gene; a splice acceptor derived from the rabbit beta-globin gene.

a

	NRP1 mRNA (2^{-dCT} (IL10/HPRT))		% increase in NRP-1 mRNA	MFI NRP1 72hrs		% increase in NRP-1 protein (cell surface MFI)
	CAG-GFP	CAG-NRP-1-GFP		CAG-GFP	CAG-NRP-1-GFP	
B209	0.008	7.255	90587.5	2110.0	2429.3	15.1
B293	0.037	3.625	9697.3	2847.0	3453.0	21.3
B347	0.022	7.765	35195.5	3136.7	2861.0	-8.8
B348	0.016	11.058	69012.5	2648.7	2956.0	11.6

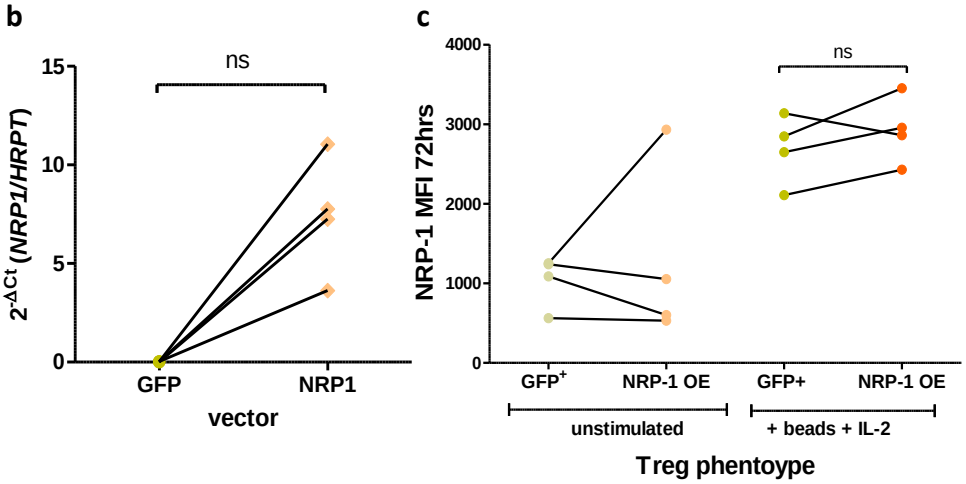


Figure 5.2 Over-expression efficiency in human Tregs transduced with CAG – driven GFP and NRP1

$CD4^{+}CD25^{+}CD127^{lo}$ Tregs transduced with lentivirus-containing plasmids encoding either CAG-GFP or CAG-NRP1-GFP. (a) NRP1 expression in NRP1-overexpressing and control Tregs (mRNA quantified by RT-PCR and protein quantified by flow cytometry) of Tregs are tabulated by cell donor. (b) qRT-PCR was performed to quantify *NRP1* mRNA in NRP1-overexpressin and control Tregs. (c) Transduced Tregs were stimulated for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL) before median fluorescence intensity of NRP1 was quantified by flow cytometry. Statistical analysis was performed using Wilcoxon Matched-Pairs Signed Ranks tests: (b) p=0.2500; (c) p=0.2500.

Nonetheless, we would predict that a moderate increase in NRP1 expression could confer a detectable enhancement of suppressive function in these Tregs. However, in an *in vitro* assay, suppression of PBMC proliferation in the presence

of NRP1 OE Tregs was comparable to that observed in the presence of control Tregs [**Figure 5.3**]. Therefore, we conclude that increased NRP1 expression is insufficient to improve the suppressive potency of Tregs.

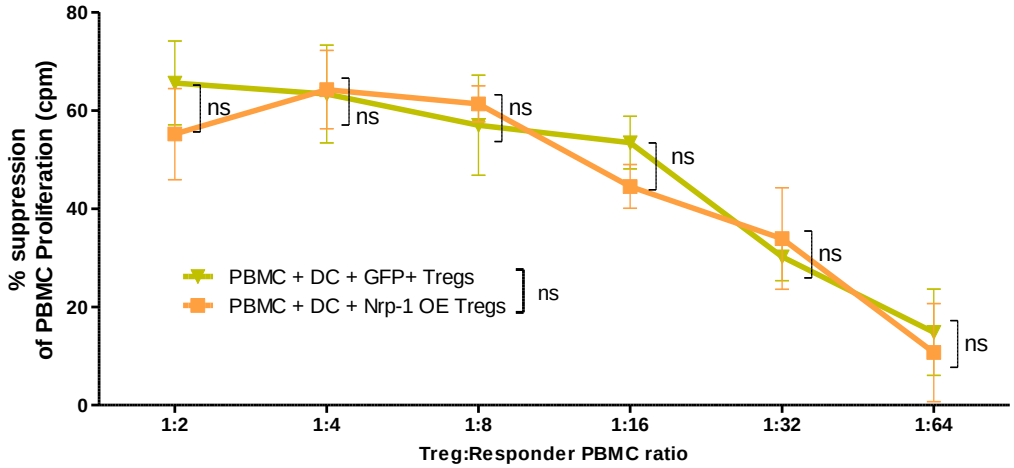


Figure 5.3 Suppression of PBMC proliferation by NRP1-overexpressing Tregs *in vitro*

PBMCs (10^5 cells per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for 6 days in the presence or absence of Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-NRP1-GFP. Tritiated thymidine was added for the final 16hours of co-culture and proliferation of responder cells was measured as uptake of tritium (cpm). Percentage suppression was calculated relative to PBMCs with DCs and no Tregs. Bars indicate mean and SEM of three assays using different cell donors, with at least 6 replicates. Statistics were derived from a two-way ANOVA with Bonferroni post-tests: (p=0.4833).

5.3.3 Tregs over-expressing NRP1 exhibit altered cytokine secretion

Although overexpression of NRP1 in Tregs failed to promote suppression *per se*, other alterations in the biology of these cells resulting from enhanced NRP1 expression might be favourable to cell therapy applications. Following TCR-stimulation, no difference was observed in the proliferation, measured as percentage of cells staining positive for Ki67, in NRP1 OE Tregs, compared to control Tregs [**Figure 5.4a**]. Likewise, the expression of activation markers CD25 and CD69 was comparable between NRP1 OE and control Tregs [**Figure5.4b-c**].

These results indicate that the proliferative capacity and activation potential of Tregs is not altered by enforced expression of NRP1.

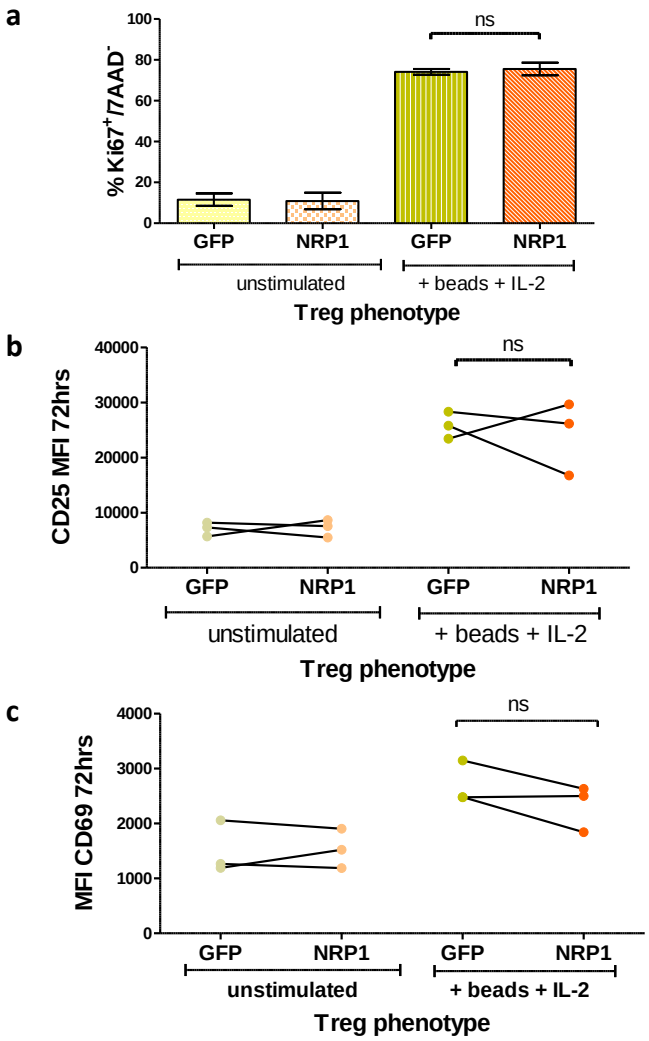


Figure 5.4 Proliferation and activation of NRP1-over-expressing Tregs
Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-NRP1-GFP, were cultured for 72hrs in the presence αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). Expression of (a) the proliferation marker Ki67 (b) activation marker CD25 and (d) activation marker CD69 was quantified by flow cytometry. Mean values of three replicates are plotted for each donor. Statistical analyses were performed using Wilcoxon matched-pairs signed ranks tests: (a) p=0.500; (b) p=0.7500; (c) p=0.5000.

The level of transcription of genes associated with Treg phenotype, *FOXP3*, *IKZF4* (EOS) *IL10*, *TGFB1* (TGFβ) and *ENTPD1* (CD39), was not significantly different between NRP1 OE Tregs and control Tregs [Figure 5.5a-e] Likewise, no significant difference was measured in the quantity of Th-associated mRNAs *TBX21* (T-bet), *GATA3* and *RORC* (RORγt) [Figure 5.5f-h]. *FOXP3* [Figure 5.5j] and *IL-10* [Figure 5.5k] proteins were also expressed at comparable levels in NRP1 OE and control Tregs. Together, these results suggest that the Treg-associated transcriptional prolife of Tregs remains broadly unaltered by forced expression of NRP1.

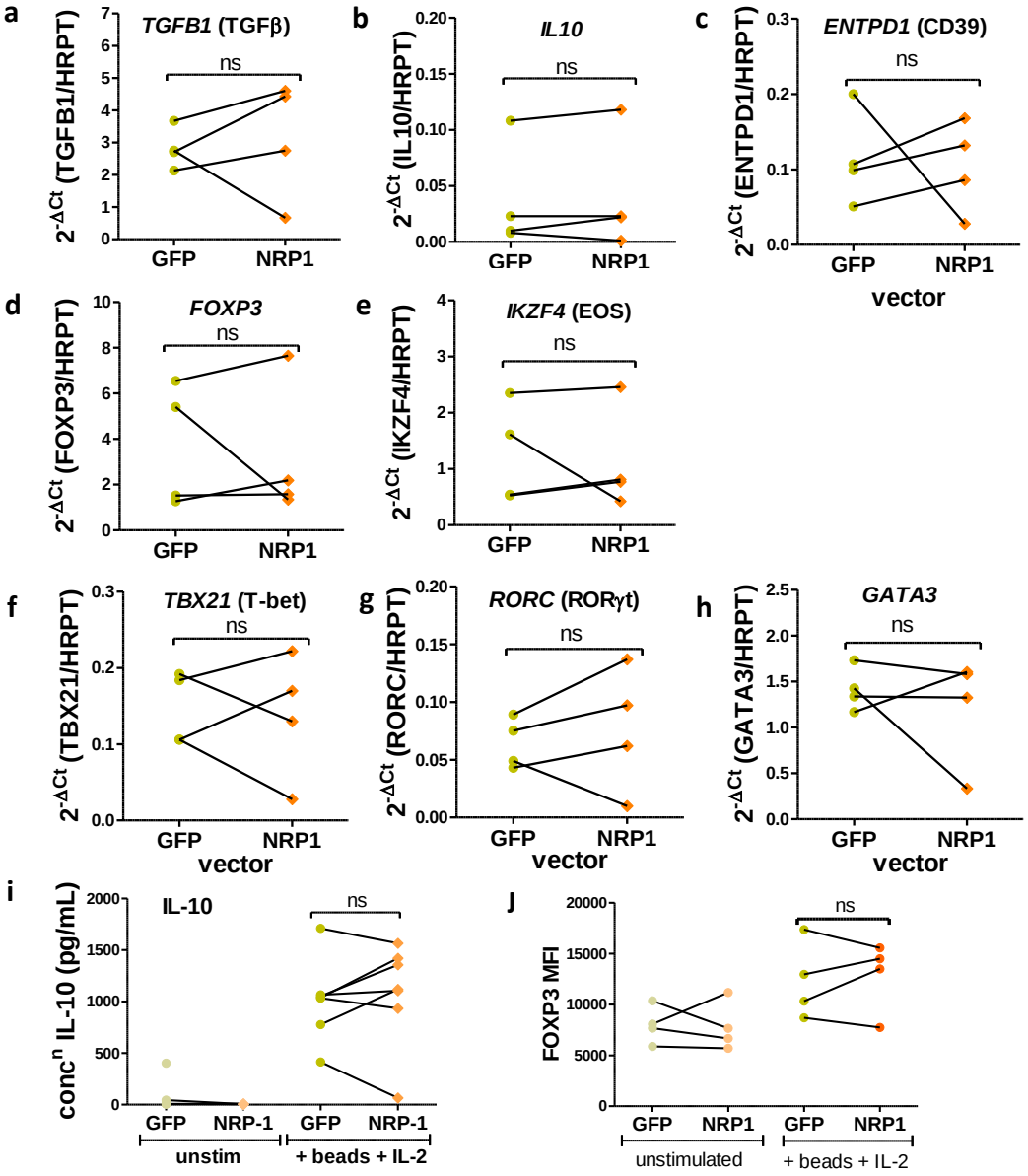


Figure 5.5 Expression of Treg and Th-associated genes by NRP1 OE Tregs

Tregs, transduced with control vector, CAG-GFP, or CAG-NRP1-GFP, were cultured for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). (a-h) qRT-PCR was performed to measure mRNA of Treg-associated genes *TGFB1* (TGFβ), *IL10, ENTPD1* (CD39), *FOXP3 and IKZF4* (Eos). (f-h) mRNA of Th-associated transcription factors *TBX21* (T-bet), *RORC* (RORγt) and *GATA3* was quantified by qRT-PCR. Mean values from two replicates are plotted for each donor. (i) IL-10 in the supernatants of Treg cultures was quantified using a flow cytometry bead assay. (j) Intracellular expression of FOXP3 protein was measured by flow cytometry. Wilcoxon Matched-Pairs Signed Rank tests were used to assess statistical significance: (a) p0.8750; (b) p=0.5000; (c) p=0.8750 ; (d) p=0.8750 ; (e) p= 0.8750; (f) p=0.8750 ; (g) p=0.6250; (h) p= 0.6250; (i) p= 0.6875; (j) p= 0.8750.

Analysing cytokine secretion in the supernatants of stimulated Tregs, concentrations of pro-inflammatory cytokines (IL-4, IL-13, IL-21, IL-22, IL17A and IL-17F, IFNγ, TNFα, IL-6 and IL-9) did not differ significantly between NRP1 OE and control Tregs [Figure 5.6]. Nonetheless, it is of interest to observe a moderate reduction in nearly all of the cytokines (excluding IFNγ and IL-9) analysed in NRP1 OE Tregs. This reduction in pro-inflammatory cytokine secretion in NRP1 OE Tregs, whilst very moderate and not statistically significant, is remarkably consistent between cytokines and between cell donors. We speculate that the sum of this differential cytokine secretion might prove to be biologically significant, particularly if a higher overexpression efficiency were achieved.

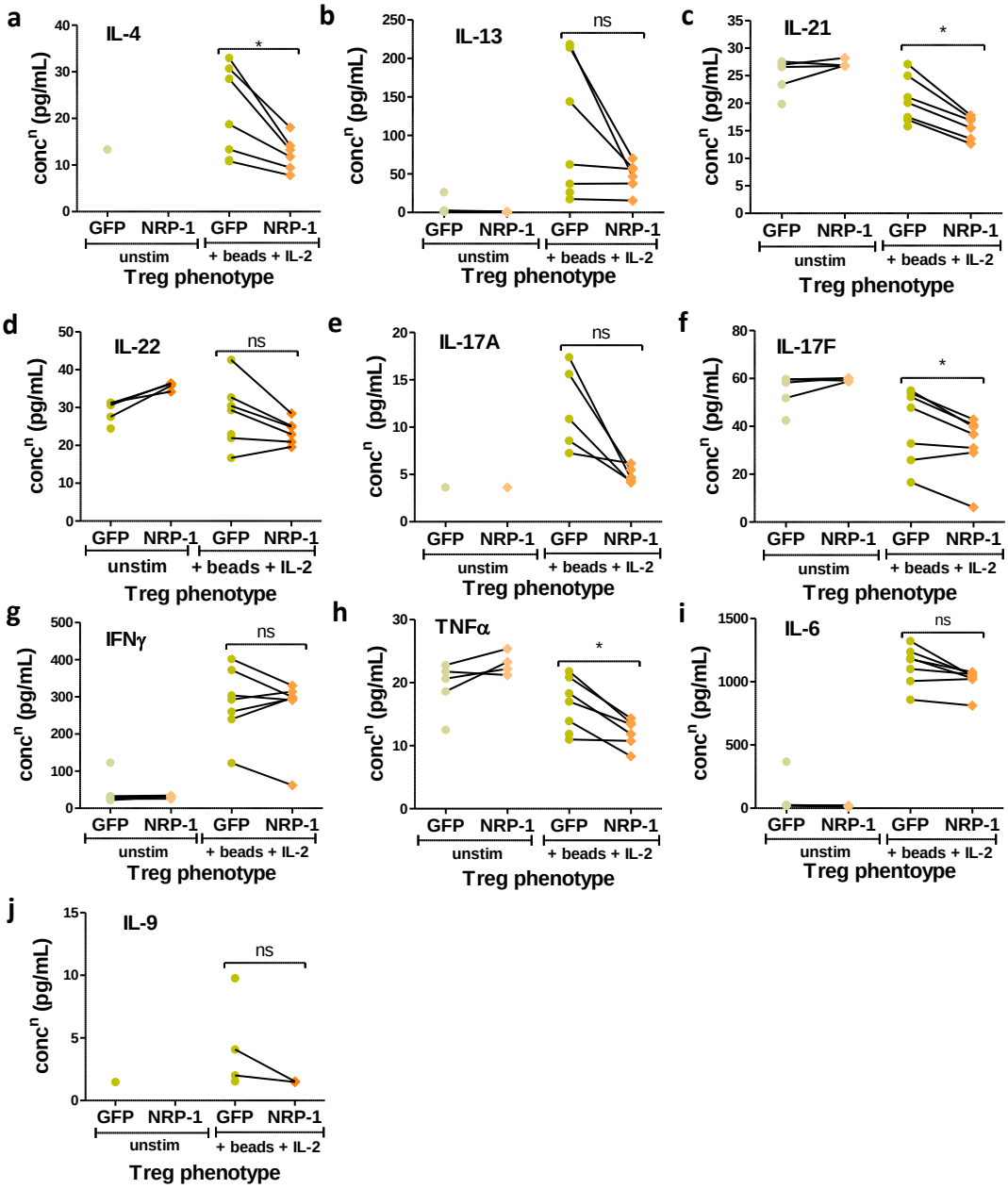


Figure 5.6 Expression of cytokines by NRP1- over-expressing Tregs

Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-NRP1-GFP, were cultured for 72hrs with or without αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). A flow cytometric bead assay was performed on supernatants of Treg cultures to measure concentration of secreted cytokines. The mean of three replicates is plotted for each donor. Wilcoxon matched-pairs signed rank tests were used to assess statistical significance: (a) p=0.0313; (b) p=0.0625; (c) p=0.2188; (d) p=0.0938; (e) p=0.0625; (f) p=0.0469; (g) p=0.4688; (h) p=0.0313 ; (i) p=0.5000.

5.3.4 Substantial over-expression of IL-10 in Tregs can be achieved via transduction with CAG-IL10 *in vitro*

Following our discovery that forced expression of NRP1 was not sufficient to enhance Treg suppressive capacity, we investigated the possibility that targeting an effector molecule downstream of NRP1 might be a more efficient method of promoting Treg function than forcing expression of NRP1. Specifically, we selected IL-10 as a candidate for genetic modification in Tregs, due to its established anti-inflammatory properties[386].

After transduction with *IL10* driven by a strong promoter, we measured increased expression of *IL10* mRNA [Figure 5.7a.b] and of IL-10 protein secreted in Treg culture supernatants [Figure 5.7c.d], up to double the concentration secreted by control Tregs. The efficiency of IL-10 overexpression was variable between cell cultures, suggesting that the protocol could be optimised further for more consistent results.

a

	IL10 mRNA (2^{-dCT} (IL10/HPRT))		% increase in IL-10 mRNA	Concentration of IL-10 secreted 72hrs (pg/mL)		% increase in IL-10 secretion (concentration in supernatant)
	CAG-GFP	CAG-IL10-GFP		CAG-GFP	CAG-IL10-GFP	
B209	0.010	9.534	95240.0	1053.2	1308.0	24.2
B293	0.108	4.400	3974.1	1710.7	3181.0	86.0
B345				1033.5	2118.7	105.0
B346				777.2	2432.8	213.0
B347	0.023	5.786	25056.5	1036.8	1213.2	17.0
B348	0.008			1066.1	1279.7	20.0

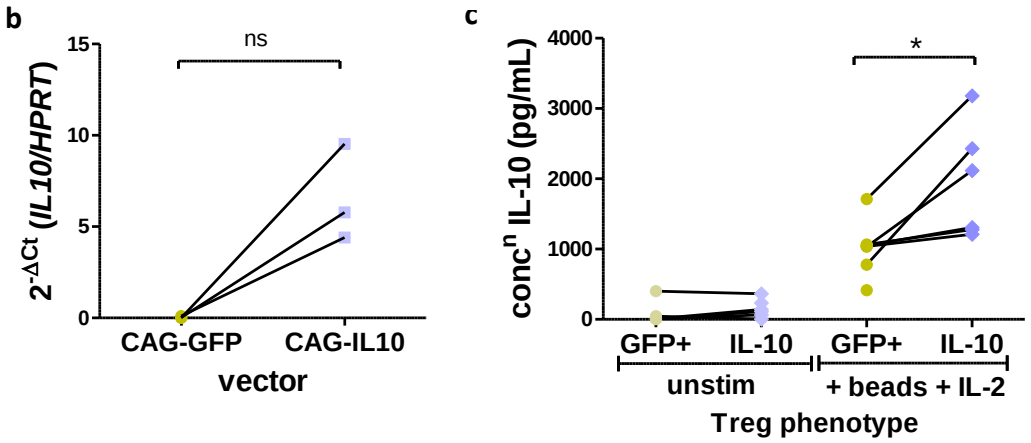


Figure 5.7 Over-expression efficiency in human Tregs transduced with CAG –driven GFP and IL-10

$CD4^{+}CD25^{+}CD127^{lo}$ Tregs transduced with lentivirus containing plasmids encoding either CAG-GFP or CAG-NRP1-GFP were FACS-sorted upon expression of GFP. (a) IL-10 expression in Tregs (mRNA quantified by RT-PCR and protein quantified by flow cytometry) is tabulated by cell donor for IL-10-overexpressing and control Tregs. (b) qRT-PCR was performed to quantify *IL10* mRNA. (c) Transduced Tregs were stimulated for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL) and concentration of IL-10 in the culture supernatants was quantified by flow cytometry. Wilcoxon Matched-Pairs Signed Rank tests were used to assess statistical significance: (b) p=0.2500; (c) p=0.0313.

5.3.5 Tregs over-expressing IL-10 mediate more potent suppression of PBMC proliferation *in vitro*

In an *in vitro* assay for PBMC proliferation, cell proliferation was suppressed to a greater extent in the presence of IL-10 OE Tregs, compared with control Tregs [Figure 5.8]. Therefore, we conclude that augmenting IL-10 expression in Tregs is a viable strategy to improve the suppressive potency of these cells.

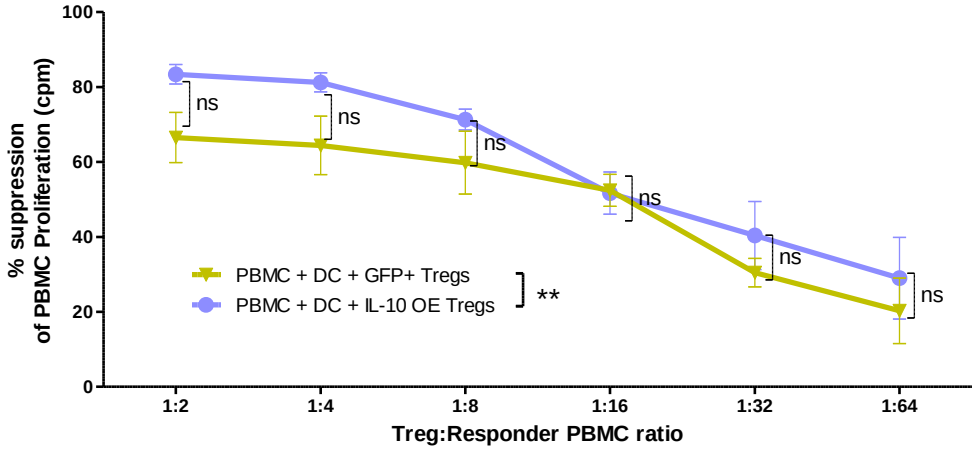


Figure 5.8 Suppression of PBMC proliferation by IL-10-overexpressing Tregs in vitro
PBMCs (10^5 cells per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for 6 days in the presence or absence of Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP. Tritiated thymidine was added for the final 16 hours of co-culture and proliferation of responder cells was measured as uptake of tritium (cpm). Percentage suppression was calculated relative to PBMCs with DCs and no Tregs. Bars indicate mean and SEM of three assays using different cell donors, with at least 6 replicates. Statistics were derived from a two-way ANOVA with Bonferroni post-tests: (p=0.0084).

5.3.6 IL-10-over-expressing Tregs retain a normal phenotype

The proliferation of IL-10 OE Tregs, measured as expression of Ki67, was not significantly different from that of control Tregs [Figure 5.9a]. IL-10 OE Tregs expressed comparable levels of activation markers CD25 [Figure 5.9b] and CD69

[Figure 5.9c] to control Tregs. These results suggest that IL-10 over-expression does not alter the activation or proliferative potential of Tregs.

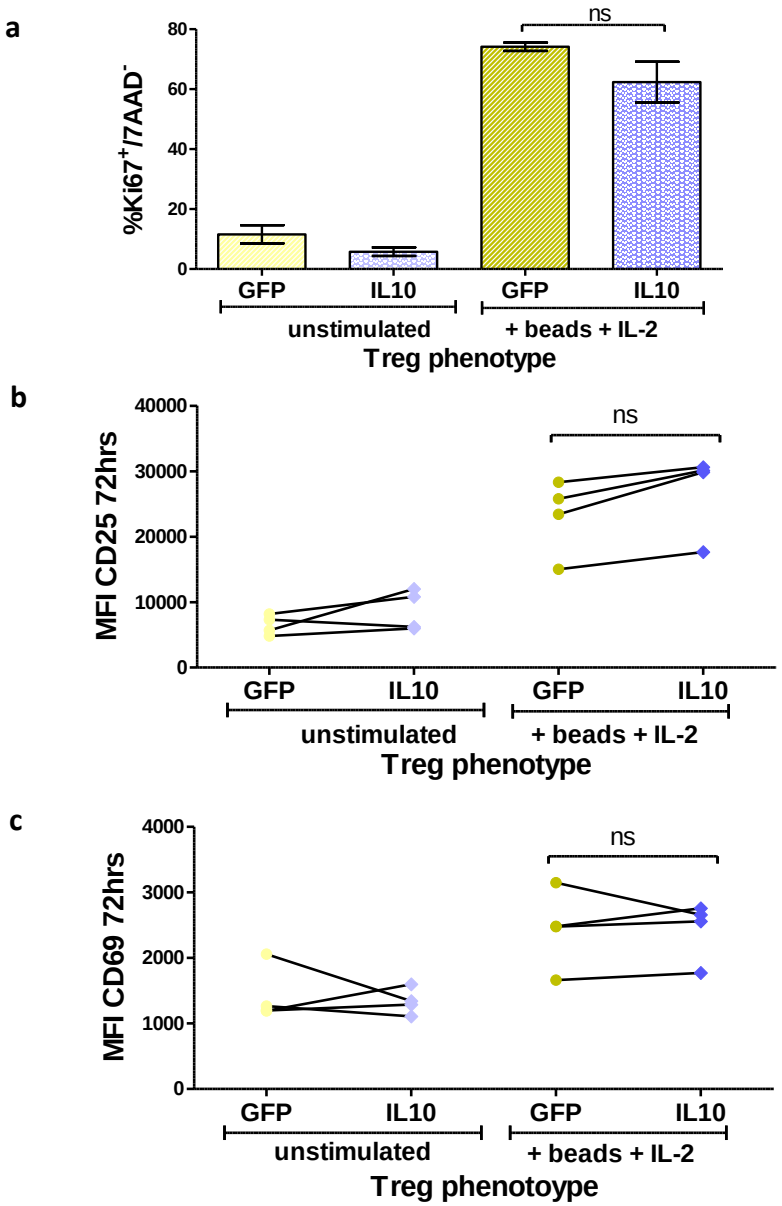


Figure 5.9 Proliferation and activation in IL-10-over-expressing Tregs
Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP, were cultured for 72hrs in the presence αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). Expression of (a) the proliferation marker Ki67 (b) activation marker CD25 and (c) activation marker CD69 was quantified by flow cytometry. Mean values of three replicates are plotted for each donor. Statistical analyses were performed using Wilcoxon matched-pairs signed ranks tests: (a) p=0.1250; (b) p=0.1250 ; (c) p=1.000.

Other than elevated transcription of *IL10*, we observed no significant differences between IL-10 OE Tregs and control Tregs with respect to transcription of Treg-associated genes [Figure 5.10a, b] or Th-associated genes [Figure 5.10 d-f]. Nor was the expression of FOXP3 protein altered in IL-10 OE Tregs, compared to control Tregs [Figure 5.10c]. These data indicate that the phenotype of Tregs is stable after genetic modification to drive IL-10 expression.

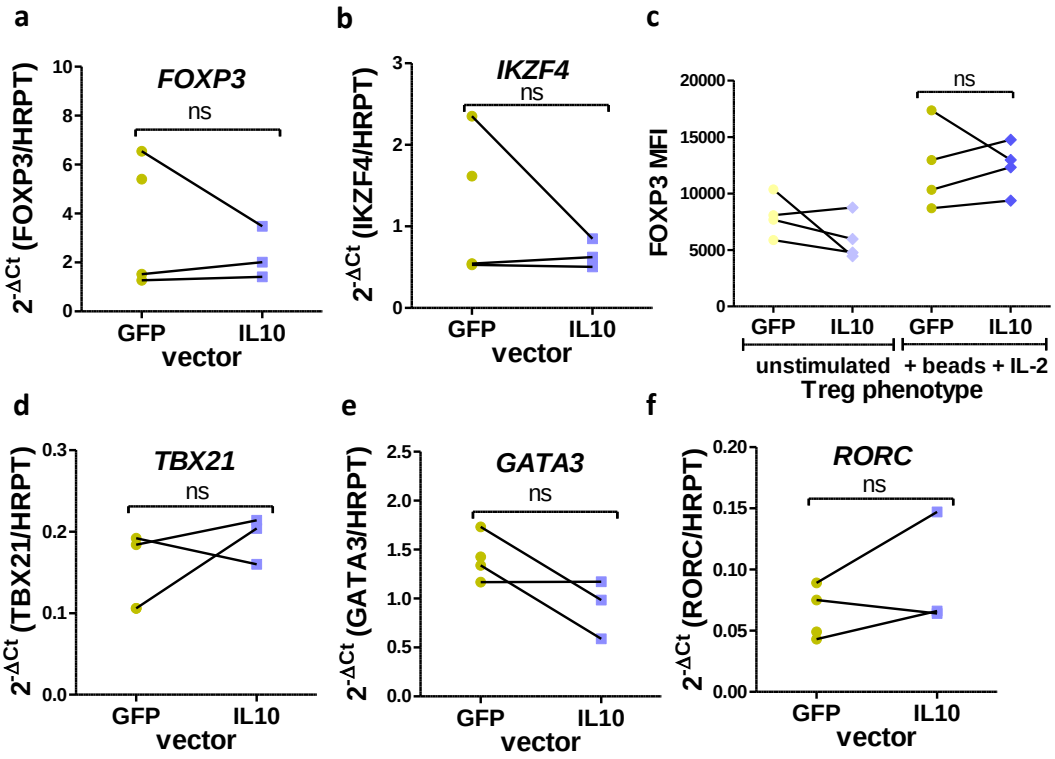


Figure 5.10 Expression of Treg- and Th-associated genes by IL-10 over-expressing Tregs

(a-b, d-f) qRT-PCR was performed on cDNA synthesised from Tregs transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP. mRNA transcripts were quantified for *HPRT,* the Treg-associated transcription factors (a) *FOXP3* and (b) *IKZF4 (EOS),* and for the Th-associated transcription factors (d) *TBX21 (T-bet),* (e) *GATA3* and (f) *RORC* (RORγt). Mean values from two replicates are plotted for each donor, with lines joining data points from the same cell donor. (c) Tregs were cultured for 72hrs with or without αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL) prior to measurement of FOXP3 protein expression by flow cytometry. Mean values from three replicates are presented for each donor. Wilcoxon signed-rank tests were used to assess statistical significance: (a) p=1.000; (b) p= 0.7500; (c) p=0.8750; (d) p=0.7500 ;(e) p=0.5000 ; (f) p=.0.5000.

All pro-inflammatory cytokines measured in the supernatants of Treg cultures (IL-4, IL-13, IL-21, IL-22, IL-17, INFγ, TNFα and IL-6) were secreted at equivalent concentrations by IL-10 OE and control Tregs [Figure 5.11]. Hence, we conclude that IL-10 can be over-expressed in Tregs without compromising the phenotype of those Tregs.

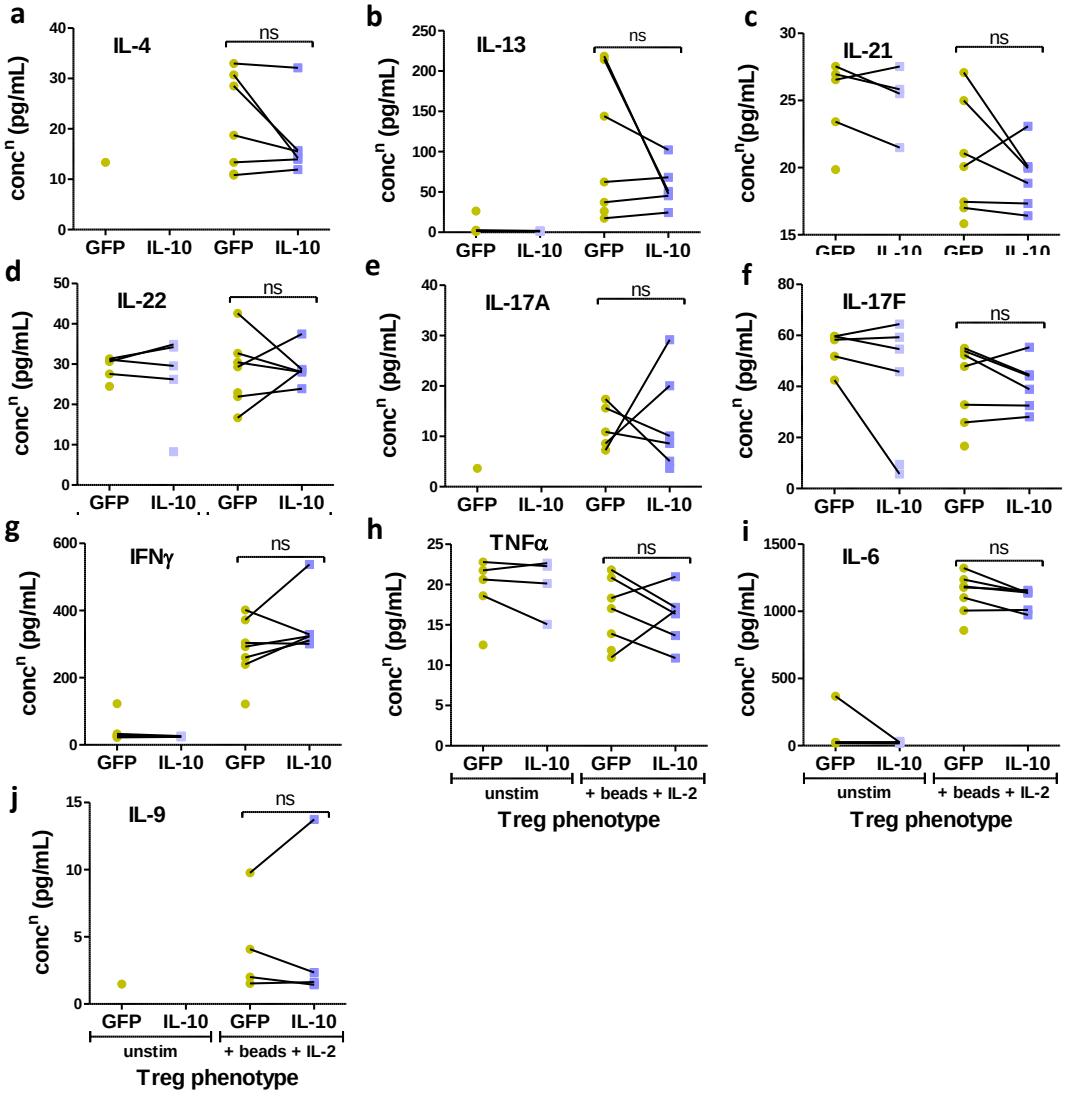


Figure 5.11 Cytokine secretion by IL-10 over-expressing Tregs

Tregs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP, were cultured for 72hrs in the presence αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (200U/mL). A flow cytometric bead assay was performed on supernatants of Treg cultures to measure concentration of secreted cytokines (a) IL-4, (b) IL-13, (c) IL-21, (d) IL022, (e) IL-17A, (f) IL-17F, (g) IFNγ, (h) TNFα, (i) IL-6 and (j) IL-9. The mean of three replicates is plotted for each donor. Wilcoxon matched-pairs signed rank tests were used to assess statistical significance: (a) p=0.2188; (b) p=0.4375; (c) p=0.2188; (d) p=1.0000; (e) p=1.0000; (f) p=0.3125; (g) p=0.3125; (h) p=0.5625; (i) p=0.0625; (j) p=1.0000.

5.3.7 IL-10 secretion can be induced efficiently in Teffs by transduction with IL10

As an extension of our experiments on IL-10 OE Tregs, we were interested to examine whether forcing IL-10 expression in Teffs, that do not normally secrete substantial amounts of this cytokine, could confer a less pro-inflammatory phenotype upon these cells. Substantially augmented levels of *IL10* mRNA expression were confirmed in Teffs transduced with the CAG-IL10 construct, compared to control (CAG-GFP- transduced) Teffs [Figure 5.12].

a

	IL10 mRNA ($2^{-dCT\ (IL10/HPRT)}$)		% increase in IL-10 mRNA
	CAG-GFP	CAG-IL10-GFP	
B344	1.000	11.158	1015.8
B345	0.002	10.625	531150.0
B346	0.001	15.964	1596300.0

b

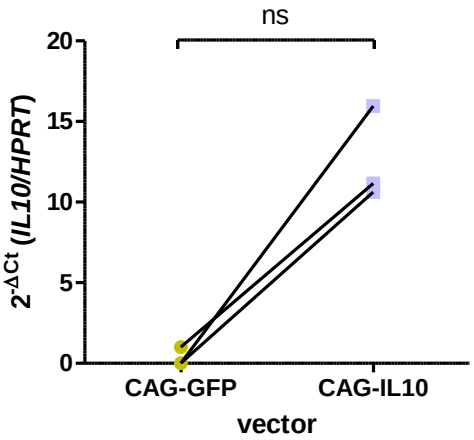


Figure 5.12 Over-expression efficiency in human $CD4^+CD25^-$ Teffs transduced with CAG-IL10

$CD4^+CD25^-$ Teffs were transduced with either CAG-GFP, CAG-IL10 -GFP. Transduced Teffs were stimulated for 72hrs with αCD3αCD28 beads (1 bead: 5 cells) and IL-2 (100U/mL) before quantifying *IL10* mRNA expression by qRT-PCR. qRT-PCR data are (a) tabulated by cell donor (b) plotted for each donor separately, with lines adjoining data from the same donor. Statistical significance was assessed using a Wilcoxon Matched-Pairs Signed Rank test (b) p= 0.2500.

5.3.8 IL-10-expressing Teffs can suppress PBMC proliferation *in vitro*

Unexpectedly, PBMC proliferation was suppressed considerably in the presence of IL-10 OE Teffs [**Figure 5.13a**]. Moreover, expression of the activation marker CD69 was also suppressed in PBMCs cultured with IL-10 OE Teffs [**Figure 5.13b**]. These results suggest that secretion of IL-10 alone was sufficient to confer immune suppressive properties upon Teffs. Considering the range of pro-

inflammatory function of Teffs, it is quite remarkable that a single molecule can counteract this behaviour.

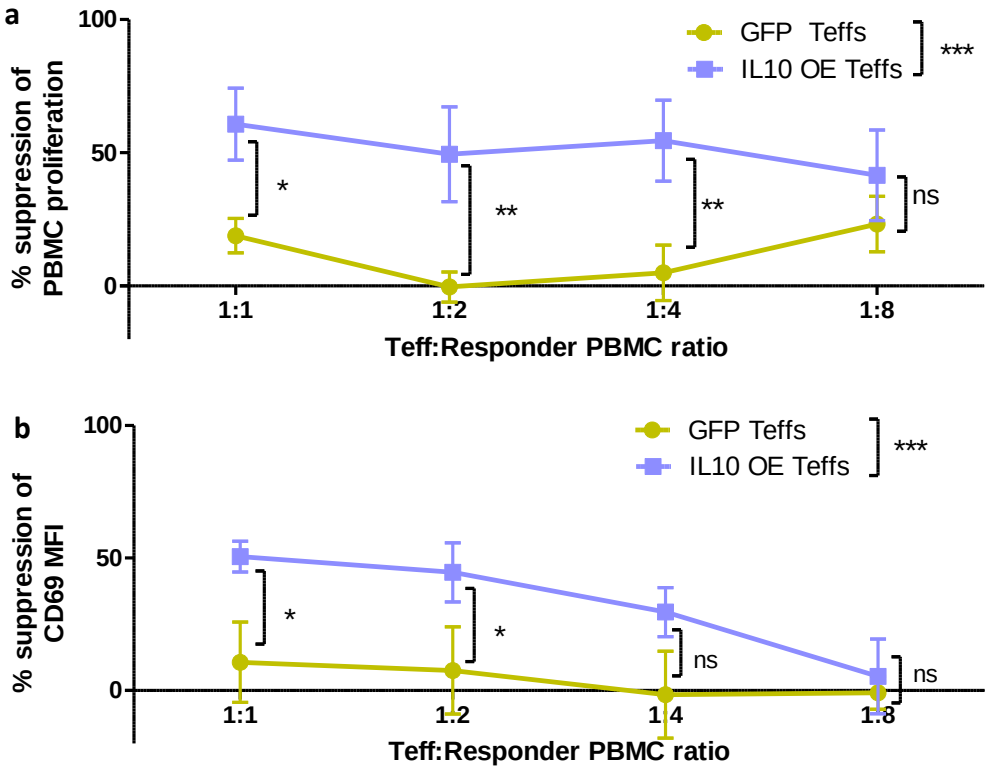


Figure 5.13 PBMC proliferation in the presence of IL-10-overexpressing Teffs *in vitro*

VPD-stained PBMCs (10^5 cells per well) stimulated with allogeneic moDCs (10^4 per well) were cultured for 4 days in the presence or absence of $CD4^+CD25^-$ Teffs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP. Division index was calculated from VPD dilution, measured by flow cytometry. MFI of CD69 expressed on the surface of responder cells was quantified by flow cytometry. Statistical analyses were performed using a two-way ANOVA with Bonferroni post-tests: (a) $p<0.0001$; (b) $p=0.0006$.

5.3.9 IL-10 expression in Teffs does not alter the transcription of Treg- and Th-associated genes

Since IL-10 is secreted into the culture supernatant, IL-10 OE Teffs have been exposed to paracrine and autocrine IL-10 for the duration of their expansion. Given that IL-10 is known to have tolerogenic properties, it is highly plausible this cytokine would induce changes in the phenotype of IL-10 OE Teffs that might contribute to their suppressive behaviour in the suppression assay. In order to assess to what extent the broader phenotype of Teffs is altered by IL-10 expression, we repeated the qRT-PCR analysis that we performed previously on genetically-engineered Tregs. Contrary to our predictions, neither genes encoding the Treg-associated transcription factors FOXP3 and EOS [**Figure 5.14a,b**], nor genes encoding Treg-associated immunoregulatory molecules TGFβ, NRP1 and CD39 [**Figure 5.14c-e**], were significantly differentially expressed between IL-10 OE Teffs and control Teffs. Furthermore, genes encoding Th-associated transcription factors T-bet, GATA3 and RORγt were transcribed at similar levels in IL-10 OE Teffs and control Teffs [**Figure 5.14f-h**]. We conclude that expression of IL-10 in Teffs does not deviate the transcriptional program of Teffs towards a Treg-like transcriptional signature. Rather, the suppression of PBMC proliferation mediated by IL-10-expressing Teffs is most likely attributable to the direct effects of IL-10 upon the responder cells.

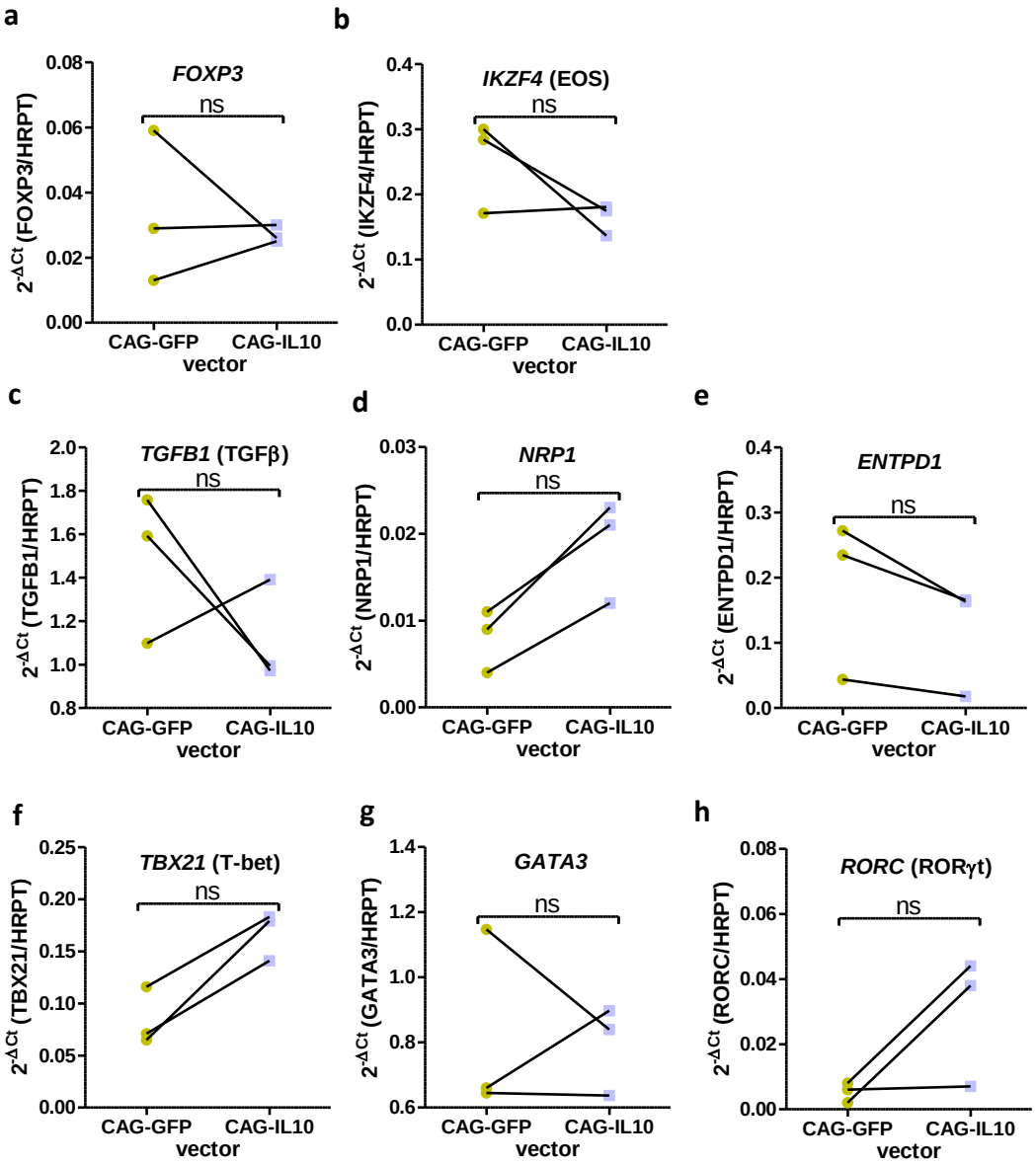


Figure 5.14 Transcription of Treg and Th-associated proteins by IL-10 over-expressing Teffs

RNA was isolated from $CD4^{+}CD25^{-}$ Teffs, transduced with either control lentiviral vector, CAG-GFP, or with CAG-IL10-GFP and cDNA was synthesised from this RNA. Taqman assays were used to quantify trasncripts for for *HPRT,* (a) *FOXP3,* (b) *IKZF4,* (EOS) (c) *TGFB1,* (d) *NRP1,* (e) *ENTPD1,* (f) *TBX21,* (g) *GATA3 and (*h) *RORC.* Mean values from two replicates are plotted for each donor, with lines joining data points from the same cell donor. Wilcoxon signed-rank tests were used to assess statistical significance: (a) p=1.0000; (b) p=0.5000; (c) p=0.5000; (d) p=0.2500; (e) p=0.2500; (f) p=0.2500; (g) p=0.7500; (h) p=0.1736.

5.4 Discussion

Given that we identified NRP1-dependent transcriptional targets in human Tregs, we predicted that increasing the magnitude of NRP1-dependent signalling by augmenting the availability of NRP1 ligands might improve the function of these cells.

From the very broad repertoire of NRP1-associated ligands, we selected three that have been most strongly implicated in immune regulation, particularly in association with T cells, namely VEGF[334,369], SEMA3[321,322,370] and SEMA4[291,372]. Disappointingly, short-term incubation of Tregs with any these ligands failed to confer any elevation in the suppression of cell proliferation mediated by these cells. These assays were by no means exhaustive, being restricted by the expense of procuring more ligands or larger volumes of ligand that would be required for long-term culture. Thus, our results do not preclude the possibility that long-term exposure to these ligands, or others, might promote the function of Tregs. These would likely be rewarding avenues to pursue in the future. It is also worth considering that soluble ligands can act as receptor antagonists, so reduced Treg activity might also be an outcome of adding NRP-associated ligands to co-cultures.

Having developed a protocol for genetic engineering of human Tregs during *in vitro* expansion, we decided to explore the possibility of targeting NRP1 in Tregs through genetic modification. Specifically, we introduced into Tregs a genetic construct in which NRP1 expression was driven by a strong, constitutive promoter. *In vitro* suppression assays revealed no augmentation of suppressive

capacity in NRP1-overexpressing Tregs. These results appear to be contrary to our finding that NRP1-deficient Tregs are impaired in their suppressive function. However, there are several explanations for this apparent discrepancy. Firstly, the increase in NRP1 expression after transduction with CAG-NRP1 was not as strong as we had anticipated. These cells were selected upon GFP, which should be co-transcribed with NRP1 at stoichiometric ratios. Therefore, we attribute the low expression of NRP1 protein to post-transcriptional regulation. As to whether a more efficient over-expression of NRP1 would have revealed a consequent improvement in Treg-mediated suppression, we can only speculate. It is also entirely possible that this result is not an artefact of poor overexpression; increasing the expression of NRP1 alone might well be insufficient to improve Treg function. It is quite probable that the native abundance of this molecule is not a limiting factor in its downstream biochemical signalling. Adding more of the molecule might not achieve the outcome of increasing the intensity of NRP1-dependent signalling.

As a molecule with a well-established role in immune regulations that we identified as a transcriptional target of NRP1, we selected IL-10 as a favourable candidate for genetic targeting in Tregs[386]. Aberrations in IL-10 have been causally associated with immune-mediated pathologies[387]. IL-10 could be administered directly as a therapeutic agent, separate to the Tregs. However, there is some evidence that activity of cytokines is more potent when secreted by a cell, creating a sustained, localised, moderate concentration gradient in proximity to a target cell, compared to the broad distribution and high concentration

generated by systemic delivery of the same cytokine[388]. Intuitively, it might seem inadvisable to target a molecule that is already expressed by Tregs, as there is less "room for improvement". However, in our experience, Tregs isolated on the basis of $CD4^+CD25^+CD127^{lo}$ phenotype secrete relatively little IL-10, particularly in their resting state. Meanwhile Tr1 cells, which are highly potent immune regulators, are believed to rely upon IL-10 as their principle mechanism of immune regulation[389]. By combining in a single cell population the functionalities of $FOXP3^+$ Tregs with those of Tr1 cells, synergy between their mechanisms might yield superior suppressive function compared to either cell subset alone.

To achieve elevated and sustained expression of IL-10, we introduced a genetic construct containing the *IL10* ORF driven by a strong, constitutively-active promoter, CAG. Indeed, increasing the expression of IL-10 in Tregs by transduction was sufficient to improve the *in vitro* suppressive capacity of these cells demonstrably. Thus, we demonstrated that one simple genetic modification could enhance the efficacy of a cell therapy product. It must be acknowledged that improved function in this *in vitro* assay does not necessarily translate into improved efficacy *in vivo*, where more factors will impinge upon the activity of these cells. Additionally, constitutive expression of an immunoregulatory molecule may improve the suppressive potency of a cell at the expense of its specificity. Given more time, ideally this result should be validated in an animal model.

Furthermore, we demonstrated that secretion of IL-10 by T cells is sufficient to confer some regulatory capacity upon these cells, in an *in vitro* assay of cell

proliferation. In the form that we have designed in this study, we would not necessarily advocate IL-10 OE Teffs as cell therapy for immune regulation, as these cells retain their effector phenotype that would most likely exacerbate existing inflammation. Rather, the purpose of our experiments on IL-10-expressing Teffs was to determine the potency of cell-derived IL-10 as an immunoregulatory mediator, in isolation from the other suppressive mechanisms of Tregs. In fact, our findings are corroborated by a published study utilising a similar approach[390]. Interestingly, when compared with a Tr1 phenotype, rather than a FOXP3$^+$ phenotype, the IL-10-transduced Teffs described by the authors of this study did appear to have acquired a regulatory T cell phenotype. We did not examine Tr1 markers in our IL-10-transduced Teffs. However, had the IL-10-transduced Teffs acquired a Tr1 phenotype, one would expect these cells to be anergic, which we demonstrated our IL-10-expressing Teffs were not. Intriguingly, there is some precedent in nature for native Teff-derived IL-10 playing a role in immune modulation. A subset of memory T cells can exhibit context-dependent regulatory capacity, secreting IL-10 under steady-state conditions, whilst secreting IL-2 and contributing to immunity upon exposure to recall antigen[391].

Whilst we have demonstrated promising efficacy of driving IL-10 expression to increase the suppressive potency of human Tregs, we feel that in order to be clinically applicable, certain refinements in the design of this engineering strategy would be desirable. Augmenting native immunoregulatory molecules might improve the *potency* of regulatory cells but, in order to reduce off-target effects, enhancing the *specificity* of these cells would be desirable. Cell activation,

antigen-specificity or tissue-homing have all been the subject of genetic manipulation to confer specificity upon putative cellular therapeutics. The idea of controlling cell migration is particularly appealing in light of emerging evidence of tissue-restricted activity of lymphocytes, including Tregs[186,211,221]. By co-opting endogenous chemokine receptor signalling pathways, synthetic regulatory circuits have been engineered to allow lymphocyte migration to be induced by a synthetic stimulus, such as light[381].

More sophisticated genetic engineering strategies incorporate regulatory circuits, such that the expression or activity of the transgene is inducible. It is now becoming relatively common practice to employ synthetic biology for cell therapy applications[392,393]. One ingenious example of this type of genetic switch was recently described in a study by Wu et al[394], in which a chimeric antigen receptor was engineered such that its intracellular signalling capacity was dependent upon a synthetic small molecule drug, as well as ligation of its cognate antigen. This genetic circuit constituted a Boolean "AND" logic gate, which ultimately placed the activity of the CAR-expressing T cells under the control of a clinician administering the small molecule. Safety switches could also be introduced into cell therapy products, to enable rapid reversal of the treatment if adverse effects are detected[293,395].

It must be acknowledged that there are certain drawbacks to the application of genetic engineering to cell therapy products, compared with pharmacological manipulation. Genetic engineering involves a more cumbersome procedure than simply adding a compound to the culture, as generation of the viral vector takes

several days, additional reagents and a host cell line. More importantly, the risks (though often perceived rather than actual) associated with genetic modification *per se,* and with introduction of a viral vehicle[310,396], are not inconsequential. Off-target effects upon the genome are always a concern with any genetic engineering strategy. The vector used in this study, the lentivirus, integrates into the genome in a non-site-specific manner. In theory, the random integration of a virus into a locus that encodes or regulates an oncoprotein or tumour suppressor gene could result in cancerous transformation of the cell culture. Infusion of a cancerous cell therapy product would be of particular danger to an immunosuppressed patient.

Nonetheless, there are indisputable benefits to the genetic engineering approach to enhance cell therapy products, compared with pharmacological methods. The biological effects of the genetic modification are stable, not dependent upon continued exposure to a compound. Thus, modification of the therapeutic cells, in isolation, can be conducted entirely *in vitro*, without collateral effects upon other cells within the body that might result from attempts to sustain the treatment of therapeutic cells *in vivo* after infusion. From an economic perspective, it would also be cheaper to apply a single treatment of cells *in vitro* than to administer repeated, large doses of chemical agents. Furthermore, genetic engineering can be more specific with respect to its molecular target than pharmacological interventions. Of course, the specificity of the outcome still depends upon intelligent target selection or transgene design.

With the advance of site-specific genome editing platforms, such as zinc-finger nucleases, Transcription Activator-Like Effector Nucleases (TALENs) and Cas9-CRISPR[397], the requirement for virus-mediated transduction to achieve stable genetic modification may become obsolete. Only transient transfection is required to introduce the enzymes and templates required for genome editing. Once the genome editing has occurred, the modification is permanent. Genome editing technologies theoretically overcome the risk of insertional mutagenesis by virtue of their sequence-specificity. The Cas9-CRISPR system, for example, utilises oligonucleotides to direct enzymatic DNA repair to a unique DNA sequence, which could be a target locus, or to a safe harbour. For lymphocytes, transient transfection remains technically challenging[300] but the development of new vehicles for both oligonucleotide and protein transfection promise to ameliorate these restrictions in the near future[398]. In other respects, genome editing methodologies can outperform traditional genetic engineering techniques by permitting modification of regulatory modules, as well as protein-coding loci, to achieve more subtle or controlled outcomes. Additionally, genome editing can be used to inactivate endogenous genes, which would be particularly useful for eliminating the native TCR when re-directing lymphocyte specificity. Naturally, these new technologies are also subject to limitations and concerns.

Chapter 6: General Summary and Discussion

6.1 Results Summaries

6.1.1 Results Summary Chapter 3: Optimisation and Application of Genetic Engineering in Human Tregs

The initial goal of the study detailed in Chapter 3 was to optimise a protocol for stable genetic modification of human Tregs during *in vitro* expansion. We identified the cell culture conditions that yielded the largest number of stably-transduced human Tregs using a lentiviral vector: stronger TCR-stimulation (using a higher ratio of αCD3αCD28-coated beads), relatively high concentration of viral vector, and addition of virus on the day of isolation, at the same time as the stimulatory beads [**Figure 3.2**]. After 50 days in culture, GFP expression in these cells was confirmed to be stable [**Figure 3.3**]. Neither the viability, the activation [**Figure 3.4**] nor the suppressive function was impaired in GFP-expressing Tregs [**Figure 3.5**].

Using this optimised protocol, we were able to generate GFP-labelled Tregs for infusion in a humanised mouse model of allotransplantation. In this experiment, we aimed to determine the feasibility of spatial and temporal tracking of GFP-labelled human Tregs *in vivo*. Examining mouse tissues several weeks after infusion of GFP-expressing Tregs, we confirmed that human Tregs do persist in this humanised mouse model [**Figure 3.7**]. Moreover, we developed a method for non-invasively imaging a human skin graft situated on the flank of a mouse. Using

two-photon microscopy, we were able to visualise the dynamic behaviour of GFP-labelled Tregs within the graft, *in situ*, in real-time [Figure 3.8]. However, these cells could not be detected reliably by flow cytometry in the lymphoid organs [Figure 3.6]. The failure to detect GFP-labelled Tregs in lymphoid organs at the same time point by flow cytometry may be attributed to a combination of factors: the cells may be present at such low abundance that they do not breach the threshold of detection for this detection method, or the signal from GFP may be attenuated by the processing of these tissues.

Finally, we explored a reverse genetics approach to investigate molecular mechanisms of Treg-mediated suppression. Achieving shRNA-mediated knockdown of two candidate genes, LGALS1 and TRAIL [Figure 3.9], we were able to confirm that TRAIL contributes minimally to the optimal suppressive function in human Tregs. Meanwhile, LGALS1 expression proved to be dispensable for *in vitro* suppression of PBMC proliferation by human Tregs [Figure 3.10].

6.1.2 Results Summary Chapter 4: Investigating the Role of Neuropilin-1 in Human Tregs

In Chapter 4, we demonstrated that, despite low basal expression [Figure 4.1] NRP1 expression is required for Treg-mediated suppression of PBMC proliferation *in vitro* [Figure 4.3-4.4] and for skin allograft rejection *in vivo* [Figure 4.5]. The functional defect in NRP1 KD Tregs could not be attributed to compromised survival, proliferation or activation in NRP1 KD Tregs [Figure 4.7 & 4.8]. Nor was the expression of Treg-associated or Teff-associated genes perturbed in NRP1 KD Tregs [Figure 4.10 & 4.11]. Secretion of proinflammatory cytokines was not

significantly increased in NRP1 KD Tregs [**Figure 4.12**]. Rather, in human Tregs, the expression of the anti-inflammatory cytokine IL-10, at the transcriptional level, was dependent upon NRP1 expression [**Figure 4.12g**]. Since IL-10 is a well-established mediator of immune suppression, the promotion of IL-10 expression by NRP1 could account for the dependency of Tregs upon NRP1. Additionally, an analysis of genome-wide gene expression revealed that there were a large number of genes that were differentially expressed between NRP1 KD Tregs and control Tregs [**Figure 4.13**]. In particular, the transcription of three genes involved in the induction of autophagy, BECN1, COPS4 and MAP1ILC3B, was dependent upon NRP1 expression [**Figure 4.15**]. Currently, the link between the expression of autophagy-associated genes and Treg suppressive function is purely associative. However, recent studies implicating autophagy in the maintenance of metabolic homeostasis in Tregs[353–356] lead us to speculate that facilitation of autophagy might be a mechanism by which NRP1 potentiates Tregs suppressive function.

Successful knockdown of NRP1 was also achieved in $CD4^{+}CD25^{-}$ Teffs [**Figure 4.16**] and resulted in no apparent alterations in the physiology of these cells, with respect to proliferation [**Figure 4.17**] activation [**Figure 4.18**] or FOXP3 expression [**Figure 4.19**] The subset of genes that were differentially expressed between NRP1 KD and control Teffs only partially overlapped with the genes differentially expressed between NRP1 KD and control Tregs [**Figure 4.13**]. The genes validated in Treg samples by qRT-PCR were typically also downregulated in NRP1 KD Teffs, but not always with statistical significance [**Figure 4.20**].

6.1.3 Results Summary Chapter 5: Investigating Strategies to Enhance Human Tregs during *in vitro* expansion

Having implicated NRP1 in the function of human Tregs, we investigated strategies by which NRP1 activity might be manipulated in Tregs during *in vitro* expansion, with the goal of improving Treg suppressive function. Short-term exposure of Tregs to NRP1-associated ligands VEGF, SEMA3A or SEMA4A failed to enhance the ability of these cells to suppress PBMC proliferation *in vitro* [Figure 5.1].

As an alternative approach to the augmentation of NRP1 activity, we sought to determine the effect of enforcing overexpression of NRP1 upon the phenotype of Tregs. A moderate elevation in NRP1 expression was achieved by transduction of Tregs with a construct that drives expression of NRP1 from a CAG promoter [Figure 5.2]. Counterintuitively, the potency of suppression mediated by Tregs that over-expressed NRP1 was not enhanced relative to control Tregs [Figure 5.3]. No significant difference between NRP1 over-expressing Tregs and control Tregs was observed with respect to proliferation or activation *in vitro* [Figure 5.4]. Nor did we detect any significant alterations in the transcription of various Treg-associated and Th-associated genes [Figure 5.5]. However, secretion of anti-inflammatory cytokines IL-4, Il-21, IL-17A and TNFα was markedly diminished in NRP1 OE Tregs, compared to control Tregs [Figure 5.6].

In contrast, Tregs engineered to over-express IL-10 [Figure 5.7] exerted significantly stronger suppression of PBMC proliferation *in vitro* [Figure 5.8]. As a potent anti-inflammatory mediator[386], the enhanced suppressive capacity of IL-

10 OE Tregs is most likely attributable to the direct activity of IL-10 upon responder cells. This conclusion was supported by observations that the broad phenotype of IL-10 OE Tregs, with respect to *in vitro* proliferation and activation, was not altered relative to control Tregs [**Figure 5.9**]. Whilst some autocrine effects of IL-10 upon IL-10 OE Treg cultures might be expected to promote a Treg molecular signature, we observed no significant differences in the expression of Treg-associated and Th-associated genes [**Figure 5.10**] or in secretion of cytokines, other than IL-10 [**Figure 5.11**].

IL-10 OE Teffs [**Figure 5.12**] also proved to be capable of mediating suppression of PBMC proliferation *in vitro* [**Figure 5.13**]. As for IL-10 OE Tregs, the phenotype of IL-10 OE Teffs was not polarised towards a Treg transcriptional signature [**Figure 5.14**]. Rather, we infer that IL-10 expression *per se* is sufficient to confer suppressive capacity upon Teffs.

These experiments also demonstrate that the suppressive potency of human Tregs can be enhanced by a relatively elementary genetic engineering protocol that is compatible with the *in vitro* expansion procedure required for generation of Treg-based cellular therapeutics.

6.2 General Discussion

Although genetic engineering has been applied to human Tregs in other labs, it is not common practice. Therefore, we felt it important to develop a method for genetic modification of Tregs that would be compatible both with our current lab practices and with clinical Treg expansion protocols. Whilst the protocol that we developed turned out to be broadly similar to those already published[171,273,277],

we did incorporate certain refinements, in particular, addition of lentivirus on the first day of Treg activation. No doubt, these methods will be improved iteratively but the protocol described herein makes the generation of useable numbers of modified Tregs feasible and, indeed, relatively facile.

The ability to visualise human Tregs, and indeed other lymphocyte subsets, *in situ* during an immune response will, we hope, prove to be invaluable to our understanding of the biology of these cells after adoptive transfer. Our venture into intravital imaging of Tregs is still at a preliminary phase but we have demonstrated that this approach is feasible in this prevalent humanised mouse model. Furthermore, we have developed a technique for non-invasive imaging of skin grafts situated on the flank of a mouse. This technique should serve to enable longitudinal imaging studies without the artefacts that might be incurred by surgical interference and without the need to perfect the relatively challenging technique of ear skin grafting[318]. With some optimisation, this methodology should yield a depth of information that cannot be obtained by other methods. We envisage this set-up being used to analyse dynamic physiological and immunological processes within the skin in different immunological contexts.

An understanding of the cues that potentiate Treg function will facilitate the design of clinical strategies that make the best use of these cells in the clinic. In Chapter 4, we identified a protein, NRP1, that seems to transduce such environmental cues that potentiate Treg-mediated suppression. Whilst we identified some of the molecular mediators regulated by this NRP1-dependent pathway, namely IL10, BECN1 and MAP1ILC3B, the signalling pathway between

NRP1 and its nuclear targets remains to be characterised. In particular, the identification of salient NRP1-associated molecular stimuli would be informative for the development of adjuncts to Treg therapy.

Once more is understood about the way in which the activity is dictated by their environment, future strategies could incorporate pre-treatment of therapeutic cells or combination therapy, with agents known to prime Treg function. Alternatively, the responsiveness of Tregs to different cues could be modulated genetically. In the most extreme permutation, synthetic biology could be used to incorporate native signalling pathways known to regulate Treg function into novel regulatory circuits, thus allowing therapeutic Tregs to be controlled via synthetic stimuli. Such synthetic circuits would offer an exclusive line of communication between a clinician and the modified cells. Some very creative synthetic biology constructs have already been applied successfully to putative cellular therapeutic agents[381,392,399], which could be applied to Treg therapy.

Our overexpression studies show how simply the suppressive potency of Tregs can be modulated genetically. However, in the application of genetic engineering or genome editing to therapeutic Tregs, we would endorse an alternative approach, focussing on the conditional induction of Treg activity, rather than simply augmenting constitutive, ubiquitous activity.

In my opinion, the two properties possessed by Tregs that most recommend these cells for application as an immune cell therapy are specificity and tolerogenic potential. Indeed, the principle rationale for developing cellular therapies for immune-mediated pathologies is to impose localised immune

regulation, and perhaps tolerance induction, rather than global immune suppression. Therefore, I would advocate future research focussing on understanding, and ultimately enhancing, these two properties in Tregs.

Without specificity, cell therapies would be subject to the same limitations as pharmacological immunosuppression; namely, increased risk of infection and malignancy. As such, ensuring the specificity of regulatory cells will be critical to realising the theoretical benefits of immune cell therapies. It is thought that, rather than imposing systemic immune suppression, regulatory cells possess a degree of specificity that restricts their activity to a pathological immune response, sparing physiological immunity. The nature of this specificity is not straightforward. Generally, when people refer to the specificity of Tregs, they are alluding to specificity for a cognate antigen, conferred by the TCR. Consistent with this semantic bias, most clinical strategies to enhance Treg function have focussed upon refining the antigen-specificity of these cells. This has been achieved through selection of antigen-reactive cells in culture[267,268,400] and by introducing artificial antigen receptor of the desired antigen-specificity into Tregs [274,277,377,379,401].

Treg suppressive activity can also be restricted or specialised with respect to the anatomical location of their activity, the nature of the immune response that they regulate, or to a particular target cell phenotype. When developing strategies to reduce off-target suppression by therapeutic Tregs, therefore, there is scope to exploit all of these varieties of specificity, not only antigen-specificity.

Abundant evidence is emerging for specialised functions of Tregs resident within different lymphoid and non-lymphoid tissues[185,186,194,221]. Thus, localisation of Tregs might be a mechanism by which function of adoptively-transferred Tregs can be controlled. For example, in mice, exposure to the vitamin A metabolite All Trans Retinoic Acid (ATRA) induces Tregs expressing mucosal-homing receptors CCR9 and $\alpha_4\beta_7$ that tend to home to the intestine[223], where these gut-tropic Tregs promote oral tolerance[402]. Meanwhile, Tregs with a memory-type phenotype are enriched in the skin[194]. Enforcing expression of tissue-homing molecules on the surface of Tregs might help to enrich these cells within a particular target tissue, such that their activity is, to some extent, spatially restricted.

Similarly, different Treg suppressive mechanisms, of which there are a multitude, are adapted to suit different requirements. For example, IL-10 appears to be the predominant mediator exploited by Tregs in the maintenance of tolerance to environmental antigens in the mucosae[403]. For example, in mice, Treg-derived IL-10 was shown to be required for Treg-mediated suppression of skin graft rejection[404], and protection from experimental colitis[235], and for tolerance to leishamania[405]. Yet IL-10 is dispensable for *in vitro* suppression of T cell proliferation[238]. The particular complement of suppressive mechanisms employed by a given Treg is most likely determined by the life history and prevailing microenvironment of that cell. Thus, modulating the predominance of different suppressive mechanisms might be another means to direct the activity of Tregs.

Molecules other than those acting directly as immunoregulatory mediators can be utilised by Tregs to modify their microenvironment in a manner that potentiates Treg-mediated suppression. Such molecules might also be useful targets to modulate the activity of therapeutic Tregs *in vivo*. A recent study illuminated that, by secreting the chemokines CCL3 and CL4, Tregs attract Teffs into close proximity and this proximity is permissive to Treg-mediated suppression of those Teffs[236]. Altering the repertoire of chemokines secreted by therapeutic Tregs might help to target preferentially the cellular subsets that are thought to be the major protagonists in a particular immune pathology.

The second appealing property of Tregs is their ability to propagate their suppressive activity to other cells. One of the major mechanisms by which Tregs discourage the activation of T cells is by impeding the maturation of DCs. Whilst mature DCs prime Teffs and promote immunity, immature DCs exhibit a more tolerogenic phenotype, producing higher levels of anti-inflammatory cytokine and co-inhibitory molecules[406]. Via modulation of DCs and establishment of an anti-inflammatory milieu (including IL-10, TGFβ[407] and adenosine[187]), Tregs can promote expansion of other Tregs and induction of Tregs from Teff precursors in the periphery[408], thereby propagating the tolerogenic microenvironment[409]. By enhancing the ability of Tregs to establish this self-propagating feed-forward mechanism, the effects of a small number of precursor Tregs could be amplified[410].

6.3 Future Directions

During the course of this project, additional avenues of research were disclosed that were intriguing but beyond the scope of this project to explore fully and questions arose that we lacked the time or resources to pursue further. Additionally, some of the methodology, despite refinements during the course of the project, remains imperfect. Whilst this is always frustrating, we feel that identifying the limitations of these studies will provide a substrate for future developments and improvements.

6.3.1 Optimisation of protocols to detect of scarce GFP-expressing cells in lymphoid tissues by flow cytometry

In Chapter 3, none of the methods that we used to detect GFP-expressing cells *in vivo* had the combination of sensitivity and reliability for which we would wish. PCR provided the best sensitivity for detecting the presence of small numbers of GFP-transduced cells *in vivo*. Another benefit of the PCR method is that it is not constrained to detection of fluorescent proteins, but could be applied to detect any genetic marker. However, the richness of the data obtained by PCR does not compare to that of flow cytometry or microscopy, which permit analysis of cell phenotype and absolute quantification of cells. For this reason, flow cytometry would be our preferred method for analysing the infiltration of cells of interest into tissues *in vivo*. Since we could not create a definite positive control for this analysis, we cannot eliminate the possibility that none of the GFP-expressing Tregs that we infused were present in the blood or spleen at the time we analysed these tissues. However, we know that the GFP^+ Tregs are present in these animals

because these cells were detected in skin grafts from the same animals at the same time point [Figure 3.7]. In previous experiments using the same model, $FOXP3^+$ cells were detected in the blood and spleen, so we infer that human Tregs can reside in these tissues. Therefore, most likely our flow cytometric analysis is insufficiently sensitive to detect the GFP signal from these cells, perhaps because this signal is not preserved with sufficient fidelity during the preparation of the tissues.

As a surrogate for a true positive control, GFP-transduced $CD4^+$Teffs could be used. Since we know that human $CD4^+$ Teffs are abundant in the blood and spleen of these mice, failure to detect any GFP^+ Teffs in these samples would confirm that the method is inadequate. Should this be found to be the case, it would be worth investigating alternative tissue preparation protocols that might better preserve GFP. Since the abundance of Tregs in these tissues might also be a limiting factor to successful detection, it would be advisable to acquire a larger portion of the sample.

6.3.2 Examination of developmental origins and phenotypic plasticity of human Tregs in the context of allotransplantation

As mentioned in Chapter 3, one of the main incentives for developing a method of stable fluorescent labelling of human Tregs was to examine the origins of the $FOXP3^+$ cells that we have observed in allografts in the humanised mouse model[306]. It is not known whether these cells are derived from the PBCMs with which these mice were reconstituted, or from the expanded Tregs that were co-injected. Theory suggests that, in an inflammatory context, such as

allotransplantation, induction of peripheral Tregs will account for a large proportion of the Treg pool. In order to ascertain the developmental origin of these cells, we had proposed to immunostain FOXP3 in grafts and lymphoid organs from mice receiving Treg-depleted PBMCs and GFP^+ expanded Tregs. Then, by immunohistochemistry or flow cytometry, we could determine the proportion of $FOXP3^+$ cells that were also GFP^+ (indicating derivation from the expanded Tregs, as opposed to the non-Treg PBCMs). However, cellular permeabilisation, which is required to stain the intracellular protein FOXP3 allows GFP to leach out of the cells, confounding the analysis. We would like to investigate protocols for FOXP3 immunostaining that preserve GFP expression for this purpose. Alternatively, cells could be transduced with a FOXP3 reporter construct, whereby expression of a secondary fluorescent protein was driven by the FOXP3 promoter, to allow analysis of FOXP3 expression without immunostaining.

6.3.3 Exploration of skin-homing properties of human Tregs

Additionally, we would like to use genetic engineering to investigate the homing of Tregs to skin. In a study published in 2012[306], we provided evidence that skin allografts from Treg-treated mice were tolerated upon re-transplantation onto a fresh donor. Deducing that the tolerance was conferred by passenger Tregs residing within the graft, we demonstrated that the infiltration of human Tregs into skin allografts was dependent upon Cutaneous Lymphocyte Antigen (CLA). Labelling of Tregs with GFP would enable us to track the kinetics of graft infiltration by different lymphocyte subsets. Moreover, genetic knockdown would

permit us to probe the molecular requirements for skin-homing in Tregs more deeply, by examining the relative abundance within skin grafts of Tregs deficient for, or over-expressing, candidate skin-homing molecules.

6.3.4 *Intravital* imaging of dynamic cellular behaviour of human Tregs in skin allografts

The ability to visualise GFP^+ human Tregs *intravital* by two-photon microscopy also offers scope for future investigations. Combining this imaging platform with the humanised mouse model, we envisage using this method to analyse dynamic interactions between different cell types in rejecting versus tolerated grafts. With this experimental design in mind, we have successfully transduced Teffs with a dsTomato fluorescent protein, to enable simultaneous imaging of green Tregs and red Teffs.

6.3.5 Genetic engineering of other human lymphocyte subsets

Following on from the protocol for Treg transduction, another avenue that might be fruitful to pursue is genetic engineering of B cells. Preliminary attempts in our lab to transduce cultures of human regulatory B cells (Bregs), using lentivirus, yielded no stably transduced cells. This result was not wholly unexpected, as the *in vitro* culture conditions used for Bregs in our lab do not promote cell proliferation, which appears to aid the transduction process. If we could adapt these cultures conditions to induce even modest B cell proliferation without altering the function of the cells, lentivirus-mediated transduction of these cells might be more feasible.

6.3.6 Application of genome editing for molecular studies in human lymphocytes

With advances in genome editing technologies, many of the applications that we had foreseen for genetic engineering, outlined above, might be accomplished more efficiently using a genome editing platform. Most likely the relative difficulty of transfecting lymphocytes may prove to be restrictive but published studies[411] already bear testament to the feasibility of this approach.

6.3.7 Determination of NRP1 ligands that potentiate human Tregs suppressive function

Our assays, assessing the effect of pre-exposure to NRP1 ligands upon Treg suppressive potency, failed to identify a ligand that would potentiate Treg suppressive function after short-term exposure. Economic considerations prevented us from exploring the effects of long-term exposure to these ligands. One means of overcoming this restriction would be to engineer APCs to express these ligands constitutively and examine the suppressive potency of Tregs co-cultured with these APCs. This approach would have the added advantage of more accurately reproducing physiological presentation of NRP1 ligands, in both soluble and membrane-bound forms. Alternatively, we could attempt to identify the salient NRP1-dependent intracellular signalling pathways in Tregs by examining the suppressive capacity of Tregs in which various NRP1-associated receptors are downregulated. Our attentions were focussed on three candidate NRP1 ligands but there is a plethora of other ligands that might contribute to the NRP1-dependency of Tregs[324,412].

The source of NRP1-dependent ligands is also worth considering. The NRP1-depemdemt suppression that we observed in a humanised mouse model suggests that salient human ligands are present in this model. Assuming that human NRP1 does not cross-react with mouse ligands, either the human haematopoietic compartment or part the skin graft (such as endothelial cells) might be putative sources.

6.3.8 Functional analysis of autophagy in human Tregs

In the study of Neuropilin-1 and its role in human Tregs, we have yet to define the entirety of the mechanism by which NRP1 modulated Treg function. Our results implicate NRP1 in the regulation of two cellular processes that impact upon Treg physiology; namely, cytokine transcription and autophagy. From the wealth of existing evidence for the role of IL-10 as a mediator of immune suppression[386], it is reasonable to conclude that by promoting *IL10* transcription, NRP1 could directly enhance the suppressive capacity of Tregs. Indeed, our results from Chapter 6 demonstrate that increasing the transcription of *IL10* in Tregs is sufficient to improve their suppressive potency. However, so far, the link that we have drawn between Treg function and autophagy is purely associative. Determining the effects of dysregulation in autophagy upon the function of Tregs will be a priority, in order to confirm that NRP1-dependent regulation of autophagy does potentiate Treg function. In particular, we plan to assess whether Treg-mediated suppression is impaired after exposure of Tregs to an autophagy inhibitor, such as chloroquine or Bafilomycin A1, and an autophagy promoter, such as spermidine[413]. If we discover that Treg suppressive function is

impaired by autophagy inhibitors, we would perform the same assay on NRP1KD Tregs, to ascertain whether this effect is NRP1-dependent.

6.3.9 Enforced expression of tissue-homing proteins to re-direct Tregs to grafted tissue

By over-expressing molecular mediators of Treg-mediated suppression, in this case IL-10, we have demonstrated that the suppressive potency of Tregs can be enhanced, via a protocol that is compatible with *in vitro* expansion. Whilst enhancing suppressive potency is desirable in Tregs destined for cellular therapy applications, this approach might be better applied to a more pressing concern within the field of immune cell therapy, namely specificity. The idea of conferring antigen-specificity upon therapeutic Tregs is already the subject of advanced research. However, there is strong evidence to suggest that the target specificity of Tregs is not exclusively a property of their antigen specificity. Rather, the targeting of Treg-mediated suppression depends upon the activation state of the Tregs and the proximity of potential target cells coincident with activation of the Treg. Thus, Treg-mediated suppression might be favoured at a particular site if the adoptively-transferred Tregs were re-directed to home to, infiltrate into, and be retained within that tissue. Additionally, if Tregs bore specificity for tissue-specific ligands, this might encourage activation of these Tregs exclusively within the target tissue, thereby conferring some spatial restriction of their suppressive activity. One appealing strategy is enforced expression of tissue-homing receptors, such as chemokine receptors. The tissue-tropism of these Tregs could then be assessed by adoptively-transferring them into a humanised mouse model. Enumerating the infiltration of these cells in the graft and in the lymphoid

tissue, could reveal whether this engineering approach results in enrichment of tissue-homing Tregs, and particularly activated Tregs, in the graft. Furthermore, by measuring the duration of graft survival in mice receiving tissue-homing Tregs versus un-modified Tregs, we could examine whether such a strategy might be used to improve the efficacy of Treg therapies.

Publications

Book Chapters

Milward, K., Hester, J & Wood, K.J. *Isolation of Human Regulatory T Cells by Fluorescence-Activated Cell Sorting*. Methods in Molecular Biology. Ed. Ashleigh Boyd. {Reviewed and pending publication 2016}.

Abstracts

Milward, K., Hester, J, Issa, F. & Wood, K.J. *Neuropilin-1 is Required for Suppressive Function in Human Regulatory T Cells*. British Society of Immunology & Annual Congress, Liverpool, UK. Oral Presentation.

Milward, K., Hester, J, & Wood, K.J. *Genetic Engineering of Human Regulatory T Cells* European Cooperation in Science & Technology AFACTT Workshop 2016, London, U.K. Oral presentation.

Milward, K., Hester, J, Issa, F. & Wood, K.J. *Insights into Treg Biology from Genetic Engineering of Primary Human Tregs.* British Society of Immunology Advanced Cell Therapy Symposium 2016, London, U.K. Oral presentation.

Milward, K., Hester, J & Wood, K.J. *Neuroplin-1 is Required to Sustain the Anti-Inflammatory Cytokine Profile of Human Regulatory T Cells*. British Heart Foundation Fellows' Meeting 2015, Cambridge, UK. Poster.

Milward, K., Issa, F., Hester, J & Wood, K.J. *Humanised Mouse Models to Study Human Tregs*. UK Humanised Mouse Symposium 2015, Cambridge, UK. Oral presentation.

Milward, K., Hester, J & Wood, K.J. *Neuropilin-1 is Required in Human Regulatory T Cells for Optimal Suppressive Function*. British Society of Immunology Oxford Immunology Group Meeting, Oxford, UK. Poster.

Milward, K., Hester, J & Wood, K.J. *The Role of Neuropilin-1 in Human Regulatory T Cells*. British Transplantation Society Annual Congress 2015, Bournemouth, UK. Oral presentation

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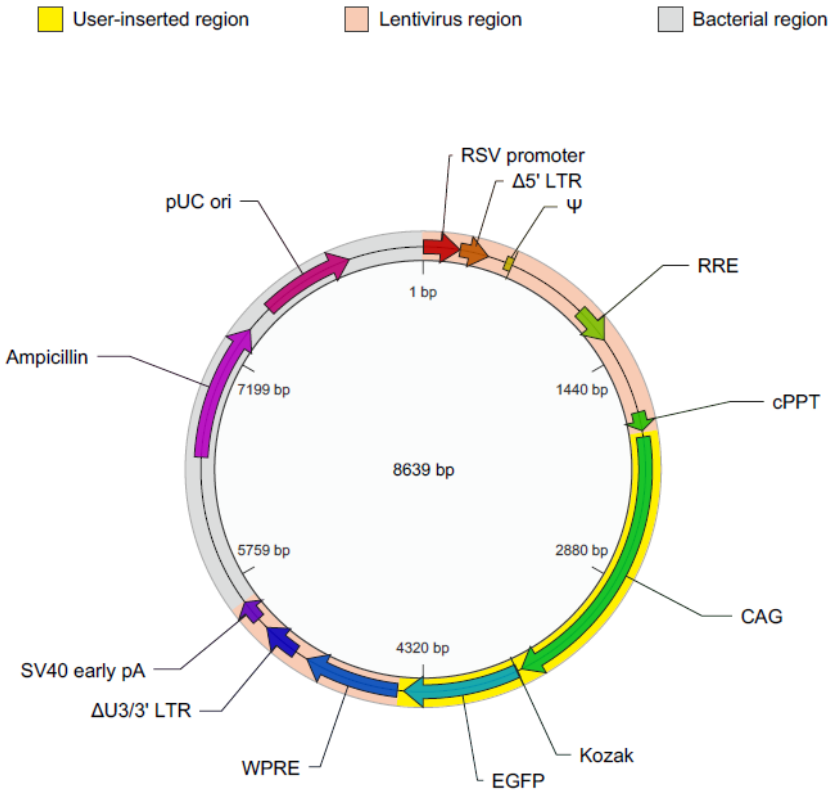
Appendices

Appendix 1:: plasmid map CAG-GFP (provided by Cyagen Biosciences)



Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTR	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
CAG	1959-3691	CAG	Component entered by user
Kozak	3716-3721	Kozak	Component entered by user
EGFP	3722-4441	EGFP	Component entered by user
WPRE	4480-5077	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
ΔU3/3' LTR	5159-5393	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	5466-5600	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	6554-7414	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	7585-8173	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

Appendix 2: sequence CAG-GFP (provided by Cyagen Biosciences)

```
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 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
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 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601 ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
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 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
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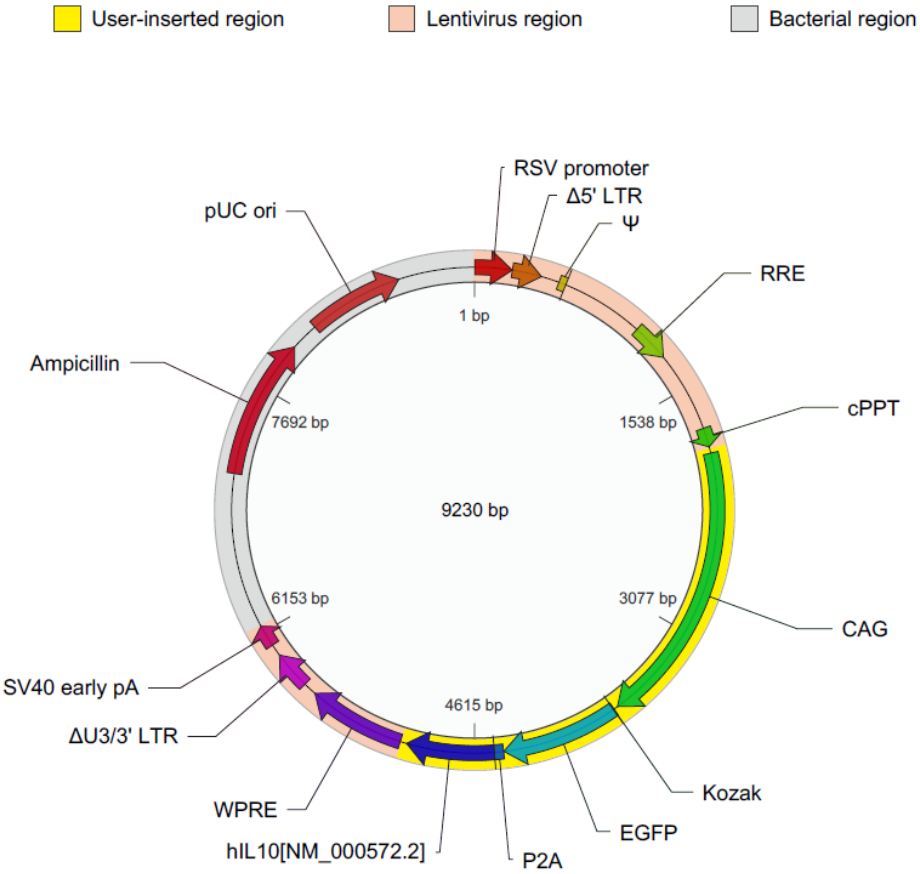
Appendix 3: plasmid map CAG-IL10 (provided by Cyagen Biosciences)



Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTR	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
CAG	1959-3691	CAG	Component entered by user
Kozak	3716-3721	Kozak	Component entered by user
EGFP	3722-4438	EGFP	Component entered by user
P2A	4439-4495	P2A	Component entered by user
hIL10[NM_000572.2]	4496-5032	hIL10[NM_000572.2]	Component entered by user
WPRE	5071-5668	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
ΔU3/3' LTR	5750-5984	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	6057-6191	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	7145-8005	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	8176-8764	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

Note: (c) denotes complementary strand.

Appendix 4: plasmid sequence CAG-IL10 (provided by Cyagen Biosciences)

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   1 AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61 TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601 ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661 AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721 GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841 ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901 AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961 CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021 ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081 TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141 GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201 GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261 GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
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1381 ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
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1681 TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741 TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801 GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861 ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
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1981 TTATTAATAG TAATCAATTA CGGGGTCATT AGTTCATAGC CCATATATGG AGTTCCGCGT
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2101 GTCAATAATG ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG
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2221 TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGCC TGGCATTATG CCCAGTACAT
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2701 GTTACTCCCA CAGGTGAGCG GGCGGGACGG CCCTTCTCCT CCGGGCTGTA ATTAGCGCTT
2761 GGTTTAATGA CGGCTTGTTT CTTTTCTGTG GCTGCGTGAA AGCCTTGAGG GGCTCCGGGA
2821 GGGCCCTTTG TGCGGGGGGA GCGGCTCGGG GGGTGCGTGC GTGTGTGTGT GCGTGGGGAG
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3121  GCACGGCCCG GCTTCGGGTG CGGGGCTCCG TACGGGGCGT GGCGCGGGGC TCGCCGTGCC
3181  GGGCGGGGGG TGGCGGCAGG TGGGGGTGCC GGGCGGGGCG GGGCCGCCTC GGGCCGGGGA
3241  GGGCTCGGGG GAGGGGCGCG GCGGCCCCCG GAGCGCCGGC GGCTGTCGAG GCGCGGCGAG
3301  CCGCAGCCAT TGCCTTTTAT GGTAATCGTG CGAGAGGGCG CAGGGACTTC CTTTGTCCCA
3361  AATCTGTGCG GAGCCGAAAT CTGGGAGGCG CCGCCGCACC CCCTCTAGCG GGCGCGGGGC
3421  GAAGCGGTGC GGCGCCGGCA GGAAGGAAAT GGGCGGGGAG GGCCTTCGTG CGTCGCCGCG
3481  CCGCCGTCCC CTTCTCCCTC TCCAGCCTCG GGGCTGTCCG CGGGGGGACG GCTGCCTTCG
3541  GGGGGGACGG GGCAGGGCGG GGTTCGGCTT CTGGCGTGTG ACCGGCGGCT CTAGAGCCTC
3601  TGCTAACCAT GTTCATGCCT TCTTCTTTTT CCTACAGCTC CTGGGCAACG TGCTGGTTAT
3661  TGTGCTGTCT CATCATTTTG GCAAAGAATT GCAAGTTTGT ACAAAAAAGC AGGCTGCCAC
3721  CATGGTGAGC AAGGGCGAGG AGCTGTTCAC CGGGGTGGTG CCCATCCTGG TCGAGCTGGA
3781  CGGCGACGTA AACGGCCACA AGTTCAGCGT GTCCGGCGAG GGCGAGGGCG ATGCCACCTA
3841  CGGCAAGCTG ACCCTGAAGT TCATCTGCAC CACCGGCAAG CTGCCCGTGC CCTGGCCCAC
3901  CCTCGTGACC ACCCTGACCT ACGGCGTGCA GTGCTTCAGC CGCTACCCCG ACCACATGAA
3961  GCAGCACGAC TTCTTCAAGT CCGCCATGCC CGAAGGCTAC GTCCAGGAGC GCACCATCTT
4021  CTTCAAGGAC GACGGCAACT ACAAGACCCG CGCCGAGGTG AAGTTCGAGG GCGACACCCT
4081  GGTGAACCGC ATCGAGCTGA AGGGCATCGA CTTCAAGGAG GACGGCAACA TCCTGGGGCA
4141  CAAGCTGGAG TACAACTACA ACAGCCACAA CGTCTATATC ATGGCCGACA AGCAGAAGAA
4201  CGGCATCAAG GTGAACTTCA AGATCCGCCA CAACATCGAG GACGGCAGCG TGCAGCTCGC
4261  CGACCACTAC CAGCAGAACA CCCCCATCGG CGACGGCCCC GTGCTGCTGC CCGACAACCA
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4381  CCTGCTGGAG TTCGTGACCG CCGCCGGGAT CACTCTCGGC ATGGACGAGC TGTACAAGGC
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4561  GGGCACCCAG TCTGAGAACA GCTGCACCCA CTTCCCAGGC AACCTGCCTA ACATGCTTCG
4621  AGATCTCCGA GATGCCTTCA GCAGAGTGAA GACTTTCTTT CAAATGAAGG ATCAGCTGGA
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4861  GCTACGGCGC TGTCATCGAT TTCTTCCCTG TGAAAACAAG AGCAAGGCCG TGGAGCAGGT
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5161  TTTAATGCCT TTGTATCATG CTATTGCTTC CCGTATGGCT TTCATTTTCT CCTCCTTGTA
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5281  GGTGTGCACT GTGTTTGCTG ACGCAACCCC CACTGGTTGG GGCATTGCCA CCACCTGTCA
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5461  GTCGGGGAAG CTGACGTCCT TTCCATGGCT GCTCGCCTGT GTTGCCACCT GGATTCTGCG
5521  CGGGACGTCC TTCTGCTACG TCCCTTCGGC CCTCAATCCA GCGGACCTTC CTTCCCGCGG
5581  CCTGCTGCCG GCTCTGCGGC CTCTTCCGCG TCTTCGCCTT CGCCCTCAGA CGAGTCGGAT
5641  CTCCCTTTGG GCCGCCTCCC CGCATCGGGA ATTCCCGCGG TTCGCTTTAA GACCAATGAC
5701  TTACAAGGCA GCTGTAGATC TTAGCCACTT TTTAAAAGAA AAGGGGGGAC TGGAAGGGCT
5761  AATTCACTCC CAACGAAGAC AAGATCTGCT TTTTGCTTGT ACTGGGTCTC TCTGGTTAGA
5821  CCAGATCTGA GCCTGGGAGC TCTCTGGCTA ACTAGGGAAC CCACTGCTTA AGCCTCAATA
5881  AAGCTTGCCT TGAGTGCTTC AAGTAGTGTG TGCCCGTCTG TTGTGTGACT CTGGTAACTA
5941  GAGATCCCTC AGACCCTTTT AGTCAGTGTG GAAAATCTCT AGCAGTAGTA GTTCATGTCA
6001  TCTTATTATT CAGTATTTAT AACTTGCAAA GAAATGAATA TCAGAGAGTG AGAGGAACTT
6061  GTTTATTGCA GCTTATAATG GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA
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6121  AGCATTTTTT TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA
6181  TGTCTGGCTC TAGCTATCCC GCCCCTAACT CCGCCCATCC CGCCCCTAAC TCCGCCCAGT
6241  TCCGCCCATT CTCCGCCCCA TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC
6301  GCCTCGGCCT CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGGACG
6361  TACCCAATTC GCCCTATAGT GAGTCGTATT ACGCGCGCTC ACTGGCCGTC GTTTTACAAC
6421  GTCGTGACTG GGAAAACCCT GGCGTTACCC AACTTAATCG CCTTGCAGCA CATCCCCCTT
6481  TCGCCAGCTG GCGTAATAGC GAAGAGGCCC GCACCGATCG CCCTTCCCAA CAGTTGCGCA
6541  GCCTGAATGG CGAATGGGAC GCGCCCTGTA GCGGCGCATT AAGCGCGGCG GGTGTGGTGG
6601  TTACGCGCAG CGTGACCGCT ACACTTGCCA GCGCCCTAGC GCCCGCTCCT TTCGCTTTCT
6661  TCCCTTCCTT TCTCGCCACG TTCGCCGGCT TTCCCCGTCA AGCTCTAAAT CGGGGGCTCC
6721  CTTTAGGGTT CCGATTTAGT GCTTTACGGC ACCTCGACCC CAAAAAACTT GATTAGGGTG
6781  ATGGTTCACG TAGTGGGCCA TCGCCCTGAT AGACGGTTTT TCGCCCTTTG ACGTTGGAGT
6841  CCACGTTCTT TAATAGTGGA CTCTTGTTCC AAACTGGAAC AACACTCAAC CCTATCTCGG
6901  TCTATTCTTT TGATTTATAA GGGATTTTGC CGATTTCGGC CTATTGGTTA AAAAATGAGC
6961  TGATTTAACA AAAATTTAAC GCGAATTTTA ACAAAATATT AACGCTTACA ATTTAGGTGG
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7081  TATGTATCCG CTCATGAGAC AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA
7141  GAGTATGAGT ATTCAACATT TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT
7201  TCCTGTTTTT GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG
7261  TGCACGAGTG GGTTACATCG AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTTCG
7321  CCCCGAAGAA CGTTTTCCAA TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT
7381  ATCCCGTATT GACGCCGGGC AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA
7441  CTTGGTTGAG TACTCACCAG TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA
7501  ATTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC
7561  GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG
7621  CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC
7681  GATGCCTGTA GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT
7741  AGCTTCCCGG CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT
7801  GCGCTCGGCC CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG
7861  GTCTCGCGGT ATCATTGCAG CACTGGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT
7921  CTACACGACG GGGAGTCAGG CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG
7981  TGCCTCACTG ATTAAGCATT GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT
8041  TGATTTAAAA CTTCATTTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT
8101  CATGACCAAA ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA
8161  GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA
8221  AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA GAGCTACCAA CTCTTTTTCC
8281  GAAGGTAACT GGCTTCAGCA GAGCGCAGAT ACCAAATACT GTTCTTCTAG TGTAGCCGTA
8341  GTTAGGCCAC CACTTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT
8401  GTTACCAGTG GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG
8461  ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG
8521  CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAAGCGC
8581  CACGCTTCCC GAAGGGAGAA AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG
8641  AGAGCGCACG AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT
8701  TCGCCACCTC TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG
8761  GAAAAACGCC AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC CTTTTGCTCA
8821  CATGTTCTTT CCTGCGTTAT CCCCTGATTC TGTGGATAAC CGTATTACCG CCTTTGAGTG
8881  AGCTGATACC GCTCGCCGCA GCCGAACGAC CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC
8941  GGAAGAGCGC CCAATACGCA AACCGCCTCT CCCCGCGCGT TGGCCGATTC ATTAATGCAG
9001  CTGGCACGAC AGGTTTCCCG ACTGGAAAGC GGGCAGTGAG CGCAACGCAA TTAATGTGAG
9061  TTAGCTCACT CATTAGGCAC CCCAGGCTTT ACACTTTATG CTTCCGGCTC GTATGTTGTG
9121  TGGAATTGTG AGCGGATAAC AATTTCACAC AGGAAACAGC TATGACCATG ATTACGCCAA
9181  GCGCGCAATT AACCCTCACT AAAGGGAACA AAAGCTGGAG CTGCAAGCTT
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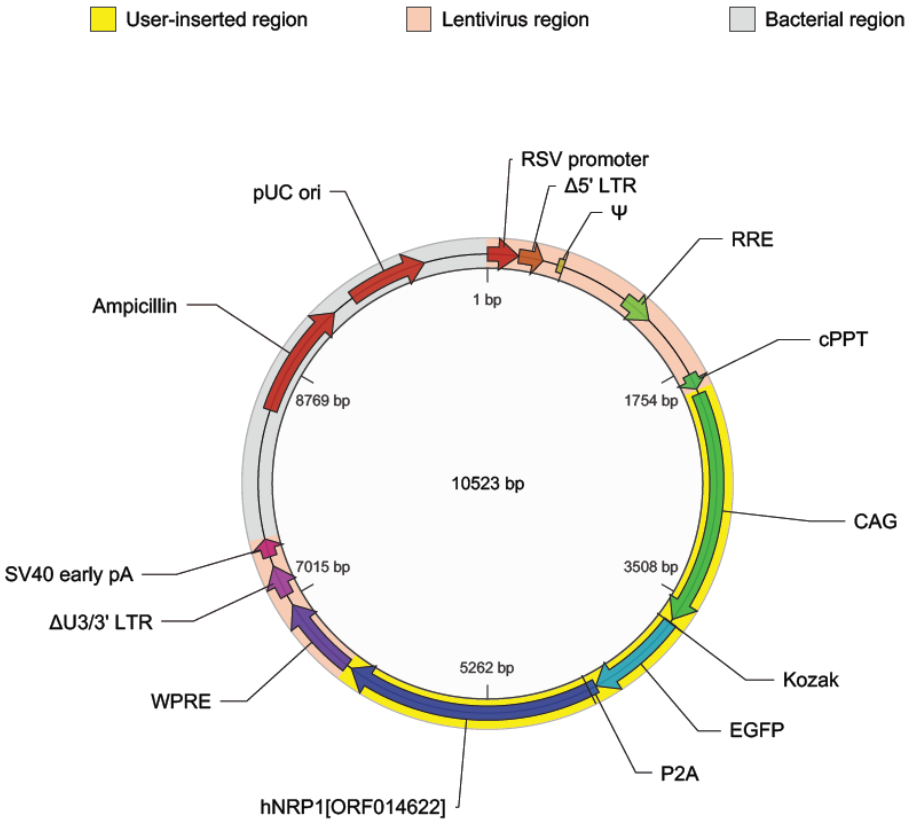
Appendix 5: plasmid map CAG-NRP1 (provided by Cyagen Biosciences)



Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTR	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
CAG	1959-3691	CAG	Component entered by user
Kozak	3716-3721	Kozak	Component entered by user
EGFP	3722-4438	EGFP	Component entered by user
P2A	4439-4495	P2A	Component entered by user
hNRP1[ORF014622]	4496-6325	hNRP1[ORF014622]	Component entered by user
WPRE	6364-6961	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
ΔU3/3' LTR	7043-7277	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	7350-7484	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	8438-9298	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	9469-10057	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

Appendix 6: plasmid sequence CAG-NRP1 (provided by Cyagen Biosciences)

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   1 AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61 TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
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 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
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 661 AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
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 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
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1021 ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
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1321 TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381 ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441 TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
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1561 TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621 AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681 TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741 TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801 GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861 ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921 ACTAGTGATT ATCGGATCAA CTTTGTATAG AAAAGTTGCT CGACATTGAT TATTGACTAG
1981 TTATTAATAG TAATCAATTA CGGGGTCATT AGTTCATAGC CCATATATGG AGTTCCGCGT
2041 TACATAACTT ACGGTAAATG GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC
2101 GTCAATAATG ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG
2161 GGTGGAGTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT CAAGTGTATC ATATGCCAAG
2221 TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGCC TGGCATTATG CCCAGTACAT
2281 GACCTTATGG GACTTTCCTA CTTGGCAGTA CATCTACGTA TTAGTCATCG CTATTACCAT
2341 GGTCGAGGTG AGCCCCACGT TCTGCTTCAC TCTCCCCATC TCCCCCCCCT CCCCACCCCC
2401 AATTTTGTAT TTATTTATTT TTTAATTATT TTGTGCAGCG ATGGGGGCGG GGGGGGGGGG
2461 GGGGCGCGCG CCAGGCGGGG CGGGGCGGGG CGAGGGGCGG GGCGGGGCGA GGCGGAGAGG
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2641 TCGCCCCGTG CCCCGCTCCG CCGCCGCCTC GCGCCGCCCG CCCCGGCTCT GACTGACCGC
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2821 GGGCCCTTTG TGCGGGGGGA GCGGCTCGGG GGGTGCGTGC GTGTGTGTGT GCGTGGGGAG
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2941 TTGTGCGCTC CGCAGTGTGC GCGAGGGGAG CGCGGCCGGG GGCGGTGCCC CGCGGTGCGG
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3061  GGGTGTGGGC GCGTCGGTCG GGCTGCAACC CCCCCTGCAC CCCCCTCCCC GAGTTGCTGA
3121  GCACGGCCCG GCTTCGGGTG CGGGGCTCCG TACGGGGCGT GGCGCGGGGC TCGCCGTGCC
3181  GGGCGGGGGG TGGCGGCAGG TGGGGGTGCC GGGCGGGGCG GGGCCGCCTC GGGCCGGGGA
3241  GGGCTCGGGG GAGGGGCGCG GCGGCCCCCG GAGCGCCGGC GGCTGTCGAG GCGCGGCGAG
3301  CCGCAGCCAT TGCCTTTTAT GGTAATCGTG CGAGAGGGCG CAGGGACTTC CTTTGTCCCA
3361  AATCTGTGCG GAGCCGAAAT CTGGGAGGCG CCGCCGCACC CCCTCTAGCG GGCGCGGGGC
3421  GAAGCGGTGC GGCGCCGGCA GGAAGGAAAT GGGCGGGGAG GGCCTTCGTG CGTCGCCGCG
3481  CCGCCGTCCC CTTCTCCCTC TCCAGCCTCG GGGCTGTCCG CGGGGGGACG GCTGCCTTCG
3541  GGGGGGACGG GGCAGGGCGG GGTTCGGCTT CTGGCGTGTG ACCGGCGGCT CTAGAGCCTC
3601  TGCTAACCAT GTTCATGCCT TCTTCTTTTT CCTACAGCTC CTGGGCAACG TGCTGGTTAT
3661  TGTGCTGTCT CATCATTTTG GCAAAGAATT GCAAGTTTGT ACAAAAAAGC AGGCTGCCAC
3721  CATGGTGAGC AAGGGCGAGG AGCTGTTCAC CGGGGTGGTG CCCATCCTGG TCGAGCTGGA
3781  CGGCGACGTA AACGGCCACA AGTTCAGCGT GTCCGGCGAG GGCGAGGGCG ATGCCACCTA
3841  CGGCAAGCTG ACCCTGAAGT TCATCTGCAC CACCGGCAAG CTGCCCGTGC CCTGGCCCAC
3901  CCTCGTGACC ACCCTGACCT ACGGCGTGCA GTGCTTCAGC CGCTACCCCG ACCACATGAA
3961  GCAGCACGAC TTCTTCAAGT CCGCCATGCC CGAAGGCTAC GTCCAGGAGC GCACCATCTT
4021  CTTCAAGGAC GACGGCAACT ACAAGACCCG CGCCGAGGTG AAGTTCGAGG GCGACACCCT
4081  GGTGAACCGC ATCGAGCTGA AGGGCATCGA CTTCAAGGAG GACGGCAACA TCCTGGGGCA
4141  CAAGCTGGAG TACAACTACA ACAGCCACAA CGTCTATATC ATGGCCGACA AGCAGAAGAA
4201  CGGCATCAAG GTGAACTTCA AGATCCGCCA CAACATCGAG GACGGCAGCG TGCAGCTCGC
4261  CGACCACTAC CAGCAGAACA CCCCCATCGG CGACGGCCCC GTGCTGCTGC CCGACAACCA
4321  CTACCTGAGC ACCCAGTCCG CCCTGAGCAA AGACCCCAAC GAGAAGCGCG ATCACATGGT
4381  CCTGCTGGAG TTCGTGACCG CCGCCGGGAT CACTCTCGGC ATGGACGAGC TGTACAAGGC
4441  CACGAACTTC TCTCTGTTAA AGCAAGCAGG AGATGTTGAA GAAAACCCCG GGCCTATGGA
4501  GAGGGGGCTG CCGCTCCTCT GCGCCGTGCT CGCCCTCGTC CTCGCCCCGG CCGGCGCTTT
4561  TCGCAACGAT AAATGTGGCG ATACTATAAA AATTGAAAGC CCCGGGTACC TTACATCTCC
4621  TGGTTATCCT CATTCTTATC ACCCAAGTGA AAAATGCGAA TGGCTGATTC AGGCTCCGGA
4681  CCCATACCAG AGAATTATGA TCAACTTCAA CCCTCACTTC GATTTGGAGG ACAGAGACTG
4741  CAAGTATGAC TACGTGGAAG TCTTCGATGG AGAAAATGAA AATGGACATT TTAGGGGAAA
4801  GTTCTGTGGA AAGATAGCCC CTCCTCCTGT TGTGTCTTCA GGGCCATTTC TTTTTATCAA
4861  ATTTGTCTCT GACTACGAAA CACATGGTGC AGGATTTTCC ATACGTTATG AAATTTTCAA
4921  GAGAGGTCCT GAATGTTCCC AGAACTACAC AACACCTAGT GGAGTGATAA AGTCCCCCGG
4981  ATTCCCTGAA AAATATCCCA ACAGCCTTGA ATGCACTTAT ATTGTCTTTG CGCCAAAGAT
5041  GTCAGAGATT ATCCTGGAAT TTGAAAGCTT TGACCTGGAG CCTGACTCAA ATCCTCCAGG
5101  GGGGATGTTC TGTCGCTACG ACCGGCTAGA AATCTGGGAT GGATTCCCTG ATGTTGGCCC
5161  TCACATTGGG CGTTACTGTG GACAGAAAAC ACCAGGTCGA ATCCGATCCT CATCGGGCAT
5221  TCTCTCCATG GTTTTTTACA CCGACAGCGC GATAGCAAAA GAAGGTTTCT CAGCAAACTA
5281  CAGTGTCTTG CAGAGCAGTG TCTCAGAAGA TTTCAAATGT ATGGAAGCTC TGGGCATGGA
5341  ATCAGGAGAA ATTCATTCTG ACCAGATCAC AGCTTCTTCC CAGTATAGCA CCAACTGGTC
5401  TGCAGAGCGC TCCCGCCTGA ACTACCCTGA GAATGGGTGG ACTCCCGGAG AGGATTCCTA
5461  CCGAGAGTGG ATACAGGTAG ACTTGGGCCT TCTGCGCTTT GTCACGGCTG TCGGGACACA
5521  GGGCGCCATT TCAAAAGAAA CCAAGAAGAA ATATTATGTC AAGACTTACA AGATCGACGT
5581  TAGCTCCAAC GGGGAAGACT GGATCACCAT AAAAGAAGGA AACAAACCTG TTCTCTTTCA
5641  GGGAAACACC AACCCCACAG ATGTTGTGGT TGCAGTATTC CCCAAACCAC TGATAACTCG
5701  ATTTGTCCGA ATCAAGCCTG CAACTTGGGA AACTGGCATA TCTATGAGAT TTGAAGTATA
5761  CGGTTGCAAG ATAACAGATT ATCCTTGCTC TGGAATGTTG GGTATGGTGT CTGGACTTAT
5821  TTCTGACTCC CAGATCACAT CATCCAACCA AGGGGACAGA AACTGGATGC CTGAAAACAT
5881  CCGCCTGGTA ACCAGTCGCT CTGGCTGGGC ACTTCCACCC GCACCTCATT CCTACATCAA
5941  TGAGTGGCTC CAAATAGACC TGGGGGAGGA GAAGATCGTG AGGGGCATCA TCATTCAGGG
6001  TGGGAAGCAC CGAGAGAACA AGGTGTTCAT GAGGAAGTTC AAGATCGGGT ACAGCAACAA
6061  CGGCTCGGAC TGGAAGATGA TCATGGATGA CAGCAAACGC AAGGCGAAGT CTTTTGAGGG
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6121  CAACAACAAC TATGATACAC CTGAGCTGCG GACTTTTCCA GCTCTCTCCA CGCGATTCAT
6181  CAGGATCTAC CCCGAGAGAG CCACTCATGG CGGACTGGGG CTCAGAATGG AGCTGCTGGG
6241  CTGTGAAGTG GAAGGTGGCA CCACTGTGCT GGCCACAGAA AAGCCCACGG TCATAGACAG
6301  CACCATACAA TCAGGTATCA AATAAACCCA GCTTTCTTGT ACAAAGTGGT GATAATCGAA
6361  TTCCGATAAT CAACCTCTGG ATTACAAAAT TTGTGAAAGA TTGACTGGTA TTCTTAACTA
6421  TGTTGCTCCT TTTACGCTAT GTGGATACGC TGCTTTAATG CCTTTGTATC ATGCTATTGC
6481  TTCCCGTATG GCTTTCATTT TCTCCTCCTT GTATAAATCC TGGTTGCTGT CTCTTTATGA
6541  GGAGTTGTGG CCCGTTGTCA GGCAACGTGG CGTGGTGTGC ACTGTGTTTG CTGACGCAAC
6601  CCCCACTGGT TGGGGCATTG CCACCACCTG TCAGCTCCTT TCCGGGACTT TCGCTTTCCC
6661  CCTCCCTATT GCCACGGCGG AACTCATCGC CGCCTGCCTT GCCCGCTGCT GGACAGGGGC
6721  TCGGCTGTTG GGCACTGACA ATTCCGTGGT GTTGTCGGGG AAGCTGACGT CCTTTCCATG
6781  GCTGCTCGCC TGTGTTGCCA CCTGGATTCT GCGCGGGACG TCCTTCTGCT ACGTCCCTTC
6841  GGCCCTCAAT CCAGCGGACC TTCCTTCCCG CGGCCTGCTG CCGGCTCTGC GGCCTCTTCC
6901  GCGTCTTCGC CTTCGCCCTC AGACGAGTCG GATCTCCCTT TGGGCCGCCT CCCCGCATCG
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7021  CTTTTTAAAA GAAAAGGGGG GACTGGAAGG GCTAATTCAC TCCCAACGAA GACAAGATCT
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7201  GTGTGCCCGT CTGTTGTGTG ACTCTGGTAA CTAGAGATCC CTCAGACCCT TTTAGTCAGT
7261  GTGGAAAATC TCTAGCAGTA GTAGTTCATG TCATCTTATT ATTCAGTATT TATAACTTGC
7321  AAAGAAATGA ATATCAGAGA GTGAGAGGAA CTTGTTTATT GCAGCTTATA ATGGTTACAA
7381  ATAAAGCAAT AGCATCACAA ATTTCACAAA TAAAGCATTT TTTTCACTGC ATTCTAGTTG
7441  TGGTTTGTCC AAACTCATCA ATGTATCTTA TCATGTCTGG CTCTAGCTAT CCCGCCCCTA
7501  ACTCCGCCCA TCCCGCCCCT AACTCCGCCC AGTTCCGCCC ATTCTCCGCC CCATGGCTGA
7561  CTAATTTTTT TTATTTATGC AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG
7621  TAGTGAGGAG GCTTTTTTGG AGGCCTAGGG ACGTACCCAA TTCGCCCTAT AGTGAGTCGT
7681  ATTACGCGCG CTCACTGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC CCTGGCGTTA
7741  CCCAACTTAA TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG
7801  CCCGCACCGA TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG GACGCGCCCT
7861  GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG
7921  CCAGCGCCCT AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG
7981  GCTTTCCCCG TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC
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8101  GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTTAATAGT GGACTCTTGT
8161  TCCAAACTGG AACAACACTC AACCCTATCT CGGTCTATTC TTTTGATTTA TAAGGGATTT
8221  TGCCGATTTC GGCCTATTGG TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT
8281  TTAACAAAAT ATTAACGCTT ACAATTTAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC
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8401  CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT
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8641  CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA
8701  ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA
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9121  TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG
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