

EXAMINING A ROLE FOR HISTONE
DEACETYLASE INHIBITORS AS
IMMUNOSUPPRESSANTS IN ORGAN
TRANSPLANTATION

by

Jonathan Ellis

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Department of Infection & Immunity
School of Medicine
University of Birmingham Medical School

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Abstract

Current transplant immunosuppression regimens have numerous limitations. Recent evidence suggests histone deacetylase inhibitors (HDACis) may represent a class of drug with immunosuppressive properties. This study initially assessed a panel of experimental and established HDACis and identified a novel HDAC6-specific inhibitor (KA1010) to compare with cyclosporin (CyA) with the pan-HDACi suberoylanilide hydroxamic acid (SAHA) further in models of alloreactivity.

Proliferation and MLR-based assays were used to determine the immunosuppressive effect of compounds, and a murine model of allogeneic skin transplantation was adopted to assess the in vivo effects of HDAC6 inhibition.

KA1010 displayed superior inhibitory effects on the activation of peripheral mononuclear cells using in vitro models of transplantation. In a one-way MLR, KA1010 (5 μ M) reduced parent cell proliferation from 92% to 64% ($p=0.001$). A two-way MLR, adopting IFN- γ production as a marker of alloresponse, resulted in up to 91% reduction. Dose-response curves revealed dose-dependent profiles with a greater potency of HDACis over CyA (IC₅₀ values of 82.0nM and 13.4nM for KA1010 and SAHA).

Mice treated with KA1010 displayed no significant features of skin allograft rejection upon histological analysis at 70 days and graft survival of 80% in subjects treated with 160mg/kg. Immunological assessment, revealed a significant increase in CD4+CD25+FoxP3+ regulatory T cells (from 18% to 25%, $p=0.0002$) and a corresponding reduction in CD4+ T cells (from 58% to 42%, $p=0.0009$).

HDAC6 may represent an optimal target for future immunosuppressant therapeutics with a particular role in transplantation. In this thesis, we have demonstrated a superior immunosuppressive effect of KA1010 over both CyA and SAHA, in the models of allotransplantation adopted.

The salient findings in this study have been collated in an article paper which has been accepted for publication in the peer-reviewed journal, Transplantation (see Appendix 1).

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Abbreviations

ACTH	adrenocorticotrophic hormone
ADCC	activation and antibody-dependent cellular cytotoxicity
APC	antigen-presenting cell
AR	acute rejection
ATP	adenosine triphosphate
cAMP	cyclic AMP
CFSE	carboxyfluorescein diacetate succinimidyl ester
ConA	concanavalin A
CR	chronic rejection
CTCL	cutaneous T-cell lymphoma
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic lymphocyte antigen-4
CyA	cyclosporin A
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum

GVHD	graft-versus-host disease
HAR	hyperacute rejection
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HLA	human leucocyte antigen
HSF-1	heat shock factor protein 1
HSP	heat shock proteins
IDO	indoleamine 2,3-dioxygenase
IFN- γ	interferon-gamma
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
LCA	leucocyte common antigen
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MACS	magnetic-activated cell sorting
MAPK	mitogen-associated protein kinase
MeTIMP	methyl-thioinosine monophosphate

MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NES	nuclear exportation signal
NFAT	nuclear factor of activated T-cells
NF-Kb	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
PHA	phytohaemagglutinin
PKC	protein kinase C
PMA	phorbol myristate acetate
PBMC	peripheral blood mononuclear cell
PI	propidium iodide
PS	phosphatidylserine
RPMI	Roswell Park Memorial Institute
SAHA	suberoylanilide hydroxamic acid
STAT-3	signal transducer and activator of transcription 3
TCR	T cell receptor
TGF- β	transforming growth factor beta
TGTP	thioguanosine triphosphate
TLR-2	toll-like receptor 2

TNF- α	tumour necrosis factor-alpha
TSA	trichostatin
UBP	ubiquitin binding protein
VSA	sodium valproate
ZAP-70	zeta-associated protein of 70 kDa
6-MP	6-mercaptopurine
7-AAD	7-aminoactinomycin D

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1 Introduction

1.1 Historical Background

In 1902, Emerich Ullman performed the first reported successful experimental transplantation of a kidney between dogs (Ullman 1914). The graft survived 5 days. Subsequent attempted xenografts proved unsuccessful, and Ullman abandoned further renal transplant research.

In 1912, Alexis Carrel, ‘the father of transplantation’, was awarded the Nobel Prize in Physiology or Medicine. He pioneered surgical techniques, particularly in vascular anastomosis that are still in common practice today. He also demonstrated techniques for preserving organs and blood vessels and later developed an early perfusion pump. He famously went on to say in 1914 “the technical problems of transplantation were essentially solved, but until some method was developed to prevent the reaction of the organism to the foreign tissue, there would be no clinical application of organ transplantation” (Morris 2004). Following Carrels’ work, a number of experimental transplants were attempted but failed, primarily due to graft rejection.

In 1933, the Ukrainian surgeon, Professor Yurii Voronoy performed the first human to human cadaveric transplant (Matevossian et al. 2009). He grafted a kidney from a 60-year-old deceased brain trauma male (Blood Group B) into a 26-year-old female (Blood Group O). The recipient was in end-stage renal failure as a result of mercury intoxication, and despite the ABO blood group incompatibility, produced a urine output of 5mls/hr up to the second post-operative day. The recipient subsequently died on the fourth post-operative day, though there was no vascular thrombosis of the graft vessels.



**Figure 1-1 Implantation in the groin of the first homotransplantation performed by
Voronoy in 1933**

During the Second World War, a zoologist named Peter Medawar was studying burn and wound healing. He recognised that skin grafts only worked when the graft came from the recipient and researched extensively the mechanisms of transplant rejection. His work would represent the start of an evolving area of research; that of transplant immunology.

In conjunction with Thomas Gibson, Medawar published a series of papers on graft rejection and how it was mediated by immune cells (Gibson & Medawar 1943), (Medawar 1944). They demonstrated that the process of rejection in both human and rat tissue was characterised by specificity and memory for donor tissue and that it was accompanied by infiltration of leucocytes. However, it was an Australian virologist, Frank Macfarlane Burnet who first introduced the concept of self and non-self, to immunology (the ability of the immune system to discriminate between cells of its own body and foreign antigens). In his landmark text – ‘The Production of Antibodies’ he postulated that ‘self’ was defined during

embryogenesis. He later went on to describe his clonal selection theory in order to explain why antibodies are not produced against antigens on our own cells.

1.1.1 Early Immunosuppression

In 1948, Merck Company produced the first commercially available adrenal cortical hormone, Cortone (cortisone). Initially, Medawar et al. suggested that the immunosuppressive effects of cortone were of limited significant benefit. By the early 1950's, a number of groups were again attempting human kidney transplantation. The surgical technique had by now changed somewhat and grafts were implanted into the right iliac fossa with vascular anastomosis to the iliac vessels. The results were modest with grafts surviving up to 3 weeks on the immunosuppressive regimen of adrenocorticotrophic hormone (ACTH) or cortone. However subsequent studies by Medawar et al. demonstrated that long-term graft acceptance was dependent upon the induction of tolerance. It was generally accepted that the weak steroid effect of cortone was simply insufficient and its use was side-lined.

One of the centres carrying out transplants in the early 1950's was the Peter Bent Brigham Hospital in Boston. Here, David Hume's group reported on a series of nine transplants performed (Hume et al. 1955). They made several important conclusions, principally that pre-operative blood transfusion and the matching of donor and recipient blood groups were important. However, with little confidence in the pharmacological agents used at the time, it was believed that the immunosuppressive effect of endogenous uraemia was more likely to be responsible for the short periods of function before rejection. Effective immunosuppression remained elusive.

In December 1954, Joseph Murray performed the first successful human kidney transplant (in terms of long term survival) at Peter Bent Brigham Hospital in Boston (Merrill et al. 1956). By applying the knowledge gleaned by E. C. Padgett (Padgett 1932) and J. B. Brown (Brown 1937) in the 1930's which showed that genetic identity was necessary for successful skin grafting between monozygotic twins, he transplanted the kidney of a 23 year old male into his twin brother, who was suffering advanced glomerulonephritis. The recipient survived for eight years before succumbing to a recurrence of glomerulonephritis in the originally unaffected kidney. This success provided a catalyst for a series of successful transplants between twins (Murray, Merrill, & Harrison 1958) and highlighted several important lessons on the technical and ethical issues, however significant deficiencies in the immunological treatment of patients remained and the future of transplantation had essentially hit an immunosuppressive 'brick wall'.

By the late 1950's total body irradiation with allograft bone marrow rescue was gaining acceptance as a viable immunosuppressive therapy. This involved pre-conditioning the recipients with total body irradiation (without donor bone marrow, instead their own bone marrow was recovered and infused back post-transplant). Unfortunately, there were difficulties in controlling total body irradiation and as a consequence, unacceptably high levels of graft-versus-host disease were occurring. Though this technique was refined to a sublethal irradiation alone regimen, there remained a high incidence of overwhelming sepsis and mortality. It did, however, provide a significant breakthrough, allowing the first successful allotransplants, one by the Boston group (Merrill et al. 1960) and five by a group in France (Hamburger et al. 1962; Kuss et al. 1962). Despite these being isolated successes among a large number of failures, it soon became apparent that myelotoxicity needed to be

avoided and the momentum was thus provided to continue research, with the resulting emergence of chemical immunotherapy.

The first major breakthrough in the development of cytostatic drugs (that is drugs which inhibit cell growth and multiplication) came in 1959 with the discovery by Robert Schwartz and William Dameshek in Boston, that 6-Mercaptopurine (6-MP) reduced the antibody response in rabbits to human serum albumin (Schwartz & Dameshek 1959). They further reported that a short course of 6-MP in addition to administration of antigen, induced a long-term failure to respond to this antigen. Effectively they had induced tolerance. However, as they stated, its clinical use in human transplantation was prevented by 'development of resistance' to the drugs and its 'toxicity in humans'.

1.1.2 Azathioprine

During this period, a young surgeon by the name of Roy Calne was unsuccessfully using radiation in a series of canine renal transplants. He, fortunately, sought the advice of Kendrick Porter, a professor of pathology at St Marys Hospital London who had recently worked with Schwartz. Porter introduced Calne to the potential benefits of 6-MP and in 1960, Calne published his results demonstrating its effectiveness in prolonging renal graft survival in dogs (Calne 1960). He postulated the mechanism of action was 'either by a non-specific toxic effect on the immune system or by a true specific production of immune tolerance' [i.e., by the graft as antigen]. Shortly after this, at the suggestion of Peter Medawar, Calne travelled to Boston to join Joseph Murray at the Brigham Hospital. On his way, he visited George Hitchings and Gertrude Elion at the Burroughs Wellcome Research Laboratories in New York. They had originally provided Schwartz and Dameshek with 6-MP, and encouraged Calne to study a new derivative called BW57-322 (later termed

Azathioprine). Murray and Calne quickly demonstrated that Azathioprine was a more successful immunosuppressant than 6-MP, with fewer toxic side-effects (Calne 1961; Calne & Murray 1961). Though their results were far from conclusive, several groups around the world began to trial azathioprine as a chemical immunosuppressant in human renal transplantation, with variable success. The Boston group treated 27 transplant recipients, nine of whom survived greater than a year, including the notable case of Melvin Doucette, a 24-year old accountant who was transplanted in April 1962 (Murray et al. 1963); (Murray, Wilson, & O'Connor 1967). He survived 18 months and was considered to be the first successful unrelated renal allograft. Calne himself, who had returned to London, treated eight recipients with Azathioprine, with only two surviving. In addition, two transplant teams from Paris, Professor Jean Hamburger and Dr Rene Kuss, reported only one long term survivor out of 28 transplants. There was a general feeling amongst the transplant community that the wider use of Azathioprine as an immunosuppressant was going to be limited by a narrow and difficult to manage therapeutic range, resulting ultimately in a similar fate to that of irradiation.

However, a major breakthrough in correlating these variable results into an effective clinical strategy came in September 1963 at the National Research Council Conference in Washington. Here almost everyone involved in transplantation met (a mere 25 people) and presented their results. Amongst them was Thomas Starzl from the Veterans Administration Hospital in Colorado. Despite having only been involved in transplantation for less than a year, he presented impressive results on a series of 33 patients, twenty-seven of which were still alive with functioning kidneys (Starzl, Marchioro, & Waddell 1963). He had found that episodes of acute rejection could be effectively managed with pulses of high-dose steroids.

There were additional, practical observations made at the meeting that would ultimately contribute to the rapid proliferation of transplant programs around the world. Amongst them, it was noted that blood-type mismatch was incompatible with graft survival and that the flushing and cooling of donor organs was beneficial. There was also a broad acceptance that patient mortality was not a direct result of graft rejection in a significant number of cases. As a result of this landmark conference, over the next few years, transplant centres expanded in number from less than ten to nearly one hundred around the world. The use of Azathioprine and steroids were further refined and became standard practice for the next twenty years with a resulting proliferation in successful transplantation internationally.

1.1.3 Cyclosporin

There were few further developments in transplant immunology until the advent of cyclosporin in the early 1980's. The Swiss pharmaceutical company, Sandoz (now Novartis) Laboratories encouraged their employees to collect naturally occurring organisms which could potentially be developed as antifungal antibiotics. A soil sample, collected by a Sandoz employee while on holiday in Norway in 1970 was presented to Jean-Francois Borel for further testing. Borel found this sample contained the fungi *Tolypocladium flatum* and one of its metabolites, a peptide labelled #24-556. This compound displayed little anti-microbial activity; however it was found to have an immunosuppressive effect with an unusually low level of toxicity. In contrast to the cytostatic immunosuppressants in use at the time, #24-556 had a selective activity on lymphocytes. This specificity resulted in fewer side effects, in particular, the myelotoxic effects immunosuppressed patients were experiencing with current regimens (Azathioprine and steroids). Given Borel had an immunology background, he recognised this immunosuppressive effect and was keen to investigate further. By the

end of 1973, the potential immunosuppressive effects of #24-556 had been confirmed in a number of animal studies. At this stage, Sandoz did not deem transplantation as an area lucrative enough to justify further investment. However, the potential of #24-556 was recognised as a possible treatment for rheumatoid arthritis, and further research was therefore funded. Compound #24-556 was subsequently named cyclosporin A (or CyA, originating from cyclical peptide of spore origin).

By 1976, Borel had published his first paper on the subject (Borel et al. 1976) in which he succinctly summarised the effects of cyclosporin A:

1. Selective for lymphocytes, mainly for T-helper cells but sometimes for T-effector cells
2. Suppressed antibody- and cell-mediated immunity and chronic (but not acute) inflammation
3. Inhibited the induction phase of lymphoid cell proliferation, affecting early mitogenic triggering but not mitosis
4. Not toxic to lymphocytes (its effect being reversible)
5. Was effective on all mammals tested (mouse, rat, guinea pig, rabbit, monkey, dog)
6. Had no cardiovascular, psychotropic or other pharmacological effect which would seriously limit its effectiveness in man

In April 1976, Borel presented these findings at the spring meeting of the British Society for Immunology. Amongst the delegates were Roy Calne and David White, a member of Calne's transplant group in Cambridge. Calne and White obtained samples of cyclosporin from Borel and began carrying out their own transplantation studies in rats. Their initial results were remarkable, and they rapidly began testing in dogs (Calne et al. 1978a; Calne & White 1977)

in which orthotopic allograft survival was ten times greater compared to azathioprine and steroid treatment. Encouraged by these results, Calne commenced trials in humans. His initial results published in *The Lancet* (Calne et al. 1978b) reported 5 out of 7 mismatched cadaveric transplant recipients were successfully discharged from hospital. These initial results were encouraging; however nephrotoxic and hepato-toxic side effects had been noted. The next year, he published a landmark paper in the history of transplant immunology (Calne et al. 1979), though several negative issues were highlighted. Principally, that Cyclosporin use resulted in i) a high incidence of lymphoma, ii) normal graft function was not achieved in any recipients and iii) a high patient mortality. These effects were initially treated as rejection, but it eventually transpired that they were actually secondary to over-suppression. International interest in cyclosporin had already been stimulated. The following year, Thomas Starzl published results combining Cyclosporin with a steroid, as he had done with Azathioprine (Starzl et al. 1980). This allowed the dose of cyclosporin to be reduced resulting in far less nephrotoxic side effects while retaining its immunosuppressive properties. The results were impressive with 21 out of 22 kidney transplant patients surviving more than a year. Following further refinement, cyclosporin was approved for use as a transplant immunosuppressant by the US Food and Drugs Agency in November 1983. It rapidly became the cornerstone of immunosuppressant regimens around the world.

1.1.4 Tacrolimus

During 1984, in remarkably similar fashion to the discovery of cyclosporin, another macrolide immunosuppressant was isolated from the fungus *Streptomyces tsukubaensis*, in soil collected from Mount Tsukuba, Japan. Isolated by Kino et al. (Kino et al. 1987), it was originally named FK506 and was subsequently given the generic name, tacrolimus.

Initial experiments involving tacrolimus quickly established that it has a similar mechanism of action to cyclosporin by inhibiting interleukin-2 (IL-2) production, but in a far more potent manner (30-100 times more potent). Subsequent *in vitro* animal testing, however, divided opinion on its potential clinical application. Scientists at the University of Pittsburgh concluded that tacrolimus was a remarkably effective drug with minimal side effects, in contrast to results published from the UK condemning the drug for its 'intolerable' side effects ((Calne, Collier, & Thiru 1987), (Collier, Thiru, & Calne 1987), (Todo et al. 1987), (Murase et al. 1987)). Unsurprisingly, the first clinical trials involving tacrolimus were therefore carried out in Pittsburgh and began in January 1989, led by Starzl. Initially, the use of tacrolimus was restricted to rescue therapy for liver allograft recipients undergoing chronic rejection or those in whom cyclosporin caused significant side effects. The results of this trial, presented at the 1990 Congress of the Transplantation Society in San Francisco, were so impressive, that in the spring of the same year Starzl extended the use of tacrolimus to first-line therapy in liver transplant recipients. Of 120 transplant recipients treated with tacrolimus and low-dose prednisolone, there was an 87.5% graft survival (Todo et al. 1990). They also reported fewer side effects and a more advantageous therapeutic index when compared with cyclosporin.

The potential of tacrolimus was now gaining momentum with the establishment of multicentre trials in both the USA (The US Multicentre FK506 Liver Study Group) and Europe (European FK506 Multicentre Liver Study Group). Both published similar findings in 1994 (1994a; 1994b) and found that, compared with cyclosporin, patients receiving tacrolimus (and low-dose steroid) had significantly fewer episodes of acute rejection and the episodes that did occur were far less severe. As a result, tacrolimus was approved for commercial use in the US in June 1994 and two months later in the UK.

1.2 Mechanisms of Action

The agents described have varied mechanisms in which they act as an immunosuppressant in the transplant setting (Figure 1-2).

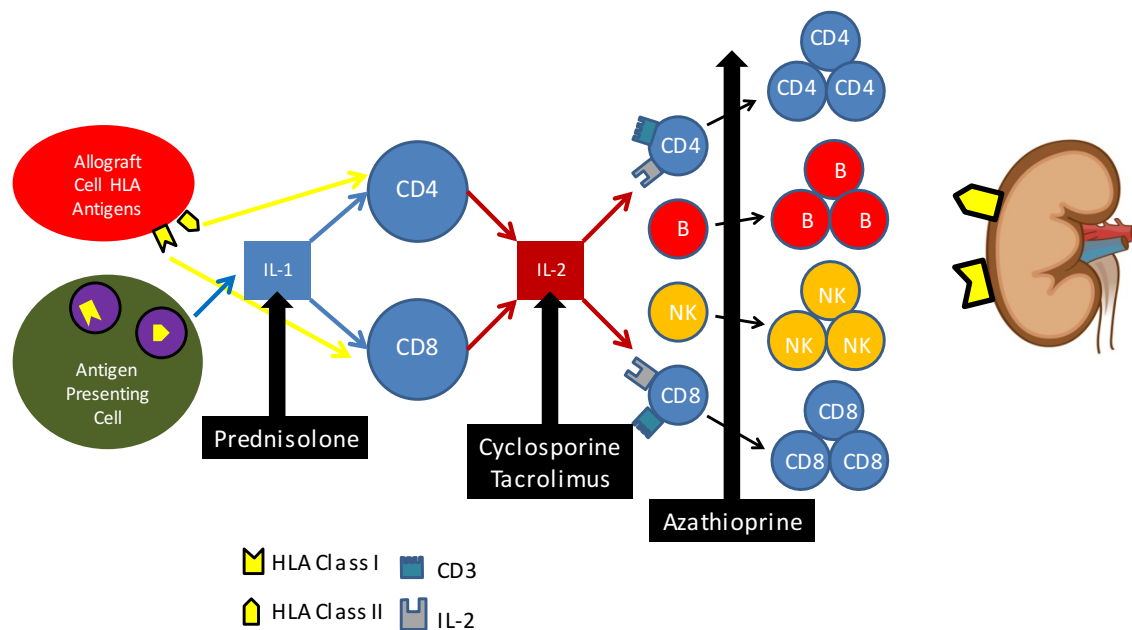


Figure 1-2 Mechanisms of action of established immunosuppressants

1.2.1 Prednisolone

Prednisolone is a non-specific anti-inflammatory and immunosuppressive synthetic glucocorticoid steroid. Its effect is mediated primarily by binding to intracellular glucocorticoid receptors. These receptors in the absence of glucocorticoid exist as an oligomeric complex bound to heat shock proteins (HSP's) in the cytoplasm. By binding to these complexes, glucocorticoids initiate a conformational change which results in dissociation from the HSP. The complex then translocates to the nucleus where it binds to glucocorticoid-responsive genes resulting in increased transcription. One of the genes up-

regulated is the interleukin-1 receptor antagonist (IL-1RA). One of the functions of IL-1 is to act as a co-stimulator for helper T-cell activation. By inhibiting antigen-presenting cell (APC) IL-1 production, helper T-cells are unable to proliferate into Th1 cells and thus activate cytotoxic T cell proliferation, a key aspect of cell-mediated immunity. Prednisolone also dampens the humoral arm of the immune system by inhibiting IL-6 production (an inducer of B-cell activation). As a non-specific agent, prednisolone has many other immunological (e.g., inhibition of several other cytokines including IL-2, IL-3, IL-4, etc.) and non-immunological targets. As a result, there are many varied side effects associated with steroid therapy, and therefore, its use is kept to a minimum, with an increasing number of steroid-free regimens being used in transplantation.

1.2.2 Azathioprine

Azathioprine is a pro-drug of 6-mercaptopurine (6-MP) which is increasingly being replaced with more modern immunosuppression, though it does still have a role in some regimens as an anti-proliferative agent. Once absorbed, azathioprine undergoes a reductive cleavage of the thioether portion of the molecule, which results in 6-MP. 6-MP is then further metabolised (primarily via methylation) into several active products, the most important of which is methyl-thioinosine monophosphate (MeTIMP). This active metabolite is a purine-synthesis inhibitor and thus inhibits DNA and RNA synthesis in rapidly proliferating cells, such as T- and B-cells. Since its discovery, several other metabolites of azathioprine have been found to have an immunosuppressive role, including Thioguanosine triphosphate (TGTP), which sends activated T cells into apoptosis.

As with prednisolone, azathioprine does not have a particularly specific target profile and due to its effects on DNA/RNA synthesis it has a wide variety of side effects, including

anaemia secondary to bone marrow deficiency and an increased risk of several cancers including lymphoma and skin cancers (O'Donovan et al. 2005).

1.2.3 Cyclosporin

Cyclosporin is a polypeptide of 11 amino acids which has very poor solubility in water. For this reason, it has undergone an evolutionary process in terms of its development as a suspension / emulsion formula. The first Cyclosporin formula available (Sandimmune®) had very poor bioavailability and high pharmacokinetic variability. Its successor (Neoral®) was therefore developed as a micro-emulsion with an improved profile.

Cyclosporin blockades intracellular T-cell signalling by binding to its cytoplasmic immunophilin, cyclophilin (Figure 1-3). Under normal circumstances, activated T-cells increase intracellular calcium, which binds calmodulin, and activates the enzyme calcineurin. Once activated, this calcineurin complex dephosphorylates the cytoplasmic transcription factor NFAT (nuclear factor of activated T-cells). Once dephosphorylated, NFAT translocates to the nucleus and increases IL-2 gene transcription and ultimately T-cell proliferation. By binding to cyclophilin, cyclosporin forms a complex which inhibits the function of calcineurin and thus prevents IL-2 release.

Unfortunately, cyclosporin is not without its side effects. Though generally considered less toxic than earlier immunosuppressants, there is plenty of evidence documenting the adverse effects cyclosporin can have on recipients. Among these, the primary effect is nephrotoxicity. There are a number of mechanisms by which cyclosporin damages the graft, and the true reasons are likely multifactorial though vascular dysfunction is clearly implicated and this may well be through stimulated TGF- β production leading to interstitial fibrosis (Naesens, Kuypers, & Sarwal 2009).

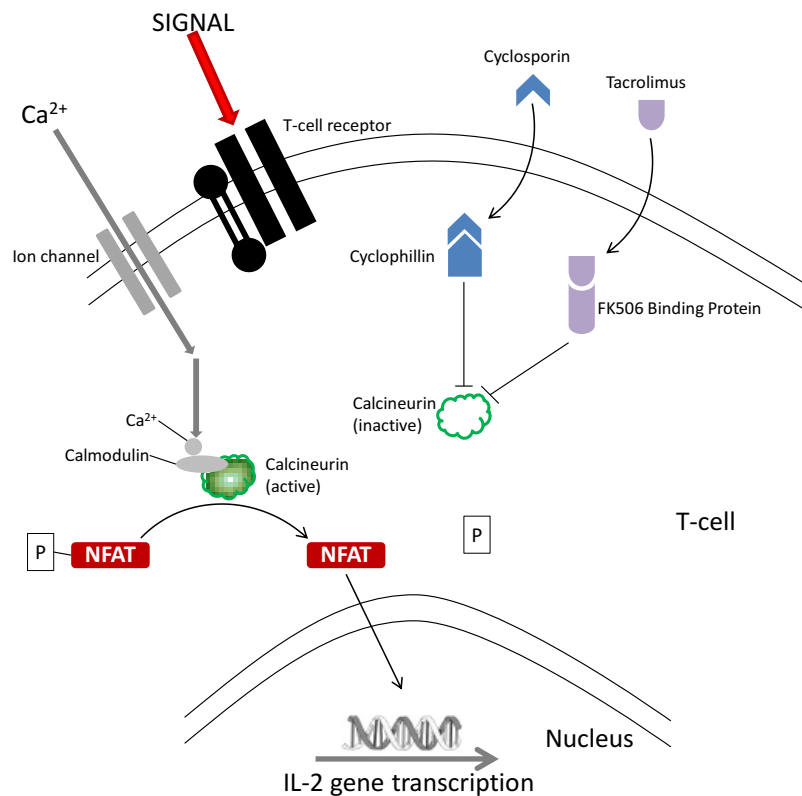


Figure 1-3 Mechanism by which cyclosporin exerts its effect on IL-2 blockade

1.2.4 Tacrolimus

The pathway by which tacrolimus acts is very similar to that of cyclosporin though it does differ significantly in terms of structure (it is a macrolide antibiotic) and potency (50-100 times more potent). As with cyclosporin, tacrolimus is a calcineurin inhibitor which prevents the dephosphorylation of NFAT. It differs in mechanism by binding to the immunophilin FK506 binding protein; its subsequent downstream effects, however, are similar (Figure 1-3).

1.3 The Current Status of Transplant Immunosuppression

Solid organ transplantation has transformed survival for patients with end-stage liver, kidney, pancreas, lung and cardiac disease. A common factor in determining the success of solid organ transplantation is the need for lifelong immunosuppression to prevent rejection by the host immunity. While the incidence of acute rejection has been markedly reduced and side effect profiles much improved, chronic rejection and, ultimately, long-term graft survival remains largely unchanged (Meier-Kriesche et al. 2004). Current immunosuppressive strategies remain associated with undesirable side effects, such as renal injury, diabetes and cardiovascular disease (Halloran 2004; Jardine et al. 2005; Ojo et al. 2003). Therapeutic approaches geared towards achieving immune tolerance have so far evaded the field, and thus new immunosuppressive strategies with better toxicity profiles continue to be sought.

1.4 T cell development

In order to fully understand the rejection process, it is first necessary to consider the role of the key mediator - T cells.

The development of T cells begins as precursors, which migrate from the bone marrow to the thymus. The thymic cortex is the major site of thymocyte proliferation, with a complete turnover of cells approximately every 72 hours. These thymic T cells (or thymocytes) undergo a process of phenotypic change which generates a diverse T-cell receptor (TCR) repertoire. Via a subsequent process of positive and negative selection, antigen-specific naive cells are generated which then move into the medulla, where they undergo further differentiation and selection. The majority (98%) of thymocytes generated will undergo

apoptosis in the thymus at this stage. Finally, surviving cells migrate into the secondary lymphoid organs and tissues of the body where they can respond to antigens.

1.4.1 Thymocyte development

Beta, Positive and Negative selection

The process of positive and negative selection within the thymus will ultimately determine T cell lineage.

Beta selection

Immature thymocytes (haematopoietic precursors that have derived from the bone marrow and colonised the thymus) lack CD4 and CD8 expression and are termed double negative cells. These double negative cells will eventually give rise to either $\alpha\beta$ (90% of the total number of cells) or $\gamma\delta$ T cell receptor expressing cells (Robey & Fowlkes 1994). Those cells destined for the $\alpha\beta$ -TCR population first express the adhesion molecule CD44 followed by CD25 (Interleukin-2 receptor α chain). As they progress through development, cells continue to express CD25 but CD44 expression reduces. This CD25 positive population subsequently undergoes beta selection (Godfrey et al. 1993).

In order to produce a diverse T cell receptor repertoire (to maximise recognition of an array of antigen peptides), the T cell receptor gene in this $CD4^-CD8^-CD25^+$ population undergoes extensive rearrangement. This first involves rearrangement of the TCR- β chain, followed by its pairing with a pre-T α chain to produce a pre-TCR. This process results in a large number of non-functional TCRs, and this population are excluded through apoptosis at this stage (termed β selection).

Having successfully undergone beta selection, thymocytes now have the ability to express a TCR on their surface. However, in order to be functional, these TCRs must be able to interact

with the major histocompatibility complex (MHC) of antigen presenting cells (APCs).

Selecting out those thymocytes with non-functional TCRs is termed positive selection.

Positive selection

To bind to MHC, the TCR requires a co-receptor (either CD8 in the case of MHC I or CD4 in the case of MHC II). In order to facilitate this, the thymocyte will express both co-receptors, therefore transforming them from a double negative to a double positive, CD4⁺CD8⁺ phenotype (Robey & Fowlkes 1994). If these cells are capable of binding to MHC (either class), they receive a survival signal via the TCR. Approximately 90% of the thymocyte population are incapable of binding MHC at this stage and undergo apoptosis.

Depending upon which MHC the thymocyte is most capable of recognising will determine which of the co-receptors (CD4 and CD8) is down regulated and ultimately determine to which T cell subset the thymocyte is destined (Kaye et al. 1989; Kruisbeek et al.

1985; Marusic-Galesic, Longo, & Kruisbeek 1989; Teh et al. 1988). Only CD4 is upregulated.

Cells which successfully bind to MHC class I will down-regulate CD4 and become cytotoxic T cells (Tc cells), those which bind MHC class II will down-regulate CD8, upregulate CD4 and become helper T cells (Th cells). This process is positive selection and the resulting cells are termed single positive thymocytes.

Negative selection

The next stage in T cell development involves the migration of these single positive thymocytes to the cortico-medullary junction of the thymus. Here they are exposed to APC cells which have a high expression of self-peptide. Those cells which have evolved TCRs which recognise and have a high affinity to this self-antigen are selected out and undergo

apoptosis (approximately 5% of the population of cells to reach this stage). Without this process, cells able to recognise self-antigen in the periphery would represent a danger to the hosts by activating its own immune system against themselves, and subsequent autoimmune disorders. Cells surviving this process, with a low affinity to self-antigens, but a high affinity to foreign antigens constitute the precursor population for conventional T cells. It is at the later end of this stage that cells with an intermediate affinity for self-peptide escape negative selection and are selected out. These cells are destined to become the regulatory T cell lineage (see below).

After undergoing these various selection steps, the single positive thymocytes reside in the thymus for another 1 to 2 weeks where they continue exposure to self-antigens and undergo additional maturational processes (the detail of which is complex and beyond the scope of this thesis). Finally upon departure from the thymus, they enter the peripheral circulation as mature T lymphocytes.

1.4.2 T cell subsets

T cells function by secreting soluble mediators through cell contact-dependent mechanisms. They can be classified into various subsets depending upon their function and the phenotypic difference in their surface markers.

Effector T cells were originally thought to have terminally differentiated phenotypes, but there is a growing body of evidence that suggests these cells have an inherent plasticity. T cells can have a mixed phenotype and have the ability to convert from one subtype to another.

Many T cell subsets have been characterised and can be differentiated by their cell surface markers, transcription factors, effector molecules and function.

	<i>Cell</i>	<i>Surface phenotype</i>	<i>Transcription factors</i>	<i>Effector molecules secreted</i>	<i>Function</i>
NAIVE	CD4 αβ T cells	αβ TCR, CD3, CD4, CCR7, CD62L, IL-7R (CD127)	THPOK		Patrol via lymph nodes scanning MHC Class II complexes on APCs for the antigen. Following activation, naive CD4 cells differentiate into effector or regulatory T cells, can also give rise to memory T cells
	CD8 αβ T cells	αβ TCR, CD3, CD8, CCR7, CD62L, IL-7R (CD127)	RUNX3		Patrol via lymph nodes scanning MHC Class I complexes on APCs for antigen. Following activation, differentiate into CTLs and memory T cells
CYTOTOXIC	Cytotoxic T cells (CTLs)	αβ TCR, CD3, CD8	EOMES, T-bet, BLIMP1	Perforin, granzyme, IFNγ	Protect host from viral infection and cancer by killing infected/transformed cells.
HELPER	T helper (Th1) cells	αβ TCR, CD3, CD4, IL-12R, IFNγR, CXCR3	T-bet, STAT4, STAT1	IFNγ, IL-2, LTα	Promote protective immunity against intracellular pathogens. By secreting IFNγ induce activation of macrophages. Development regulated by IL-12
	Th2 cells	αβ TCR, CD3, CD4, IL-4R, IL-33R, CCR4, IL-17RB, CRTH2	GATA3, STAT6, DEC2, MAF	IL-4, IL-5, IL-13, IL-10	Promote humoral response and host defence against extracellular parasites. Can also potentiate allergic reactions and asthma. Development regulated by IL-4, IL-25 & IL-33
	Th9 cells	αβ TCR, CD3, CD4	PU1	IL-9, IL-10	Involved in host defence against extracellular parasites. Despite IL-10 production (anti-inflammatory), promote allergic inflammation
	Th17 cells	αβ TCR, CD3, CD4, IL-23R,	RORγt, STAT3, RORα	IL-17A, IL-17F, IL-21,	Promote protective immunity against extracellular bacteria & fungi (also

		CCR6, IL-1R, CD161		IL-22, CCL20	autoimmune and inflammatory disease). Generated in the presence of TGFβ & IL-6/IL-21. Maintained by IL-23 & IL-1
	Th22 cells	αβ TCR, CD3, CD4, CCR10	AHR	IL-22	Involved in inflammatory skin diseases. Role in host defence remains unclear
	TFH cells	αβ TCR, CD3, CD4, CXCR5, SLAM, OX40L, CD40L, ICOS, IL-21R, PD1	BCL-6, STAT3	IL-21	Provide germinal centre responses and provide help for B cell class switching
REGULATORY	TR1 cells	αβ TCR, CD3, CD4	Not known	IL-10	Immunosuppression (mediated by IL-10). Generated from naive cells in the presence of TGF-β & IL-27 (or in the presence of immunosuppressive drugs, Vit D3 and Dexamethasone
	Natural Treg cells	αβ TCR, CD3, CD4, CD25, CTLA4, GITR	FOXP3, STAT5, FOXO1, FOXO3	IL-10, TGFβ, IL-35	Generated in the thymus. Mediate immunosuppression & tolerogenic responses
	Inducible Treg cells	αβ TCR, CD3, CD4, CD25, CTLA4, GITR	FOXP3, STAT5, FOXO1, FOXO3, SMAD2, SMAD3, SMAD4	IL-10, TGFβ	Generated from naive T cells, and in some cases depend on TGFβ & IL-2 for differentiation. Mediate immunosuppression & tolerogenic responses
MEMORY	Central memory	CCR7, CD44, CD62L, TCR,	BCL-6, BCL-6B, MBD2,	IL-2, CD40L,	Reside in secondary lymphoid organs, mounting responses to antigens. Following

	T cells	CD3, IL-7R, IL-15R	BM1	Low levels IL-4, IFN γ , IL-17A	stimulation, rapidly proliferate into effector T cells
	Effector memory T cells	CD62L, CD44, TCR, CD3, IL-7R, IL-15R, CCR7	BLIMP1	Inflammatory cytokines	Found in peripheral tissues. Provide immediate protection upon antigen challenge through production of effector cytokines
NKT	Natural Killer T cells	NK1.1, SLAMF1, SLAMF6, TGF β R	PLZF	IL-4, IFN γ , IL-17A	Both pro- & anti-inflammatory function. Modulate immune responses in cancer, autoimmunity, allergy, infection and GvHD
$\gamma\delta$	$\gamma\delta$ T cells	$\gamma\delta$ TCR, CD3		IFN γ , IL-17A, IL-17F, IL-22	Both pro- & anti-inflammatory function. Characteristics of both innate and adaptive immunity
CD8 $\alpha\alpha$	CD8$\alpha\alpha$ T cells	$\alpha\beta$ or $\gamma\delta$ TCR, CD3, CD8 $\alpha\alpha$, B220		IL-10, TGF β	Can develop intra- or extra-thymically. Express $\alpha\beta$ or $\gamma\delta$ TCRs. Can have regulatory function through production of IL-10 & TGF β

Table 1-1 T cell subsets involved in immune response

1.4.3 T cell receptor

Antigen recognition by T cells (specifically their T-cell receptors – TCRs) is the key event in the activation, proliferation and differentiation of alloreactive T cells. The TCR is a heterodimer composed of two transmembrane polypeptide chains covalently linked by a disulphide bridge (Figure 1-4).

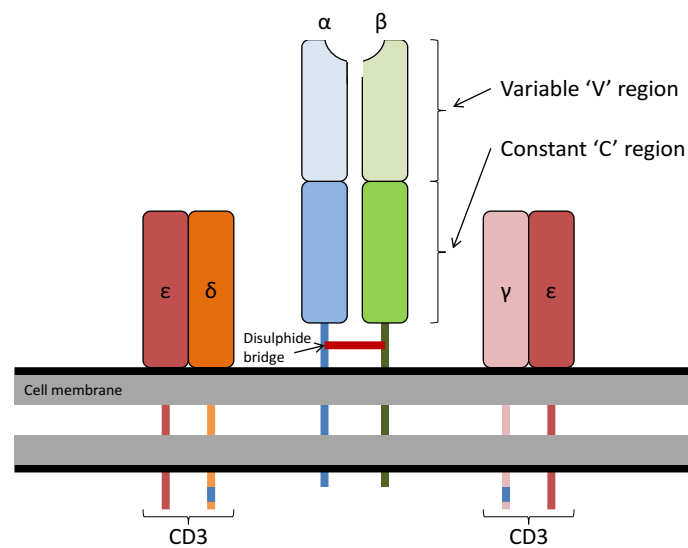


Figure 1-4 The T-cell receptor complex - highly variable alpha (α) and beta (β) chain expressed as part of a complex with the invariant CD3 chain molecules

Each chain of the TCR is composed of a Constant (C) region and a Variable (V) region. A short cytoplasmic chain on the C region anchors the TCR to the cell membrane. The V region is responsible for binding to the MHC:antigen complex. Within this region, there are short stretches of amino acids which confer variability between TCRs and allow them to recognise and bind to a diverse number of antigens.

In order to be expressed and function, the TCR requires the co-expression of an invariant complex termed CD3. Once the TCR has bound the MHC:antigen complex, CD3 facilitates signal transduction, which subsequently results in a signalling cascade and ultimately T-cell activation. This function has been exploited in terms of transplantation through the development of OKT3 (a monoclonal antibody to CD3) as a potent immunosuppressive for use in T-cell mediated rejection.

1.5 The T cell response

1.5.1 CD4/CD8

The two major subsets of mature T cells are CD4⁺ 'helper' T cells and CD8⁺ 'cytotoxic' T cells. They are defined by the presence of either the CD4 or CD8 transmembrane coreceptor protein, necessary in the activation pathway of T cells. The primary role of these coreceptors is to bind their respective MHC class II (CD4) or I (CD8) molecule inferring a degree of stability on the TCR:MHC/antigen complex and facilitating downstream signalling during the activation process.

In terms of transplantation, once activated, the CD4⁺ T cells differentiate into effector cells which have the potential to cause graft damage through cytokine production, most likely in a chronic setting. CD8⁺ T cells differentiate into cytotoxic T cells (CTLs) either through the direct or indirect pathway. If, via the indirect pathway, the CTLs are self-MHC restricted and, therefore, do not affect the graft. However, if they are generated via the direct pathway, they are able to target class I MHC nucleated cells within the graft. These T cells are likely to play a key role in acute rejection.

1.5.2 Costimulation

In addition to the TCR complex described (signal 1), T cells require a second signal in order to fully activate (signal 2). This second signal is in the form of a costimulatory molecule, found on the T cell surface, which binds to its own specific ligand on APCs (Li, Rothstein, & Sayegh 2009). It has become increasingly evident that as well as positive signals which promote T-cell activation (co-stimulatory pathway), there are a number of co-pathways which have the converse effect and can attenuate or even terminate the immune response (co-inhibitory pathway) (Boenisch, Sayegh, & Najafian 2008). Without this costimulatory

signal, T cells activated via the TCR alone become apoptotic or anergic. The costimulatory pathway also promotes stabilisation of the TCR:MHC/antigen complex allowing the downstream transduction of signals which activate the calcineurin, MAP kinase and nuclear factor κ B pathways.

Over the last decade, a number of costimulatory molecules (Table 1-2) which have both negative and positive impacts on T cell fate have been identified. One of the most studied pathways and most relevant to transplantation is the interaction of the B7 molecules (also known as CD80 and CD86) on the APC, with the CD28 molecule of the T-cell. CD28 is expressed on the surface of all CD4⁺ cells and approximately 50% of CD8⁺ T cells and its interaction with B7 molecules leads to increased transcription of the pro-inflammatory cytokine, interleukin-2 (IL-2) (Jenkins et al. 1991).

T Cell	APC	Effect	Comments	Study
CD28	B7-1 (CD86)	+	B7-1 and B7-2 can both provide T cell costimulation through CD28	(Acuto & Michel 2003)
	B7-2 (CD80)	+		
PD1	PDL-1, PDL-2	-	PD-1 can be induced on CD4 ⁺ , CD8 ⁺ T, NK, and B cells and monocytes, and ligation delivers an T cell inhibitory signal	(Durrbach et al. 2010)
CTLA4	B7-1(CD86)	-	CTLA-4 binding to B7-1 and B7-2 inhibits T cell proliferation	(Larsen et al. 2006)
	B7-2 (CD80)	-		
ICOS	ICOS-L, B7h	+	In experimental studies, anti-ICOS blocking mAb can significantly prolong allograft survival	(Ozkaynak et al. 2001)
CD28	B7-H3	+	B7-H3 is induced in dendritic cells; monocytes; and T, B, and NK cells and increases T cell proliferation	(Steinberger et al. 2004)

TNF-R	TNF	+	The TNF-R regulates dual T cell functions through apoptosis and also TRAF-mediated T cell activation.	(Durrbach et al. 2010)
CD40L, CD154	CD40	+	CD40 engagement activates B cell antibody production and upregulates expression of the B7 family	(Durrbach et al. 2010)
OX40	OX40L	+	OX40 is expressed on activated CD4 and the Th2 T cells and activates B cell proliferation	(Acuto & Michel 2003)
CD27	CD70	+	CD27 activation induces high levels of IL-2 and TNF- α	(Yamada et al. 2005)
		+	Blockade of CD27 may prevent chronic allograft vasculopathy	
CD = cluster of differentiation; CTLA = cytolytic T lymphocyte antigen; ICOS = induced costimulatory molecule; IL-2 = interleukin-2; mAb = monoclonal antibody; NK = natural killer; PD = programmed death; Th2 = T helper type 2; TNF- α = tumor necrosis factor- α ; TNF-R = tumor necrosis factor receptor; TRAF = tumor necrosis factor receptor associated factor.				

Table 1-2 Costimulatory molecules involved in a T cell response

Adapted from (Souba et al. 2013)

Following this interaction and activation, the co-inhibitory molecule, cytotoxic lymphocyte antigen-4 (CTLA-4) is rapidly upregulated by the T-cell. CTLA-4 also binds to the B7 molecule, with an affinity 10-20 times more than that of CD28. Through this mechanism, CTLA-4 inhibits T-cell activation and thus limits proliferation and the alloresponse. In 2011, Belatacept, a human fusion protein which combines a fragment of the human IgG1 molecule with an extracellular portion of CTLA-4 was approved for therapeutic use as an

immunosuppressant in renal transplantation. Early results on its effectiveness are mixed but, on the whole, encouraging (Vincenti et al. 2012).

1.5.3 Signal Transduction

Activation of the TCR through antigen recognition is followed by a phosphorylation cascade which ultimately results in the upregulation of certain transcription factors and determines the fate of the activated T cell (Figure 1-5 T cell activation signal transduction pathway).

Following TCR activation, cytoplasmic protein kinases, phosphorylate the TCR/CD3 complex resulting in recruitment of the ZAP-70 (zeta-associated protein of 70 kDa) tyrosine kinase which further phosphorylates an array of adapter molecules. From here a number of divergent pathways are facilitated including the calcineurin, protein kinase C (PKC) and mitogen-activated protein kinase (MAP kinase) pathways. These pathways eventually reconverge to generate a number of transcription factors (NFAT, NFκB and AP-1) leading to upregulation of genes involved in proliferation and differentiation.

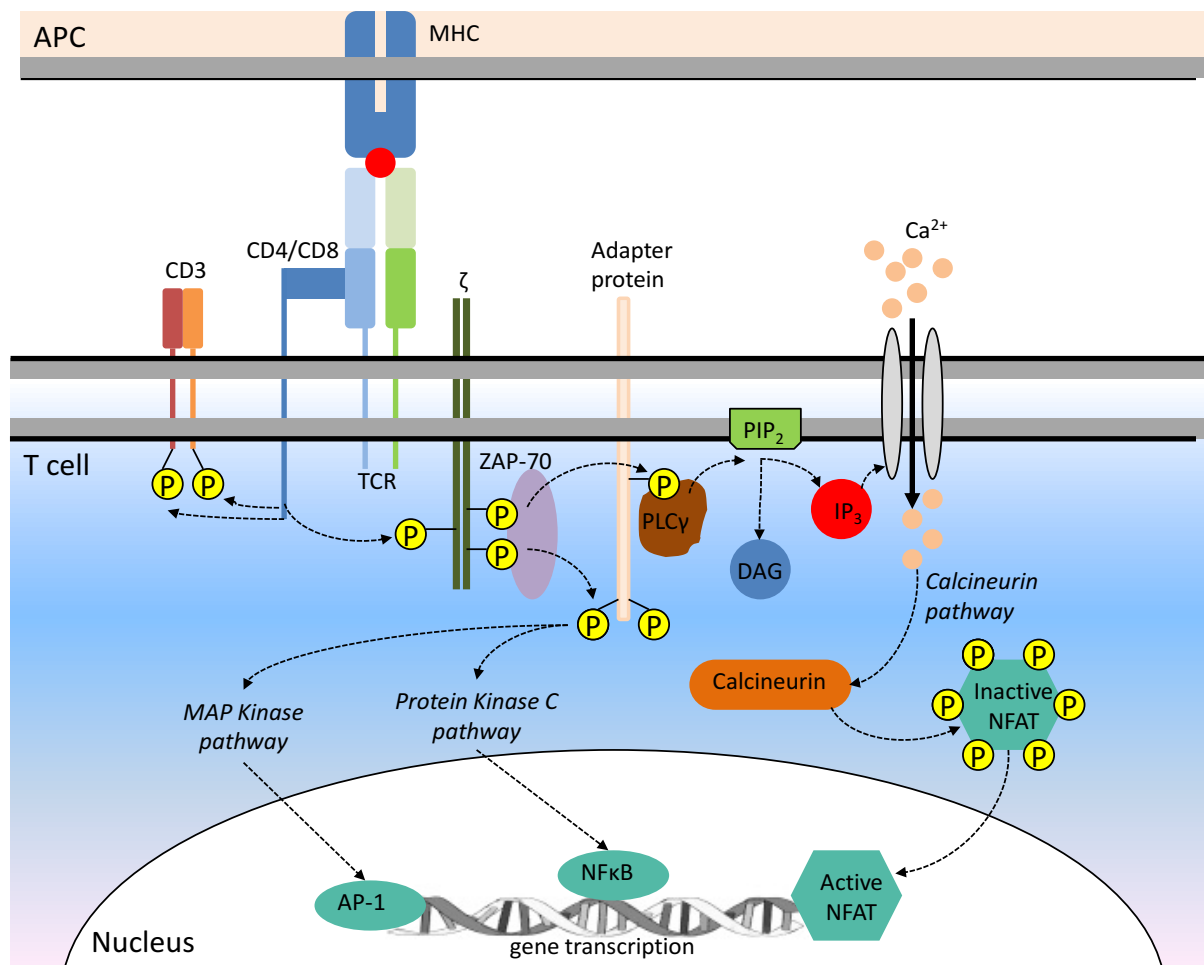


Figure 1-5 T cell activation signal transduction pathway

Adapted from (Chandraker, Sayegh, & Singh 2012)

1.5.4 Cytokine production

Many of the genes upregulated as a consequence of TCR activation result in the production of numerous cytokines which act as mediators of effector T cell function. These cytokines determine the fate of the naive T cell and ultimately their differentiation into various helper T-cell subsets.

1.6 Allorecognition

The first step in the allorecognition response to a transplanted graft is to recognise that the tissue is foreign, through the presentation of non-self antigens (as opposed to recognising host 'self' antigens) achieved through two distinct mechanisms (Figure 1-6). This is dependent on whether or not donor antigen is presented through recipient APCs or donor APCs. If presentation is via the donor APC, this is termed the 'direct pathway' and is the dominant mechanism in the acute rejection process. Conversely, if antigen is presented via the recipient APC, it is termed the 'indirect pathway', this mechanism predominates in chronic rejection (Game & Lechler 2002). The direct pathway is significantly more potent (~100:1) than the indirect pathway in terms of T cell activation (Lechler et al. 1990).

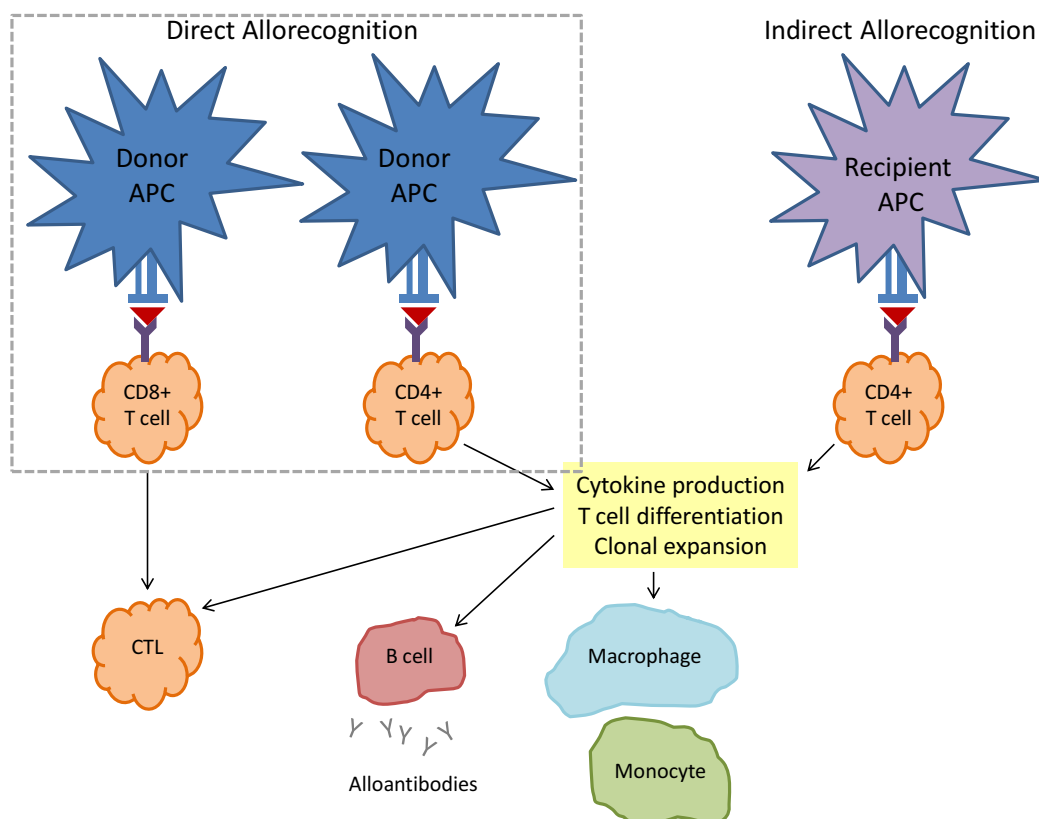


Figure 1-6 CD8⁺ cytotoxic T cells (CTLs) are generated via the direct pathway. CD4⁺ helper T cells, generated via the direct and indirect pathway enhance CTL activity and alloantibody production by B cells

A third mechanism has been proposed, termed the 'semi-direct pathway'. This pathway involves the transfer of intact MHC molecules between donor and recipient APCs, rather than just antigen alone (Herrera et al. 2004). These intact molecules are then presented directly to host T cells, thus stimulating the immune alloresponse.

1.6.1 Direct Pathway

Most episodes of acute rejection are mediated by the direct pathway. Also termed the 'endogenous class I pathway', it is active in nearly all cells. When allogeneic T cells are cultured together (MLR or Mixed Lymphocyte Reaction) they will respond with extreme potency (Bach & Hirschhorn 1964). This response is due in part to the extensive number of precursor T cells and results in the activation of a large number of cytotoxic T cells. In transplantation, the direct pathway relies on the allorecognition of donor leucocytes (principally dendritic cells) expressing class I (recognised by CD8⁺ T cells) and class II (recognised by CD4⁺ T cells) endogenous peptides.

This direct pathway is of particular importance in the early stages of transplantation during which a large number of APCs, with the potential to initiate an alloresponse, are present within the donor graft. However, their numbers are finite and if a response is not maintained the relative importance of this mechanism in terms of rejection recedes. However, the indirect response is able to maintain a long-term level of activity through a renewed pool of recipient APCs, which can result in chronic rejection.

1.6.2 Indirect Pathway

The indirect pathway, in contrast to the direct pathway, describes antigen presentation via recipients APCs and is essentially the 'normal' mechanism of T-cell stimulation. Therefore, in

theory, all proteins in the donor graft (different to the recipient) have the potential to stimulate an immune response. Three mechanisms, by which donor antigen is presented, have been postulated:

- Graft antigen is deposited into the host circulation and taken up by host DCs within secondary lymphoid tissue
- Donor cells migrate directly to host lymphoid tissue and are engulfed by host APCs
- Recipient APCs migrate through the graft 'picking up' donor antigen en route to secondary lymphoid tissue

Seminal studies by Lechler and Batchelor (Lechler & Batchelor 1982a; Lechler & Batchelor 1982b) demonstrated that DCs were the most likely cell responsible for presenting donor antigen and thus activating naive host T cells (as in the direct pathway). They further demonstrated that by depleting rat MHC-incompatible renal grafts of leucocyte passenger cells (by temporarily implanting the graft in an interim host) the grafts were ultimately accepted without immunosuppression, in certain donor/recipient combinations, but not in others, thus suggesting that it was not the recipient APCs stimulating an immune response (Lechler & Batchelor 1982b). Further evidence for this mechanism of antigen presentation by migrating DCs from the donor graft to secondary lymphoid tissue were demonstrated in studies by Larsen et al. (Larsen et al. 1990; Larsen, Morris, & Austyn 1990).

1.7 Patterns of rejection

Graft rejection has classically been defined by the period of time following implantation, at which it occurs, with immediate losses termed hyperacute (HAR), early losses termed acute (AR) and long-term / late losses termed chronic (CR). Although these terms continue to be

used, the events are not necessarily time specific and AR can occur at any stage after transplantation (Busch, Galvanek, & Reynolds, Jr. 1971). Though indistinct in terms of timing, these terms of rejection can be characterised based on specific mechanisms.

Hyperacute rejection (HAR) will usually occur within minutes to hours following reperfusion and is mediated by pre-existing humoral immunity. Acute rejection, which happens to a greater or lesser degree in all transplants, occurs anytime from one week to several years following reimplantation, though most commonly within the first 3 months (Humar et al. 2000) and is predominantly mediated by the formation of cellular immunity. Chronic rejection often occurs months to years after the onset of acute rejection and is mediated by both the humoral and cellular immune responses, usually manifesting as fibrosis.

1.7.1 Hyperacute Rejection

Hyperacute rejection is an antibody-mediated process which, fortunately, due to the advent of crossmatching, is a relatively rare occurrence. It is mediated by pre-formed antibodies within the host recognising donor HLA antigen on the vascular endothelium of the graft. This results in activation of the complement cascade leading to necrosis, platelet deposition and localised coagulation (Colvin 2007).

1.7.2 Acute Rejection

Acute rejection typically occurs within the first six months after transplantation and can be divided into two mechanistic categories – a) Acute cellular rejection (T-cell mediated) or b) Humoral rejection (antibody mediated or acute vascular rejection).

Acute cellular rejection – Up to 90% of rejection episodes are classified as cellular (Bates et al. 1999). Immature dendritic cells from within the graft, transport antigen to the recipient's

secondary lymphoid tissue. Along the way, these dendritic cells mature into APCs (Larsen, Morris, & Austyn 1990). Though the donor graft will have an extensive supply of DCs, recipient DCs also contribute to the process by circulating through the graft. The mature dendritic cells subsequently activate the recipient T cells within the host secondary lymphoid tissue, resulting in T cell differentiation and ultimately graft damage.

Humoral rejection – The main feature of this type of rejection is inflammation causing rapid graft dysfunction. Antibodies against HLA-molecules, endothelial cell antigens and ABO blood group antigens on endothelial and red cells can all mediate rejection (Nankivell & Alexander 2010). These donor-specific antibodies (DSAs) may be either preformed or develop *de novo* (Terasaki & Mizutani 2006). The process is characterised by the presence of arteritis secondary to the binding of donor-specific IgG antibodies against endothelial cell alloantigens. This leads to complement activation and antibody-dependent cellular cytotoxicity (ADCC) resulting in vascular injury.

With modern immunosuppression (induction therapy, calcineurin inhibitor, mycophenolate and steroids) the incidence of acute rejection is currently between 10-20% (Ekberg et al. 2007; Gaston et al. 2009; Vincenti et al. 2005).

1.7.3 Chronic Rejection

Chronic rejection is less well defined than the other types of rejection. It is a multi-factorial process and involves both antibody- and cell-mediated pathways as well as non-immunological factors. It affects 30-40% of renal grafts within 5 years. The most widely accepted cause of CR is prior episodes of AR. Non-immune causes are numerous and include

delayed-graft function, calcineurin inhibitor toxicity, hypertension, diabetes and hyperlipidaemia.

Immunologically, the indirect pathway of allorecognition dominates and CR may also reflect antibody responses to antigen mismatches not suppressed by immunosuppressive agents. This is supported by several studies demonstrating the presence of circulating anti-HLA antibodies against Class I and II MHC molecules and complement deposition in graft vessels (Colvin 2009).

1.8 Rejection of the Transplanted Graft

The rejection of transplanted organs remains one of the biggest barriers to modern transplantation. Despite, the huge advances in matching donors and recipients and the development of immunosuppressive pharmaceuticals, there still exists a degree of rejection in all transplanted grafts (unless identical twins). The mechanism by which rejection occurs is complex and is mediated by both an adaptive (cellular and humoral) and innate immune response.

1.8.1 Innate Immunity

The innate immune response in isolation is not capable of mediating graft rejection. It represents the first line of defence and in contrast, to the adaptive response which takes several days to become effective can mount an immediate response against pathogens. It is made up of cells and molecules, highly conserved between species, which can differentiate between self and non-self and does not require the recognition of specific antigens.

As a consequence of the organ retrieval process and subsequent reimplantation, certain ischemia-reperfusion injuries occur within the graft with the result that molecules termed damage-associated molecular patterns (DAMPs), and pathogen-associated molecular

patterns (PAMPs) are released. These molecules are recognised by specific pattern-recognition receptors (PRRs) such as Toll-like receptors, expressed by host macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DCs). Once DAMPs such as heat shock protein (HSP) activate these PRRs, several responses are stimulated. In the first instance, gene transcription is induced which results in the production of pro-inflammatory cytokines, chemokines and adhesion molecules, the result of which is an early inflammatory response. For example, the release of tumour necrosis factor-alpha (TNF- α) causes vascular endothelial activation and subsequent leucocyte transmigration into tissues. In addition to this direct effect, ligands of TLR receptors also stimulate DCs to act as antigen-presenting cells, and via this mechanism contribute to the adaptive immune response.

1.8.2 Adaptive Immunity

The adaptive and innate immune responses are not separate entities and in the rejection process are co-dependent through several different mechanisms. For example, cytokine / chemokine production following T cell activation can recruit cells of the innate response such as NK cells and macrophages (Pratt, Basheer, & Sacks 2002). Conversely, some of the associated molecular pattern molecules have chemoattractant properties on T cells (Guani-Guerra et al. 2010).

Adaptive immunity involves recipient T (cell-mediated response) and B cells (humoral response) recognising specific donor antigen and is considered the primary immune response to transplanted tissue. It was first recognised in transplantation by Snell and Gorer, who described skin grafts transplanted between different strains (allogeneic) of inbred mice led to graft rejection (Gorer, Lyman, & Snell 1948). This finding led to the identification of the major histocompatibility complex (MHC). In humans, this is located on

chromosome 6 and its protein products are termed human leucocyte antigens (HLA). The HLA genes are an extremely polymorphic region within the MHC and are divided into three groups, depending upon their structure, function and distribution (Klein & Sato 2000a). HLA Class I genes encode for HLA-A, HLA-B and HLA-C antigens, normally expressed on almost all nucleated cells. These antigens are recognised exclusively by CD8⁺ T cells and regulate direct antigen presentation. HLA Class II genes encode for HLA-DP, HLA-DQ and HLA-DR antigens and are only expressed on B lymphocytes, activated T lymphocytes and antigen presenting cells (APCs) – dendritic cells, monocytes and macrophages (Klein & Sato 2000). They are recognised by CD4⁺ T cells and regulate the indirect antigen pathway. The Class III genes encode for several components of the complement system.

In transplantation, the effects of HLA-A, HLA-B and HLA-DR antigens have the greatest impact on graft rejection (Opelz et al. 1991). In the first 6 months, an HLA-DR mismatch is most important, with HLA-B emerging as significant over the initial 2 years and HLA-A mismatch resulting in a long term effect (Morris et al. 1999; Opelz et al. 1999; Takemoto et al. 2000; Zantvoort et al. 1996).

1.9 Histone Deacetylase (HDAC)

Central to this study is the potential role histone deacetylase inhibitors have in modifying the immune response to a transplanted organ. The primary substrate for these compounds are HDACs, though their effects are not limited exclusively to these targets, as will be explained.

Histones are a family of important chromosomal proteins which are integral to the organisation of eukaryotic DNA in chromosomes. The organisation and function of DNA is

achieved through the addition of proteins including core histones (H2A, H2B, H3 and H4) which together form the complex structure, chromatin. Conformational changes in chromatin (and thus changes in protein function) can be affected by altering their acetylation status.

In the early 1960s, it was first discovered that histone proteins undergo acetylation (Allfrey, Faulkner, & Mirsky 1964; Phillips 1963). However, it was not until the mid-1990s with the identification of histone acetyl transferases (HATs) and histone deacetylases (HDACs) that the link between histone modification and the resultant change in gene activity was established. Acetylation of histones are a responsibility of HATs and this process often results in transcriptional activation. Conversely, HDACs cleave acetyl groups resulting in deacetylation and generally an opposite effect on transcription (Pazin & Kadonaga 1997). Specifically, the process of deacetylation takes place by the removal of acetyl groups from the lysine amino acids of the histone tail. This conformational change results in a positive charge which in turn allows a high-affinity bond to form between the histone molecule and DNA – effectively a ‘closed’ state. In this ‘closed’ deacetylated state, transcription factors are unable to gain access to DNA binding sites, with a resultant reduction in gene expression. This role of HDACs has led to them being extensively studied as targets for pharmacological manipulation in a wide range of indications.

The first HDACs were identified in 1996 (Taunton, Hassig, & Schreiber 1996). Currently, 18 human HDACs have been at least partially characterised; of which 11 are zinc-dependent (Table 1-3, Figure 1-7). They are classified according to their homology to yeast HDACs (as

well as location and enzymatic activity). The remaining 7 are NAD⁺ dependent HDACs and are termed Sirtuins. They are classified as Class III HDACs.

	<i>Yeast Counterpart</i>	<i>Size</i>	<i>Location</i>	<i>Expression</i>	<i>aa</i>
Class I					
HDAC1	RPD3	58	Nucleus	Ubiquitous	482
HADC2		58	Nucleus		488
HDAC3		50	Nucleus/Cytoplasm		428
HDAC8		44	Nucleus		377
Class IIa					
HDAC4	HDA1	120	Nucleus/Cytoplasm	Specific	1084
HDAC5		130	Nucleus/Cytoplasm		1122
HDAC7		110	Nucleus/Cytoplasm		855
HDAC9a		160	Nucleus/Cytoplasm		1011
ClassIIb					
HDAC6	HDA2	160	Nucleus/Cytoplasm	Specific	1215
HDAC10		70	Nucleus/Cytoplasm		669
Class IV					
HDAC11	RPD3/HDA1	39	Nucleus	Ubiquitous	347

Table 1-3 Classification of histone deacteylases

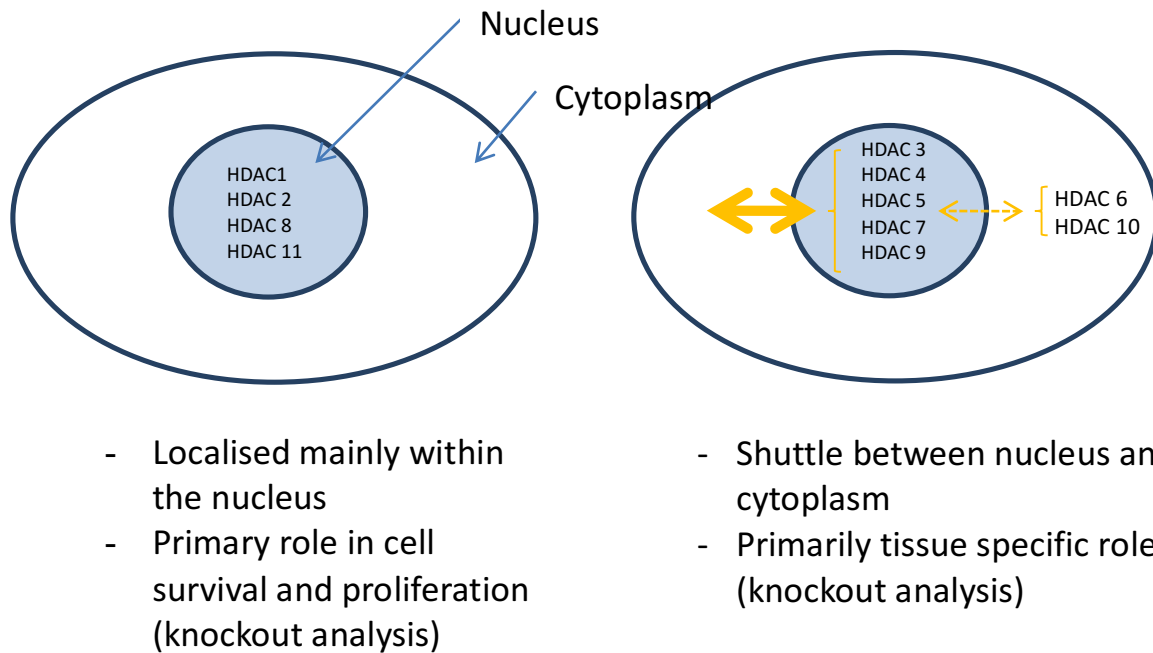


Figure 1-7 Cellular distribution of histone deacetylases

Though, they have maintained their traditional name the effect of HATs and HDACs are not limited exclusively to the deacetylation of histones and they, in fact, have a wide range of non-histone targets (Minucci & Pelicci 2006). As mentioned previously, the specific target of HDACs is not the histone protein itself but is, in fact, a lysine amino acid. Therefore HDACs, given their broad distribution can affect the function of an enormous number of non-histone, lysine-containing proteins. These non-histone proteins, including transcription factors and regulators of DNA repair, replication and recombination are all affected by acetylation (Yang & Seto 2007). HDACs (and HATs) should, therefore, be more accurately termed lysine deacetylases (lysine acetylase transferases).

Acetylation (and deacetylation) can have a number of effects on non-histone proteins.

- Protein stability. Ubiquitination is a similar post-translational process to acetylation whereby the lysine amino acid residue is targeted. In this case, however, ubiquitin is

attached to the substrate protein. The result, as with acetylation, is an alteration in protein structure and consequently function. As both these processes compete for the same target, they can, therefore, interact and confer instability upon the substrate protein. For example, HDAC deacetylation may lead to the exposure of lysine residues and ubiquitination resulting in protein instability.

- Protein – protein interactions. One such example involves the acetylation of signal transducer and activator of transcription factor 3 (STAT3) which is activated by cytosolic, cytokine signalling. This acetylation results in dimerisation of STAT3 and nuclear translocation.
- Protein localisation. The acetylation status of a protein can have an effect on its geographical localisation within the cell. For example, the multi-functional high-mobility group box 1 (HMGB1) protein (a cytokine mediator of inflammation secreted by immune cells such as macrophages) will undergo acetylation during the inflammatory process. In this acetylated state the protein is shuttled from the nucleus to the cytoplasm in preparation of the inflammatory response.
- DNA binding. Hyper-acetylation of transcription factor histones can often result in increased DNA binding which can subsequently affect transcriptional activity.

Though these examples have highlighted the upregulating effects of acetylation, this is not always the case and often the binding of transcriptional factors to DNA can be impaired. This therefore, would suggest that HATs and HDACs work in a synergistic manner in order to coordinate a number of cellular functions.

1.10 The role of histone deacetylases in immunity

A more detailed description of the role of HDACs and inhibitors on the innate and adaptive immune systems can be found in the Discussion section

1.10.1 Innate immunity

A role for HDACs has been implicated in a number of aspects of the innate immune system. Of particular note, however, in the innate immune system is the influence HDACs can have over the Toll-like receptor (TLR) and interferon (IFN) signalling pathways.

Though current evidence using pan-HDACis (Roger et al. 2011) suggests that HDACs may have a positive effect on TLR and IFN signalling pathways, it conversely also provides support for the investigation of HDAC specific inhibitors in manipulating targeted beneficial mechanisms to the dampening of the allograft immune response.

1.10.2 Adaptive immunity

HDACs can influence a broad range of features of the adaptive immune system through the modulation of signalling pathways (Shakespear et al. 2011).

B lymphocyte and function

HDACs have a direct effect on the development and function of lymphocytes. For example, murine studies have shown that if one or both of either HDAC1 or HDAC2 are eliminated in conditionally targeted mice, B cell development is arrested at an early stage. However if either of these enzymes are present, then development proceeds as normal (Yamaguchi et al. 2010). Interestingly, it is not only the early development of B cells that is dependent on HDAC 1 and/or 2 and the same study also found that selective deletion of both enzymes in mature B cells resulted in cellular apoptosis under stimulating conditions.

Of particular relevance to the work presented in this study is the effect knocking out HDAC6 has on immunoglobulin levels in mice. It was found that HDAC6^{-/-} mice subjected to an antigen challenge produced lower levels of IgG and IgM (Zhang et al. 2008). However, it is unclear as to whether or not this is a result of a direct effect on B cells or due to an indirect effect on immune synapse formation (Serrador et al. 2004) and/or lymphocyte migration (Cabrero et al. 2006) (see below for effects of HDAC6).

1.11 Histone deacetylase 6

Of increasing interest throughout this study is the targeted pharmacological inhibition of HDAC6. As mentioned previously, one of the unique features of this class IIb enzyme is that it is predominantly located in the cytoplasm (Verdel et al. 2000), but retains the ability to undergo nuclear translocation. As it is predominantly located in the cytoplasm, histone is unlikely to be its primary substrate.

First discovered in humans in the late 1990s by Grozinger et al. (Grozinger, Hassig, & Schreiber 1999) and in mice by Verdel (Verdel & Khochbin 1999), it is the largest of the HDAC proteins with 1,216 amino acids. It is unique among the class II HDACs (and indeed among the zinc-dependent HDACs) in that it contains two active, functionally independent catalytic domains (Figure 1-8). The role and integration of these two domains have been the subject of differing views among researchers. Early data suggested that they acted independently (Grozinger, Hassig, & Schreiber 1999), however subsequent research challenged this and suggested that these two domains act synergistically (Zhang et al. 2003; Zhang et al. 2006) or that activity is at least mediated by the second domain (Zou et al. 2006). Though no clear evidence has been produced to delineate the roles of these

domains, it would appear that the second domain (DD2), has the principal role in terms of function.

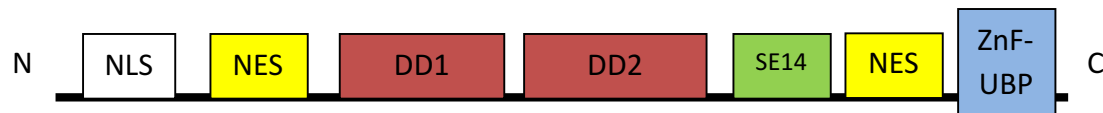


Figure 1-8 Structure of HDAC6. NES – nuclear exportation signal, DD1 & DD2 – deacetylating domain, SE14 – cytoplasmic anchoring signal, ZnF-UBP – ubiquitin binding domain

HDAC6 has a C-terminal zinc-finger ubiquitin binding domain (ZnF-UBP) and a specific tertadecapeptide repeat domain (SE14) which in combination with two leucine-rich nuclear export sequences (NES), promote cytosolic retention (Bertos et al. 2004). The discovery of this ZnF-UBP domain (Seigneurin-Berny et al. 2001) has implicated its role in a number of cell signalling pathways that are not only dependent upon acetylation but also upon ubiquitination. Indeed, HDAC6 has been shown to have the highest affinity yet discovered for ubiquitin (Boyault et al. 2006).

Given its unique structure and location (cytoplasmic) among the HDAC family, HDAC6 is well placed as a novel target for manipulation, and further research into its role is well justified. To date, three key substrates of HDAC6 have been identified; α -tubulin, HSP90 and cortactin.

1.11.1 Molecular functions of HDAC6

α -tubulin

HDAC6 has an important role in the deacetylation of a number of proteins. Its earliest and probably its most widely researched function is in regulating cytoskeletal dynamics through the acetylation of α -tubulin (Hubbert et al. 2002), which together with β -tubulin make up microtubules. These microtubules are intracellular structures which have a number of roles including nucleic and cell division, intracellular transport and organisation of intracellular structure as well as cell motility. The effect of HDAC6 deacetylation on the α -tubulin substrate is very much dependent upon the type and location of the cell expressing the substrate and results in changes in cell division, intracellular trafficking, cell-cell interactions and cell migration (Janke & Bulinski 2011).

Both *in-vitro* and *in vivo* evidence has demonstrated that over expression of HDAC6 results in complete deacetylation of microtubules and that its inhibition results in increased levels of α -tubulin (Hubbert et al. 2002; Zilberman et al. 2009)

HSP-90

By demonstrating its reversible deacetylation, heat shock protein 90 (HSP90) was first discovered to be a substrate of HDAC6 in 2005 (Kovacs et al. 2005).

HSP90 is a well-characterised, chaperone protein which has a critical role in intracellular transport, cell signalling, maintenance, protein folding and degradation. In the unstressed cell, it accounts for 1-2% of cellular proteins and maintains a key role in cellular homeostasis. However, in the stressed cell, it can account for 4-6% of cellular proteins (Crevel et al. 2001) and is integral to a cells stress response.

In the unstressed cell, HSP90 binds to and regulates the enzyme heat-shock transcription factor 1 (HSF1) and maintains it in its inactive form. However, in times of stress, an increase in misfolded ubiquitinated proteins results in dissociation of the complex as HDAC6 aims to

bind the free protein complexes. This results in activation of HSF1 and subsequent activation of HSP90 (and a protective cellular response).

Cortactin

Cortactin is a monomeric, cytoplasmic protein, which when stimulated (deacetylated by HDAC6) is involved in actin remodelling and cell motility / migration (Wu & Parsons 1993).

Studies have demonstrated that the over expression of HDAC6 results in polymerisation of actin, whereas HDAC6 knockout results in the opposite effect and reduced cell motility (Zhang et al. 2007).

1.11.2 Anti-inflammatory effects of HDAC6

During periods of infection or inflammation, a pro-inflammatory response is mounted in order to deal with the toxic insult. This response is often limited, in the acute setting by an anti-inflammatory response, once the stimulating source has been dealt with. However, in the setting of a chronic stimulus (such as an allograft), the pro-inflammatory state can be persistent. One of the key modulators of the pro-inflammatory response are FoxP3 expressing regulatory T cells (see below for the role of T_{regs} in the transplant setting). These cells play a key role in controlling the expression of inflammatory cytokines and dampen the activation of effector T cells, thus promoting immunological homoeostasis (Fontenot, Gavin, & Rudensky 2003b). There now exists an increasing body of evidence which is attempting to delineate the role that HDACs play in this process, by promoting the expression of pro-inflammatory genes (Shakespeare et al. 2011) and the potential therapeutic benefits histone deacetylase inhibition may have in terms of anti-inflammation.

In addition to this apparent histone modulated effect of histone deacetylases on inflammation, the potential role of deacetylating non-histone targets in this setting have

also been examined and contribute to the core hypothesis of the evidence provided in this thesis. Principally, the ability of HDAC6 to regulate FoxP3⁺ regulatory T cells through HSP90 modulation (de Zoeten et al. 2011).

1.12 Histone deacetylase inhibitors (HDACis)

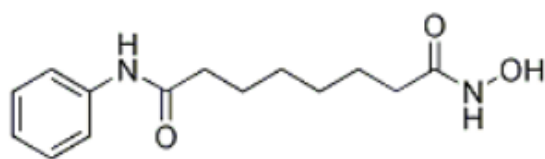
A class of drugs attracting increasing attention for their immunomodulatory effects are the histone deacetylase inhibitors (HDACis). HDACis were first identified over a decade ago as potential anti-cancer agents (Richon & O'Brien 2002), and the majority of research on their function has been carried out in this area. The most notable of these, the pan-HDACi suberoylanilide hydroxamic acid (SAHA, Vorinostat) was approved by the FDA in 2006 for the treatment of T cell lymphoma. Subsequently, in 2009, Romidepsin (FK228, Istodax) achieved FDA approval for the same indication, and more recently Panobinostat (LBH-589, Farydak) was approved for the treatment of multiple myeloma. Through the development of these drugs, the effect of HDACi on the cell cycle and the subsequent impact on reduced tumour growth is relatively well documented. However, the potential effects of HDACi on the immune system are far less well characterised. In preclinical models of organ transplantation, pan-HDACis have been shown to confer efficacy (Kinugasa et al. 2008; Kinugasa et al. 2009), and many of these have been shown to potentiate the suppressive function of T_{regs} (Akimova et al. 2010; Lucas et al. 2009; Tao et al. 2007; Tao et al. 2007). However, the broad mechanism of action of pan-HDACis and their resultant side effect profiles, such as thrombocytopenia, have limited their clinical application thus far to cancer only (Kelly et al. 2003; Strevel, Ing, & Siu 2007)

More recently however, isoform-selective HDACis have been developed, which retain target potency but which have an improved toxicity profile. Such efforts have rekindled research in identifying the role of specific histone deacetylases (HDACs) in the pathogenesis of disease. Hancock and colleagues have recently demonstrated that inhibition of class II HDACs (HDAC4-7, HDAC9 and HDAC10) results in the mediation of immunosuppression. Of particular note, is the inhibition of HDAC7, HDAC9 (class IIa) (Tao & Hancock 2008) and HDAC6 (Class IIb) (Beier et al. 2012; de Zoeten et al. 2011) which appear to have the most significant effect on T_{reg} function. In the case of HDAC6, inhibition leads to an apparent increased expression of FoxP3, which is critical to the development and immunosuppressive function of T_{regs} . It is this potential effect of HDACis on T_{regs} which is central to the hypothesis of this project that they may have benefit as an immunosuppressive therapy in transplantation.

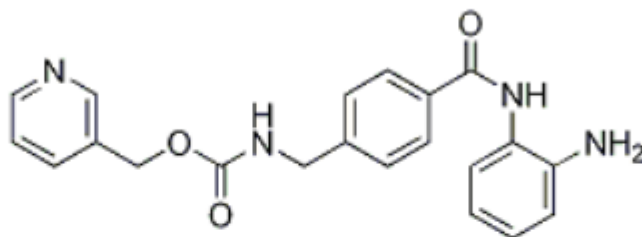
HDACis affect a conformational change through the acetylation of histone, resulting in a reduced affinity for DNA. This alteration in chromatin structure exposes the DNA and therefore facilitates enhanced gene transcription. The mechanism by which histone deacetylation occurs has already been discussed earlier in this chapter, and the long list of non-histone protein substrates of HDACs already described. The potential targets therefore of HDACis is similarly extensive and includes transcription factors, structural proteins and signalling molecules all of which may be involved in immunomodulatory processes.

HDACis are a diverse set of compounds with differing biological activities and specificities. They are generally classified into four broad structural categories (hydroxamic acids, synthetic benzamides, cyclic peptides and short chain fatty acids (Figure 1-19).

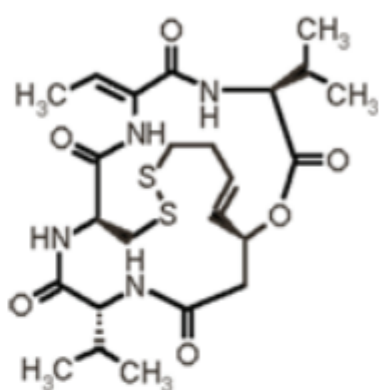
Hydroxamic Acids (eg SAHA)



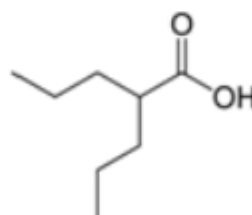
Synthetic benzamides (eg MS275)



Cyclic peptides (eg FK228)



Short chain fatty acids (eg valproic acid)

**Figure 1-9 Structural class (and example) of HDACi**

Of these HDACi subtypes, the most widely researched and currently most successful in terms of clinical application are the hydroxamic acids. In general, these historically, have tended to be non-specific in inhibiting HDACs; however due to burgeoning interest in the development of selective and specific compounds in order to improve efficacy and side effect profiles a number of 'cleaner' compounds have been developed. One of these recently engineered compounds is the HDAC6 specific inhibitor, KA1010. This thesis will describe how this compound was selected from a panel of potential HDACi compounds and assessed as a potentially superior immunosuppressant compared to current therapies and pan-HDAC inhibitors.

1.12.1 A novel HDAC6-specific inhibitor – KA1010

The hydroxamate structure of KA1010 closely resembles that of SAHA (Figure 1-10) with an additional benzene ring at the N-terminus and is highly selective for HDAC6.

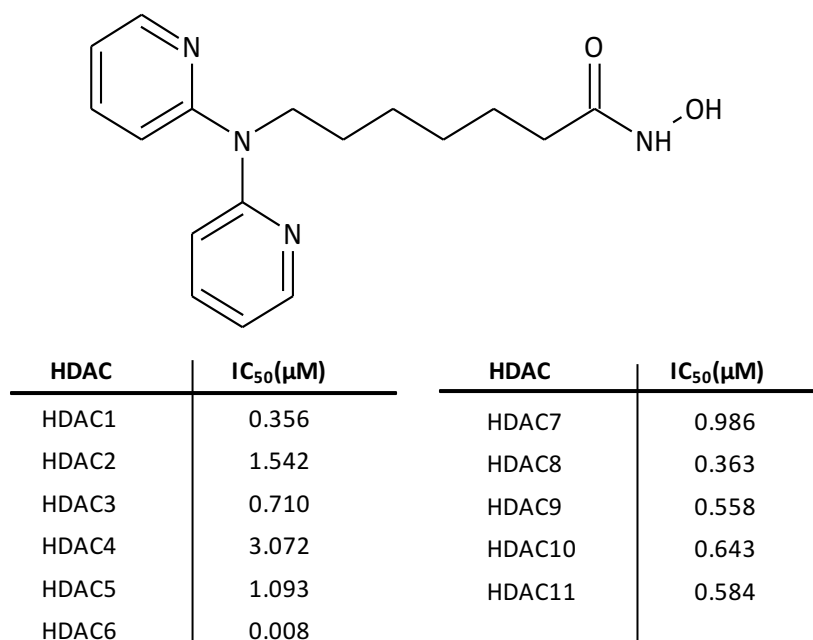


Figure 1-10 Structure and HDAC-specificity of KA1010

It is a novel, engineered compound not currently licensed for clinical use and its potential applications in other clinical settings (as with several other HDACi compounds) such as inflammation are under investigation. Its development has been undertaken by Karus therapeutics and they have kindly supplied samples of the compound for the purpose of this study.

1.13 The role of regulatory T cells in transplantation

One of the most researched areas in transplantation over recent years is the potential role FoxP3+ regulatory T cells (T_{regs}) may have in developing new immunosuppressive strategies. T_{regs} possess a unique function among the T cell population in that they are able to naturally

limit immune responses and, in the normal healthy subject prevent autoimmunity (for example, prevention of materno-fetal allograft rejection). Strategies harnessing this cellular immune compartment by promoting their function, expansion or adoptive transfer may lead to alternative immunosuppressive modalities (De Serres, Sayegh, & Najafian 2009; Feng et al. 2011; Roncarolo & Battaglia 2007; Wood & Sakaguchi 2003).

1.13.1 Discovery of Tregs

In 1970 Gershon and Kondo (Gershon & Kondo 1970), first identified a population of T cells (suppressor T cells) with a modulatory effect on immune responses. These cells were characterised by the expression of either Lyt-1 or Lyt-2 and were found to be present in both the CD4⁺ and CD8⁺ subset populations. However, attempts to identify the encoding gene region thought to be responsible for these specialised cells proved fruitless and interest in further research dwindled.

It would not be until 1995 when Shimon Sakaguchi published his seminal paper in the *Journal of Immunology* that T_{regs} were first recognised (Sakaguchi et al. 1995) as a separate immunomodulatory T cell population. He demonstrated the existence of a subpopulation of T cells, expressing the IL-2 receptor α -chain (CD25) present in the normal immune system, making up approximately 5% of the T cell population. He showed that the removal of this population of T cells from normal mice resulted in autoimmune disease analogous to conditions such as type I diabetes, autoimmune thyroiditis and autoimmune arthritis in humans. He concluded that this population of T cells were responsible for maintaining natural self-tolerance and its depletion, results in a number of autoimmune diseases. Sakaguchi's findings prompted intense research into the characteristics of T_{regs}. In 2001, it was identified that an X chromosome mutation of the FoxP3 gene in mice was responsible

for the severe autoimmune condition seen in Scurfy mice (Brunkow et al. 2001). The human equivalent of the FoxP3 gene was rapidly found to be responsible for an analogous condition known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) characterised again by severe autoimmunity and leading to type I diabetes, thyroiditis, inflammatory bowel disease and severe allergy (Bennett et al. 2001; Chatila et al. 2000; Wildin et al. 2001). Finally, following this discovery, several groups began to link the phenotypical similarities between Scurfy mice and those which had undergone T_{reg} depletion. In 2003, it was reported that FoxP3 was essential for T_{reg} development and function (Fontenot, Gavin, & Rudensky 2003; Hori, Nomura, & Sakaguchi 2003; Khattri et al. 2003). FoxP3 mRNA was found to be a feature of $CD4^+CD25^+$ cells but was not present, even despite activation in $CD4^+CD25^-$ cells. However, retroviral transduction of the FoxP3 gene into $CD4^+CD25^-$ cells transformed them into functional and phenotypical T_{reg} -like cells.

1.13.2 Types of regulatory T cells

It is now widely accepted that $CD4^+FoxP3^+ T_{regs}$ can be classified broadly into two groups, depending up on their ontology, namely natural T_{reg} (nT_{reg}) or induced (or adaptive) T_{reg} (iT_{reg}). However, these cells typically lack homogeneity and it is likely that this classification is over-simplistic.

1.13.3 Natural T_{regs}

The more homogenous of these two populations are the natural T_{regs} . They are derived in the thymus (Sakaguchi et al. 1995) from immature $CD4^+CD8^+$ T cells (see thymocyte development above) and migrate out to the periphery where they make up 5-10% of $CD4^+$ T cells. The characteristic expression of CD25 is acquired quite late in its development at the $CD4^+CD8^-$ single positive thymocyte stage. The critical factor which appears to determine

whether a thymocyte takes the conventional $CD4^+CD25^-$ lineage route of development or the T_{reg} route is the degree of affinity the TCR has to self-peptides. Typically, those thymocytes with a higher (intermediate) affinity for antigen will develop as T_{regs} (von & Jaeckel 2001).

The involvement of the NF- κ B pathway seems to have a significant role in nT_{reg} development and its level of thymic activation directly correlates with the degree of FoxP3 induction and T_{reg} differentiation (Long et al. 2009). However, its precise role remains unclear.

What is clearer is the dependence of co-stimulatory molecules on nT_{reg} development.

Murine studies looking at mice deficient in CD28 (Keir & Sharpe 2005), CD80/86, CD40 (Paust et al. 2004) and IL-2rB (Malek et al. 2002) produce fewer numbers of less suppressive nT_{regs} . IL-2 and TGF- β are also important in nT_{reg} development. Though T_{regs} express high levels of the IL-2 receptor (CD25), they are unable to produce IL-2 themselves and are therefore dependent on paracrine production for continued survival. The role of TGF- β is less clear though it has been shown to be necessary to maintain FoxP3 expression in nT_{regs} (Marie et al. 2005).

1.13.4 Induced T_{regs}

Induced T_{regs} typically arise from the conversion of naive $CD4^+FoxP3^-$ conventional T cells (T_{conv}) in the periphery following one of a number of stimuli. Two main types of iT_{reg} have been identified, based on the cytokine which induces them. Type 1 regulatory T cells ($Tr1$) are induced by IL-10 (Groux et al. 1997; Vieira et al. 2004) and T helper 3 ($Th3$) cells are induced by TGF- β (Weiner 2001). Appropriately, each subset exerts its inhibitory effect through the secretion of the same cytokine which stimulated its differentiation.

The conversion of FoxP3⁻ T cells to FoxP3⁺ iT_{regs} has also been shown to be induced in the presence of IL-4 and IL-13, without the presence of either IL-10 or TGF-β (Skapenko et al. 2005).

1.13.5 Function of T_{regs}

The role of T_{regs} in the immune system is of great interest. The therapeutic potential of these cells in terms of dampening the alloimmune response and ultimately achieving transplant tolerance is of enormous clinical interest. Applications expanding the T_{reg} population are relevant to transplantation, and their benefits in other disease processes such as the treatment of inflammation, oncology and autoimmune disease are well recognised (Kohm et al. 2002; Maloy et al. 2003; Workman et al. 2009).

Though nT_{regs} and iT_{regs} have broadly similar mechanisms in terms of suppression and are phenotypically alike, their proliferative stimuli are quite different. As described, nT_{regs} rely on the response of thymocytes to self-peptide in order to stimulate their expansion. iT_{regs} on the other hand, are stimulated through a weaker TCR mechanism in response to exogenous peripheral antigens (Apostolou & von 2004; Kretschmer et al. 2005). As a result, iT_{regs} tend to be stimulated in response to anti-inflammatory cytokines in a setting of inflammation. Another difference between the two subsets is the dependence upon CD28 as a co-factor (which is necessary for nT_{regs}, but not required for iT_{regs} (Apostolou et al. 2002; Kretschmer et al. 2005)). Of note, studies have demonstrated that the *in vitro* expansion of CD28 stimulated iT_{regs} actually results in cells with unstable FoxP3 expression and reduced survival (Floess et al. 2007). Furthermore, if these cells are developed in the absence of CD28, they remain fully functional even after *in vivo* transfer (Taylor, Lees, & Blazar 2002).

Over recent years, there has been an expansion of theories as to the mechanisms by which T_{regs} infer suppression on their targets. Many of these theories are diverse and the reality is that they are all likely to contribute to varying degrees, depending upon factors such as the environment and the cell type targeted. However, further work is still required to clarify how these various mechanisms interplay.

T_{regs} have the ability to suppress a multitude of cell types, including dendritic cells (DCs), natural killer T cells (NKT), B cells and $CD8^+$ and $CD4^+$ T cells (Miyara & Sakaguchi 2007). The resulting effect of T_{reg} suppression on target cells can be equally as varied as the mechanism by which they act. This can range from down-regulating growth factors such as IL-2 (Wing, Fehervari, & Sakaguchi 2006) to lysing targets through granzyme B and perforin (Gondek et al. 2005; Grossman et al. 2004).

The mechanisms by which T_{regs} manifest suppression can be broadly divided into five categories (Figure 1-11);

1. Cell-to-cell contact

Through TCR activation (Thornton & Shevach 2000), T_{regs} are able to suppress Teff cells by inhibiting their transcription of IL-2 (mRNA) (Thornton et al. 2004; Thornton & Shevach 1998) and by consuming IL-2 (de la et al. 2004). The precise mechanism of this effect is unclear, but it appears that TGF- β and IL-10 are not implicated.

2. Dendritic cell modulation

T_{regs} can inhibit DC function through two mechanisms. The first is to inhibit indoleamine 2,3-dioxygenase (IDO) resulting in reduced tryptophan, necessary for cell division. This is through the binding of CTLA-4 to CD80/86 on DCs (Fallarino et al.

2003). The second T_{reg} :DC interaction involves the sending of inhibitory signals via LAG-3 to MHC class II molecules (Liang et al. 2008).

3. Inhibitory cytokines

One of the most widely studied mechanisms by which T_{regs} modulate immune responses is through the release of immunosuppressive cytokines such as IL-10 (Ito et al. 2008), TGF- β (Levings, Sangregorio, & Roncarolo 2001), IL-35 (Chaturvedi et al. 2011) and galectin-1 (Shevach 2009; Sojka, Huang, & Fowell 2008).

4. Metabolic disruption

Hydrolysis of extracellular ATP via ectoenzymes CD39 and CD73 results in adenosine production (Deaglio et al. 2007). This adenosine then binds to the adenosine specific A2A receptor on Teff, disrupting the cyclic AMP (cAMP) dependent secretion of cytokines (Bopp et al. 2007).

5. Cytolysis

Activated T_{regs} have the ability to mediate lysis of Teff cells through the release of granzyme B / perforin. This mechanism requires CD18 mediated adhesion (Grossman et al. 2004).

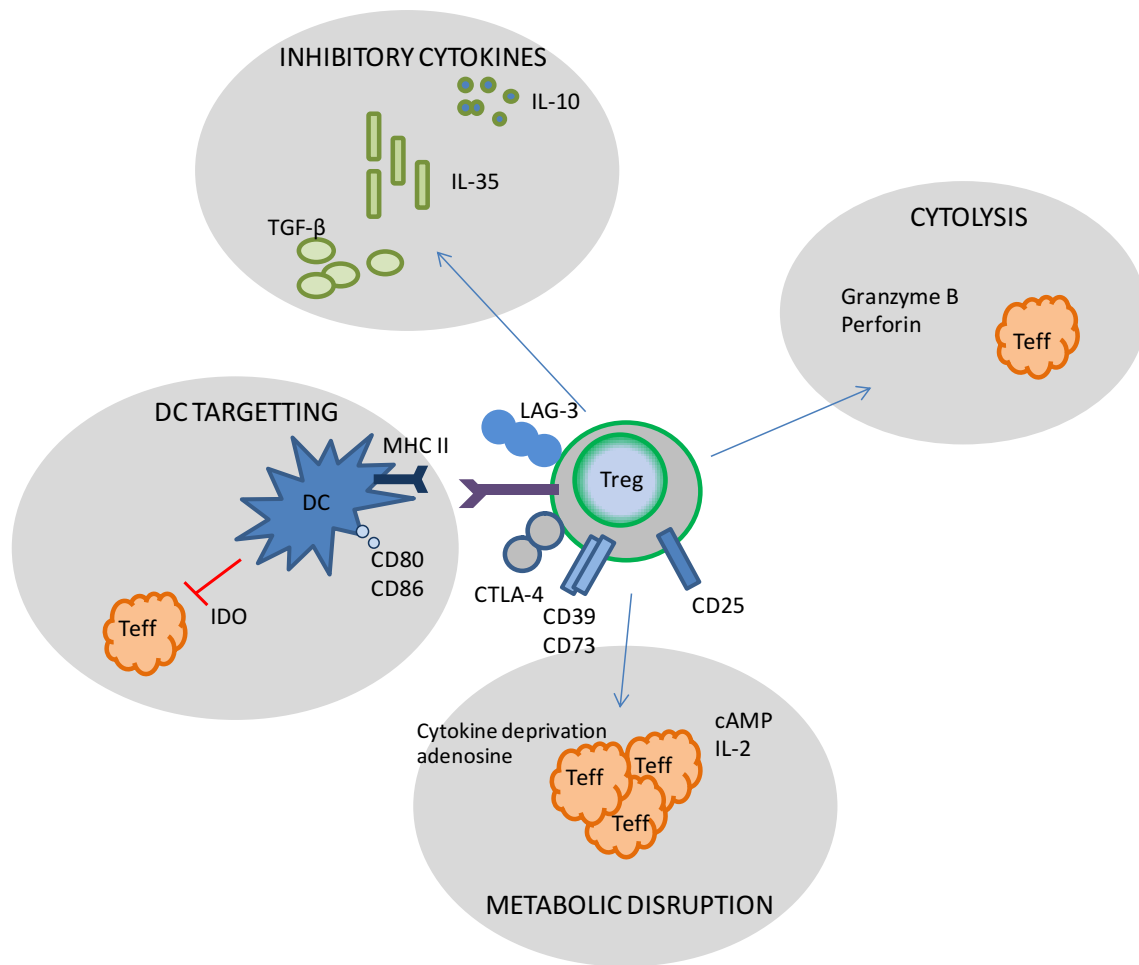


Figure 1-11 T_{reg} mechanisms of action

Adapted from Frontiers in Immunology (Caridade, Graca, & Ribeiro 2013)

1.14 Aim of the study

The aim of this study therefore was to assess the potential immunosuppressive function of histone deacetylase inhibitors through the modulation of regulatory T cells, and consider whether or not this group of compounds may have a therapeutic role in transplantation. We were able to compare the effects of the established immunosuppressant (CyA) with established pan-HDACis, in clinical use for other indications (SAHA). In addition, through an industrial partnership we were able to obtain and assess the effect of a number of cleaner, engineered HDACi compounds with more selective or specific inhibitory profiles. After considering a panel of HDACis, we were then able to focus further studies in the form of transplantation models on those compounds considered to have the greatest potential (KA1010).

1.15 Overview

Chapter 2 describes the various methods employed during the course of this study.

The findings of this study are then presented over three results chapters. They are broadly categorised into the following:

- Cellular effects of Histone deacetylase inhibitors (Chapter 3)
- Effect of histone deacetylation on *in vitro* models of allotransplantation (Chapter 4)
- *Ex vivo* effects of histone deacetylase inhibition on allotransplantation (Chapter 5)

Chapter 3 identifies the HDAC targets of interest in relevant cell types and examines the proliferative effects of a panel of novel and established HDACis on several cell lines. In addition to this toxicity and viability of treated cells are assessed. This chapter also examines the effects of HDACi on regulatory T cells and their suppressive effect in a T_{reg} suppression

assay. The chapter concludes with data on the effects of HDACi on lymphocyte subset expression.

Chapter 4 builds on findings from the previous studies and considers the effects of HDACi (KA1010 and SAHA) as well as Cyclosporin A on several *in vitro* models of transplantation. The basis for this is a mixed lymphocyte reaction (MLR) and both one- and two-way types were used. A novel adaptation of the one-way MLR was further developed in order to demonstrate autologous preservation. Finally results examining the effect of HDACi on T-cell activation markers and MLR cytokine release are presented.

Chapter 5, the final results chapter focusses on the *in vivo* effects of HDACi (specifically KA1010 +/- Rapamycin) on a murine skin model of transplantation. Data pertaining to graft survival are presented along with the physiological effects of HDACi. This chapter concludes with results demonstrating the effect KA1010 has on relevant T cell subsets *ex vivo*.

The final chapter in this thesis discusses the above results in further detail and considers how these findings add to the current evidence relating to a potential immunosuppressive role for HDAC6 inhibition. Possible mechanisms of action are also postulated and areas for further study are highlighted.

Chapter 2 - Materials and Methods

2 Materials and Methods

2.1 Compounds

HDACi compounds were supplied by Karus Therapeutics Ltd, Oxford, UK in powder form. They were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10mM and stored at -80°C. For experimental use, they were diluted in Roswell Park Memorial Institute Media (RPMI) supplemented with 10% human albumin solution (HAS). Cyclosporin A (CyA; Sigma-Aldrich, Dorset, UK) was supplied in powder form and dissolved in DMSO to make a 50mg/ml stock solution.

2.2 Isolation of PBMCs

Peripheral blood cells (PBMCs) were isolated from healthy donors by Ficoll-Paque PLUS density centrifugation (GE Healthcare, Buckinghamshire, UK) from 20ml of heparinised blood collected from healthy volunteers. Blood was diluted 1:1 at 37°C with sterile supplemented RPMI (containing 10% FCS, 1% Penicillin-Streptomycin and 1% Glutamine), gently mixed and divided into 10ml aliquots and carefully layered onto 6ml of Ficoll-Paque Plus and centrifuged at 2100rpm for 20 minutes. Due to their low density, PBMCs form a visible layer at the interface between Ficoll and blood. After carefully removing this lymphocyte-containing layer of cells, they were transferred to a fresh tube and washed with supplemented RPMI by centrifuging at 1700rpm for 10 minutes. A second wash was performed before finally suspending the cells in 10ml of supplemented RPMI. Cell number

and density was calculated using a haemocytometer after staining with filtered Tryptophan blue.

All reagents were obtained from Sigma-Aldrich unless otherwise stated and plastic labware from BD-Falcon UK.

2.3 Western Blot Analysis

10% SDS-PAGE gels were constructed. To make the resolving gel 12ml Protogel (Fisher Scientific, Ireland) was added to 10.5ml of distilled water, 7.5ml of 1.5M Tris (pH 8.8) and 300µl 10% SDS. 150µl of 10% APS and 30µl of TEMED were added to the solution before allowing the gel to set in the cassette. The stacking gel consisted of 2.6ml Protogel (Fisher Scientific, Ireland), 12.2ml of distilled water, 5ml of 0.5M Tris (pH 6.8) and 200µl 10% SDS. 100µl of 10% APS and 20µl of TEMED.

Proteins were denatured by suspending a pellet of 1×10^6 cells in 0.5ml PBS in a large eppendorf. A pellet of cells was reformed by centrifuging at 3000rpm for 5 minutes. The PBS was pipette off and the pellet allowed to dry. The pellet of cells was then resuspended in 20µl of SDS buffer (50mM Tris pH 6.8, 2% SDS, 10% Glycerol, 1% β -Mercaptoethanol, 12.5mM EDTA and 0.02% Bromophenol Blue) and boiled at 100°C for 5 minutes. After a further centrifuge at 13,000rpm for 1 minute samples were loaded onto the gel in 20µl aliquots.

Samples were resolved at 100 volts until the dye reached the bottom of the gel. The stacking gel was removed and samples were transferred onto a nitrocellulose membrane (Amersham Hybond-P membrane, GE Lifesciences) which had been pre-soaked for 5 seconds in methanol prior to being soaked in transfer buffer (25Mm Tris, 182Mm Glycine

and 10% methanol) for five minutes. Samples were then transferred at 10 volts for 90 minutes and the membrane blocked overnight in 10% blocking buffer (PBS Tween with BSA). Three washes were then carried out using blocking buffer before the addition of the primary antibody (see below).

The primary antibody was incubated with the sample overnight and an identical washing undertaken to that described previously. The secondary antibody (anti-mouse IgG horseradish peroxidase linked whole antibody at a concentration of 1/2000 (eBioscience, Hatfield, UK)) was added to the sample and incubated for a further 1 hour. The membrane was washed again and incubated for 2-3 minutes with Amersham Enhanced Chemiluminescence reagent (GE Healthcare, Uppsala, Sweden) before loading onto cassette for developing.

Western Blotting was performed to demonstrate the presence of the specific target of interest, HDAC6. Standard Western Blotting procedures were performed using a monoclonal anti-HDAC6 antibody (Sigma-Aldrich, Dorset, UK). Briefly, T cell subsets were separated using immunomagnetic negative selection kits (Stemcell Technologies, Grenoble, France; Miltenyi Biotec Ltd, Surrey, UK). One million cells were denatured in sodium dodecyl sulphate (SDS) buffer and transferred onto a Polyvinylidene fluoride (PVDF) membrane. Blots were probed using a mouse monoclonal anti-HDAC6 antibody (1/500). The secondary antibody was anti-mouse IgG horseradish peroxidase linked whole antibody – 1/2000 (eBioscience, Hatfield, UK). Detection was performed using an Amersham Enhanced Chemiluminescence reagent (GE Healthcare, Uppsala, Sweden). Consistent sample loading in each well was confirmed by reblotting with actin antibody.

2.4 Antibodies

Blots were probed using monoclonal anti-HDAC antibodies (obtained from Sigma-Aldrich, Dorset, UK) at a concentration of 1/500. The antibodies used were:

- anti-HDAC2
- anti-HDAC3
- anti-HDAC4
- anti-HDAC6
- anti-HDAC9

2.5 Toxicity assay

In order to assess toxicity a FITC Annexin V apoptosis detection kit was used (BD Pharmingen). After isolating PBMCs, cells were re-suspended at a density of 1×10^6 cells / ml in supplemented RPMI and cultured in 200 μ l aliquots in a 96 well round-bottomed plate for 72 hours at 37°C. The culture media was supplemented with compound of interest.

Following the culture period, cells were washed twice in cold PBS and re-suspended in 1x Binding Buffer at a concentration of 1×10^6 cells/ml. 100 μ l of the solution (1×10^5 cells) was then transferred to a 5 ml culture tube and 5 μ l of FITC Annexin V and 5 μ l PI added. Following gentle vortex, the cells were incubated for 15 minutes at room temperature in the dark. Finally, 400 μ l of 1x Binding Buffer was added to each tube and flow cytometry analysis undertaken within 1 hr.

Flow cytometry was undertaken using a BD FACSCanto II flow cytometer (Becton Dickinson and Co, New Jersey, USA) and FlowJo software (Tree Star Inc, Oregon, USA). PE – side scatter, FITC – forward scatter.

2.6 Jurkat Cell proliferation assay

A resazurin-based system was used to calculate Jurkat cell proliferation. Firstly, Jurkat cells were cultured in supplemented RPMI in a 96-well round-bottomed plate at a density of 5000 (or 50,000) cells / ml at 37°C for up to five days. The media was supplemented with sodium azide (negative control), PMA or LTA (positive control) or compound of interest (HDACi) at a range of doses described in the results. Untreated serial dilution controls were also undertaken so a standard curve could be produced for extrapolation purposes.

Following the culture period, 10µl of sterile 0.01% Resazurin was added to each well and after gentle shaking, the plate incubated for a further four hours. After a further brief shaking relative fluorescence units (RFU) were then measured using a spectrophotometer (filter pair - excitation = 530-570 nm and emission = 590-620 nm). The standard curve was calculated and relative cell numbers extrapolated using the best fit line equation.

2.7 PBMC proliferation assay

CFSE was used to assess the proliferation of PBMCs (CellTrace cell proliferation kit, Invitrogen, Paisley, UK). Freshly isolated PBMCs were prepared in the standard fashion and suspended in supplemented RPMI at a concentration of 1×10^6 cells / ml. A 5mM stock solution of CFSE was prepared by dissolving 50µg in 18µL of DMSO. This was further diluted in PBS before adding to the cell media at a concentration of 1µM. Cells were then incubated for 10 minutes at 37°C for ten minutes. Any free dye was then removed by washing five times in culture media. The CFSE-stained cells were then resuspended in supplemented RPMI at a concentration of 1×10^6 cells / ml and distributed in 200µl aliquots into a 96-well round-bottomed plate (media was supplemented with appropriate concentration of compound of interest).

Following the incubation period, cells were washed in RPMI and proliferation was assessed using a BD FACS Canto II flow cytometer to assess cell division.

2.8 Regulatory T cell suppression assay

Isolation of regulatory T cells was undertaken using the MACS CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi-Biotec). Numbers of freshly isolated PBMCs were calculated and suspended in 90µl cold buffer (PBS pH 7.2 + 0.5% BSA + 2Mm EDTA) per 10⁷ cells. 10µl of CD4⁺ T cell Biotin-Antibody cocktail was added per 10⁷ cells, mixed well and refrigerated for 5 minutes. 20µl of Anti-Biotin MicroBeads were then added followed by a further 10 minute refrigeration period. The cell suspension was passed through a separation column and the unlabelled CD4⁺ enriched effluent collected. After two washes with cold PBS, the cell suspension was centrifuged at 300xg for ten minutes and the supernatant aspirated. The pellet was suspended in 90µl of buffer and 10µl of CD25 MicroBeads added. The suspension was then refrigerated for 15 minutes, washed in PBS and suspended in 500µl of buffer.

The suspension was then applied to a second separation column and the unlabelled effluent collected (CD25⁻ fraction). The CD25⁺ fraction was then retrieved from the column by rinsing with 3 x 500µl washes and enriched by passing through a further column.

Once isolated, the CD4⁺CD25⁺ regulatory T cells were cultured with 1µM CFSE-labelled (as above) CD4⁺ responder cells and proliferation assessed using flow cytometry. Media was enriched with compound of interest at various doses (see Results) to assess HDACi effect.

Following assay development, stimulation was achieved using Dynabeads (Invitrogen, Paisley, UK). According to manufacturer protocol these were washed in buffer (PBS + 0.1% BSA + 2mM EDTA, pH 7.4) and re-suspended in culture medium. Cultures were undertaken

in a 96-well plate and Dynabeads were added at a cell to bead ratio of 1:1. Following the 5 day assay incubation period, beads were removed using a magnet prior to flow cytometry analysis of responder cell proliferation.

2.9 Effect of HDACi on lymphocyte subset expression

Freshly isolated PBMCs were diluted to a density of 1×10^6 cells / ml and cultured in 200 μ l aliquots for up to five days in a 96-well round bottomed plate. The media was supplemented with compound of interest where appropriate.

Following the culture period, cells were washed in RPMI and stained using the following antibodies (BD Biosciences):

<i>Antibody</i>	<i>Fluorochrome</i>	<i>Concentration</i>
CD4	APC	1:100
CD8	PerCP	1:100
CD3	FITC	1:100
CD19	PE	1:100

2.10 PBMC Irradiation Assay

Gamma irradiation causes DNA damage to PBMCs and results in cells no longer able to proliferate or produce de novo protein but with otherwise normal function. In order to achieve an irradiated population of cells, freshly isolated PBMCs were suspended in sterile supplemented RPMI (containing 10% FCS, 1% Penicillin-Streptomycin and 1% Glutamine) and exposed to varying degrees of radiation using a standard laboratory irradiator.

2.11 One-way MLR

A one-way mixed lymphocyte reaction (MLR) assay was adopted as a model of *in vitro* alloreactivity. PBMCs were prepared from two allogeneic donors. Responder cells (10^5) labelled with 1 μ M CFSE were cultured with 10^5 stimulator cells (irradiated at 20 Gy) for seven days in a 96-well round bottom plate in AIM V media supplemented with + 10% human serum together with compound of interest (CyA, SAHA and KA1010). Suppression of alloreactivity was measured by loss of CFSE staining intensity in the parent population, measured using a FACSCanto flow cytometer (Becton Dickinson and Co, New Jersey, USA) and FlowJo software (Tree Star Inc, Oregon, USA).

2.12 One-way MLR – Killing Assay

A one-way mixed lymphocyte reaction (MLR) assay was adopted as a model of *in vitro* targeted alloreactivity by assessing the response of both allogeneic and autologous responders in the same assay. PBMCs were prepared from two allogeneic donors. Allogeneic responder cells (50,000 cells) were labelled with FITC Mouse Anti-Human CD45 antibody (Becton Dickinson and Co, New Jersey, USA) at a concentration of 1/2000 or 1/100. The autologous population, in order to facilitate discrimination between the two populations, was stained with the same antibody at a concentration of 1/600 or with APC-H7 Mouse Anti-Human CD45 antibody (Becton Dickinson and Co, New Jersey, USA) at a concentration of 1/100. Unlabelled stimulator cells (100,000 cells per assay) were irradiated at 20 Gy. Populations were combined as required and cultured for varying lengths of time in a 96-well round bottom plate supplemented with RPMI (containing 10% FCS, 1% Penicillin-Streptomycin and 1% Glutamine) together with compound of interest (CyA, SAHA and KAR3000). Suppression of alloreactivity was measured by assessing the staining intensity of

target populations, measured using a FACSCanto flow cytometer (Becton Dickinson and Co, New Jersey, USA) and FlowJo software (Tree Star Inc, Oregon, USA).

2.13 Two-way MLR

For a two-way MLR, the suppressive activity of HDACis was compared with Cyclosporin for their ability to inhibit IFN- γ release by allogeneic PBMCs. The premise of the two-way MLR is similar to the one-way MLR except that targets were not irradiated, allowing allorecognition by both donor and recipient. The technique is otherwise similar. Supernatant was aspirated from two-way MLR cultures and IFN- γ release assessed using an ELISA kit (below).

2.14 Interferon- γ ELISA

IFN- γ release was measured using the eBioscience™ Human IFN- γ ELISA Ready-SET-Go® kit (eBioscience, Hatfield, UK) as per manufacturer's instructions – briefly, these consisted of the following:

A Corning Costar 9018 ELISA plate was coated with 100 μ L/well of capture antibody in 1X Coating Buffer (stock solution diluted to 1 in 10). The plate was sealed and incubated overnight at 2-8°C. After incubation, the wells were aspirated and washed 3 times with >250 μ L/well wash buffer (allowing ~ 1 minute for soaking during each wash step to increase the effectiveness of the washes). Following this, the plate was blotted on absorbent paper to remove any residual buffer.

The next step was to block the wells with 200 μ L/well of 1 x ELISA/ELISPOT Diluent (1 part 5 x ELISA/ELISPOT Diluent with 4 parts DI water) and incubate the plate at room temperature for 1 hour. The wells were then aspirated and a further wash undertaken using wash buffer. DI water was used to reconstitute lyophilized standards and allowed to sit for 15 minutes with gentle agitation prior to diluting further. A series of standard concentrations were then

plated to produce a standard curve - using 1 x ELISA/ELISPOT Diluent, reconstituted standard was diluted to prepare the maximum standard concentration (according to individual lots certificate of analysis). 100 µL/well of top standard concentration was then added to the appropriate wells and 2-fold serial dilutions of the top standards performed to make the standard curve for a total of 8 points. At least two wells with 100 µL/well of 1 x ELISA/ELISPOT Diluent only to serve as plate blanks were also used.

The plate was next sealed and incubated overnight at 2-8° followed by a repeat wash step (as above, repeated 5 times).

100 µL/well of detection antibody diluted in 1 x ELISA/ELISPOT Diluent was then added to each well, the plate sealed and incubated at room temperature for one hour. Again the plate was then washed 5 times (as above) and 100 µL/well of Avidin-HRP diluted in 1 x ELISA/ELISPOT Diluent added to each well. The plate was then sealed and incubated at room temperature for 30 minutes a further wash (as above, repeated seven times).

100 µL/well of 1 x TMB (Tetramethylbenzidine) solution was added to each well and the plate incubated at room temperature for 15 minutes after which 50 µL of Stop Solution was added to each well.

Finally, the plate was read using at 450nm using an ELISA plate reader (Promega Glomax Plate Reader) and the data was analysed using GraphPad Prism version 5.

2.15 T cell activation marker assay

A standard two-way MLR was undertaken as described above. In order to assess the effect of compound, the assays were treated by supplementing the culture media with either CyA (500nM), SAHA (20nM & 2µM) or KA1010 (10nM → 10µM). Cells were harvested from the culture at various time points (0,24,48 & 72h) and washed in RPMI. They were then stained

for cell surface markers CD3 (APC-H7), CD8 (PE), CD69 (FITC), CD25 (APC) and HLA-DR (PerCP) and assessed using standard flow cytometry protocols.

2.16 Multiplex cytokine assay

To assess cytokine release in MLR culture supernatant a standard Human 13plex flow cytomic kit from eBiosciences (Hatfield, UK) was used. In summary, the protocol consisted of preparing antigen standards as per manufacturers' guidelines. The kit consisted of premixed beads in a 96-well plate. To each well, 50µl of supernatant was added and the plate sealed and shaken at room temperature for 120 minutes. The plates were washed twice in buffer and 25µl of detection antibody added to each well. The plate was resealed and shaken for 30 minutes at room temperature. After 2 further washes 50µl of Streptavidin-PE was added to each well. The plate was resealed again and shaken at room temperature for a further 30 minutes. Two further washes were undertaken and the beads resuspended in 120µl of reading buffer. After a final shake for 5 minutes the plates were read on a Luminex 100 machine.

2.17 Flow cytometry

All flow cytometry was undertaken using standard protocols as per the manufacturers guidelines using a FACSCanto flow cytometer (Becton Dickinson and Co, New Jersey, USA) and FlowJo software (Tree Star Inc, Oregon, USA).

Antibodies for all procedures were supplied by Becton Dickinson and Co (New Jersey, USA).

2.18 Allogeneic murine skin transplant model

5-6 week old male C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice, bred

were used as recipients and donors respectively. All mice were

bred in pathogen-free conditions and housed in conventional conditions. Donor mice were euthanized in accordance with the University's standard operating procedure (SOP). Donor sites were cleaned with an alcohol-based preparation and full-thickness grafts were procured from both the ear and abdominal skin. Tissue was placed on ice. The ear graft was prepared by splitting the ventral collagenous flap from the dorsal skin and this was floated in PBS. The abdominal graft was prepared by removing fat from the subcutaneous surface and again floated in PBS.

Recipient mice were anaesthetised and post-op analgesia administered intraperitoneally. Recipient graft sites were prepared by removing a 1cm² patch of skin after shaving and skin preparation. Each recipient received an ear graft to the left dorsum and abdominal graft to the right dorsum. Grafts were removed from the PBS and placed on the graft bed. Excess PBS was removed using a sterile Q-tip and the graft trimmed so that it lied flat within the graft bed. The graft was then secured using 7-0 polypropylene sutures and sterile non-adherent gauze placed over. Finally, a sterile dressing was secured and the recipient placed in a clean cage to recover.

The sterile dressing was removed 4-5 days later and graft site examined.

Immunosuppression was administered daily, commencing on the day of surgery via the intraperitoneal route and this was combined with daily graft inspection (after dressings are removed).

Following the experimental period, recipient mice were euthanized in accordance with the University's SOP. Graft sites were excised encompassing a perimeter of normal non-transplanted recipient skin, frozen and stored at -80°C before further analysis (histology). In

addition to the retrieval of skin, recipient spleen and lymph nodes were also harvested, frozen and stored at -80°C.

2.19 Statistical analysis

All data presented as mean +/- the standard error of the mean (SEM). Statistical analysis of individual compounds was performed with a two-tailed unpaired t-test, using GraphPad Prism. When the effect of several compounds was assessed (MLR assays) a one-way or two-way ANOVA was used to evaluate the overall effect of compounds. Statistical significance of effect of compounds of interest in these assays was compared to control using a Tukeys multiple comparison test. A p value of less than 0.05 was considered to be of statistical significance.

Chapter 3 - Results

3 Cellular effects of Histone deacetylase inhibitors

3.1 Introduction

As described, histone deacetylase inhibitors comprise a large group of compounds which have effects on a range of targets (and consequently cell function). In order, therefore, to assess the suitability of such compounds as potential immunosuppressants it is important to delineate both the desirable and non-desirable cellular effects. A series of *in vitro* experiments were designed in order to evaluate some of the more pertinent effects in relation to transplantation. However, before the effects of HDACis could be evaluated in any detail, it was important to first confirm the presence of histone deacetylase proteins in human cellular tissues of interest (principally PBMCs and Jurkat cells) and specifically within relevant T cell subsets. SAGE data obtained from the Human Transcriptome Map (Caron et al. 2001) provides a broad overview of HDAC tissue distribution. This demonstrates the widespread expression of HDACs 1, 2, 3, 5, 6, 7 and 10 throughout the majority of tissues studied. HDACs 8 and 9 appear to be expressed more in tumor cells than healthy cells and this difference is observed to a greater extent in the case of HDAC4 (de Ruijter et al. 2003). In this study, the presence of HDACs in experimental tissue was achieved through established Western blot techniques.

Once the presence of HDACs in target cells had been established, more specific effects of HDACi compounds could be assessed. The first of these was to assess toxicity. The majority of data currently available relating to toxicity is from oncology trials where HDACis are generally used in higher concentrations than would be anticipated for an

immunomodulatory role. In addition to this data, further evidence supporting HDACis as well tolerated compounds would be suggested from their established use in the treatment of conditions such as epilepsy (sodium valproate) with very few recognised side effects. In this study, a toxicity assay assessing cellular apoptosis and necrosis using Propidium iodide (PI) and Annexin V staining was employed to compare the effects of both established and novel HDACis.

As described previously, HDACs can affect many substrates with a resultant alteration in a number of cell processes including proliferation (Xu, Parmigiani, & Marks 2007). It is this very function which provides HDACis with a role in oncological management. In order to compare the effects of both established and novel HDACis on proliferation a number of assays were undertaken. These involved a mitogen stimulated assay in which either Jurkat cells or PBMCs were cultured in the presence of HDACi. Two techniques were utilised in quantifying proliferation – 1) Resazurin reduction (for Jurkat cells) and 2) CFSE-dilution (for PBMCs).

The hypothesis for this study considers the potential modulatory effects of HDAC inhibition on the upregulation of regulatory T cells. It has been suggested that pan-HDAC inhibition (Wang et al. 2009), selective HDAC inhibition (Reilly et al. 2008) and specific HDAC inhibition (de Zoeten et al. 2010 ;de Zoeten et al. 2011) can affect the function of this unique T cell subset. In order to test this hypothesis, the effects of a number of HDACi compounds were assessed in a regulatory T cell suppression assay, similar to that developed by Sakaguchi (Takahashi et al. 1998) in the late 1990s. This comprised of assessing the effect of HDACi treatment on isolated regulatory T cells in autologous assays and comparing responder CD4⁺ T cell proliferation.

To further delineate the potential effects of HDACi compounds on lymphocyte physiology, a T and B cell subset marker assay was employed in which PBMCs were cultured and stained for specific markers of interest.

Limited data exists with regards to the effects of HDACi on non-regulatory T cells. Finally, to consider the non-T_{reg} modulated effect of HDACi on T cell responses, a T cell activation marker assay was developed. This consisted of culturing PBMCs in the presence of HDACi compound and staining the resulting cells with identifiable markers to establish which subsets are affected.

3.2 HDAC Expression in PBMCs

Current evidence suggests the inhibitory function of HDAC inhibition is mediated through regulatory T cells (Tao et al. 2007). In order to demonstrate the presence of the principal HDAC targets in human PBMCs, Western blot analysis was undertaken (see Materials and Methods for design). This demonstrated that CD4⁺CD25⁺ regulatory T cells (in addition to CD4⁻ and CD4⁺CD25⁻ cells) do indeed carry a number of HDAC proteins, supporting the hypothesis that HDACi action may be mediated through T_{reg} upregulation.

3.2.1 HDAC 2

HDAC2 is a member of the Class I family of HDACs. It is principally localised to the nucleus and has a fairly ubiquitous expression throughout human cells.

Western Blot analysis demonstrates expression throughout CD4⁻ and CD4⁺ T cell subsets (Figure 3-1). β -actin was used to confirm uniform protein loading.

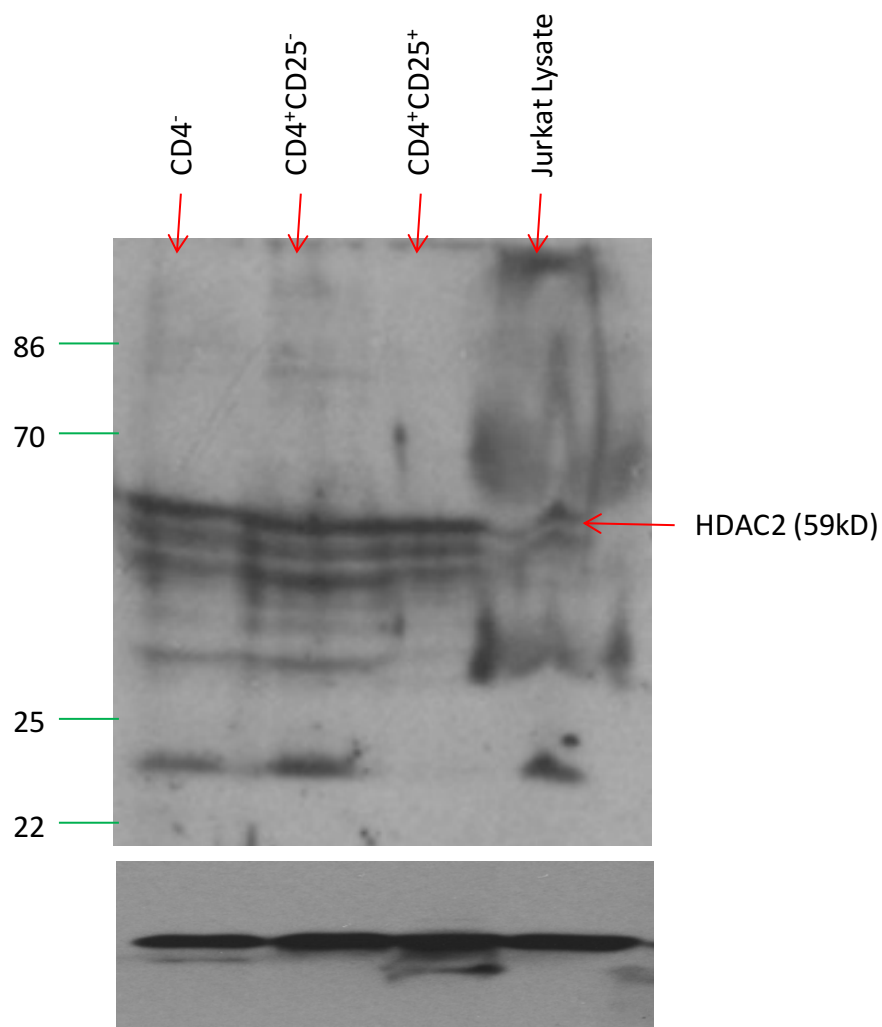


Figure 3-1 Expression of HDAC2 in Jurkat lysate and human PBMCs. (The lower panel demonstrates β -actin control samples, which were similar for all subsequent experiments.)

3.2.2 HDAC 3

Another member of the Class I family of HDACs, HDAC3 is similar to HDAC2 with respect to its widespread distribution in terms of cell type. However, though primarily localised to the nucleus of cells, it can be distributed throughout the cytoplasm.

Western blot analysis demonstrates a presence, though reduced expression of HDAC3 in human regulatory T cells compared to cytotoxic CD4⁺ T cells and CD4⁻ T cells (Figure 3-2).

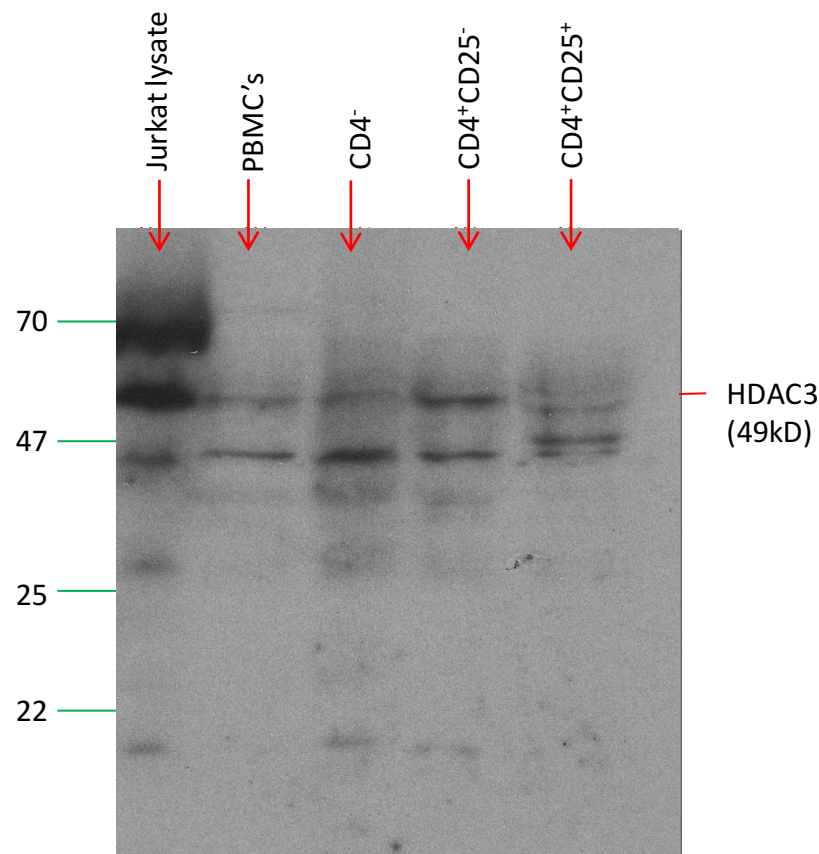


Figure 3-2 Expression of HDAC3 in Jurkat lysate and human PBMCs

3.2.3 HDAC4

HDAC4 belongs to the Class IIa group of HDACs. This group tends to be localised to muscle, heart and brain tissue. In terms of intracellular distribution, they are more diffuse than Class I HDACs and have the ability to shuttle between the nucleus and the cytoplasm (dependent upon the phosphorylation of specific serine residues (Parra & Verdin 2010)).

Western blot data suggests HDAC4 is expressed in all T cell subsets assessed (Figure 3-3).

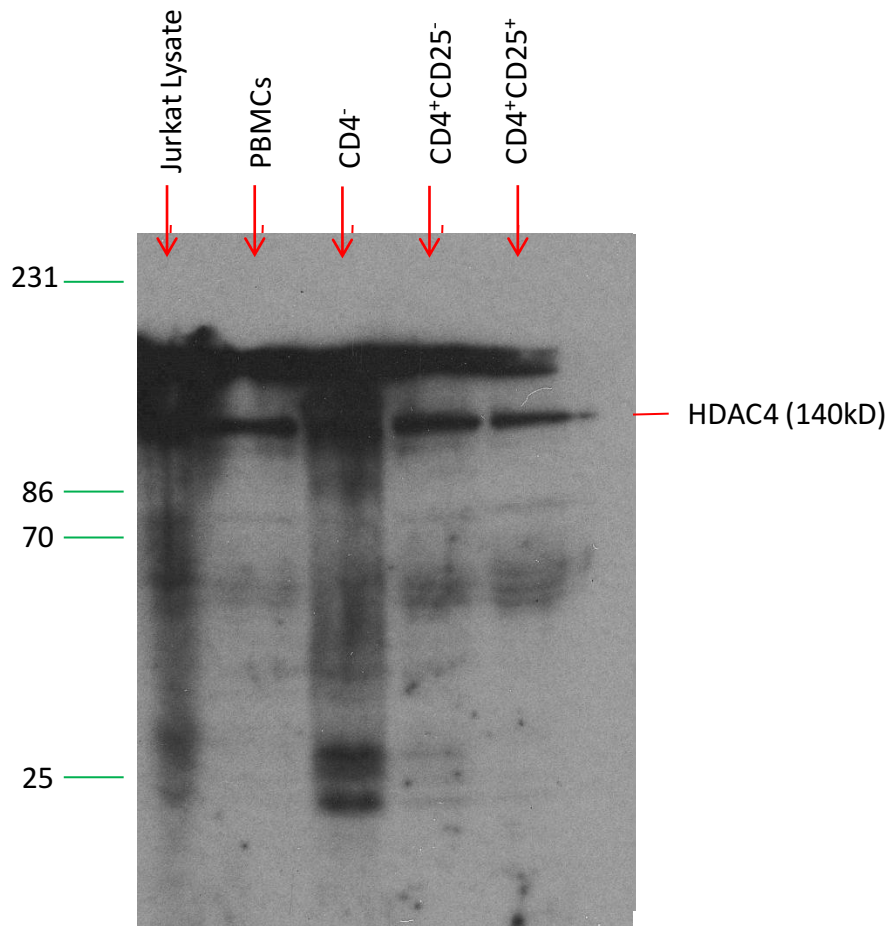


Figure 3-3 Expression of HDAC4 in Jurkat lysate and human PBMCs

3.2.4 HDAC6

HDAC6 is a member of the Class IIb family and is widely expressed, though is located primarily in heart, liver and kidney tissue. HDAC6 is unique amongst HDACs in that it is predominantly located in the cytoplasm, principally due to the presence of specific domains within the protein (NES & SE14) (na-Masangkay & Sakamoto 2011). Its reported role in modulating the suppressive activity of regulatory T cells (de Zoeten et al. 2011) and its

potential pharmacological manipulation identifies it as a possible target for immunomodulation therapy (in not only transplantation, but other clinical fields such as inflammation).

Western blot analysis confirms its presence within the T cell subsets assessed including regulatory T cells (

Figure 3-4).

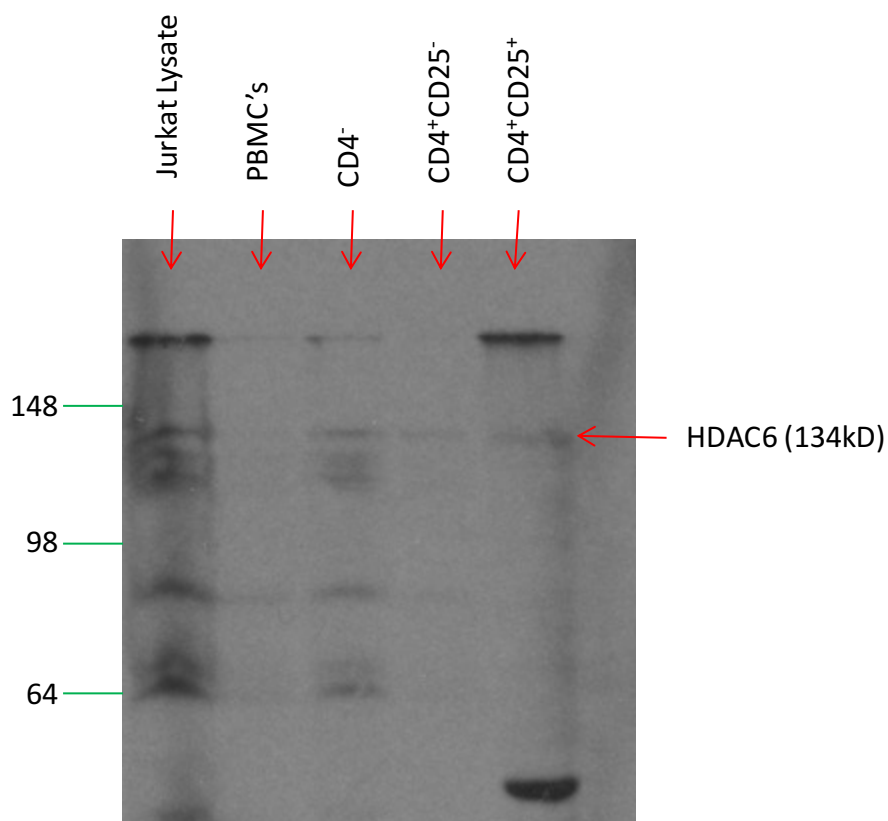


Figure 3-4 Expression of HDAC6 in Jurkat lysate and human PBMCs

3.2.5 HDAC9

As with HDAC4, HDAC9 belongs to class IIa. Characteristic of this particular group is its tissue-specific expression, and as such HDAC9 tends to be localised to cardiac and skeletal

muscle, placenta and pancreas tissue. Again its intracellular distribution is more diffuse (shuttling between nucleus and cytoplasm) than the typically localised Class I HDACs. Along with HDAC6, HDAC9 expression has also been implicated in regulatory T cell viability and function (Akimova et al. 2012).

Western blot analysis confirms the presence of HDAC9 in the cell populations studied (Figure 3-5).

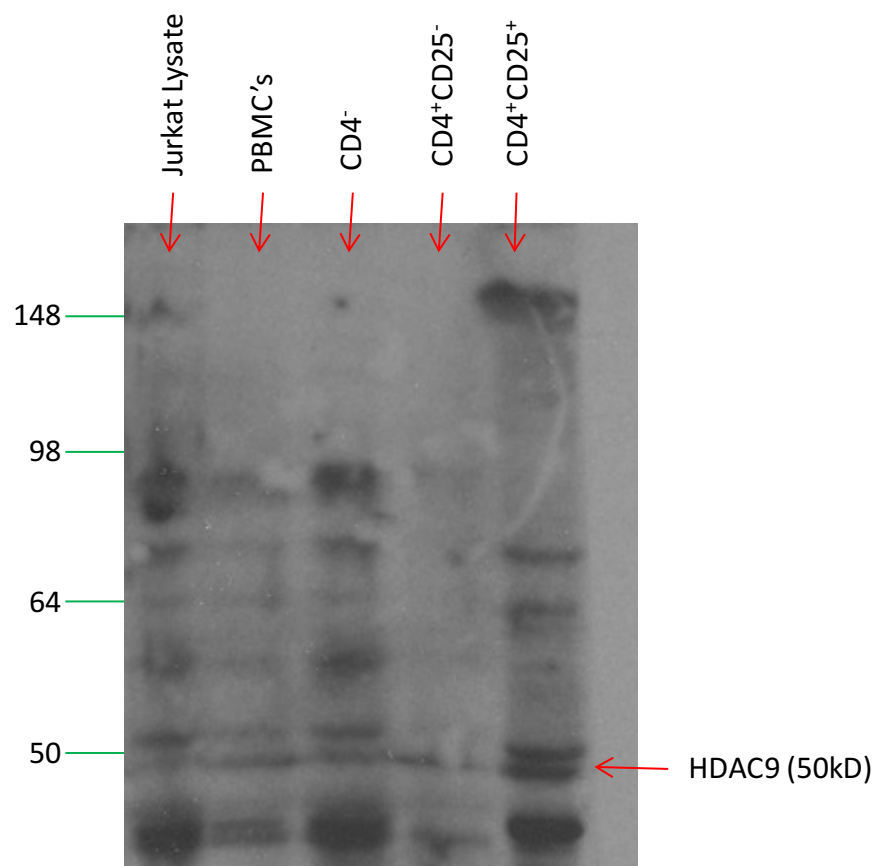


Figure 3-5 Expression of HDAC9 in Jurkat lysate and human PBMCs

3.3 HDACi Toxicity

To further assess the role of HDACi as a potential immunosuppressant, the toxic effects on cell viability were assessed. This was achieved using a well-established technique of staining treated PBMCs with Propidium iodide (PI) and Annexin V and assessing cell survival using flow cytometry.

The application of PI (a membrane impermeant dye) relies on dye exclusion. The excitation fluorescence of PI is enhanced significantly through the binding of nucleic acids (such as nuclear DNA). Therefore, for PI to become detectable, the cell membrane must be breached (Figure 3-6A). This feature makes it an eminently useful compound in identifying late apoptotic / necrotic cells.

In combination with Annexin V, flow cytometry analysis is able to differentiate the stage of cell degradation by identifying cells at an early apoptotic stage. Annexin V relies upon the externalisation of phosphatidylserine (PS) from the inner leaflet of the cell membrane during apoptosis. Fluorescently labelled Annexin V, binds strongly to PS and, therefore, allows the identification of early apoptotic cells.

After staining, flow cytometry analysis identifies the viability of treated cells (Figure 3-6B).

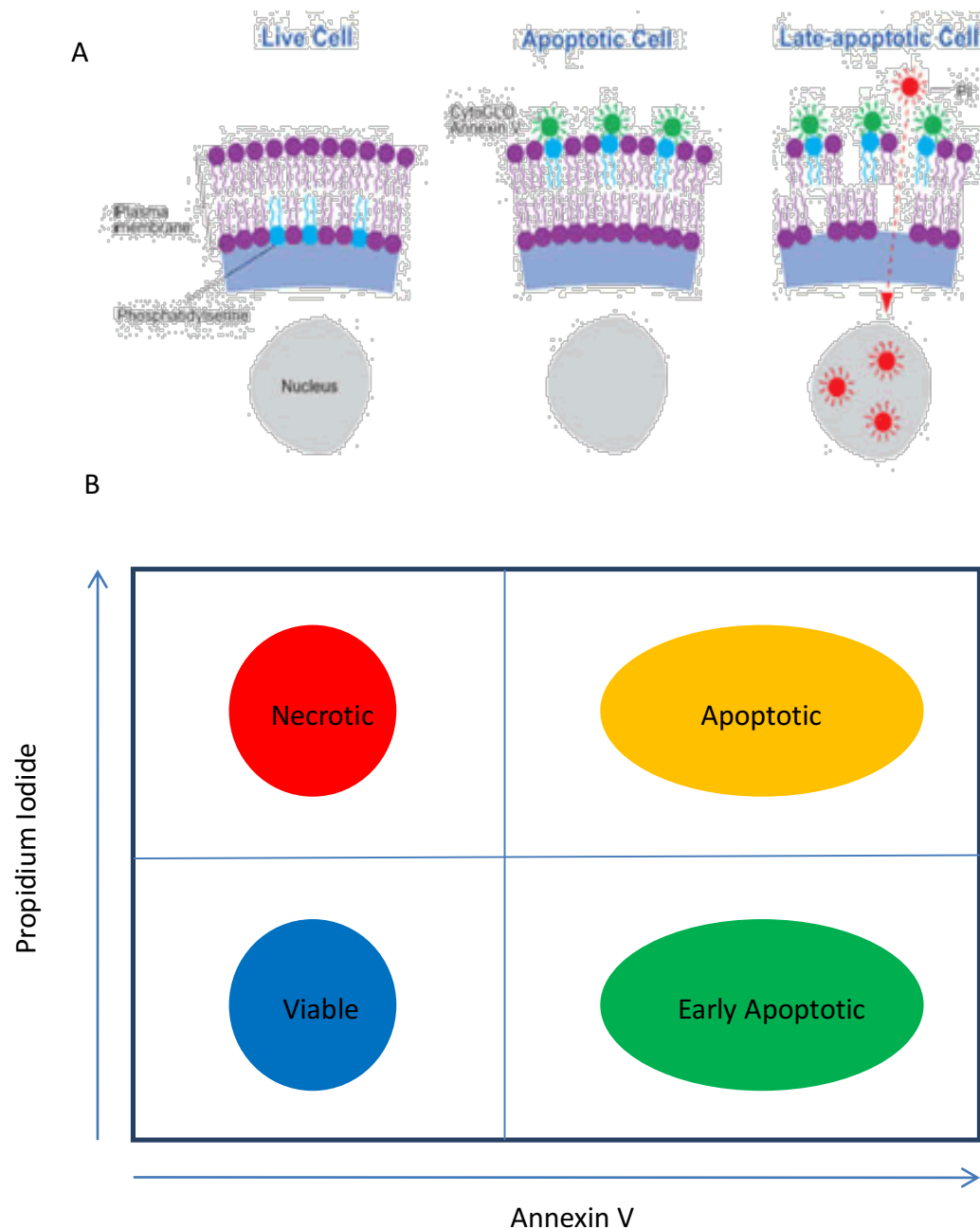


Figure 3-6 The role of PI and Annexin V in identifying non-viable cells

In this assay, freshly isolated PBMCs from individual healthy donors were either untreated (control) for 3 days, untreated and stimulated with PHA or treated for a total of 3 days with compound of interest (Cyclosporin A, SAHA, KA1010 or KA1050 at two doses). KA1050 is a novel, less specific HDAC6i and part of the panel of compounds, along with KA1010 supplied by Karus therapeutics.

Cells at day 0 demonstrated an as expected largely healthy pattern with 97-98% of cells staining viable (Figure 3-7).

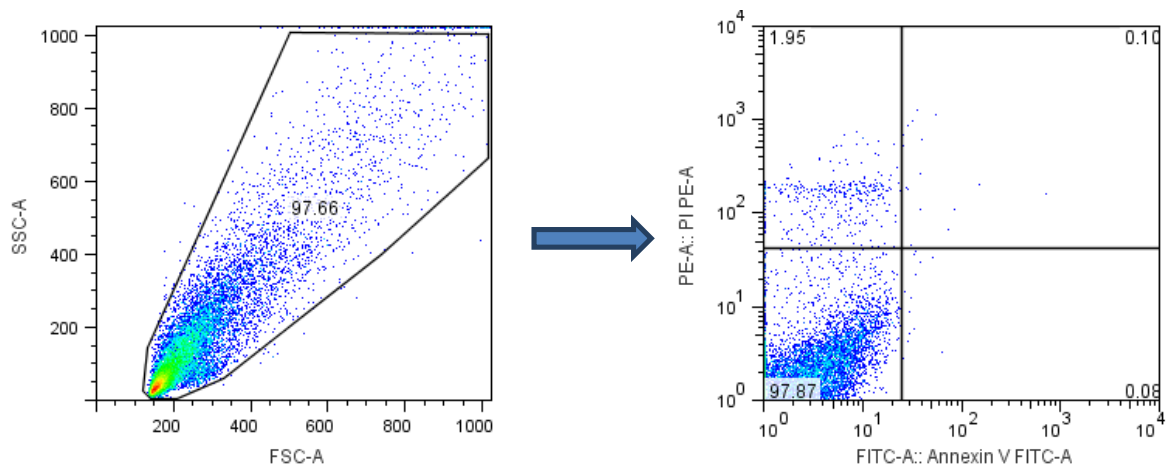


Figure 3-7 Viability staining of untreated PBMCs

Untreated / unstimulated PBMCs demonstrated a stable pattern of degradation over the initial forty eight hours with approximately 82% of cells remaining viable (Figure 3-8A). By day three, this figure had declined to 52% of cells remaining viable, with a significant number of cells progressing to an early apoptotic or apoptotic state. In comparison, untreated / stimulated PBMCs underwent a similar pattern over the initial two days; however by day three, the viable population had fallen to just 22%, with a far greater proportion of cells in the early apoptotic (50%) and apoptotic (23%) stage (Figure 3-8B)

Cyclosporin-treated unstimulated cells underwent minimal significant degradation in terms of viable population, which maintained a total proportion of approximately 70-80%, regardless of the dose used (Figure 3-8C). However at the higher dose of 5 μ M, the non-viable group of cells underwent greater progression from early apoptosis to apoptosis and necrosis when compared to those PBMCs treated at the lower dose of 500nM (Figure 3-8D).

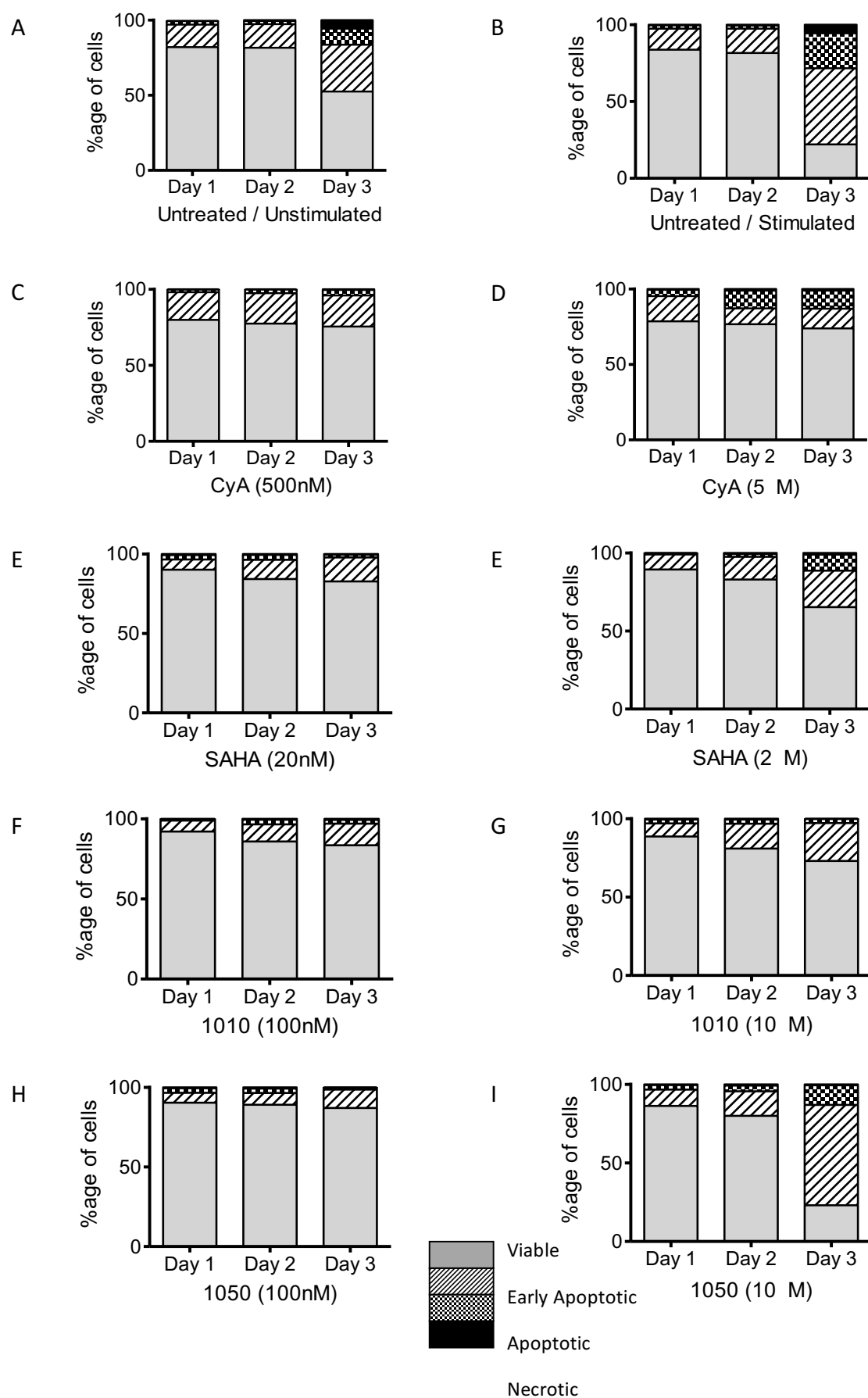


Figure 3-8 Effect of CyA or HDACi treatment on the cell viability of PBMCs over 72 hours

The addition of the pan-HDAC inhibitor, SAHA, resulted in a slight trend toward viable cell survival, particularly up to day 2. At both the lower dose of 20nM and higher dose of 2μM, this was particularly evident at day 1 with ~90% viable PBMCs compared to 80% in control conditions (Figure 3-8, E-F). By day 3, at the higher dose, however, there was significant reduction in the viable cell population with a concomitant increase in both early apoptotic and apoptotic populations. This remained less profound than control.

HDAC6-specific inhibition (KA1010 & KA1050) at the lower dose of 100nM had a similar effect on cell viability profile to SAHA (20nM). Again this was generally more favourable toward cell viability compared with control. This effect was most profound using KA1050 at the lower dose (Figure 3-8H). By day 3, at the higher doses selected KA1010 had the most favourable viability profile of the compounds tested. KA1050, at this higher dose, however, proved to have a significantly toxic effect with just 23% of cells remaining viable (Figure 3-8I).

3.4 Effect of HDAC Inhibition on Jurkat Cell Proliferation

A Resazurin-based assay was used to assess the dose-dependent effect of a panel of HDACis on a human T-cell lymphocyte (Jurkat) cell line. This assay depends upon the reduced environment of growing cells which converts the oxidised non-fluorescent blue form (resazurin) to the reduced fluorescent red form (resorufin) (Figure 3-9).

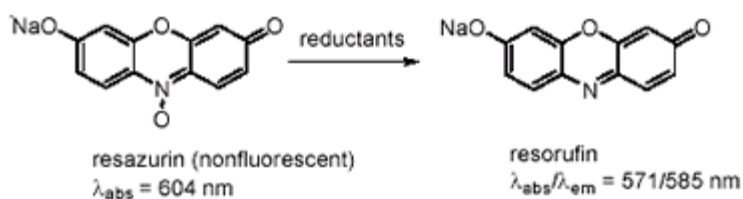


Figure 3-9 Reduction of Resazurin, resulting in the fluorescent form, resorufin

Fluorescence is then measured using a spectrophotometer. The signal generated is proportional to the number of living cells in the sample (to a sensitivity of ~80cells/well).

Firstly, a standard curve must be generated in order to calibrate the assay. This was produced by accurate serial dilution of a known number of cells (from 200,000 cells to 0 cells / well, Figure 3-10) in a 96-well plate. Resazurin (10% by volume) was then added to each well (in triplicate) and the assay incubated for twenty four hours. Absorption was measured using a spectrophotometer and a standard curve generated (Figure 3-11).

200,000 cells/well
100,000 cells/well
50,000 cells/well
25,000 cells/well
12,500 cells/well
6,250 cells/well
3,125 cells/well
0 cells/well (media only)

Figure 3-10 Serial dilution of cell number, in order to produce standard curve

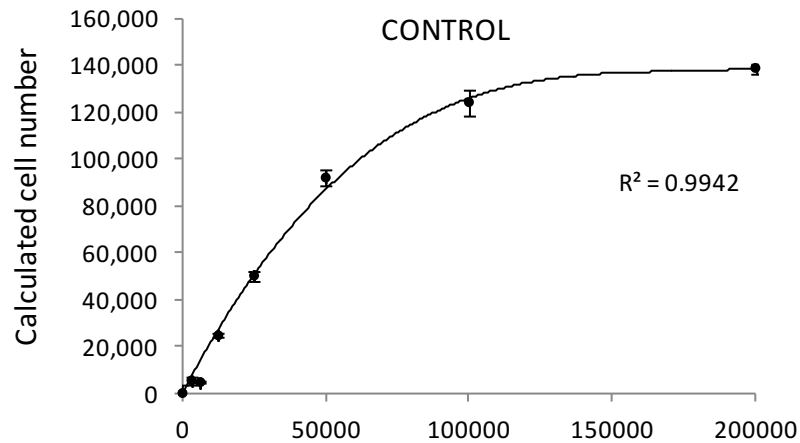


Figure 3-11 Typical Resazurin standard curve for calculating Jurkat cell number

From the best fit line for this data (with an R^2 value of >0.95) the quadratic equation of the curve was calculated and applied to the measured data from treated samples. Cell numbers were then extrapolated from this quadratic equation and plotted.

Controls

By way of control, Jurkat cells were treated with:

- Negative Control
 - Sodium Azide (NaN_3) – 0.01% - 1%
- Positive Control
 - Phorbol myristate acetate (PMA) – 1ng/ml - 50ng/ml
 - Lipoteichoic acid (LTA) – 0.5 $\mu\text{g}/\text{ml}$ - 10 $\mu\text{g}/\text{ml}$

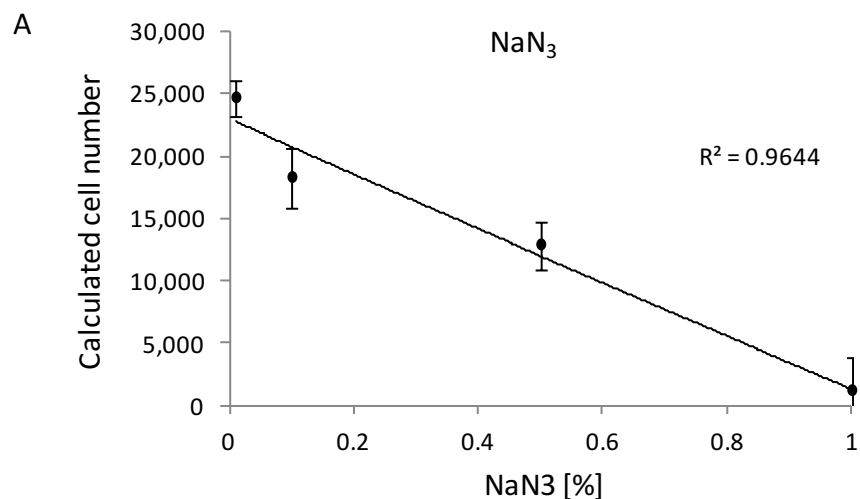
Sodium azide is a highly dissolvable cytotoxin which acts by inhibiting the mitochondrial respiratory chain and induces cell death by apoptosis (Weyermann, Lochmann, & Zimmer 2005).

PMA and LTA are potent mitogens. PMA acts as a proliferation stimulant by activating the signal transduction enzyme PKC (protein kinase C). LTA effects binding to TLR-2 receptor and induces NF- κ B and mitogen-associated PK (MAPK).

50,000 Jurkat cells / well were cultured for 24 hours in the presence of the control compounds at the concentrations below:

	NaN ₃	PMA	LTA
↓	0.01%	1ng/ml	0.5µg/ml
	0.1%	5ng/ml	1.0µg/ml
	0.5%	10ng/ml	5.0µg/ml
	1.0%	50ng/ml	10.0µg/ml

Table 3-1 Sodium azide and mitogen concentrations used to assess Jurkat cell proliferation



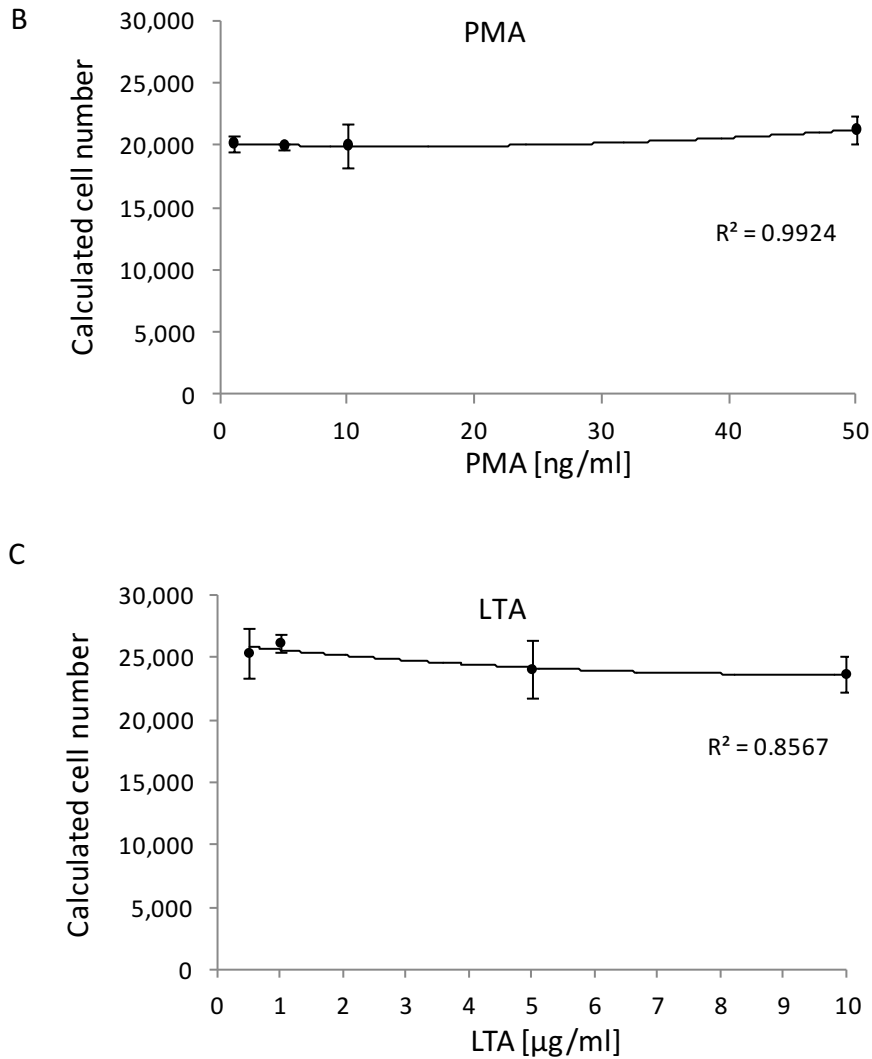


Figure 3-12 Negative (NaN3) and positive (PMA, LTA) controls

Sodium azide had a demonstratable dose-dependent negative effect on Jurkat cell number, with a concentration of 1% almost completely eliminating any cell viability (Figure 3-12A). However, the positive controls had no discernible effect on cell number regardless of concentration (Figure 3-12, B-C). This was likely due to the weak stimulatory effect PMA has on T cells in the absence of Ionomycin. In the case of LTA, though traditionally considered an activator of lymphocytes, recent evidence suggests it has a suppressive effect in terms of effector T cell proliferation and function (Son et al. 2013).

3.4.1 Established HDACis

Before assessing a panel of novel HDACi compounds, HDACis in clinical use or undergoing clinical trials for indications other than transplantation were evaluated (Table 3-2). Again, cells were treated and cultured for 24 hours before cell numbers calculated. In this assay, 100,000 cells were cultured / well in sextuplet.

<i>HDACi</i>	<i>Structure</i>	<i>Target</i>	<i>Notes</i>	<i>Concentration</i>
Sodium Valproate (VSA)	Short chain fatty acid	Class I & IIa (HDAC 1-9, except 6)	Undergoing phase II trials (AML)	1.25mM – 10mM
Trichostatin (TSA)	Hydroxamic acid	Class I, IIa, IIb & IV (HDAC 1-11)	Poor pharmacokinetics (primarily academic interest)	5nM – 500nM
Suberoylanilide hydroxamic acid (SAHA)	Hydroxamic acid	Class I, IIa, IIb & IV (HDAC 1-11)	First to be approved as treatment in certain malignant conditions (CTCL)	0.625μM – 5μM

Table 3-2 Established HDACis currently in clinical use or undergoing clinical trial

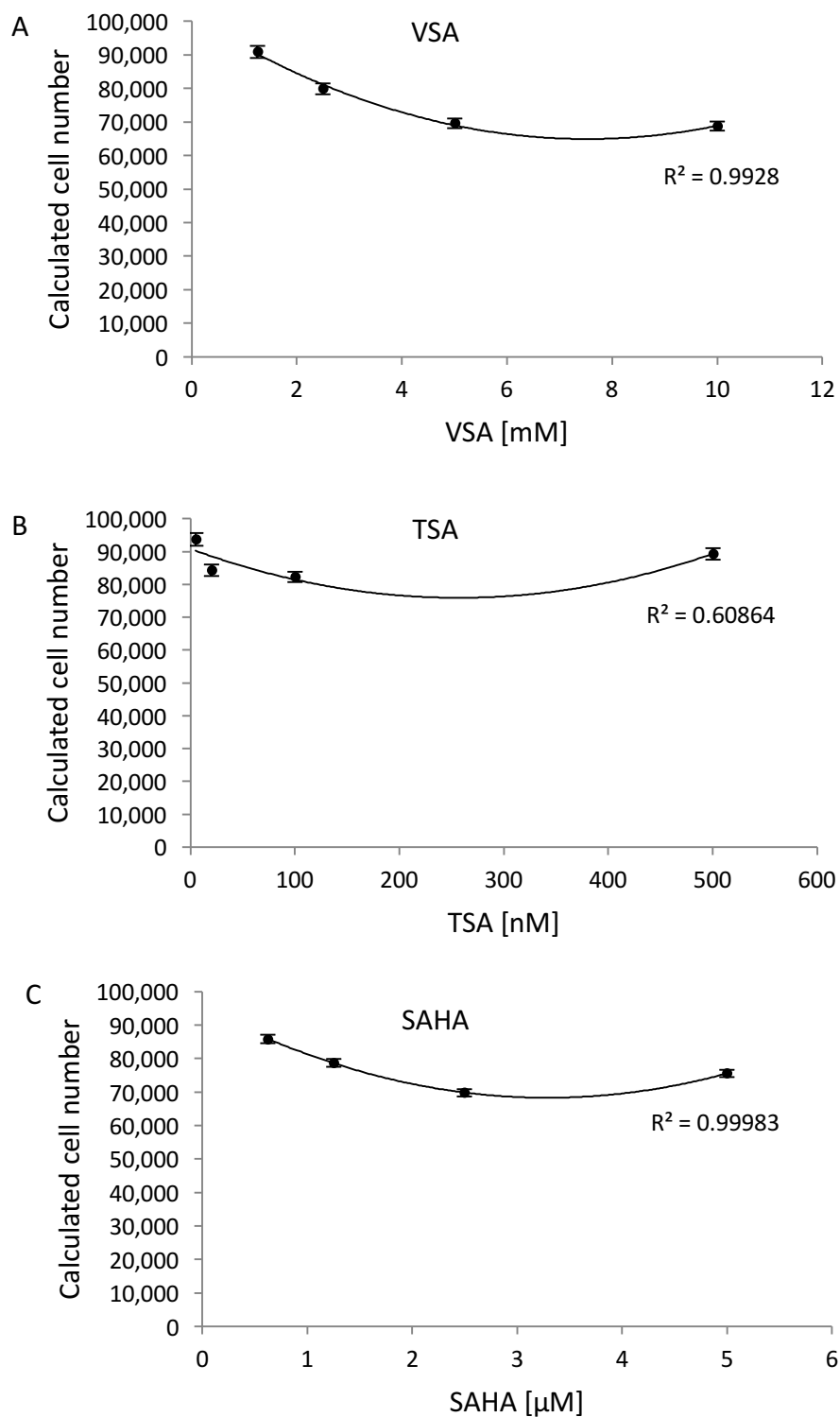


Figure 3-13 Effect of HDACi compounds on Jurkat cell proliferation

These established compounds had a moderate, dose-dependent effect on Jurkat cell number at the lower concentrations used. In the case of all three compounds, this trend was not extended to the highest dose used (Figure 3-13, A-C). Interpretation of this data would suggest that these compounds had no significant effect on Jurkat cell number when treating for 24 hours. The protocol was therefore modified to extend the culture period and alter the density of cells.

3.4.2 Experimental HDACis

In order to identify potential experimental compounds of interest, a panel of 10 HDACis were assessed. At this stage, their structure, specificity etc. were blinded and the compounds were merely identified by a unique code. The only information supplied with these compounds was their molecular weight and effective dose range, in order to enable experimental design. The compounds used are listed in Table 3-3.

<i>Code</i>	<i>MW (g.mol⁻¹)</i>	<i>Range</i>	<i>Concentration used (nM)</i>
KA001.1	486.61	1nM – 1µM	1, 10, 100, 1000
KA005.1	264.32	10nM – 10µM	10, 100, 1000, 10000
KA008.6	540.70	1nM – 1µM	1, 10, 100, 1000
KA088.2	515.69	1nM – 1µM	1, 10, 100, 1000
KA1010	314.38	10nM -10µM	10, 100, 1000, 10000
KA146.2	544.68	1nM – 1µM	1, 10, 100, 1000
KA147.3	612.68	1nM – 1µM	1, 10, 100, 1000

KA172.1	547.65	1nM – 1μM	1, 10, 100, 1000
KA185.1	546.66	1nM – 1μM	1, 10, 100, 1000
KA1005.3	297.38	10nM -10μM	10, 100, 1000, 10000

Table 3-3 Panel of experimental HDACis

Jurkat cells were cultured at a density of 5000 cells / well in a 96-well plate. Media was supplemented with compound of interest at day 0. Cell numbers were calculated at 24, 72 and 120 hours using the above described Resazurin method.

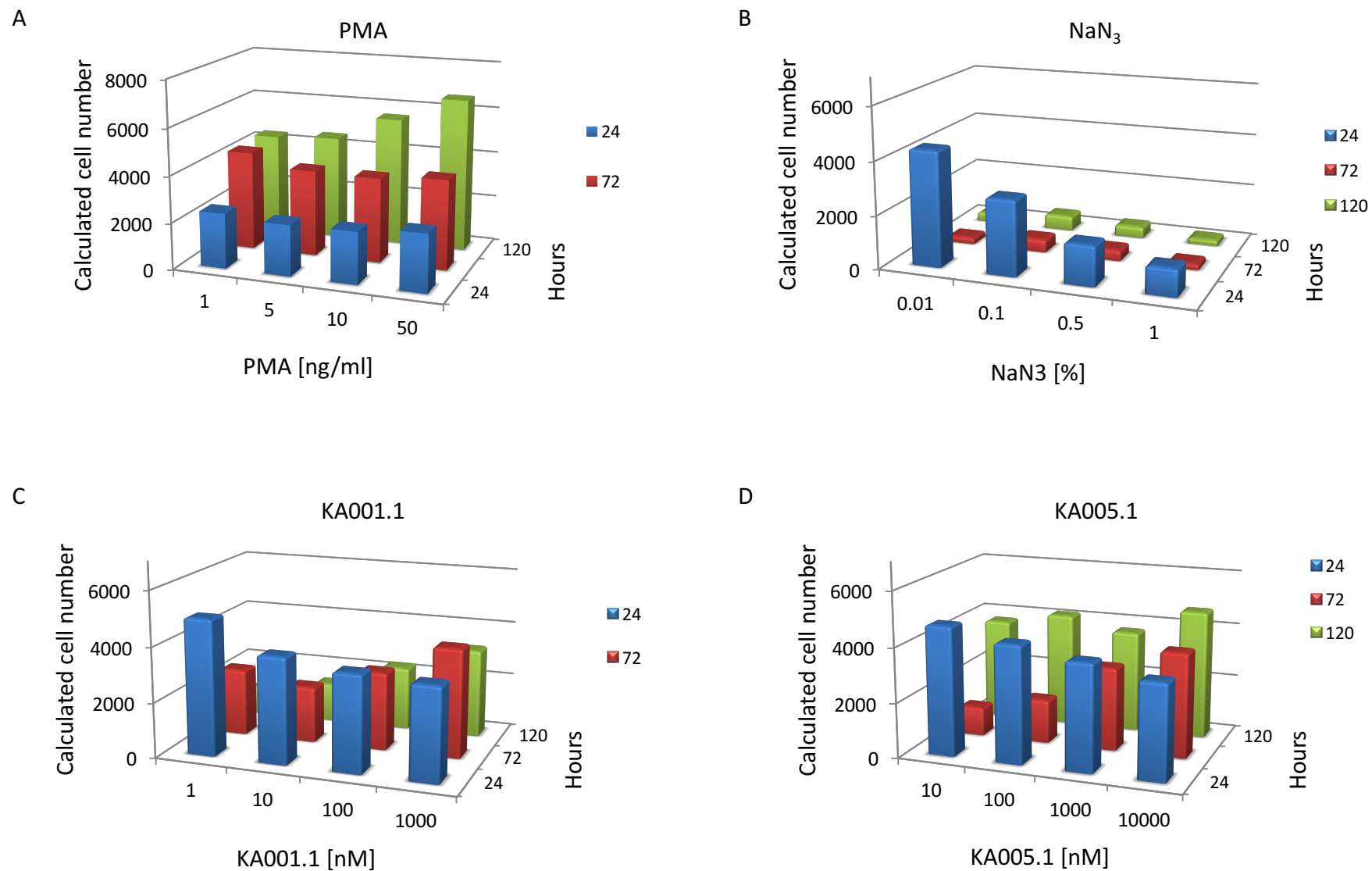


Figure 3-14 A-D Effect of controls (PMA and NaN₃) and experimental compounds on Jurkat cell proliferation over time

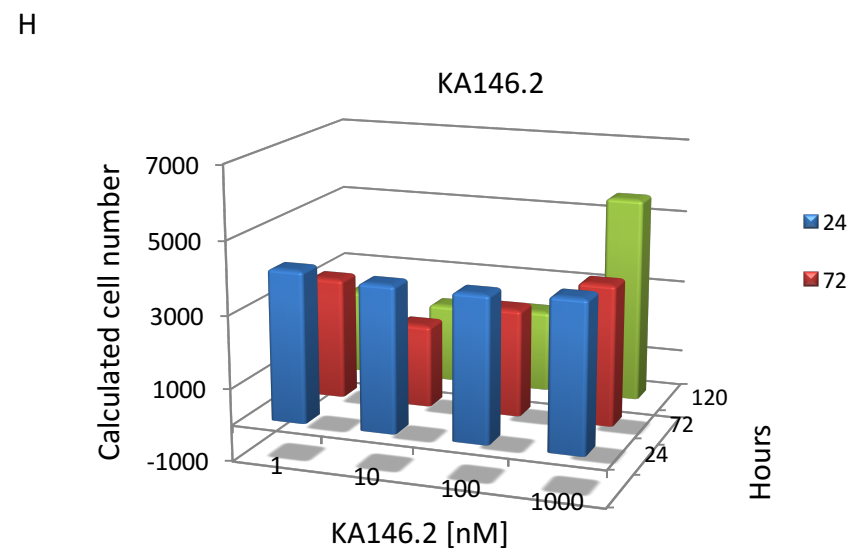
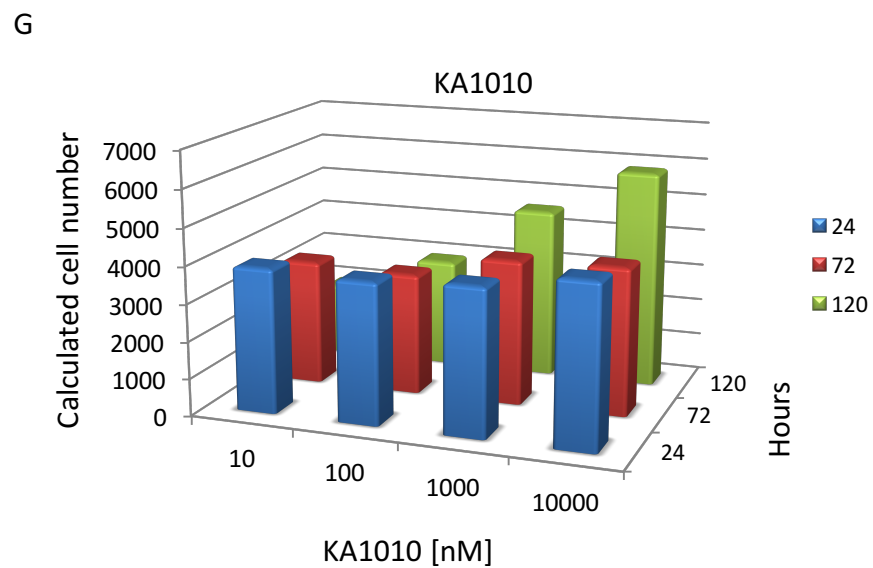
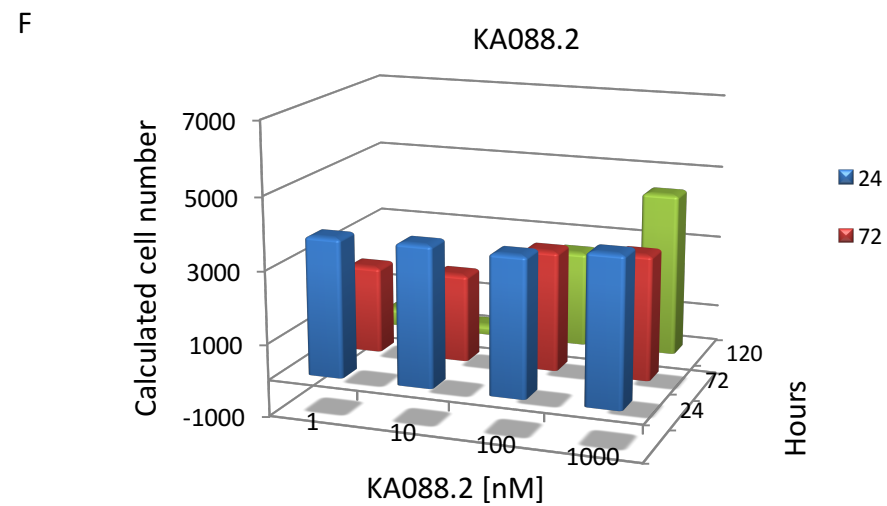
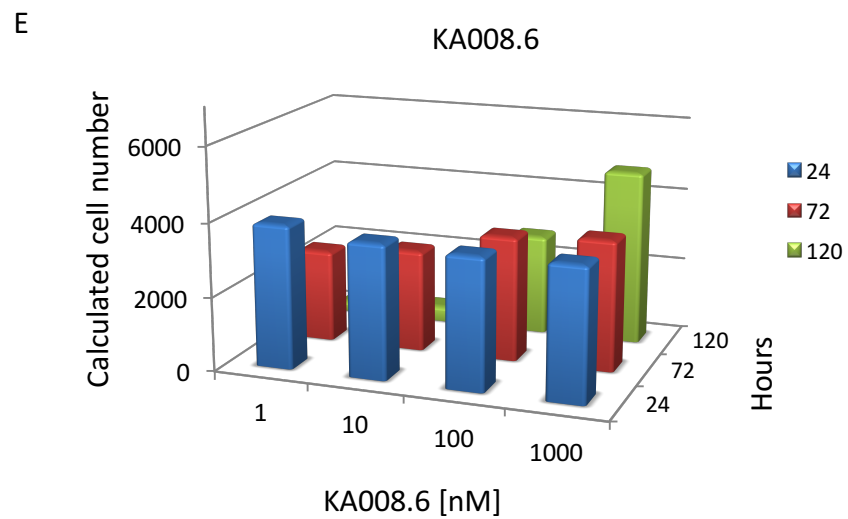


Figure 3-14 E-H Effect of experimental compounds on Jurkat cell proliferation

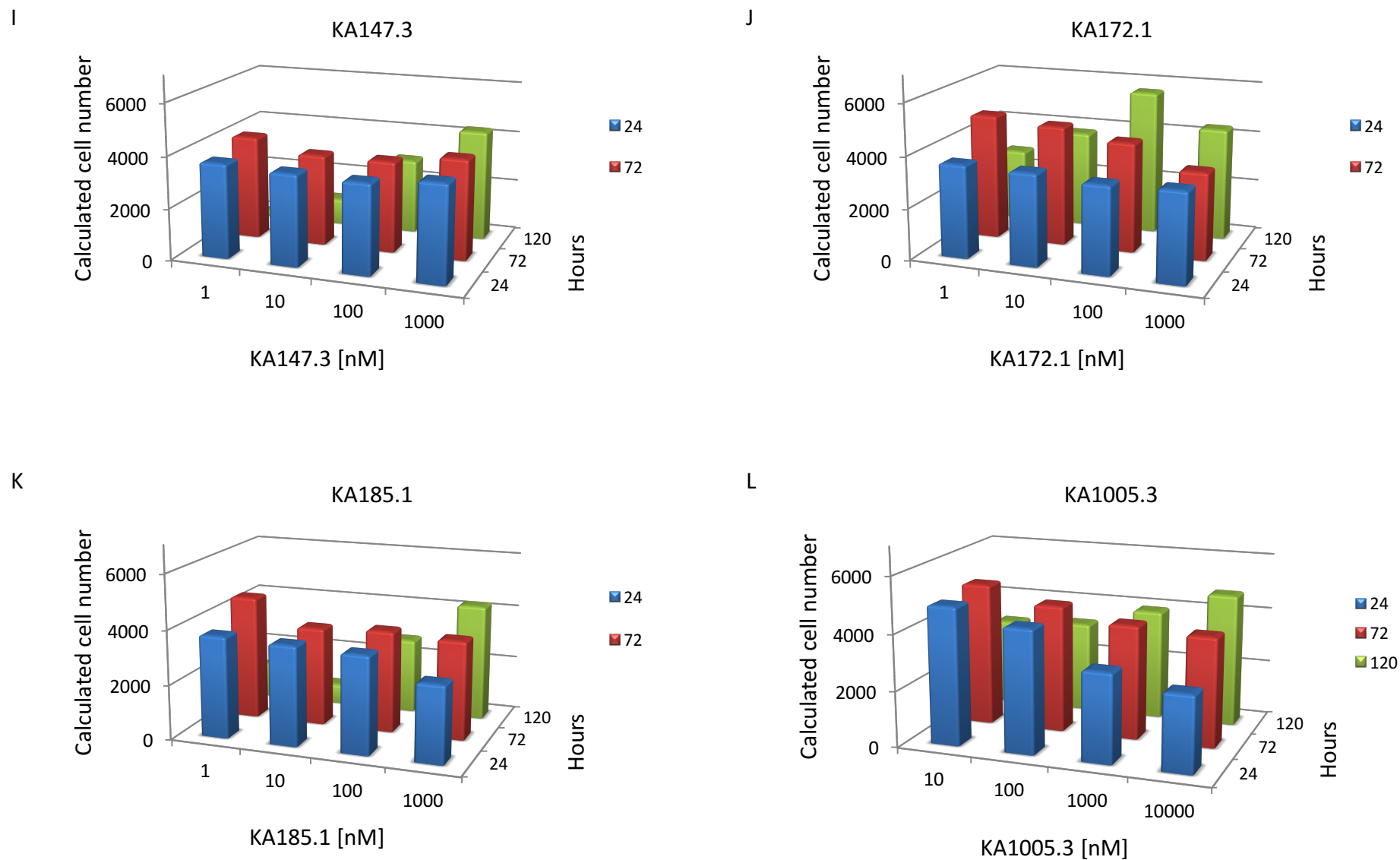


Figure 3-14 I-L Effect of experimental compounds on Jurkat cell proliferation

Positive and negative controls had an expected effect on Jurkat cell number. The addition of PMA resulted in a time-dependent trend towards cell proliferation and would suggest that the previously described controls were not cultured for a sufficient time period to demonstrate an effect. This trend was also dose dependent toward the later end of the assay (120 hours) with PMA at a concentration of 100nM resulting in a 52% increase in cell number compared to a concentration of 1nM (Figure 3-14A).

NaN₃ had a typically negative effect on cell number, with few surviving at seventy two hours and beyond, regardless of dose (Figure 3-14 B). At twenty four hours of culture, increasing concentration of NaN₃ resulted in a significant deterioration in cell survival. At a concentration of 1%, just 22% of cells survived compared to survival at 0.01% concentration.

With regards to the panel of experimental compounds (Figure 3-14, C-L) there were few general conclusions that could be made and there was little consistency in overall trends. However, at twenty four hours, most compounds had a minimal effect on cell numbers regardless of dose. This trend was also generally true at seventy two hours. By one hundred and twenty hours there is a general trend toward cell survival with increasing dose.

Increasing dose of KA001.1 had an initial negative effect on cell number (Figure 3-14 C), and numbers did not recover over time though by one hundred and twenty hours the survival of cells treated with 1000nM was 317% that of those treated with 10nM.

Cells treated with KA005.1 underwent a similar trend (Figure 3-14 D) to that of KA001.1, though there was an absence of late effect (one hundred and twenty hours) with a degree of recovery of cell numbers irrespective of dose.

The effect in terms of numbers on cells treated with compounds KA008.6, KA088.2, KA1010 and KA146.2 (Figure 3-14, E-H) was roughly similar. All four compounds had a negligible effect on cell numbers over the initial twenty-four-hour treatment period, regardless of dose. At seventy two hours there was a general trend toward increased cell survival with increased dose but this was not significant. However, at one hundred and twenty hours this effect was more profound.

Treatment with KA147.3 and KA185.1 (Figure 3-14, I & K) had no significant effect on cell numbers during the first seventy two hours of culture. At one hundred and twenty hours there was a dose-dependent protective effect in terms of cell numbers but no significantly proliferative effect.

As with KA147.3, KA172.1 (Figure 3-14 J) had no demonstrable effect on cell numbers at twenty four hours. Beyond this period, effect was less predictable than with other compounds though in general there was favourable cell survival across all concentrations compared to the majority of other compounds examined (similar to KA005.1 and KA1005.3).

Finally, KA1005.3 (Figure 3-14 L) had an initially negative dose dependent effect on cell number survival (at twenty four hours). By seventy-two hours the effects of the higher doses studied had become less profound and by the later stages (one hundred and twenty hours) had reversed with improved cell survival at the lower dose range compared with the majority of compounds assessed.

In conclusion, none of the novel compounds assessed had a significantly toxic effect on Jurkat cell survival in terms of numbers of viable cells over the treatment period. Four of the compounds (KA005.1, KA1010, KA172.1 and KA1005.3) were notable for improved cell

survival / proliferation at one hundred and twenty hours. The variable effect of the compounds in terms of cell survival / proliferation over time and dose suggests that there is significant heterogeneity between these HDACis and that there is still likely a large number of targets on which they act, despite their nominal specificity.

3.5 Effect of HDAC Inhibition on PBMC proliferation

Following the Jurkat cell proliferation assay, a number of HDACi compounds were selected in order to assess their effect on human PBMCs. As a well characterised HDACi, the effect of SAHA was compared to compounds KA1010, KA185.1 and KA146.2. These experimental compounds were selected based on their heterogenous effect on Jurkat cell proliferation.

In order to assess proliferation, 100,000 freshly isolated PBMCs per well were stained with 1 μ M of CFSE and cultured for five days at 37°C. The culture was performed in the absence or presence of IL-2 (50U/ml) to assess its effect. After five days, cells were also stained with 7-AAD in order to assess viability (7-aminoactinomycin D is a useful fluorescent intercalator that can be used to assess viability). When bound to DNA, 7-AAD undergoes a spectral shift so that it can be detected with flow cytometry. In healthy cells 7-AAD is excluded.

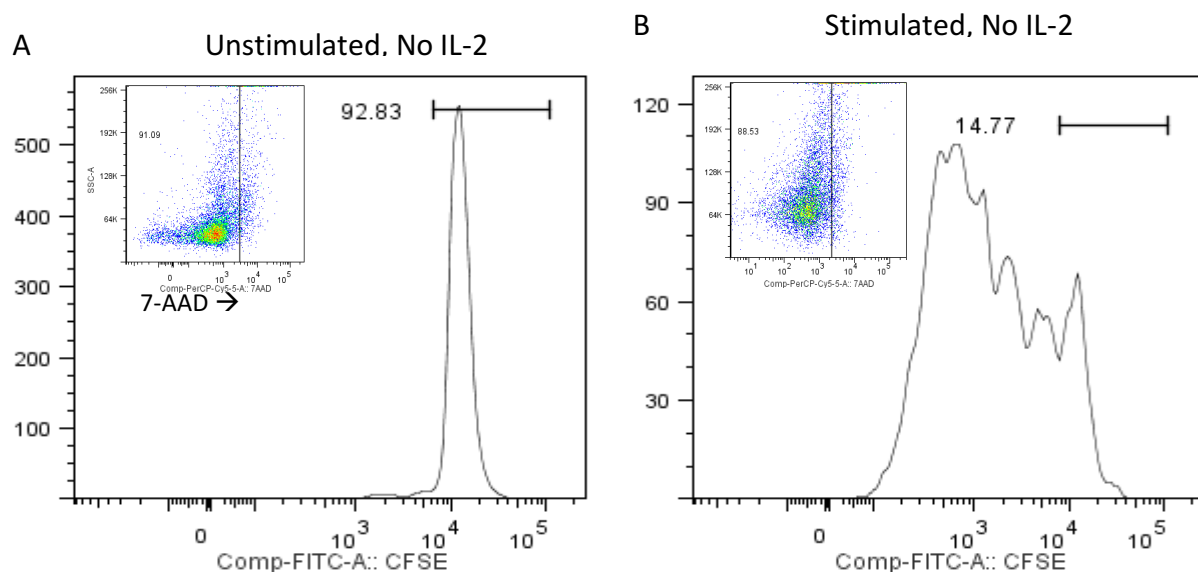
The experimental compounds were used at similar concentrations to those assessed in the Jurkat cell proliferation assay (Table 3-4);

<i>Code</i>	<i>Concentration used (nM)</i>
SAHA	1, 10, 100, 1000
KA1010	0.1, 1, 10, 100
KA185.1	0.1, 1, 10, 100
KA146.2	1, 10, 100, 1000

Table 3-4 Concentrations of HDACis

3.5.1 Controls

Firstly the effect of IL-2 was assessed in the presence or absence of stimulant (Dynabeads).



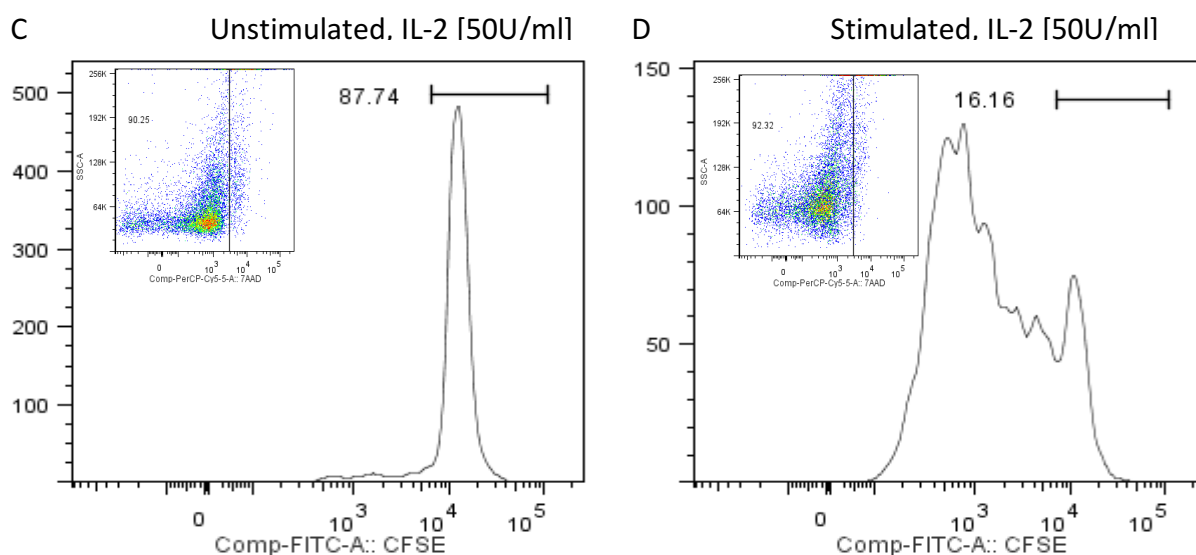


Figure 3-15 PBMC proliferation under control conditions

The effect of IL-2 on PBMC proliferation was minimal (Figure 3-15). However, as expected stimulation had a significant impact on proliferation. Cell viability was consistently around 90% regardless of conditions as demonstrated with 7-AAD staining.

3.5.2 Compounds of interest

Following condition optimisation, the effect of selective and non-selective HDACi on stimulated human leucocyte proliferation was assessed. A CFSE dilution assay was adopted using anti-CD3/CD28 stimulation beads on bulk PBMC populations (Skov et al. 2003). Cells were cultured for five days in the presence of HDACi of interest and in the presence or absence of IL-2.

As expected, anti-CD3/CD28 stimulation induced potent proliferation at five days, with an average of only 14.8% of the parental population not proliferating (Figure 3-16 C). Both HDAC6-selective (KA1010) and non-selective HDAC inhibition (SAHA) showed activity insuppressing anti-CD3/CD28-induced proliferation (Figure 3-16 & Figure 3-17).

SAHA

At doses of 1nM to 100nM, SAHA showed a similar, modest inhibitory effect on proliferation, preventing the division of an additional 5-7% of the parent population over control (Figure 3-16). However at a concentration of 1000nM, there was a significant increase in suppression of division (Figure 3-20 & Figure 3-21). This was particularly evident in the absence of IL-2 (Figure 3-16 E) where parent population remaining after five days increased from 14.8% to 55.8%. In the presence of IL-2, a dose of 1000nM was still sufficient to result in reduced proliferation but to a lesser degree (16.7% for control vs. 38.9%) (Figure 3-16 F).

KA1010

In contrast, KA1010 displayed a more pronounced dose-dependent effect on proliferation (Figure 3-17). The presence of IL-2 had a negligible effect. At a concentration of 1 μ M, KA1010 was effective at reducing division of the parent population to 65.9% (21% reduction compared to control, compared to an equivalent dose reduction with SAHA to 78%) with cell viability found to be similar to control (Figure 3-20). In the presence of IL-2, this dose was slightly less effective, reducing division from 83.3% in the untreated control to 67.3% (Figure 3-21).

At equivalent concentrations, KA1010 produced an inhibitory effect on cell division, superior to SAHA (two-way ANOVA $p=0.002$, $F=13.97$). At the lower dose of 1nM this inhibitory effect represented a 4.7% and 6.8% reduction over control for SAHA and KA1010, respectively. At the higher dose of 1000 nM, these figures increase to 9.0% and 22.6%.

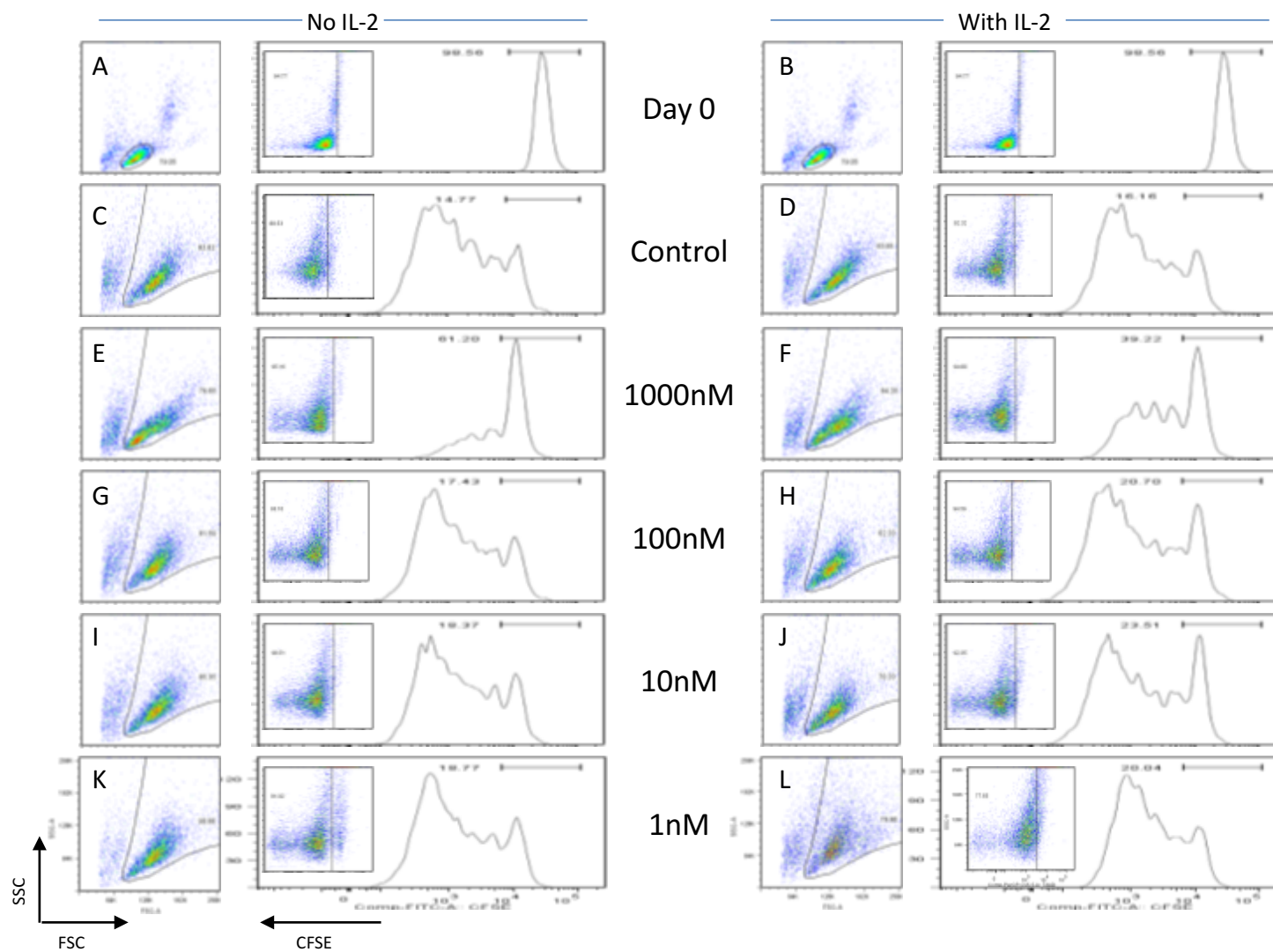


Figure 3-16 FACS data demonstrating the effect of SAHA on PBMC proliferation

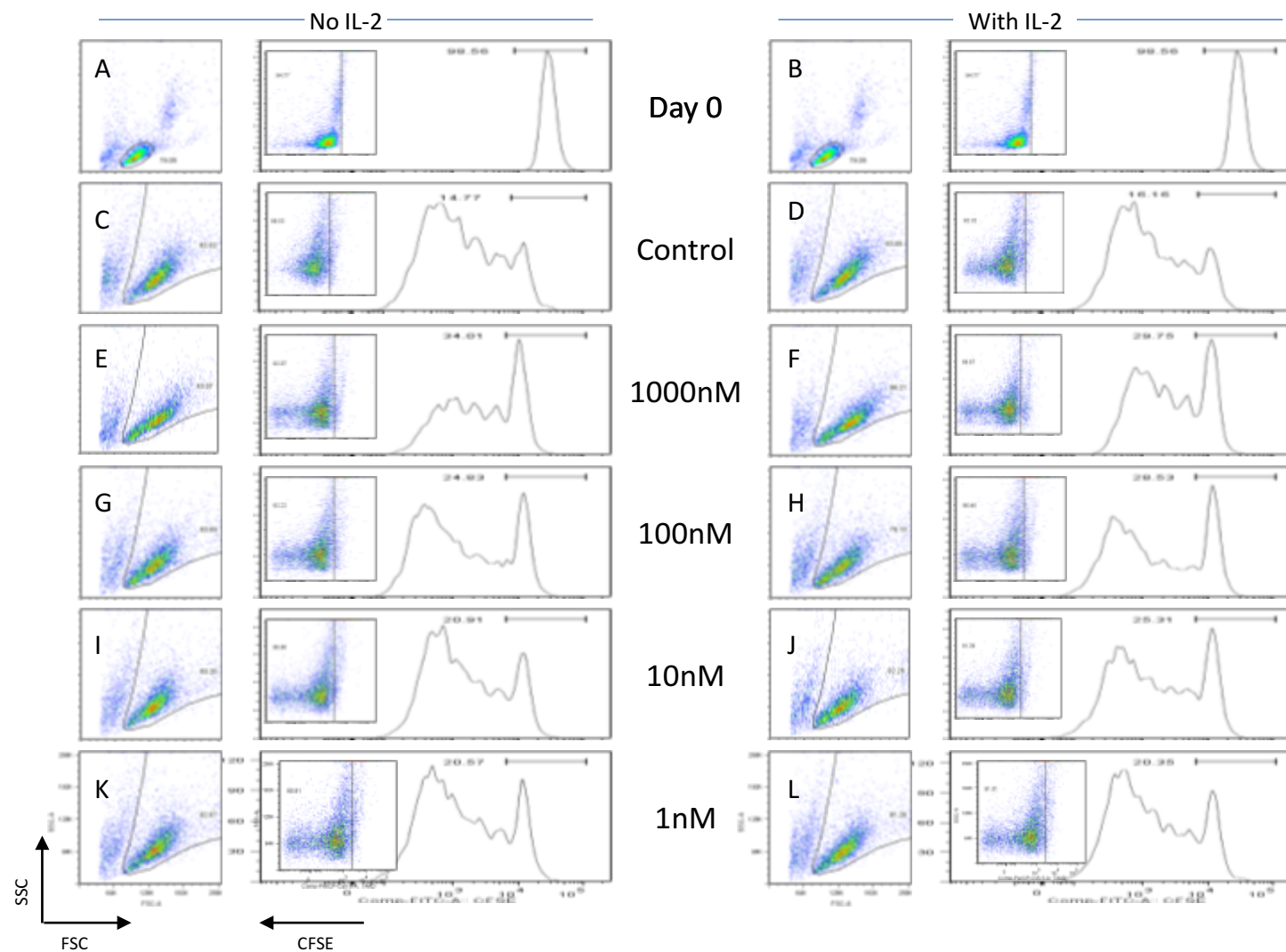


Figure 3-17 FACS data demonstrating the effect of KA1010 on PBMC proliferation

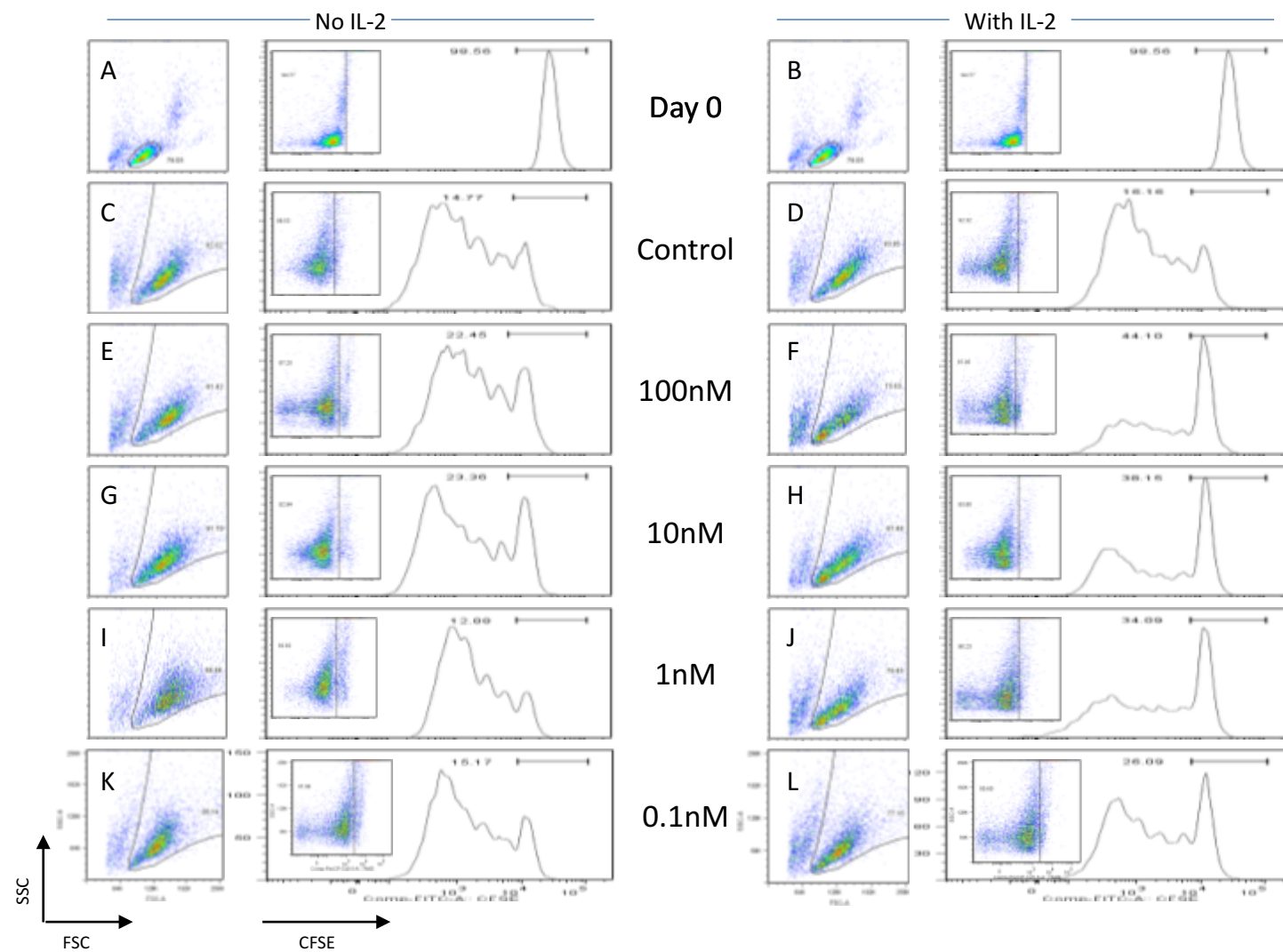


Figure 3-18 FACS data demonstrating the effect of KA185.1 on PBMC proliferation

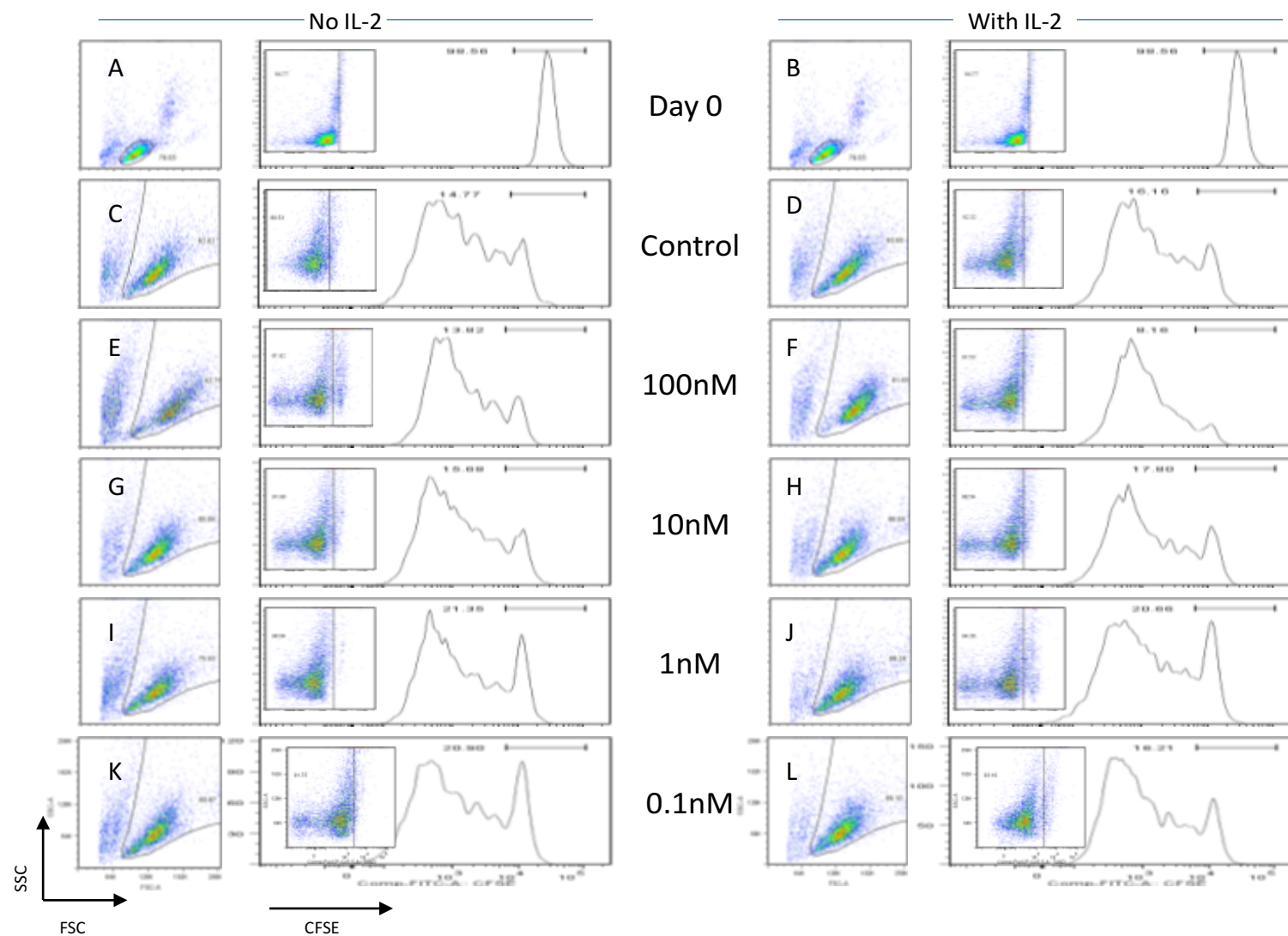


Figure 3-19 FACS data demonstrating the effect of KA146.2 on PBMC proliferation

KA185.1

In the absence of IL-2, KA185.1 had a negligible effect on suppression (Figure 3-20); however in the presence of IL-2, its effect was considerably more profound (Figure 3-21). At a dose of 100nM, under these conditions, KA185.1 suppressed an additional 27.6% of the parent population greater than control. Indeed, in the presence of IL-2, KA185.1 was the most effective HDACi compound tested.

KA146.2

KA146.2 was the least effective PBMC proliferation suppressor tested. In the non-IL-2 conditions, it had a broadly similar, minimal effect (Figure 3-20). In the presence of IL2, the suppressive effect of KA146.2 was essentially the same as control (Figure 3-21). At the higher dose of 100nM, it had a slightly proliferative effect with just 10.7% of the parent population remaining undivided after 5 days. However, this difference was not statistically significant.

This assay provides information regarding a possible mechanism of action of the compounds tested. It would suggest that the suppressive effects of SAHA and KA1010 are independent of IL-2. The suppressive effect of KA185.1 is significantly dependent upon the presence of IL-2 and KA146.2 has no notable inhibitory action on PBMC proliferation.

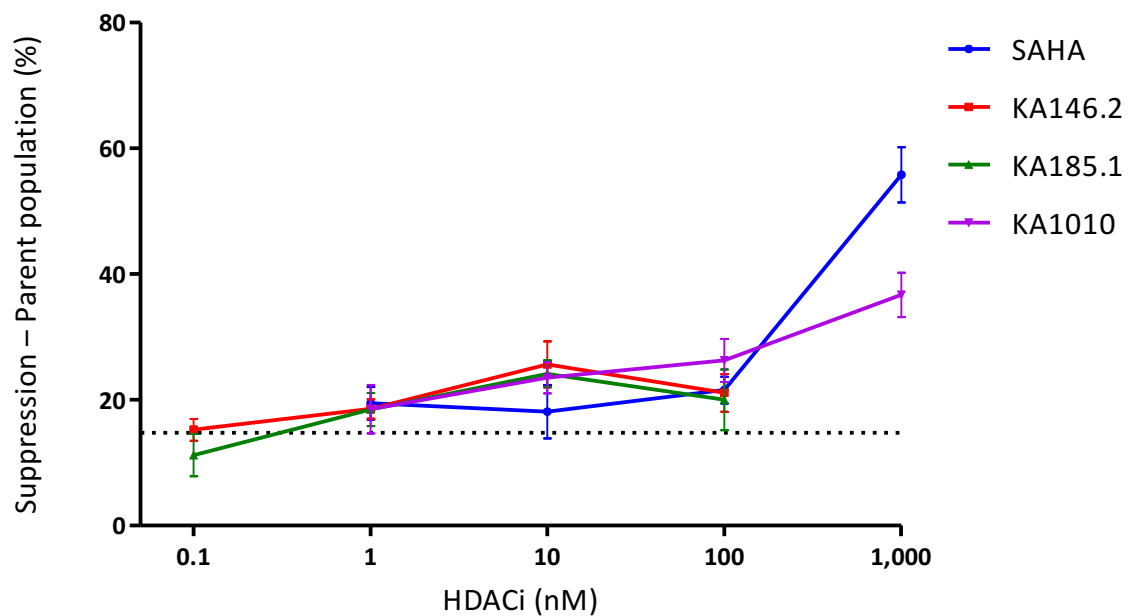


Figure 3-20 Effect of HDACi on PBMcs (no IL-2)

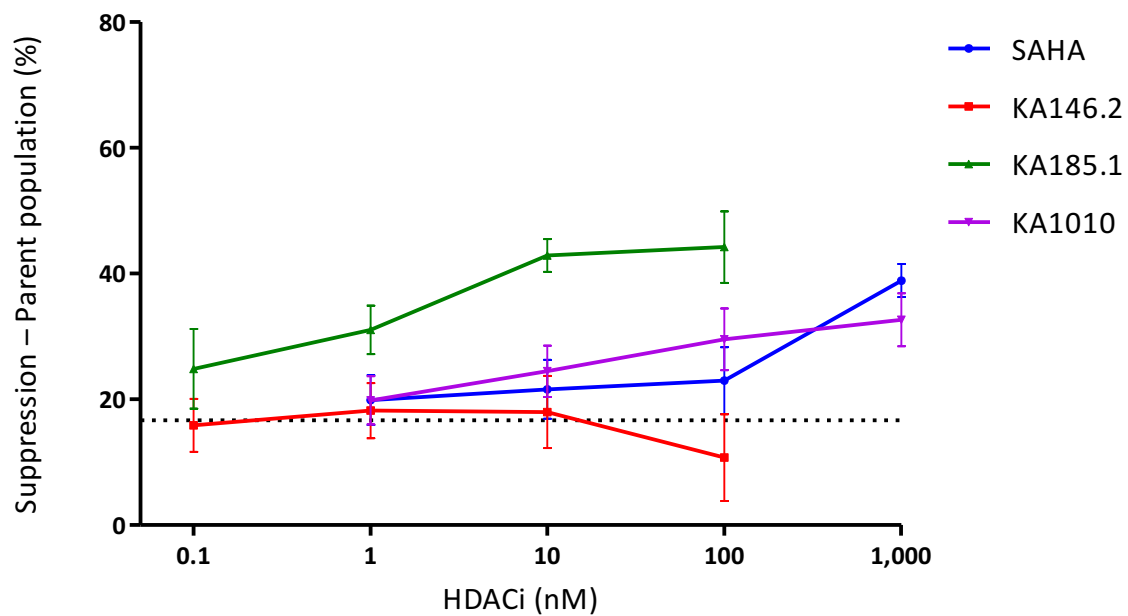
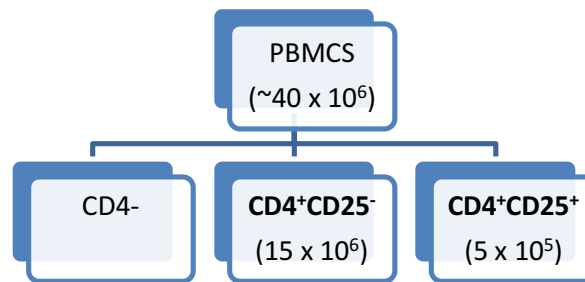


Figure 3-21 Effect of HDACi on PBMcs (with IL2)

3.6 Regulatory T cell Suppression Assay

The aim of this assay was to reproduce conditions similar to those used by Sakaguchi et al. (Miyara et al. 2009) to demonstrate the suppressive function of FoxP3⁺ regulatory T cells. Once this had been achieved the assay was then modified, with the addition of HDACis, in order to assess their effect on T_{reg} function.

In order to achieve this, it was first necessary to isolate the CD4⁺ subset of T cells and, from this subset isolate the CD4⁺CD25⁺ regulatory T cells. This was done using a magnetic-activated cell separation (MACS) method.



MACS separation involves several stages. First CD4⁻ cells are negatively selected from PBMCs using magnetic nanoparticles coated with a panel of antibodies (Table 3-5, Figure 3-22 A). The remaining CD4⁺CD25⁺ fraction is then positively selected out by labelling the cells with CD25⁺ nanoparticles (Figure 3-22 C). The retained cells from the magnetic eluting column are retrieved and tested for purity using flow cytometry (Figure 3-22 D).

Antibody	Cellular Reactivity
CD8	T cells, NK cells (MHC Class I)
CD14	Monocytes, Granulocytes, DCs, B cells
D16	Granulocytes
CD19	B cells
CD36	Monocytes, Platelets, Endothelial cells
CD56	NK cells
CD123	Myeloid precursors
TCR γ / δ	Intraepithelial lymphocytes
CD235a	Erythroid cells

Table 3-5 Antibody cocktail for negative selection

Regulatory T cells account for 2.5-4% of peripheral blood. The typically reported yield of regulatory T cells from human PBMCs using this type of isolation method is in the region of 1-2%. This was broadly true throughout this study. A purity of around 90% is typical of the published data (Tiemessen et al. 2007). Typically, 85-90% purity was experienced using this method.

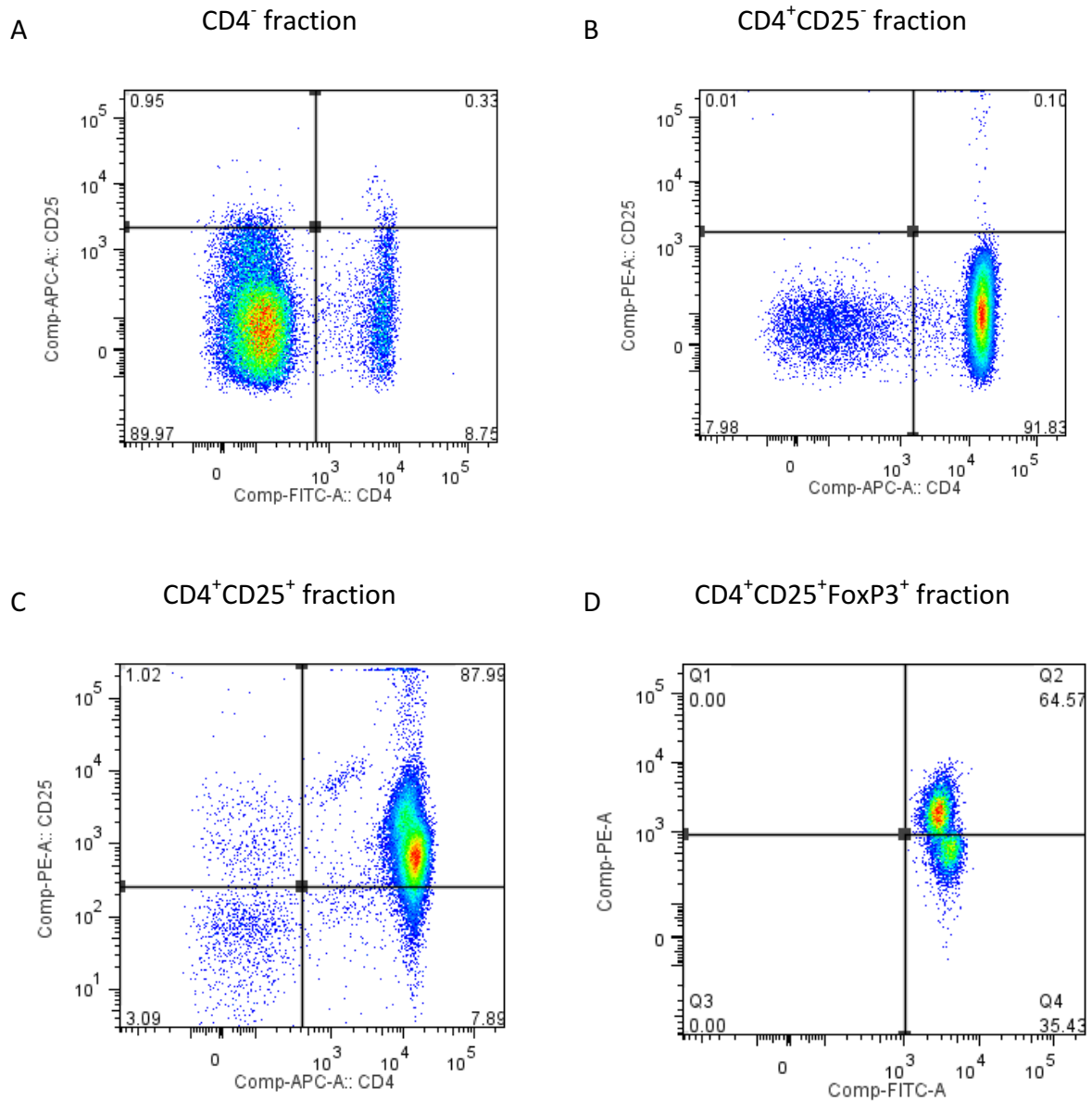


Figure 3-22 Representative data of flow cytometry analysis of isolated T cell subsets using MACS separation

The next step was to culture the CD4⁺CD25⁻ T cells (responders) with CD4⁺CD25⁺ (suppressor) cells. 1 ml of cells were cultured in a 24-well round bottomed plate at a concentration of 100,000 cells/ml. Lymphocyte proliferation was measured using carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. Fluorescent CFSE covalently binds to intracellular molecules and following cell division, distributes to daughter cells, halving in fluorescence. This protocol allows the detection of up to 8

cell divisions (Quah, Warren, & Parish 2007). Responder cells were labelled with CFSE at a concentration of 0.5 μ M.

Suppressor cells were added to the culture in increasing numbers and responder proliferation was assessed. Cells were cultured in the following fashion:

0:1	(0 : 100,000)
1:16	(6250 : 100,000)
1:8	(12,500 : 100,000)
1:4	(25,000 : 100,000)
1:2	(50,000 : 100,000)

Table 3-6 Suppressor: Responder cell numbers

Cultures were stimulated with PMA (100ng/ml) and Ionomycin (1 μ g/ml) and incubated for 3 days at 37°C. Proliferation was then assessed using flow cytometry.

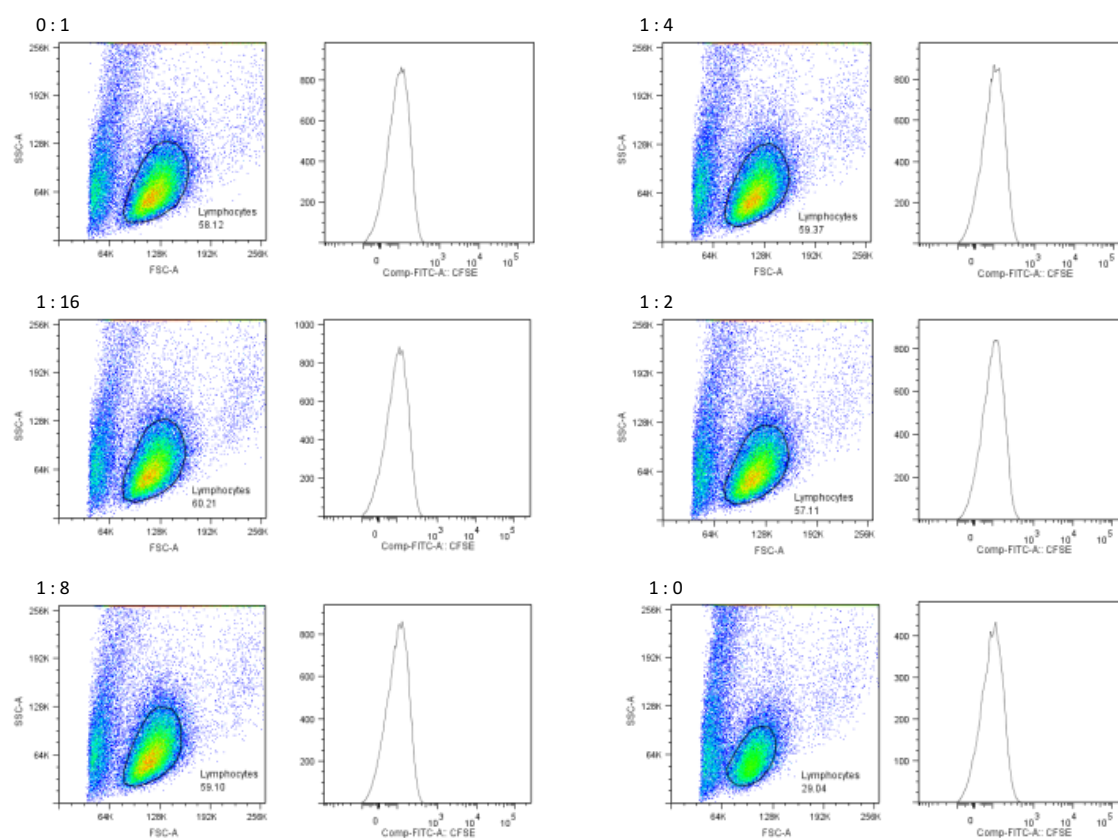


Figure 3-23 Flow cytometry data demonstrating regulatory T cell suppression of $CD4^+$ T cells (PMA/Ionomycin stimulation)

As Figure 3-23 demonstrates, increasing numbers of regulatory T cells had no effect on the proliferation of responder T cells and indeed the CFSE-labelled $CD4^+$ population failed to suggest any division had occurred. This was likely due to technical failure as responder cells did not produce a positive signal on flow cytometry, even in the responder alone population. There may also have been a lack of stimulatory effect of PMA and Ionomycin, which is an established stimulus for cytokine release assays (Han et al. 2012), but not necessarily suppression assays.

Therefore, a series of optimisation assays were undertaken in order to determine the ideal conditions for subsequently assessing the *in vitro* effect of HDACis.

3.6.1 CFSE Optimisation Assay

In order to optimise the concentration of CFSE to be used, a simple titration model was devised whereby 1×10^6 cells/ml of PBMCs were stained with one of three concentrations. CFSE 'brightness' was assessed using flow cytometry.

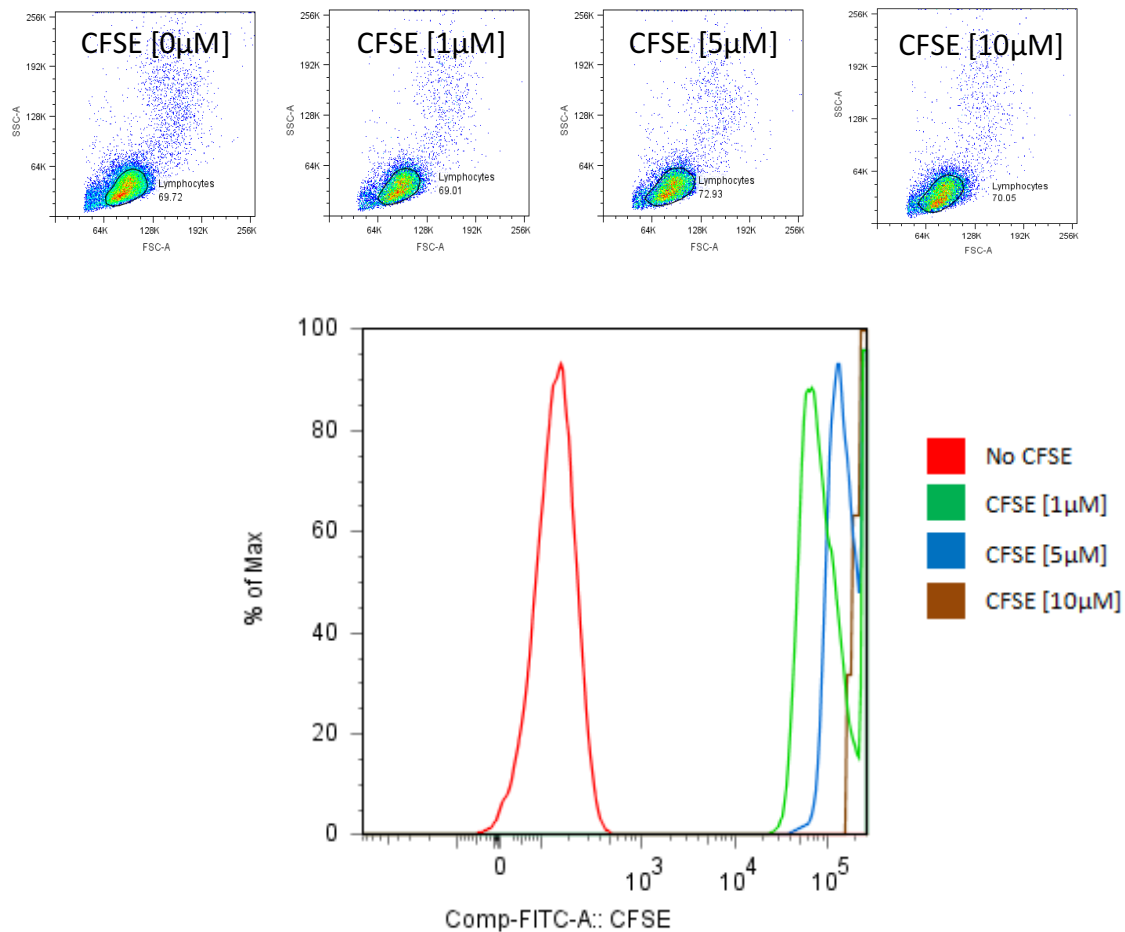


Figure 3-24 Optimisation of CFSE staining of PBMCs. Flow cytometry data demonstrating effect of varying concentrations of CFSE

All three concentrations of CFSE resulted in a bright signal (Figure 3-24) of similar intensity. A concentration of CFSE at 1 μM was therefore found to be most satisfactory and this was consistent with published data (Quah, Warren, & Parish 2007).

3.6.2 Stimulant Optimisation Assay

Optimisation studies were designed in order to compare a panel of commonly used stimulants at standard concentration. The stimulants used were:

Invitrogen T-Activator CD3/CD28 Dynabeads®

These inert beads are similar in size to antigen-presenting cells and are covalently bonded to anti-CD3 and anti-CD28 antibodies. They activate and expand the T-cell population by providing primary and co-stimulatory signals to T cells. They are commonly used at a bead-to-cell ratio of 1:1.

PMA and Ionomycin

Phorbol 12-myristate 13-acetate (PMA) is a mitogen derived from the Croton plant. It stimulates T cells by direct activation of Protein Kinase C (similar to diacylglycerol, activates PKC). Ionomycin synergizes with PMA in enhancing the activation of PKC (Chatila et al. 1989) and increasing release of IFN- γ , TNF- α , IL-2 and IL-4. Typical concentrations of PMA and Ionomycin are 10ng/ml and 1 μ g/ml respectively.

Con A

Concanavalin A (Con A) is a lectin protein originally extracted from the jack-bean, *Canavalia ensiformis* which acts as a lymphocyte mitogen. Con A has a strong affinity for cell-surface glycoproteins and once irreversibly bound, induces proliferation through a mechanism of indirectly cross-linking the T cell receptor. Typical concentration for the use of Con A as a stimulant is 2 μ g/ml.

Mitenyi-Biotec MACS T Cell Activation Kit

The MACS T Cell Activation kit works on a similar principle to the Invitrogen Dynabeads®. The kit consists of Anti-Biotin MACSiBead Particles and biotinylated antibodies against human CD2, CD3, and CD28. Anti-Biotin MACSiBead Particles loaded with the biotinylated antibodies mimic antigen-presenting cells and activate resting T cells. This kit is typically used at a bead-to-cell ratio of 1:2.

Anti-CD3 Antibody

The use of a monoclonal anti-CD3 antibody is the most direct way of inducing T cell activation and stimulation. T cell receptor recognition of anti-CD3 mAb mimics recognition of antigen and thus triggers signal transduction and proliferation (Garbrecht, Russo, & Weksler 1988). In this assay, a concentration of 0.5µg/ml was used.

PBMCs were stained with CFSE at a concentration of 1µM and cultured at 37°C for 4 days. Culture media consisted of RPMI with 10% FCS plus the stimulant under investigation at the appropriate dose. Media for the negative control did not contain any additional stimulant. Proliferation was assessed by measuring CFSE degradation using flow cytometry analysis.

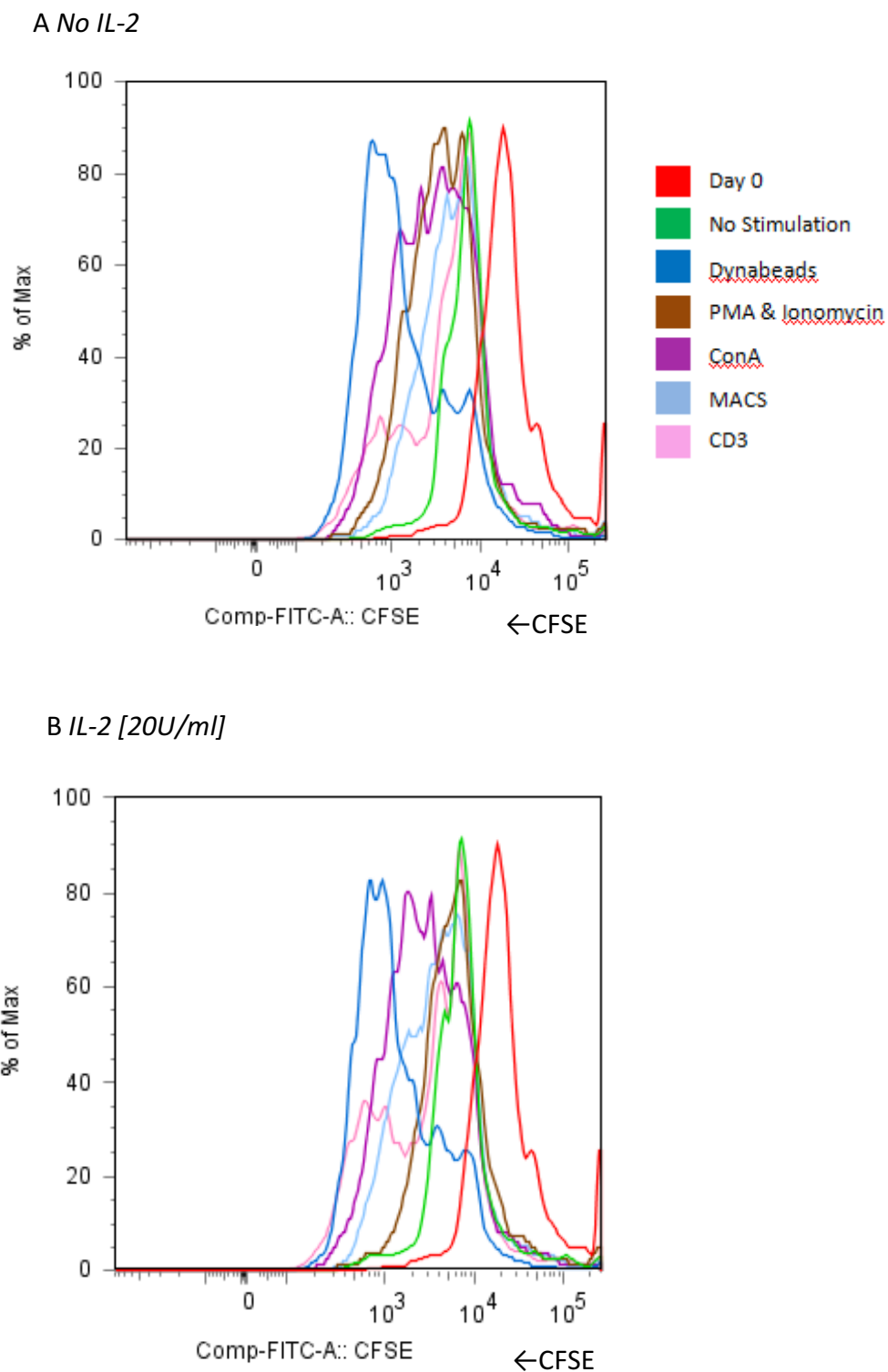


Figure 3-25 Effect of stimulant on proliferation of PBMCs with or without addition of IL-2 to culture media

All stimulants used produced a degree of proliferation though Dynabeads and ConA were the most effective. The presence of IL-2 in the culture media did not have any significant impact (consistent with the findings of previous assays).

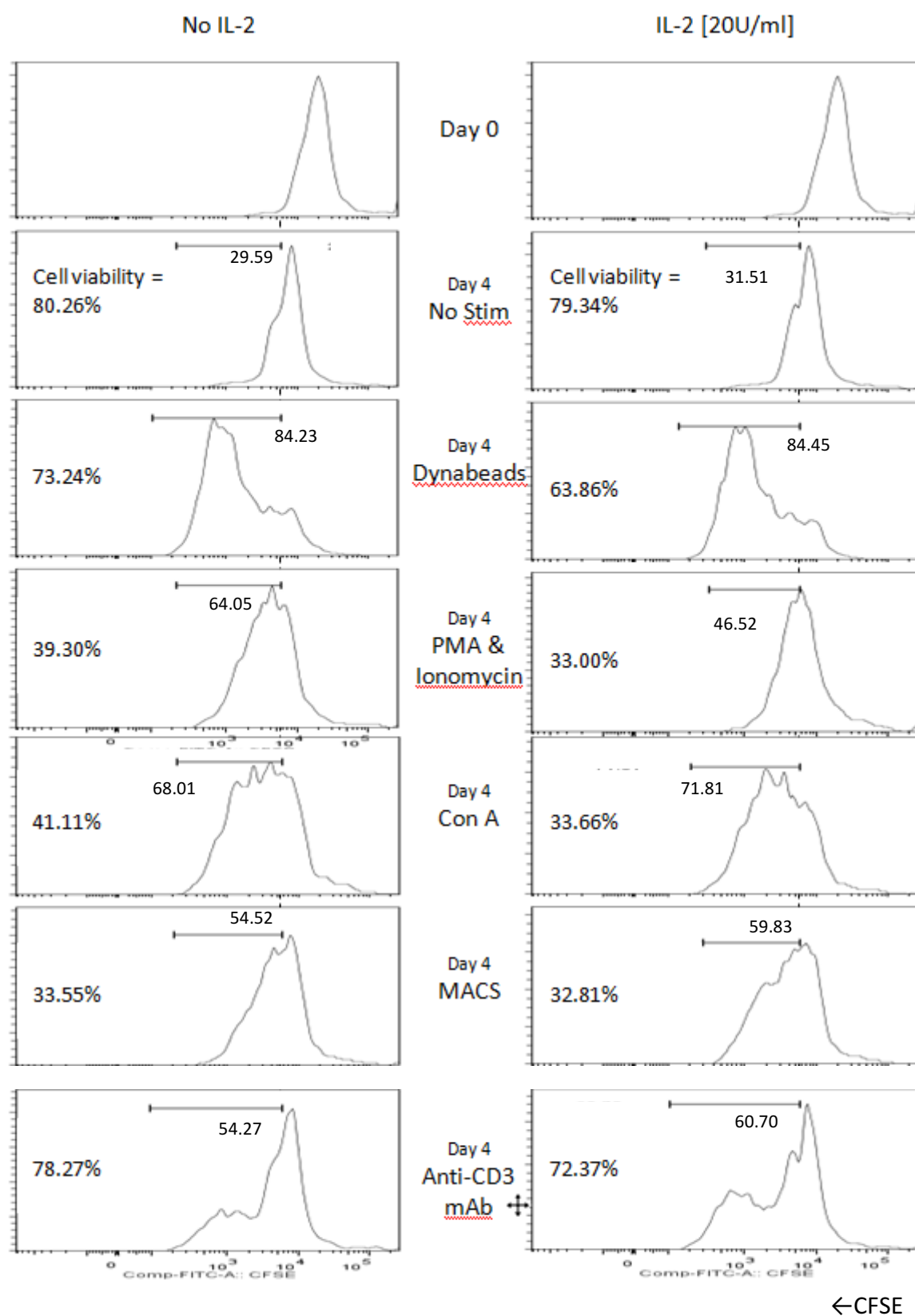


Figure 3-26 Raw FACS data demonstrating effect of stimulant on proliferation of CFSE-labelled PBMCs

The most significant proliferation occurred in those assays treated with either Dynabeads or Con A (Figure 3-25). Of the Dynabead stimulated PBMC population, 84.23% of the parent population, underwent proliferation (compared to 29.59% in the unstimulated population (Figure 3-26)). Cell viability was also comparable to the untreated population (73.24% vs. 80.26%) and was considerably better than most of the other stimulants tested.

Con A treatment consistently resulted in significant proliferation (68.01%), but further analysis demonstrated a poor level of cell viability (41.11%). Cell viabilities of <50% were also seen for the other mitogens tested (PMA/Ionomycin). Unexpectedly, the lowest degree of viability was seen in those PBMCs treated with the MACS T cell activation kit. The reasons for this are unclear, particularly as the principles of mechanism are similar to the Dynabead kit, though this was not pursued further.

Anti-CD3 mAb treatment resulted in proliferation of 54.27% of the parent population. The addition of IL-2 to the culture media only resulted in a subtle improvement in stimulation to 60.70%. This figure was expected to be somewhat higher due to the synergistic mechanism by which CD3 activation results in induction of IL-2 receptors, which can then respond to exogenous IL-2.

3.6.3 Regulatory T Cell Suppression Assay (revised)

The regulatory T cell suppression assay was therefore repeated using CFSE as a marker for proliferation and Invitrogen T-Activator CD3/CD28 Dynabeads® as stimulus. A bead-to-cell ratio of 1:1 was used and the assays were cultured for 72 hours. CFSE was added to the wells at a concentration of 1 μ M. Beads were removed prior to flow cytometry analysis.

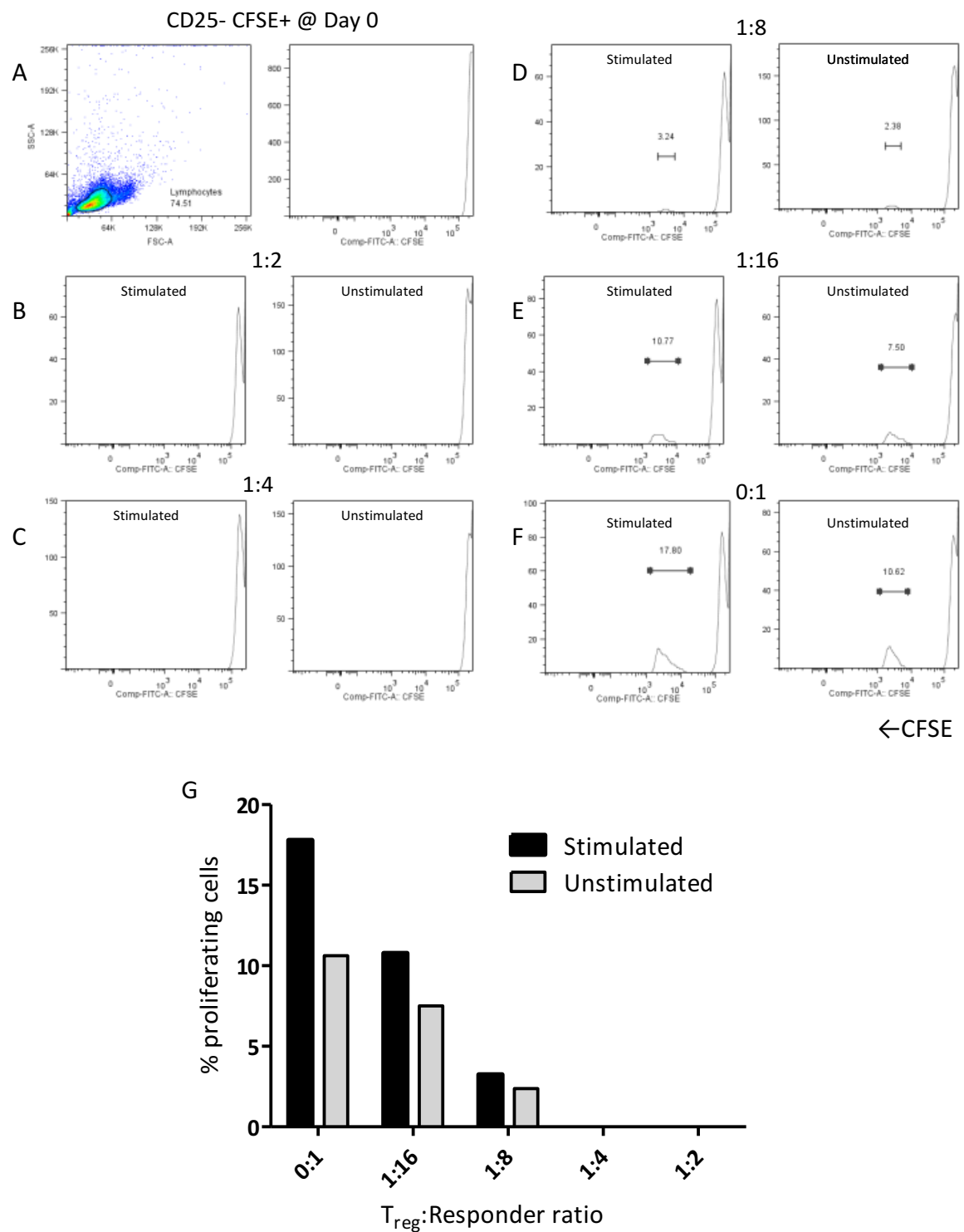


Figure 3-27 Effect of Dynabead stimulation on regulatory T cell suppression assay

CFSE at a concentration of 1 μ M effectively stained responder CD4⁺ T cells (Figure 3-27 A). Proliferation of this responder population was effectively inhibited in a ratio-dependent manner by the addition of regulatory T cells (Figure 3-27 B-F). Stimulation

of the assay using anti-CD3 anti-CD28 Dynabeads resulted in increased proliferation of responders compared to unstimulated assays (Figure 3-27 G).

3.6.4 Effect of HDACi on Regulatory T cell Suppression Assay

Following this series of optimisation studies, the above assay was repeated with the addition of HDACi compounds. Suppressor ($CD4^+CD25^+$) cells were isolated from fresh PBMCs using the MACS separation technique as above.

These cells were cultured in increasing numbers with responder cells at 37°C for five days (Table 3-7).

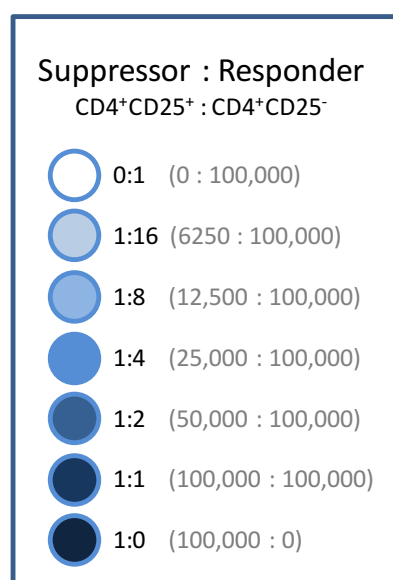


Table 3-7 Suppressor to responder ratio in T_{reg} suppression assay

Responder cells ($CD4^+CD25^-$) from the same donor were labelled with 1 μ M CFSE.

Proliferation was measured using flow cytometry. HDACis were added to the culture media at the following doses;

- SAHA (50nM)
- KA146.2 (5nM)

- KA185.1 (25nM)
- KA1010 (100nM)

Untreated stimulated and unstimulated controls demonstrated a typically suppressive effect of CD4⁺CD25⁺ on CD4⁺ cells (Figure 3-28) with stimulated cells having a significantly greater effect. The greater the proportion of T_{reg} to T_{eff} cells, the less the degree of proliferation of parent population (as per the published data).

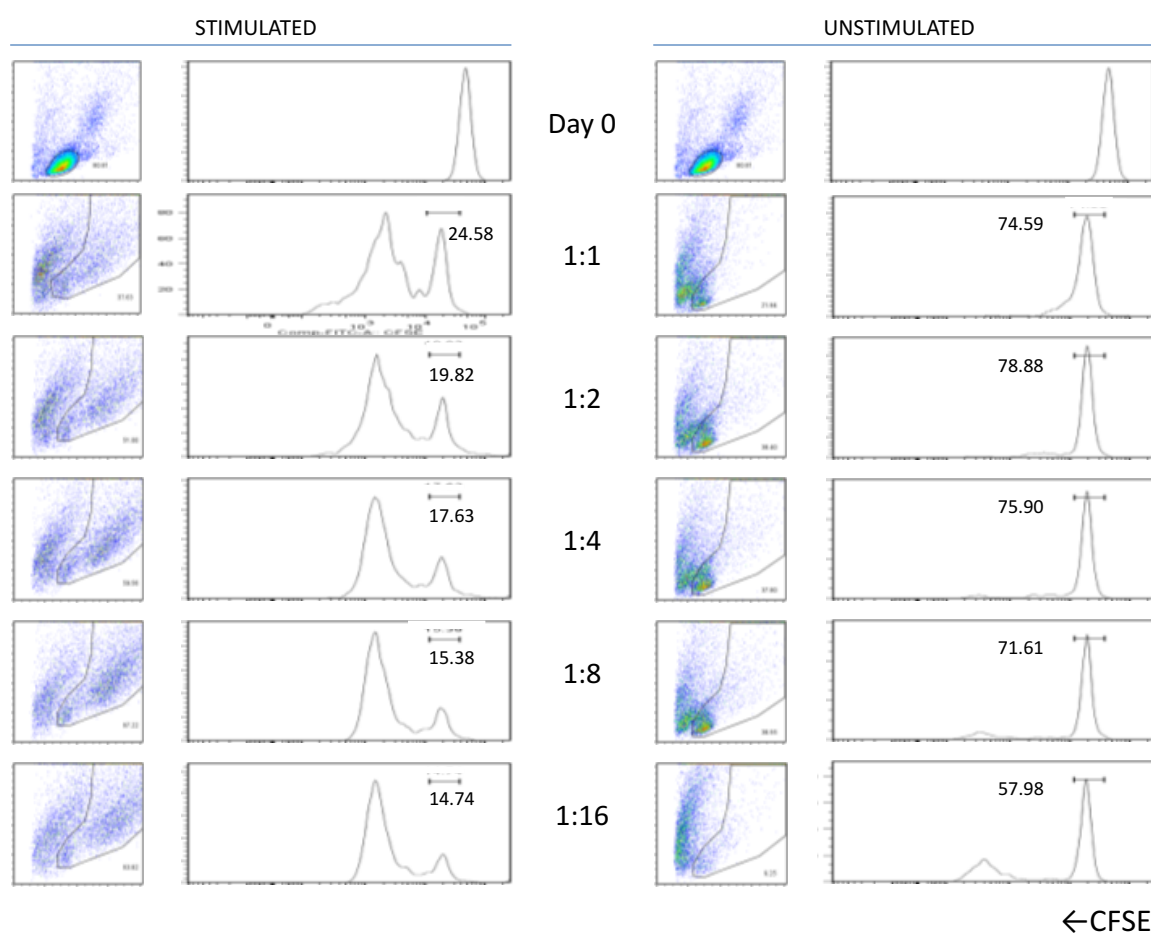
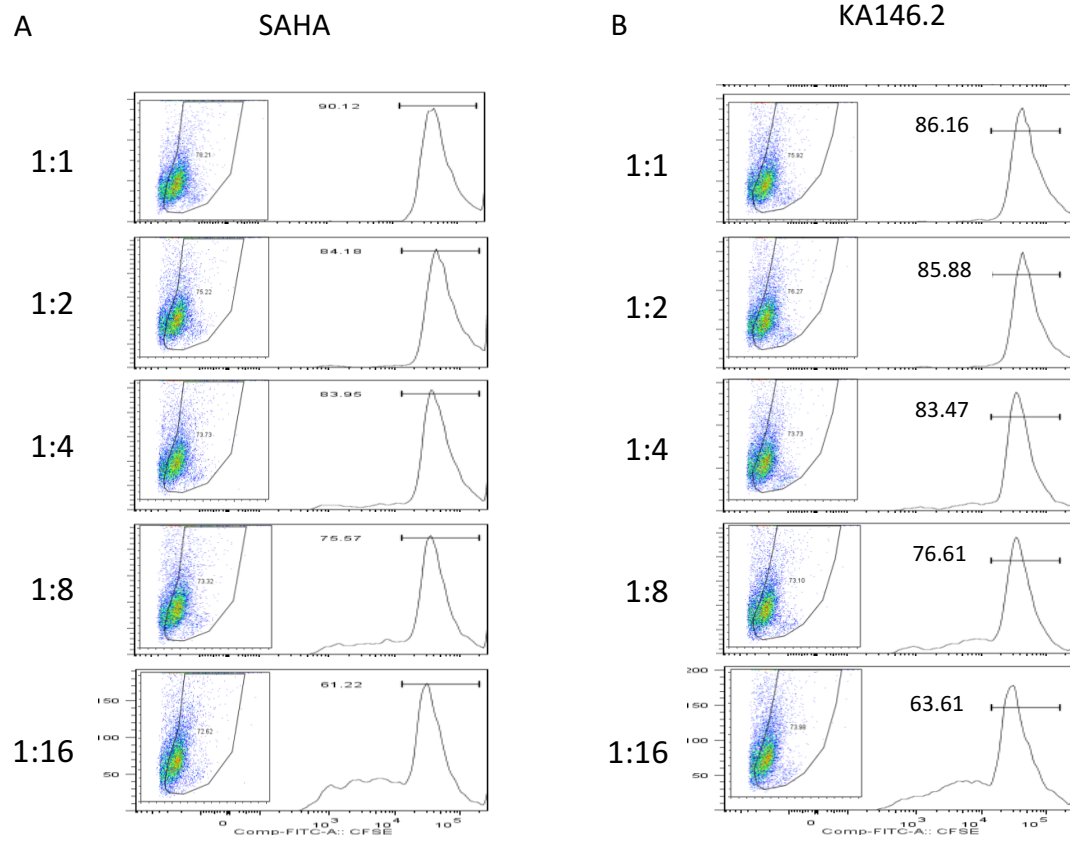


Figure 3-28 Regulatory T cell suppression assay untreated controls (stimulated and unstimulated)

The addition of a panel of HDACi compounds had broadly similar effects in terms of suppression (Figure 3-30). The general trend toward increased suppression of proliferation was maintained in the presence of all compounds. However, in these

stimulated conditions the degree of proliferation of effector T cells was significantly reduced compared to untreated stimulated control, to a degree similar to those seen in the untreated, unstimulated control.



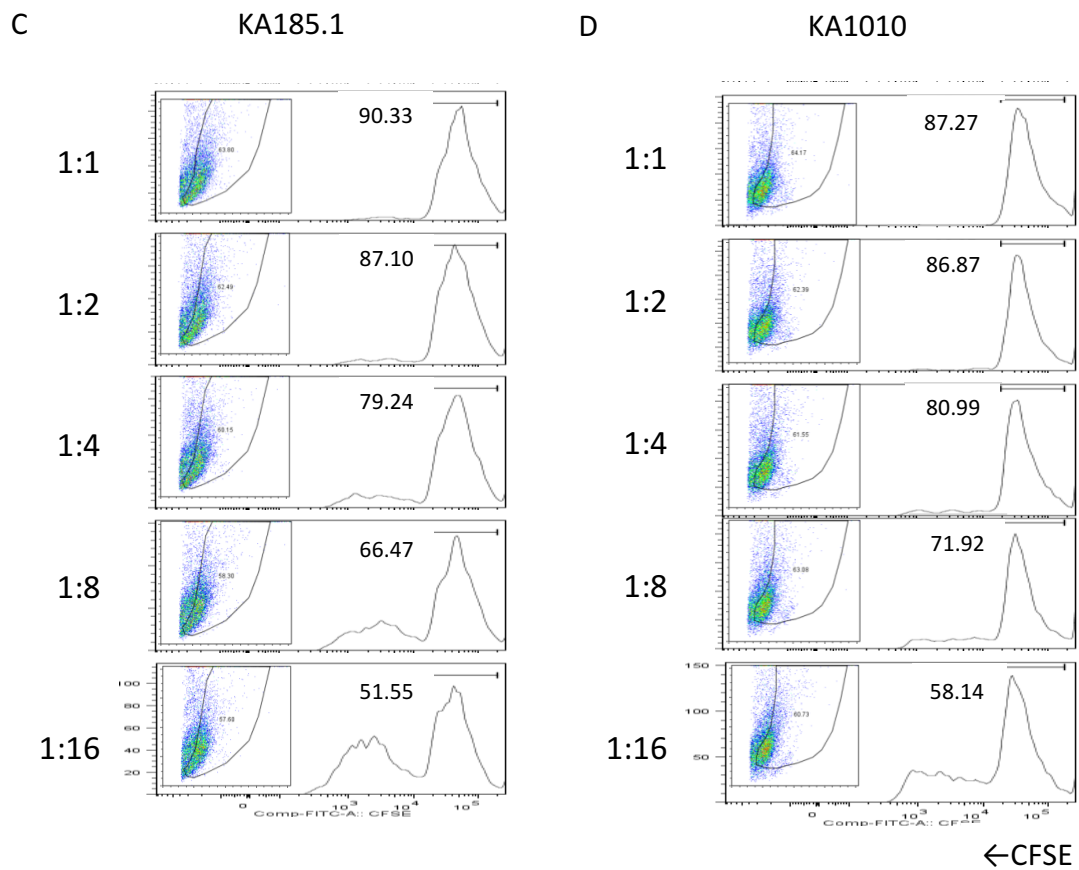


Figure 3-29 Raw data demonstrating effect of HDACi on stimulated regulatory T cell suppression assay

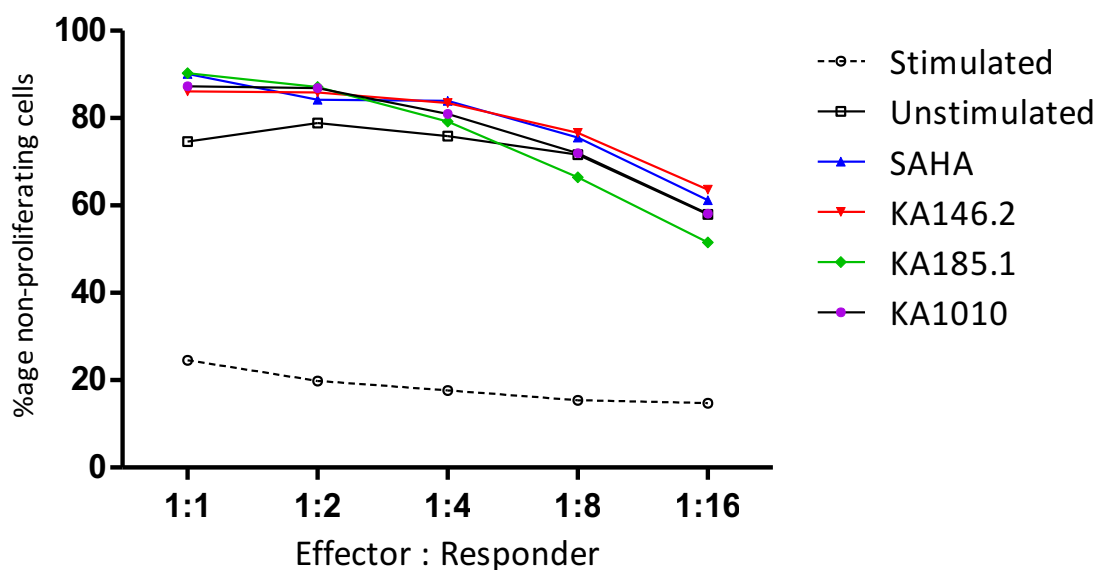


Figure 3-30 Effect of HDACi on stimulated regulatory T cell suppression assay

In the presence of KA185.1 (at a concentration of 25nM) 90.33% of the parent population remained undivided at a 1:1 effector to responder ratio (Figure 3-29 C).

This was marginally the most effective compound at this ratio.

With an effector to responder ratio of 1:16, KA146.2 was the most effective, maintaining a parent population of 63.61% (Figure 3-29 B). KA185.1 at this ratio, was however notably less effective than the other compounds tested with a non-proliferated parent population of 51.55%.

By significantly increasing the apparent suppressive function of regulatory T cells in this assay, the notion that HDACis mediate their immunosuppressive potential via their modulation is supported. It is not clear however from this assay as to whether or not this effect of HDACis is mediated through upregulation of T_{reg} function or if there is an effect on T_{reg} number.

3.7 Effect of HDAC inhibition on lymphocyte subset expression

With an apparent effect on PBMC proliferation and regulatory T cells, an assay was developed to assess the effect HDAC inhibition has on the expression of specific lymphocyte subsets. Freshly isolated PBMCs were cultured for five days in the presence of SAHA, KA146.2, KA185.1 or KA1010 at the same concentrations used previously. Following culture, the cells were stained for recognised (though not strictly specific) antibodies (Table 3-8) and subset expression assessed using flow cytometry.

<i>Antibody</i>	<i>Fluorochrome</i>	<i>Notes</i>
CD4	APC	Effector T cell population
CD8	PerCP	Cytotoxic T cell population
CD3	FITC	T cells (T cell receptor)
CD19	PE	B cells

Table 3-8 Lymphocyte subset antibody staining panel

3.7.1 Controls

The subset expression in the untreated culture was assessed. Assays were either stimulated or unstimulated and the effect of media IL-2 supplementation assessed.

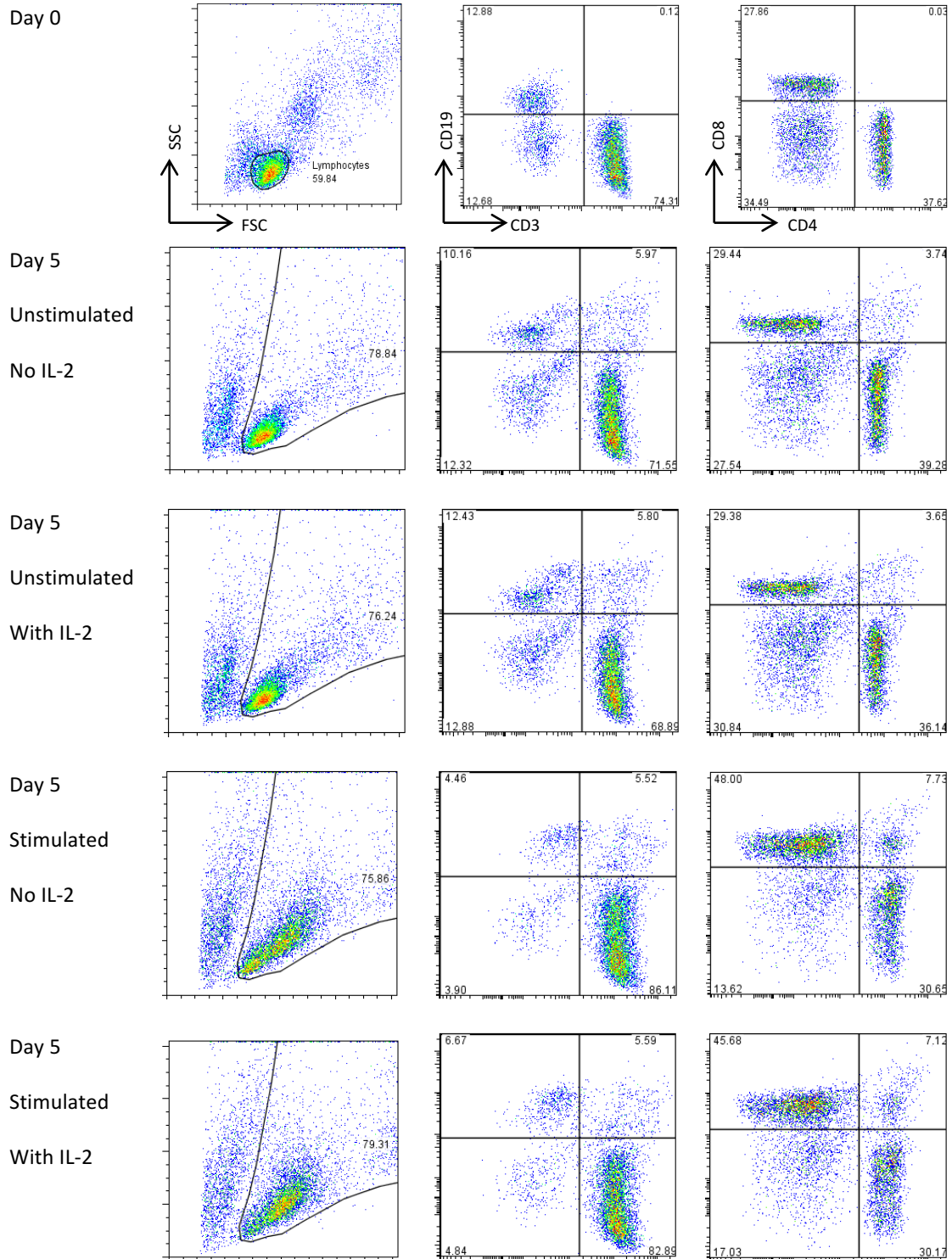


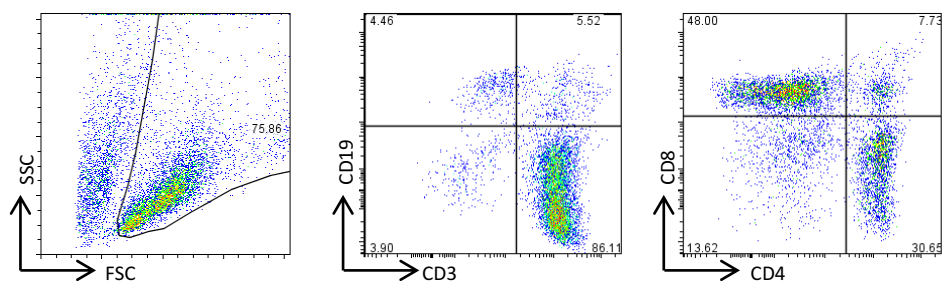
Figure 3-31 Raw data for lymphocyte subset expression under control conditions

As the control samples above demonstrate (Figure 3-31), once again, IL-2 has a negligible effect on the differentiation and expression of lymphocyte subsets over the course of a five day culture. However, stimulation with the use of Dynabeads did have a notable effect. In the presence of IL-2, stimulation resulted in a 46.3% reduction in CD19 expression and CD3 expression increased by 20.3%. Within the T cell subset, CD4⁺ T cells reduced by 16.6% and CD8⁺ T cells increased by 35.7%. This is in contrast to typical, unstimulated PBMC expression where CD19⁺ B cells number ~15% and CD3⁺ T cells ~45-70%. Of these CD3⁺ T cells, typically ~25-60% are CD4⁺ and 5-30% are CD8⁺.

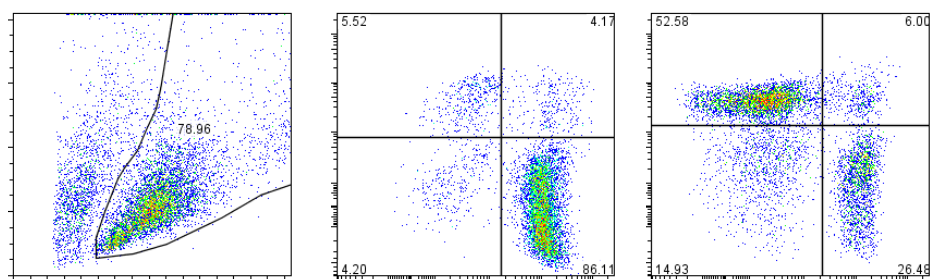
Therefore, the assay was repeated under stimulating conditions in the absence of IL-2, as this more likely reflects the immunological environment of an immune response as stimulated by allogeneic transplantation. Media was supplemented with compounds of interest added at increasing concentrations.

SAHA

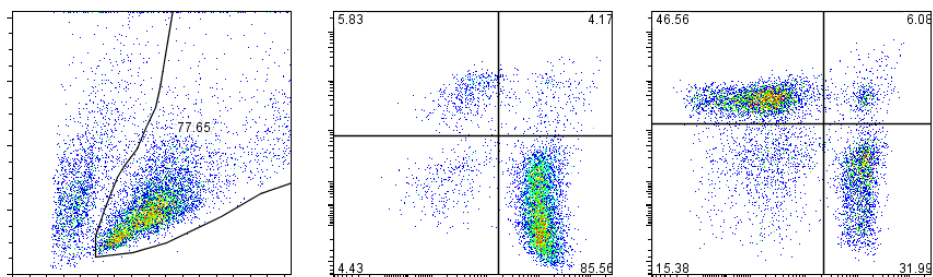
Day 0



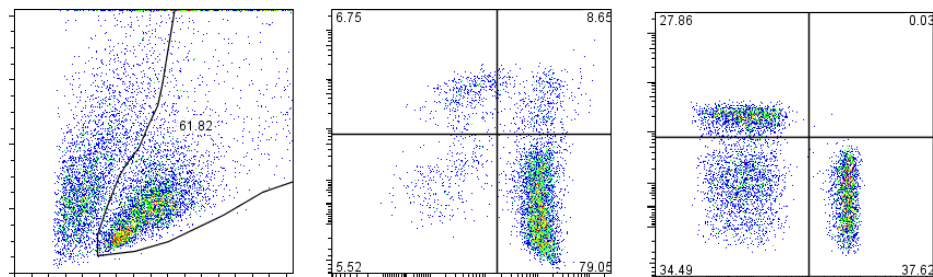
1nM



10nM



100nM



1000nM

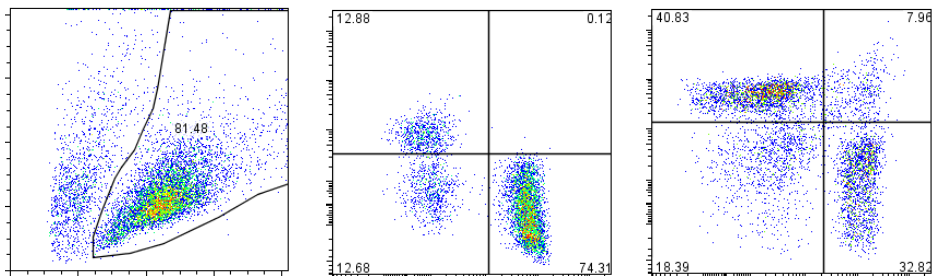
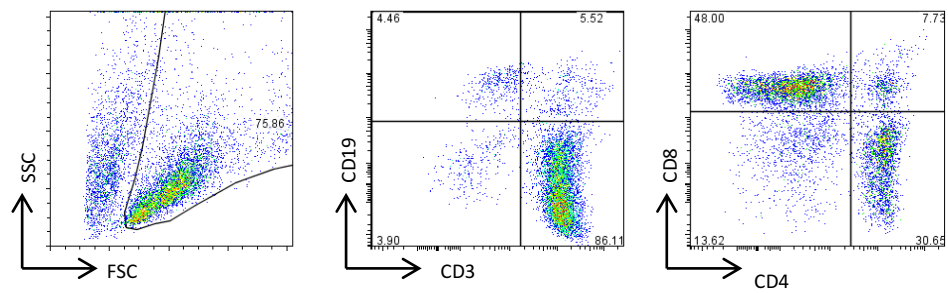


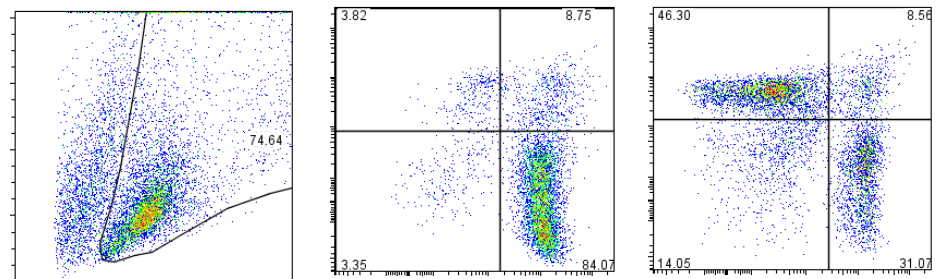
Figure 3-32 Raw data flow cytometry analysis of lymphocyte differentiation following PBMC culture in the presence of SAHA

KA146.2

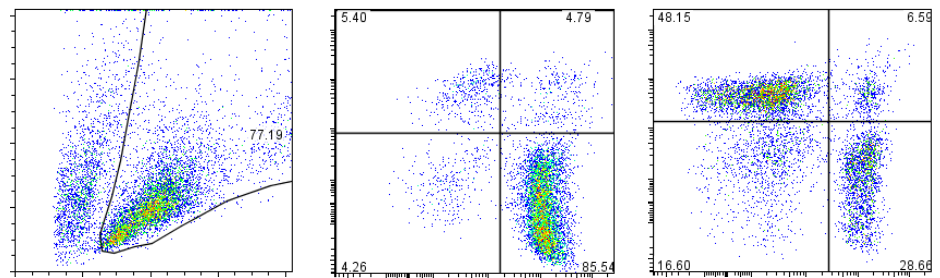
Day 0



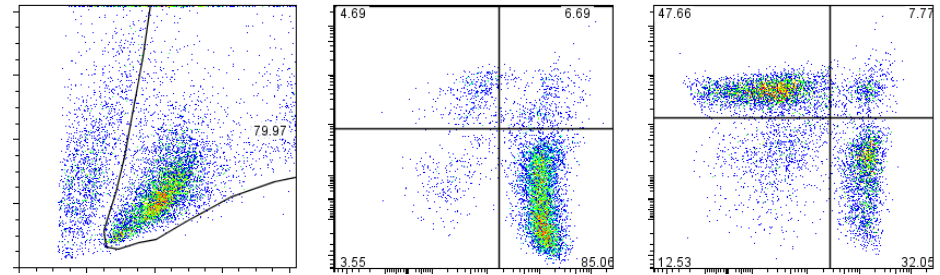
0.1nM



1nM



10nM



100nM

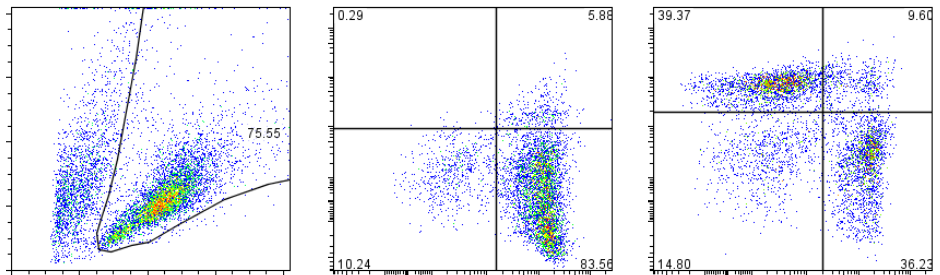
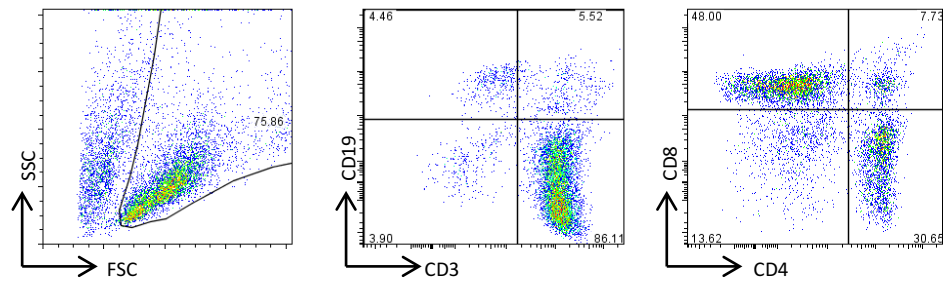


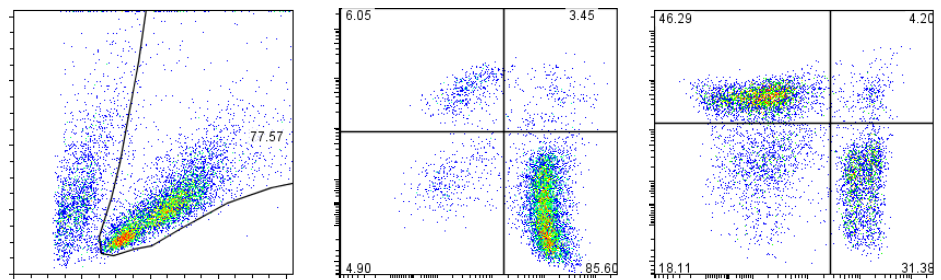
Figure 3-33 Raw data flow cytometry analysis of lymphocyte differentiation following PBMC culture in the presence of KA146

KA185.1

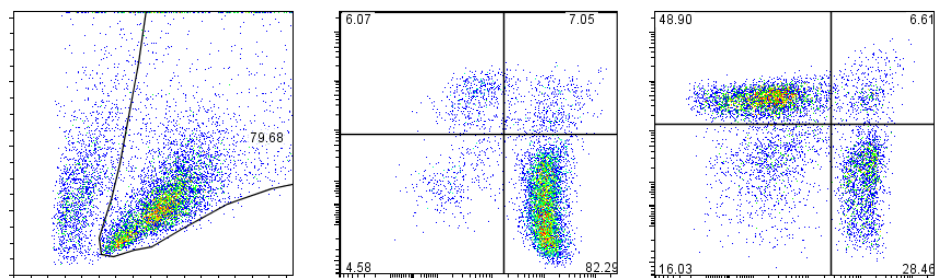
Day 0



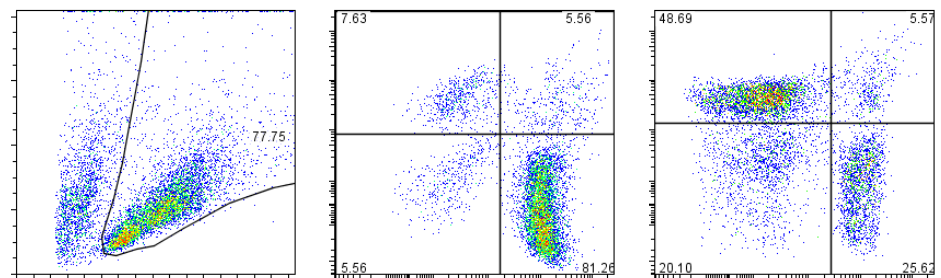
0.1nM



1nM



10nM



100nM

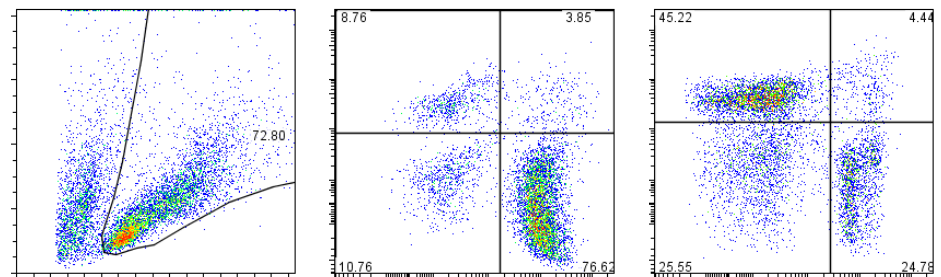
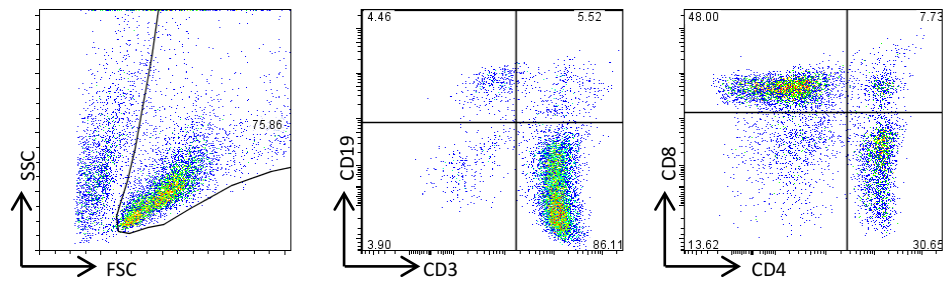


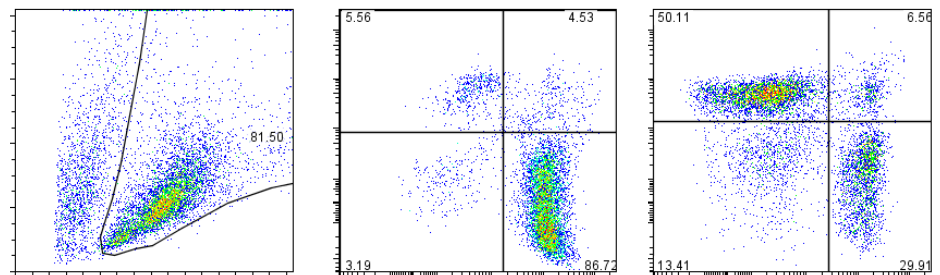
Figure 3-34 Raw data flow cytometry analysis of lymphocyte differentiation following PBMC culture in the presence of KA185.1

KA1010

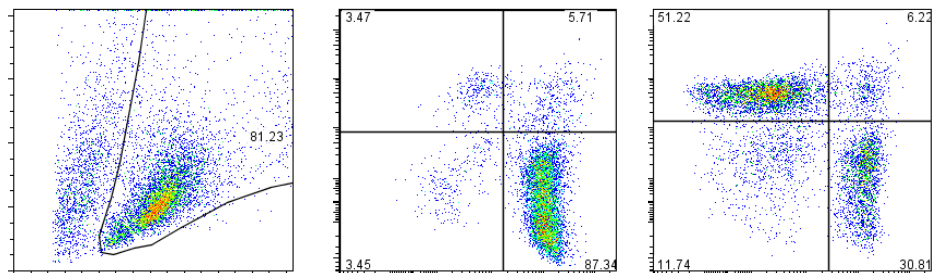
Day 0



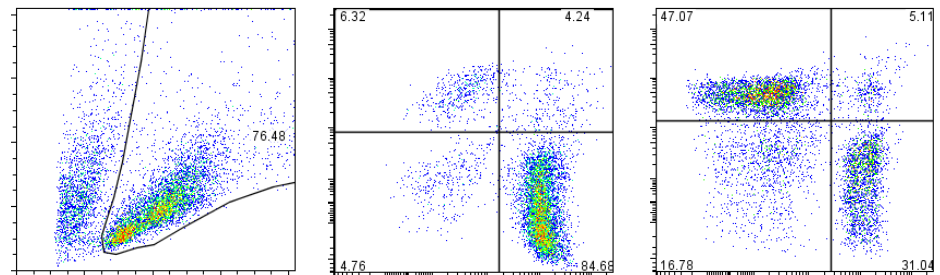
1nM



10nM



100nM



1000nM

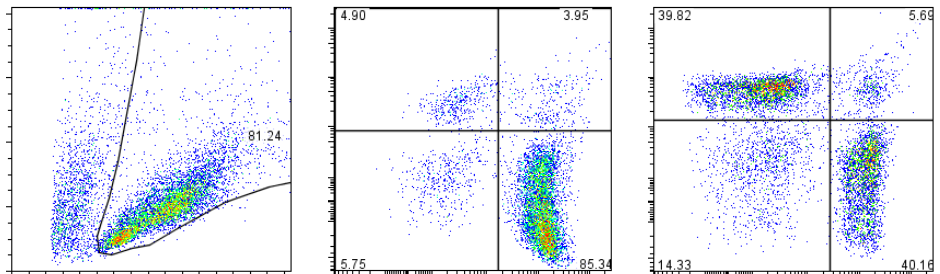


Figure 3-35 Raw data flow cytometry analysis of lymphocyte differentiation following PBMC culture in the presence of KA1010

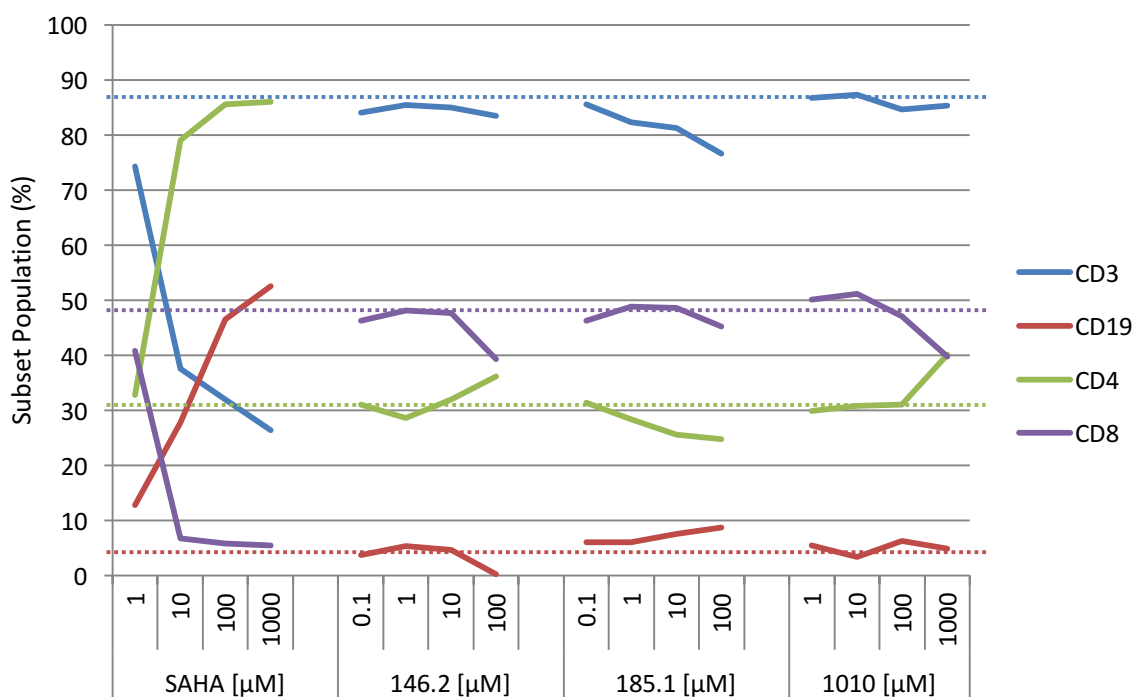


Figure 3-36 Summary of effects of HDAC inhibition (after 5 days) on B and T cell marker expression (hashed lines represent marker expression at day 0 of stimulated PBMCs)

This data demonstrates a stark contrast between the effects of SAHA and the other HDACi assessed. Even at the lowest dose of 1μM, treatment with SAHA results in a notable conformational change in subset markers. There was a reduction in CD3 and CD8⁺ expression of 13.7% and 14.9% respectively (Figure 3-32, Figure 3-36). CD19⁺ and CD4⁺ increased by 188.8% and 7.1% respectively. At the higher concentration of 1000μM, these changes were considerably more profound. CD3⁺ expression reduced by 69.2% and CD8⁺ by 88.5%. CD19⁺ expression increased by 1078.9% and CD4⁺ by 181.0%.

The effect of the other HDACi compounds assessed was more marginal (Figure 3-36). Of note, while there was relative stability in the expression of CD3⁺ T cells (~85%) in

the group of cells treated with the higher dose of KA146.2, there was an 18.0% reduction in CD8⁺ and a reciprocal 18.2% increase in CD4⁺ expression.

The effect of KA185.1 was to reduce CD3⁺ expression by 11.0% and increase CD19⁺ expression by 96.41%. This fall in T cell numbers was reflected in a 19.1% reduction in CD4⁺ expression and relative stability of CD8⁺ expression.

In many respects, the most relevant effects were seen with KA1010 treatment. There was a negligible effect on the overall expression of B cells (CD19⁺) and T cells (CD3⁺). However with increasing dose there was a reduction in CD8⁺ expression by 17.0% and concomitant increase in CD4⁺ expression by 31.1% (at a dose of 1000 µM), which partially restored the pre-stimulated status of a normal CD4:CD8 profile. Though the effect of 1010 was significantly less profound than SAHA, its effect may have more clinical relevance in an immune response dampening role, though it is unclear as to which CD4⁺ T cell subset/s may have accounted for this (i.e. was this effect a result of increased T_{regs} or another phenotype?).

Conclusion

After demonstrating effects on proliferation with relative safety in terms of toxicity, the effects of HDACi compounds were assessed in a series of *in vitro* models of allotransplantation. It was decided to rationalise the compounds based on the above experiments and their novelty and their potential application. Therefore, KA185 and KA146 (potent pan-HDACi from the synthetic depsipeptide class) were dropped from further assessment, and the novel HDAC6i, KA1010 taken forward along with the well characterised pan-HDACi, SAHA.

Chapter 4 - Results

4 Effect of histone deacetylation on *in vitro* models of allotransplantation

4.1 Introduction

The previous chapter demonstrated the basic *in vitro* effects of HDACi in terms of cellular physiology. Western blot analysis identified the presence of HDACs, the primary target of HDACis. Proliferation and toxicity studies showed that HDACis have an underlying anti-proliferative effect and in the context of these studies, these compounds are generally safe. Finally T_{reg} suppression assays revealed an immunosuppressive effect which was augmented through HDAC inhibition. With these results in mind, the next step was to assess the role of HDACi in *in vitro* models of transplantation, namely through a mixed lymphocyte reaction.

Mixed lymphocyte (or leucocyte) reactions provide an *in vitro* method for evaluating allogeneic responses and provide a robust method by which to assess the potential physiological role of HDAC inhibition in transplantation. They were first developed over 40 years ago (Bach & Hirschhorn 1964; Bain, Vas, & Lowenstein 1964) and are now a well-established immunological and pharmacological tool, often used by the pharmaceutical industry to assess the viability of potential new drugs.

The MLR relies on the MHC-T cell receptor interaction and allows evaluation of T-cell activation and proliferation in laboratory conditions (O'Flaherty et al. 2000). There

are three principal forms – one-way, two-way and three-way. In this study, only one-way and two-way have been used.

A one-way MLR consists of a responder population of lymphocytes (whose proliferation is assessed) and a stimulator population (which are rendered non-proliferative, see below). In this model the stimulator population represents the essentially passive target (graft) to the responder population (recipient). A two-way MLR differs from a one-way in that both populations are able to proliferate. It could be argued that this better represents a model of transplantation as donor organs not only present antigen but also carry immunocompetent cells such as T, B and NK cells (Richter et al. 1994; Schlitt et al. 1993). These cells can leave the graft, and it has been demonstrated that they can circulate in the host for significant periods of time (Richter et al. 1995; Schlitt et al. 1993). The ability of these cells to stimulate an immunological response is demonstrated in cases of graft-versus-host disease (GVHD) occurring after solid organ transplantation (Jamieson et al. 1991; Schlitt et al. 1995).

It may be concluded therefore that a two-way MLR could be used to represent the early stages of the immune response following transplantation and that the longer term response is better represented through a one-way assay.

The following chapter presents data assessing the effect of HDAC inhibition on MLR-type assays and compares these effects with the established immunosuppressant cyclosporin A (CyA).

In terms of MLR assay development (one-way), it was first necessary to optimise the conditions under which the stimulator population was rendered non-proliferative. γ -

irradiation was preferred over an anti-mitotic agent such as mitomycin C as the later has been shown to have significant effects on the cellular metabolism (and CD154 expression) of treated cells and subsequent stimulatory effect (Roy et al. 2001).

4.2 PBMC Irradiation Assay

In order to undertake a mixed lymphocyte reaction (MLR) assay it was first necessary to optimise a reliable method of rendering the stimulator PBMCs unable to undergo division, yet maintain cell viability (and be able to present alloantigens as well as produce cytokines). As mentioned above, there are two established methods for achieving this. The first is to treat PBMCs with an anti-proliferative drug such as mitomycin C, which acts by cross-linking DNA strands, thereby preventing cell proliferation. The alternative method is to use γ -irradiation to irreversibly damage cellular DNA and thus prevent replication. However, excessive irradiation also has the potential to cause extensive damage, such that apoptosis occurs. Therefore, it was first necessary to establish the optimal dose PBMCs could be treated at, so that they are no longer able to proliferate but still maintain viability and are thus able to act as antigen-presenting cells (APCs).

In order to achieve this, an assay was undertaken whereby freshly isolated PBMCs were irradiated at increasing doses from 0 to 50 Gy and then cultured for one to four days. Survival and viability was assessed through 7-AAD staining and flow cytometry.

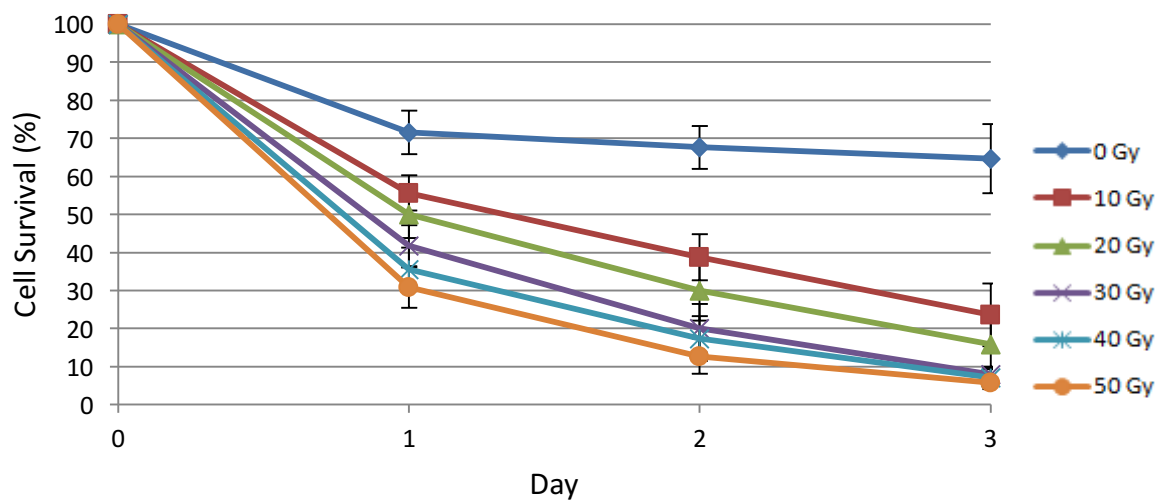


Figure 4-1 PBMC survival following irradiation

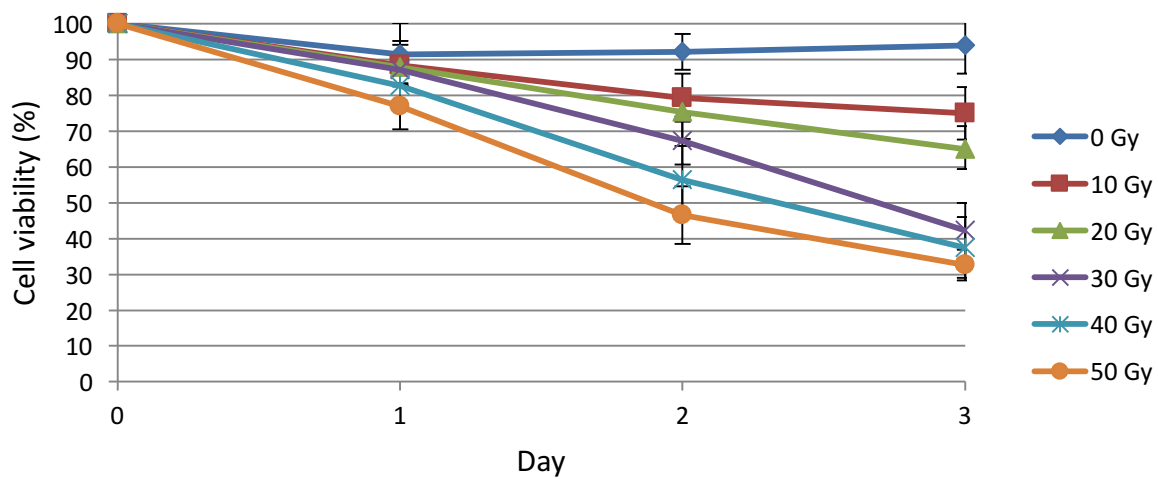


Figure 4-2 PBMC viability following irradiation

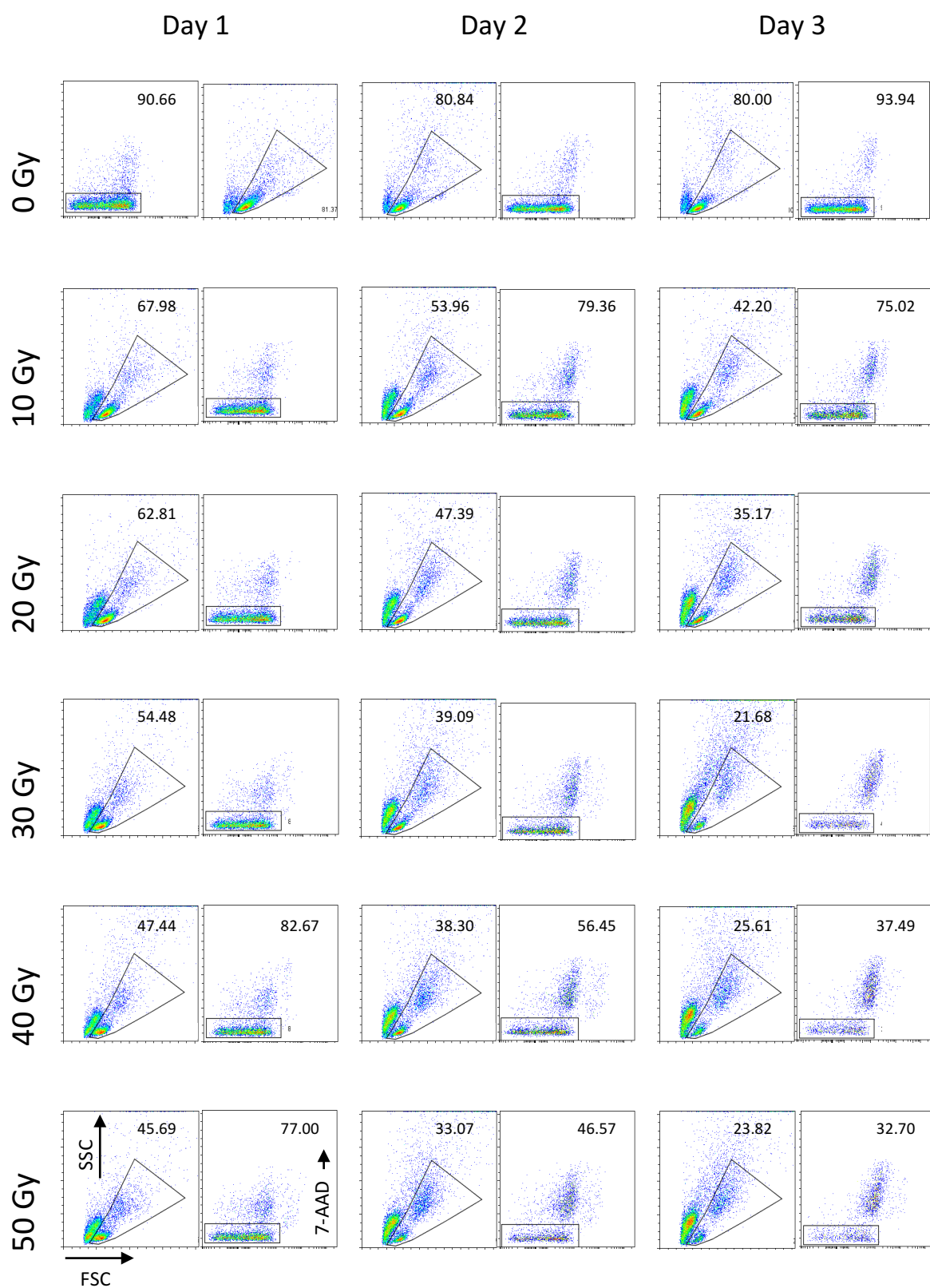


Figure 4-3 Representative raw data of effect of irradiation on PBMC survival / viability

This data demonstrates a dose/time dependent effect of γ -irradiation on PBMC survival and viability. Irradiating cells regardless of dose, resulted in a considerable reduction in terms of cell survival (Figure 4-1) to the extent that by day 3 post-exposure survival drops from 64% in the untreated population to 23% in those cells treated with the lowest dose of 10Gy. There was, however, less of a contrast between untreated and treated cells in terms of cell viability; though a dose dependent effect was still evident (Figure 4-2). Without irradiation treatment, 93% of cells retain their viability, and this reduces to 75% with treatment at a dose of 10Gy. Beyond this, there does seem to be a notable drop in cell viability from 20Gy treatment (65%) to 30Gy (42%).

Reviewing this data as a whole, a dose of 20Gy irradiation was deemed to provide the optimal condition at which to treat stimulator cells for the purpose of an MLR and this would be consistent with the majority of the published data.

4.3 Mixed Lymphocyte Reaction (MLR) – One-Way

4.3.1 Effect of compounds

The first MLR undertaken in this series of experiments was a one-way study in which the stimulating population is irradiated in order to render it viable but without the ability to undergo cell division as described above. These cells therefore merely act as foreign alloantigens.

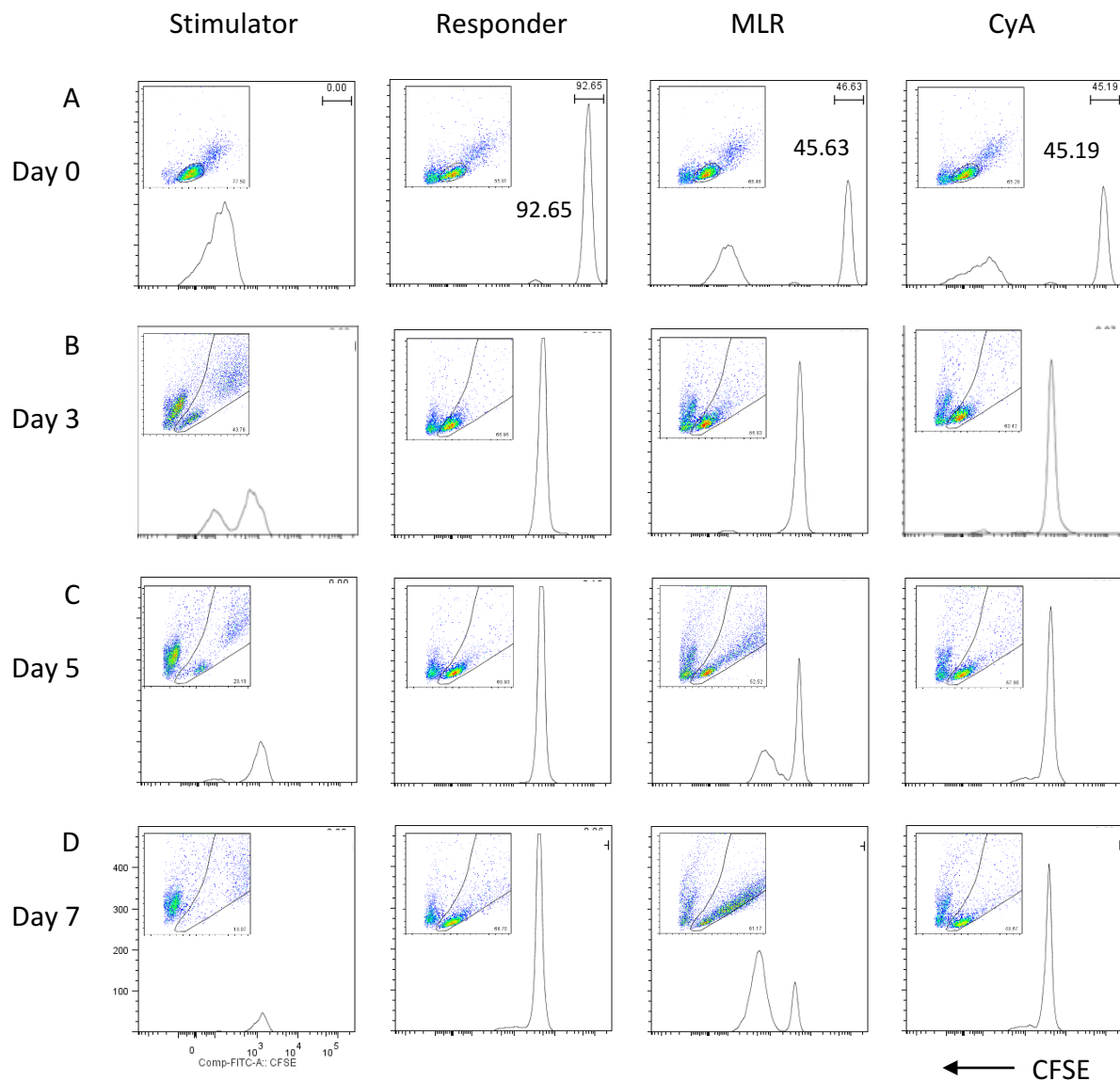


Figure 4-4 One-way MLR – PBMCs treated with CyA [200ng/ml]

In this initial experiment freshly isolated PBMCs, irradiated with 20Gy were cultured at a density of 1×10^6 cells/ml with equal numbers of CFSE-labelled [$1\mu\text{M}$] responder cells from an allogeneic donor. Responder cell proliferation was assessed at days 3, 5 and 7 in the presence or absence of CyA (at a concentration of 200ng/ml).

At day 0, as expected a non-proliferated, unstained population was observed in the stimulator only control wells (Figure 4-4 A). The responder population stained well for CFSE. In the MLR and CyA wells, just fewer than 50% of the cells stained for CFSE demonstrating an approximately 1:1 ratio of stimulators to responders.

From days 3 to 7, the stimulator only population underwent steady degradation until very few viable cells remained by the end of the assay (Figure 4-4, B-D). Conversely, the unstimulated responder population underwent negligible division as would be predicted. In the untreated MLR, there was appreciable division of the responder parent population suggesting an allogeneic response was underway. This response was significantly suppressed in the CyA assay so that minimal proliferation of the parent population occurred.

The conditions of this assay were repeated, in order to better quantify the effect of CyA as well as assess the effects of the addition of the following HDACi compounds:

- SAHA – 0.5 μ M or 5 μ M
- KA1010 – 0.5 μ M or 5 μ M

Once again the stimulator population underwent steady degradation over time without any significant division (Figure 4-5). It was also noted that there was an apparent greater degree of proliferation, suggesting a more potent alloresponse in the untreated MLR compared to the previous experiment (Figure 4-4). However, this was not quantified in the previous experiment and, therefore, cannot be commented on further.

Analysis of the MLR in the presence of CyA demonstrated reduced proliferation of the parent population (92% vs. 75%, $p=0.01$) at day seven (Figure 4-7). This observation did extend beyond, to the end of the assay – day ten (Figure 4-5).

This was also the case with the addition of HDACi to the culture media (Figure 4-6); at a dose of 500nM, SAHA reduced division of the parent population from 92% to 80% ($p=0.01$) at day seven. This was reduced further to 49% ($p=0.001$) when treated

with a higher dose of 5 μ M. However, analysis of the flow cytometry plots suggested that the vast majority of responders were apoptotic (in the region of 75-80%), and at 5 μ M SAHA had notable toxicity, which clinically would be unacceptable.

KA1010, at a dose of 0.5 μ M, had a modest inhibitory effect on proliferation, reducing the parent population by just a further 5% (to 87%, $p=0.2$). However, at a dose of 5 μ M, the non-proliferating parent population remaining after seven days was significantly inhibited, with just 64% ($p=0.001$) undergoing one or more divisions.

At this dose, KA1010 was the most effective inhibitory agent, with an acceptably non-toxic effect on PBMCs.

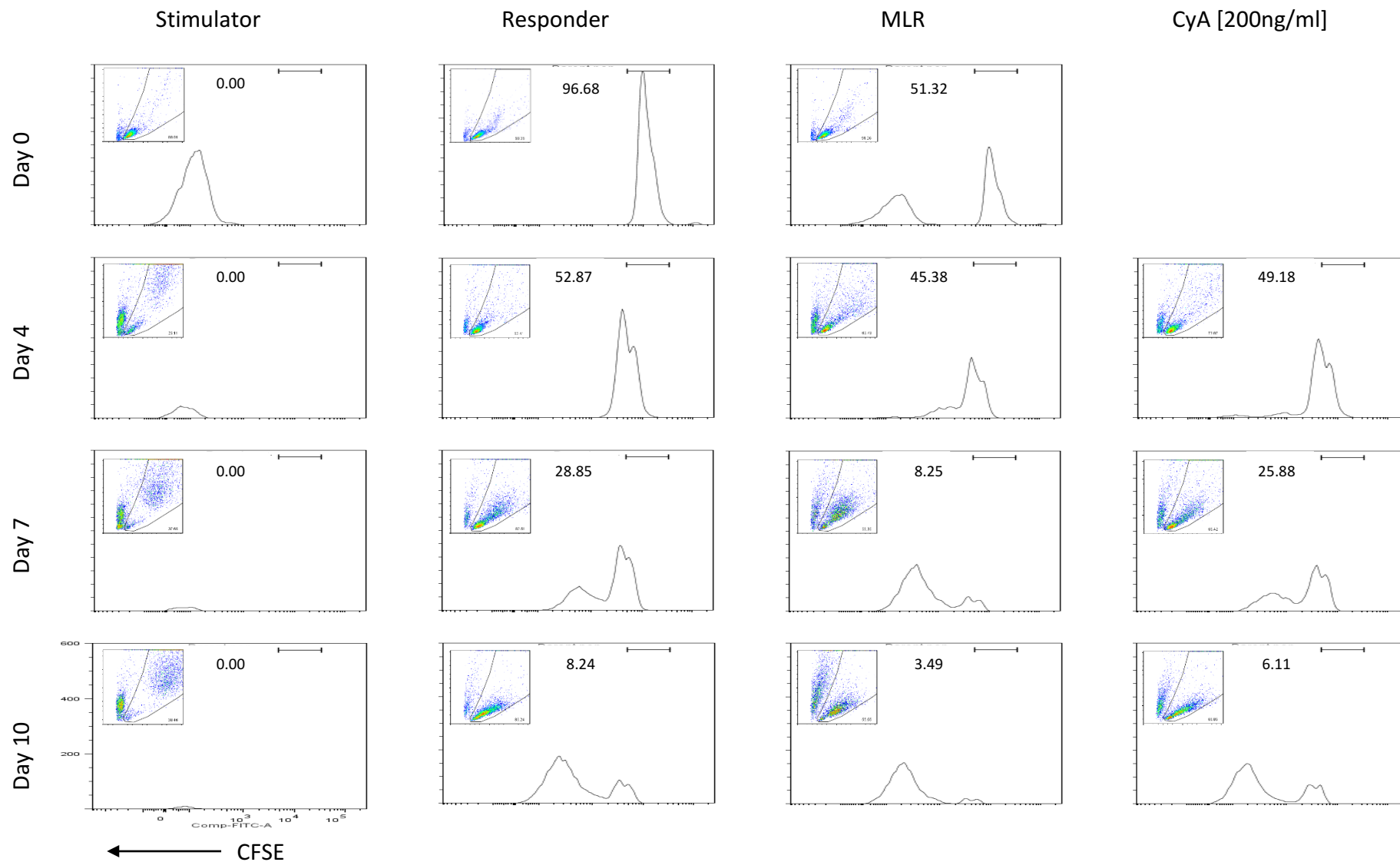


Figure 4-5 Representative data for one-way MLR – Controls and PBMCs treated with CyA [200ng/ml] for ten days

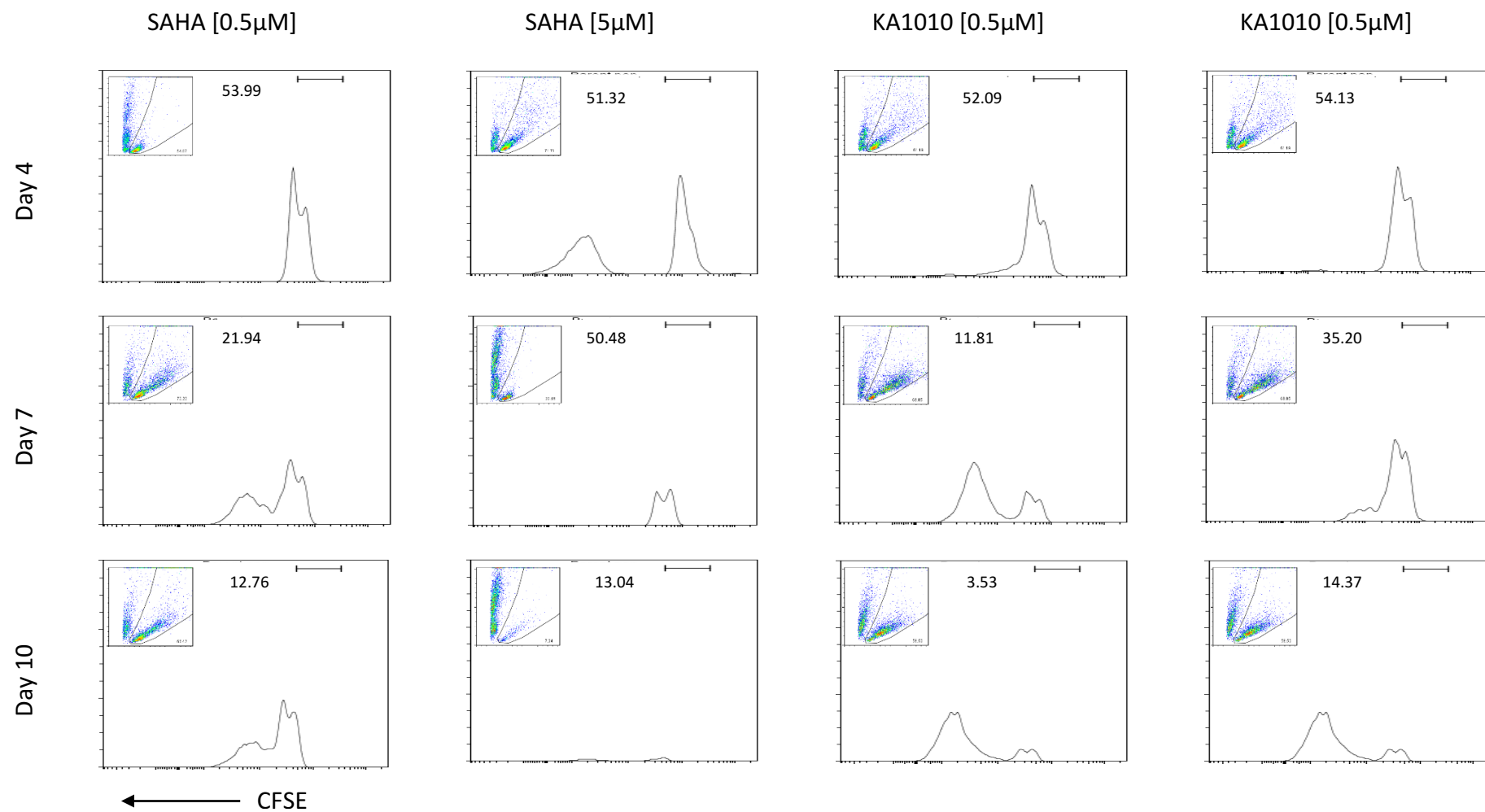


Figure 4-6 Representative data for one-way MLR – PBMCs treated with SAHA and KA1010 for ten days

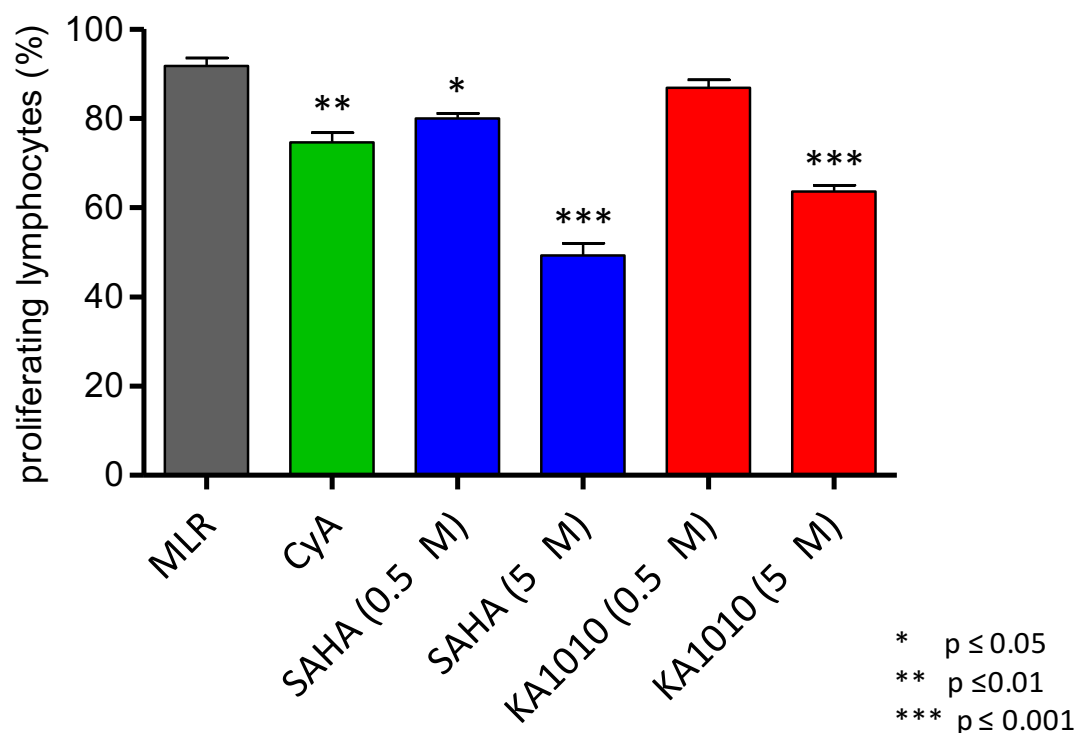


Figure 4-7 CyA and HDACi treatment (for seven days) in MLR conditions results in significant inhibition of labelled lymphocytes compared to responders in the untreated MLR (Asterisks represent p value as calculated using Tukeys multiple comparison test, n=4)

4.4 Mixed Lymphocyte Reaction – One-way Killing Assay

After demonstrating that HDAC inhibition can indeed dampen an immune response in a one way MLR, a killing assay was developed to elucidate whether or not allogeneic (as would be expected) or autologous targets are affected.

4.4.1 CD45

CD45, also known as the leucocyte common antigen (LCA) is a high molecular weight trans-membrane protein associated with all haematopoietic cells. It is required for T and B cell activation and exists in at least five isoforms which may be expressed in cells of differing

lineage or activation status. Given its abundant expression amongst leucocytes, it lends itself as a useful marker for labelling populations involved in this particular allogeneic MLR. This can be achieved by staining responder (or target) cells with anti-CD45 antibody, which will bind to a common region of the CD45 antigen, regardless of isoform.

4.4.2 Assay development

In this assay, two freshly isolated PBMC populations from allogeneic donors were labelled with an anti-CD45 antibody, conjugated to the fluorochrome FITC. Two different concentrations were used to differentiate between the two populations (Figure 4-8) on flow cytometry. These cells were then co-cultured with unlabelled, irradiated (20Gy) stimulators, autologous to one of the responder populations. The assays were cultured for 24 hours in the presence or absence of HDACi or CyA. Flow cytometry was used to assess for killing of the allogeneic responder (T1) population.

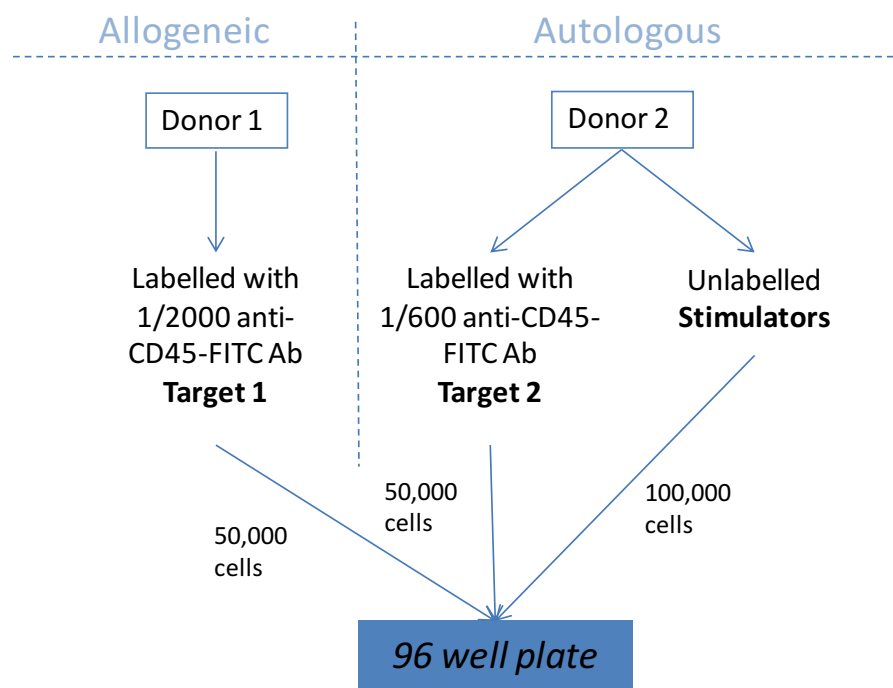


Figure 4-8 Experimental layout for one-way killing assay

Pre-culture assessment of cell staining demonstrated two distinct responder (target) populations and an unstained stimulator population. When combined (in the absence of stimulator cells), the two target populations remain distinct in terms of staining, with roughly equal numbers in each population. Analysis of the stimulator population revealed a negative staining population.

After staining, flow cytometry of the T1:T2 control samples demonstrated two distinct populations at a ratio of approximately 1:1 (Figure 4-9).

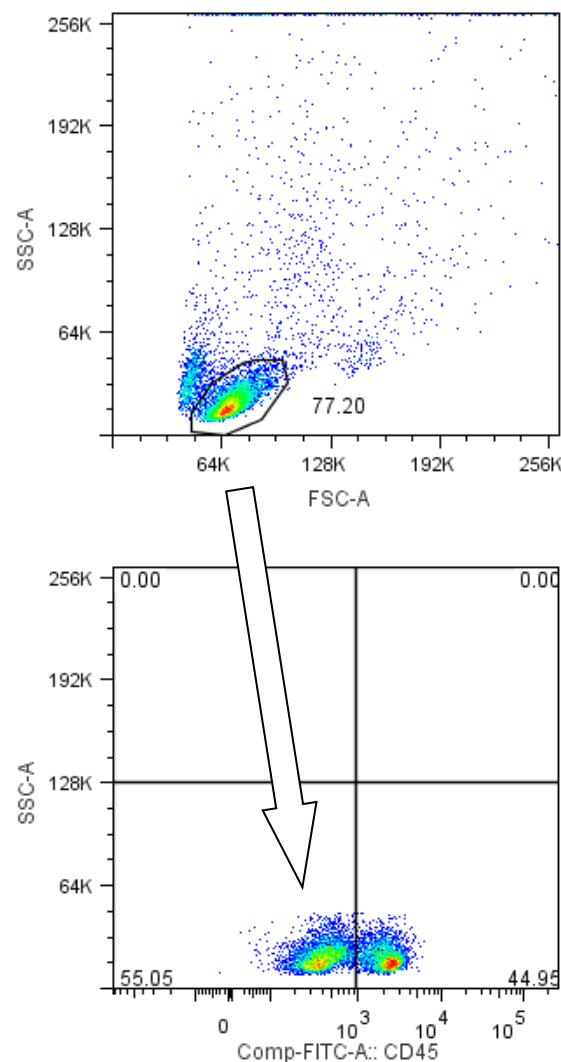


Figure 4-9 Control demonstrating two distinct responder populations based on staining antibody concentration

Following this, compounds of interest were added to the culture media and the effect on killing response assessed. KA172, a highly potent pan-HDACi was utilised in several of these experiments in order to compare effect with SAHA (and KA1010). As can be seen, from the flow cytometry plots (Figure 4-10), the addition of compounds did not result in any significant alteration in allogeneic versus autologous survival. However, these results proved difficult to interpret as the unstained stimulator population was difficult to distinguish from the allogeneic T1 population (with a weaker concentration of staining antibody).

In the untreated control assay, there was an approximately 4:1 ratio of effectors + allogeneic targets to autologous targets. From this data, there appeared to be minimal killing of autologous targets, but as mentioned assessment of the allogeneic (T1) population proved difficult. The addition of CyA or HDACi did not affect this ratio and, therefore, had no apparent immunomodulatory effect. This led to the conclusion that this assay may not be sufficiently sensitive due to the possibility that responders may be proliferating as quickly as they are being killed. In addition to this, it may also be plausible that the staining strategy adopted resulted in unstained stimulators merging with responders cells stained with low dose (1:2000) conjugated antibody, making discrimination between the two groups at analysis indiscernible.

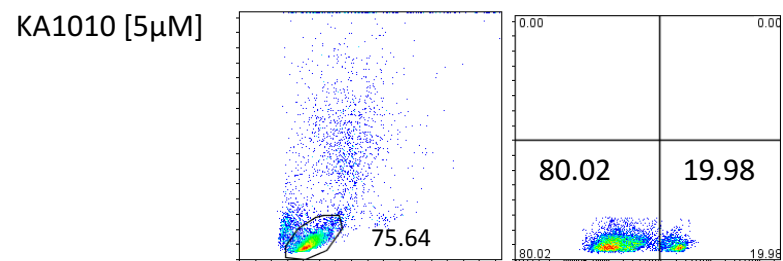
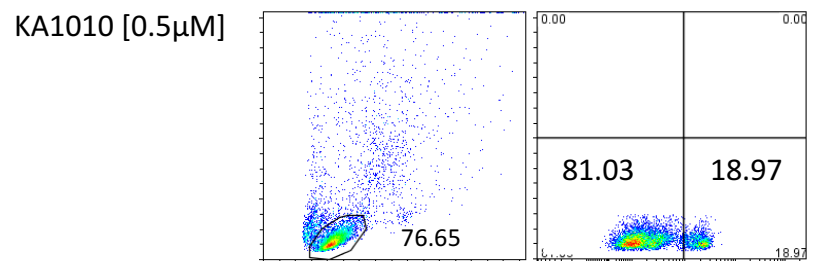
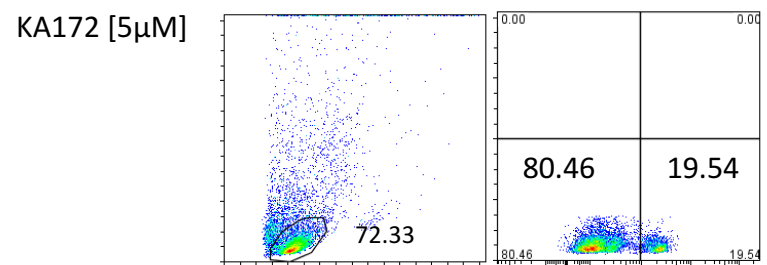
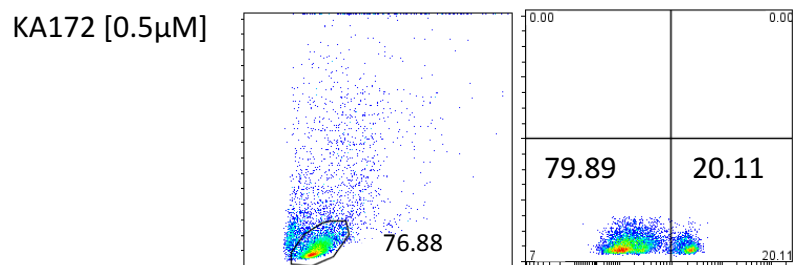
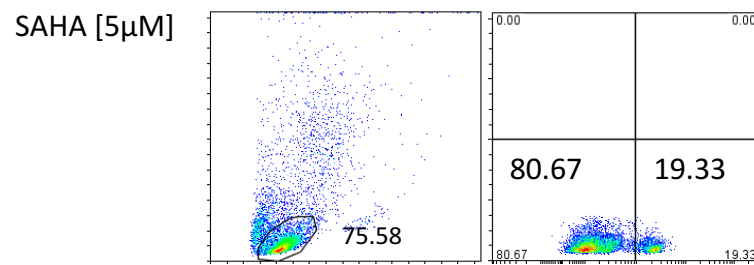
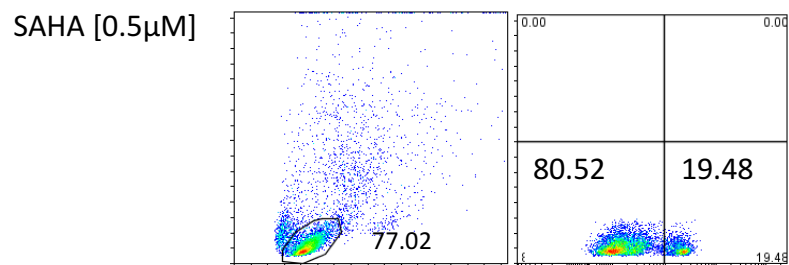
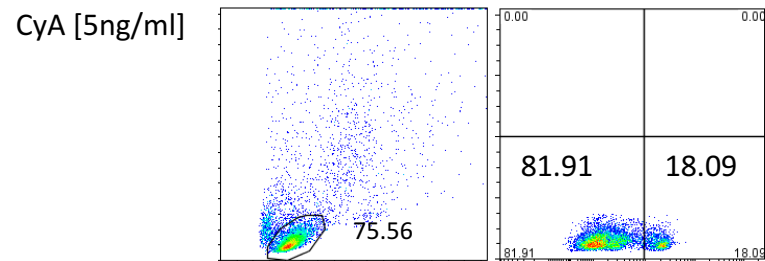
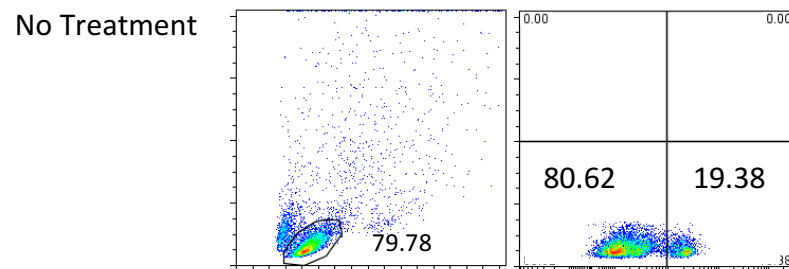


Figure 4-10 Effect compound on autologous and allogeneic responder cell population in a one-way MLR killing assay

Therefore, this assay was revised and an alternative fluorochrome used in order to better differentiate populations of interest. As previously, an allogeneic target population was isolated and stained with a conjugated anti-CD45 antibody. Again, the antibody was conjugated with the FITC fluorochrome but now a concentration of 1/100 was used. As previously, the second target population was stained with anti-CD45 antibody, but on this occasion conjugated to an alternative fluorochrome (APC-H7) with an entirely different excitation wavelength compared to FITC (and a concentration of 1/100 used). The allogeneic stimulator population was irradiated at 20Gy. The assay was cultured for 24 hours and a second time point at 18 hours incorporated.

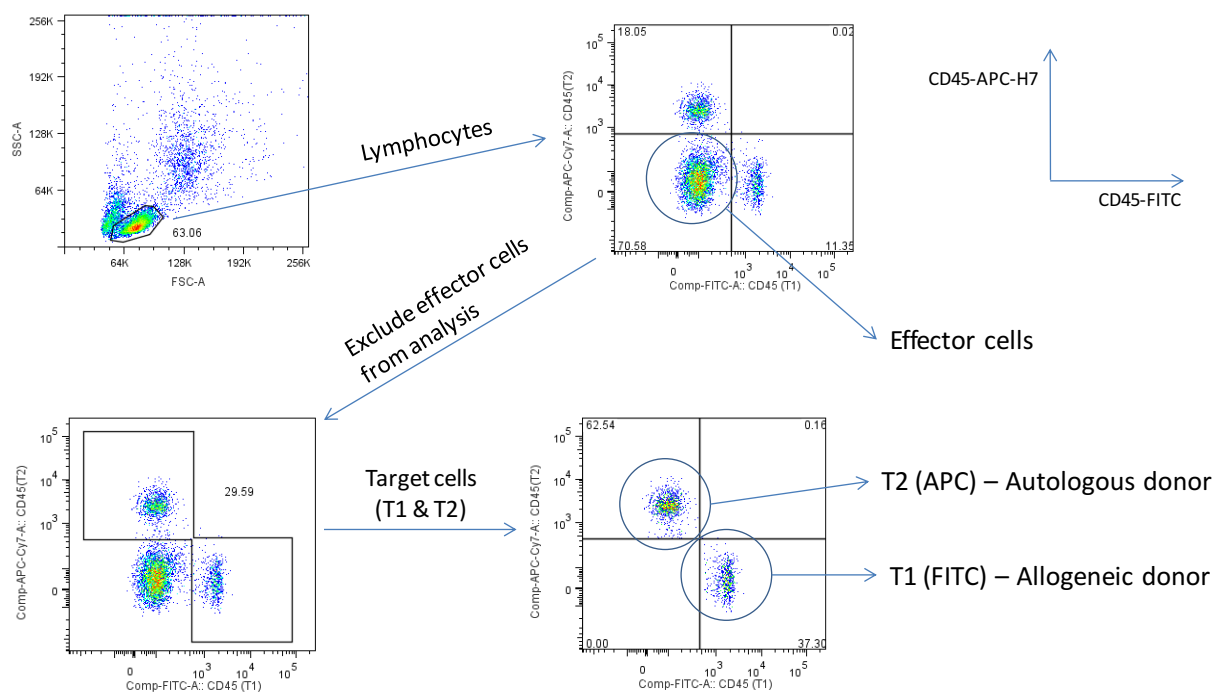


Figure 4-11 Gating strategy for modified killing assay using untreated culture (assessed at 18 hours)

Flow cytometry analysis of the untreated control sample demonstrated an improved delineation between target cell populations, making analysis more reliable (Figure 4-11).

Analysis of the control groups at 18 and 24 hours demonstrates that this delineation is maintained.

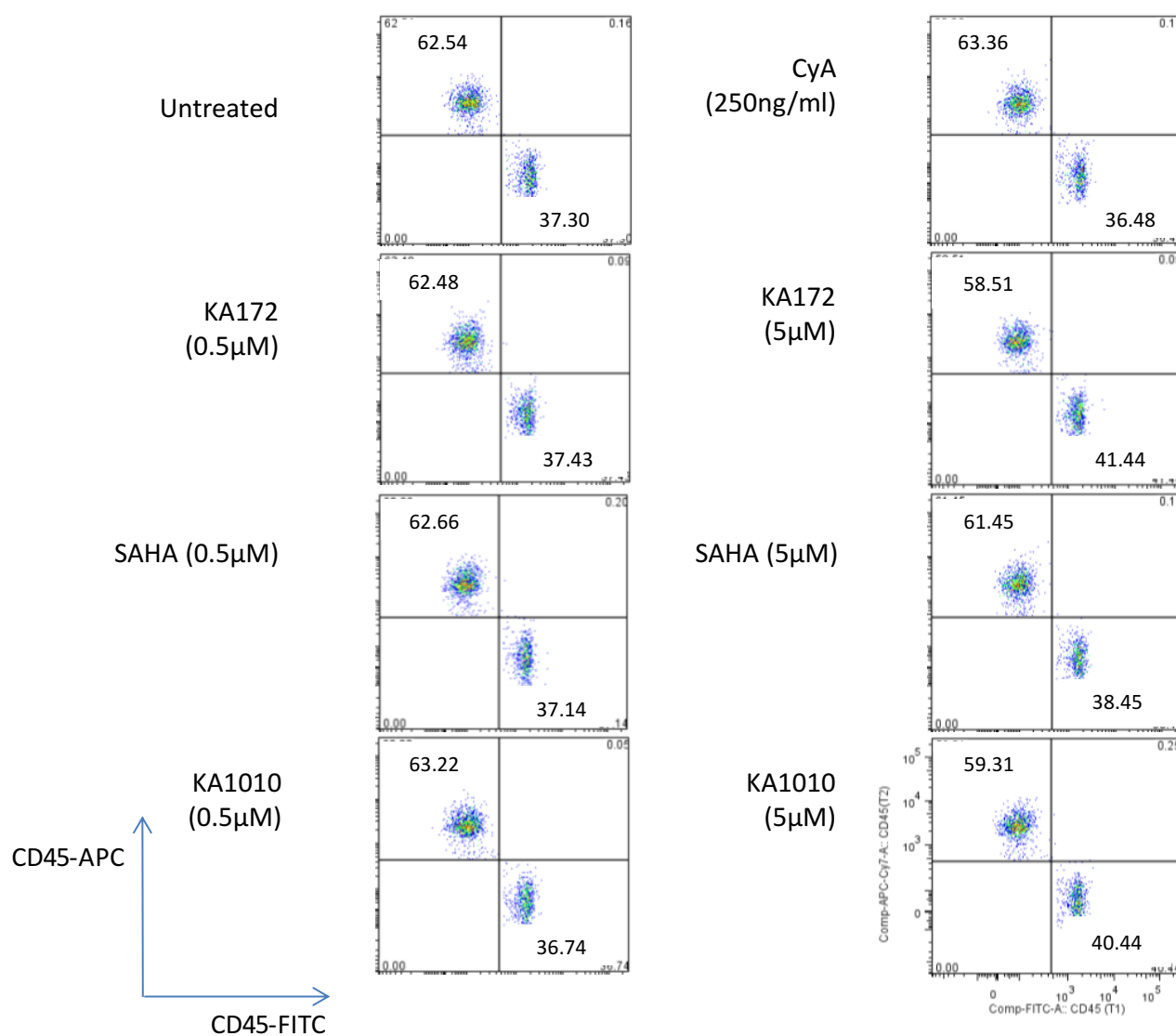


Figure 4-12 Effect of compound on one-way killing assay at 18 hours

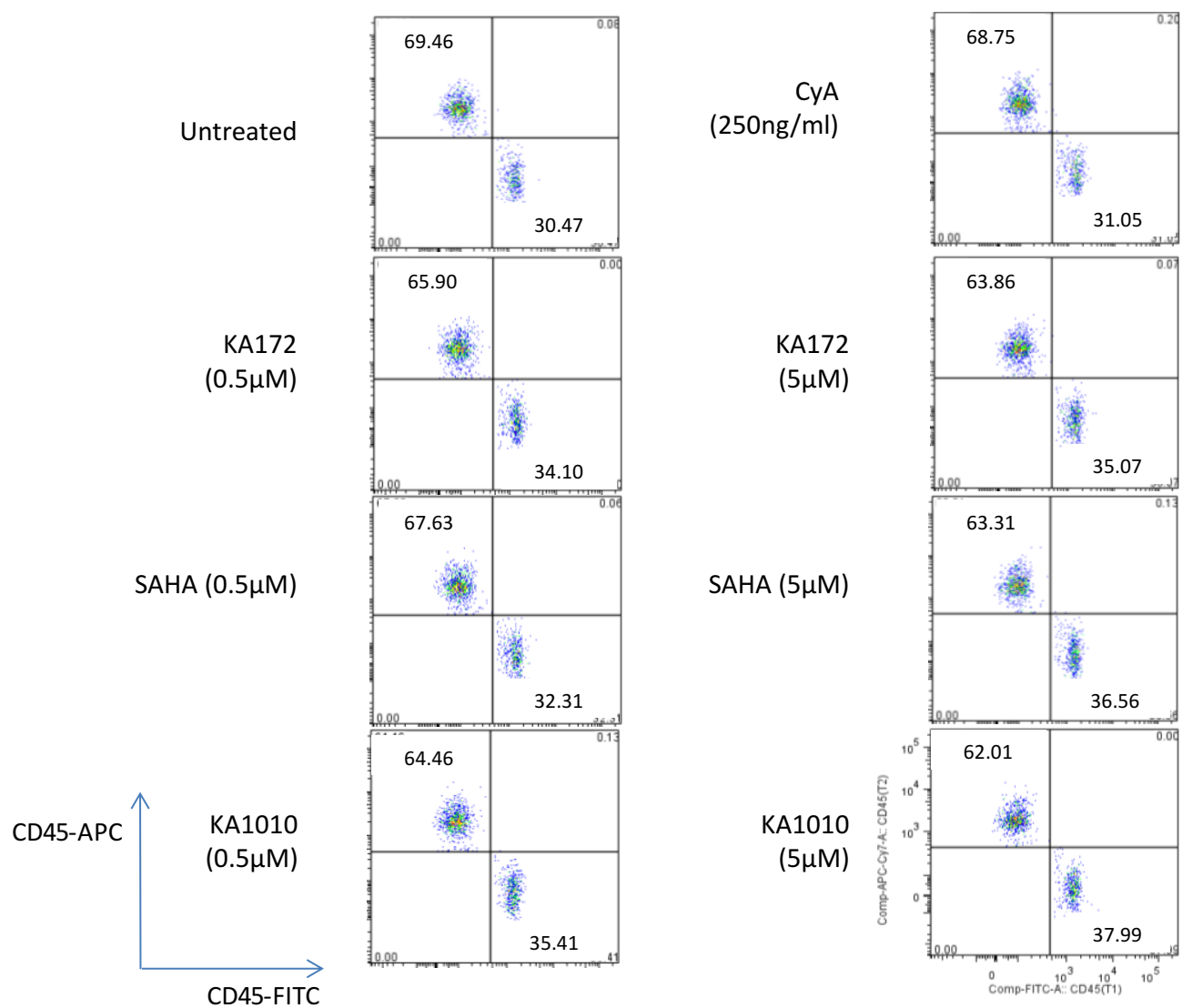


Figure 4-13 Effect of compound on one-way killing assay at 24 hours

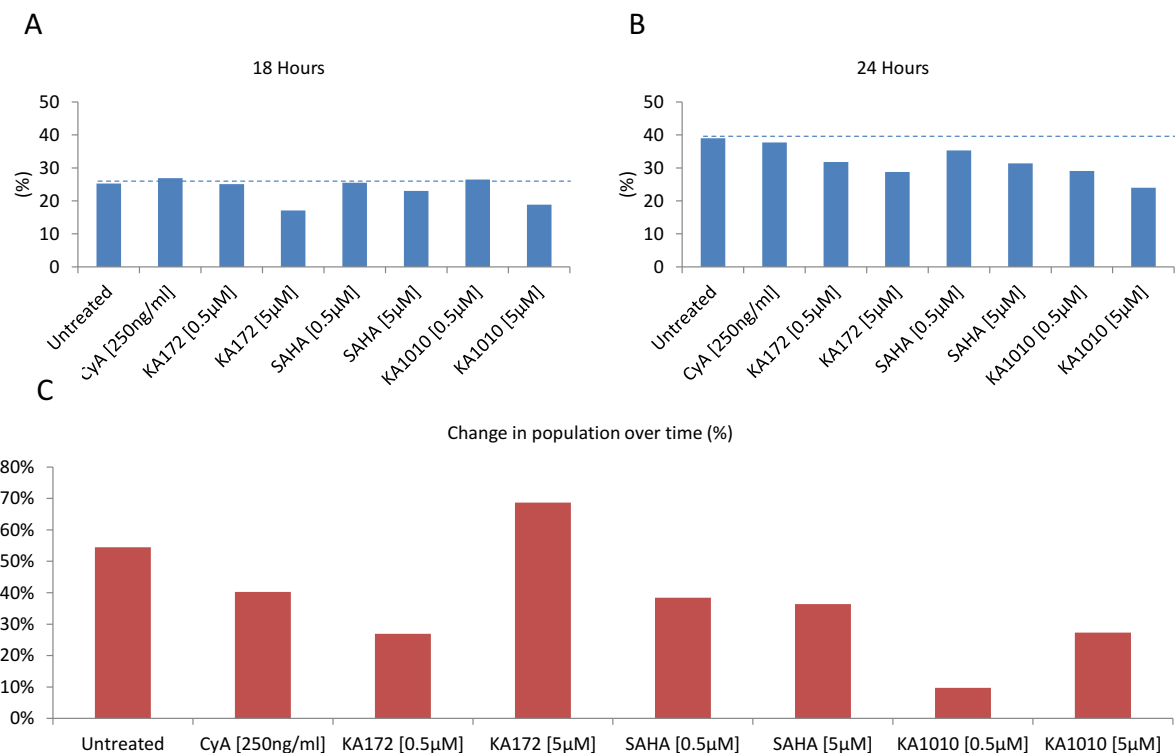


Figure 4-14 Comparative effects of compound on target cell population in one-way MLR.

Difference in relative number of cells at 18 hours (A) and at 24 hours (B). Change over time in target cell population differential (C)

As expected throughout all treatment conditions the autologous T2 population predominated over the allogeneic T1 population in terms of numbers. The relative number of allogeneic cells also reduced over time (24 hours compared to 18 hours). This is represented in Figure 4-14 by calculating the difference between T2 and T1 at each time point. This difference represents relative killing of the allogeneic population over the autologous. Therefore, a calculated difference which is less than the untreated control can be interpreted as relative preservation of the allogeneic population and may represent a degree of immunosuppression by the compound of interest.

At 18 hours, only KA172 [5µM] and KA1010 [5µM] resulted in any apparent preservation of the allogeneic population (Figure 4-14 A). At 24 hours, however, though these same compounds represented the most effective immunomodulatory agents. All the HDACis assessed resulted in a degree of allogeneic population preservation.

The dynamic effect of compounds was also assessed by calculating the percentage change in target cell population between the two timepoints (Figure 4-14 C). This demonstrated that the effect of most compounds assessed (all, with the exception of KA172 [5µM]) was to slow down the rate of differential population killing and this may also represent an immunomodulatory effect.

4.4.3 Effect of compounds on one-way killing assay

Given this positive finding, the above assay was repeated under similar conditions with an extended time frame by assessing target cell population killing at days 1, 2 and 3. In order to improve accuracy of the assay, counting beads were used in the flow cytometry analysis. This involved calibrating a known number of counting beads in the form of red carboxylated spheres. Once a concentration for these beads was established, it was possible to extrapolate numbers of cells / well, using the formula;

$$A/B \times C/D = \text{concentration of sample as cells}/\mu\text{L}$$

Where A = number of cell events, B = number of bead events, C = assigned bead count of the lot (beads/50 µL) and D = volume of sample (µL).

Flow cytometry analysis of control samples demonstrated a definable staining of target populations (Figure 4-15) and no staining of effector population.

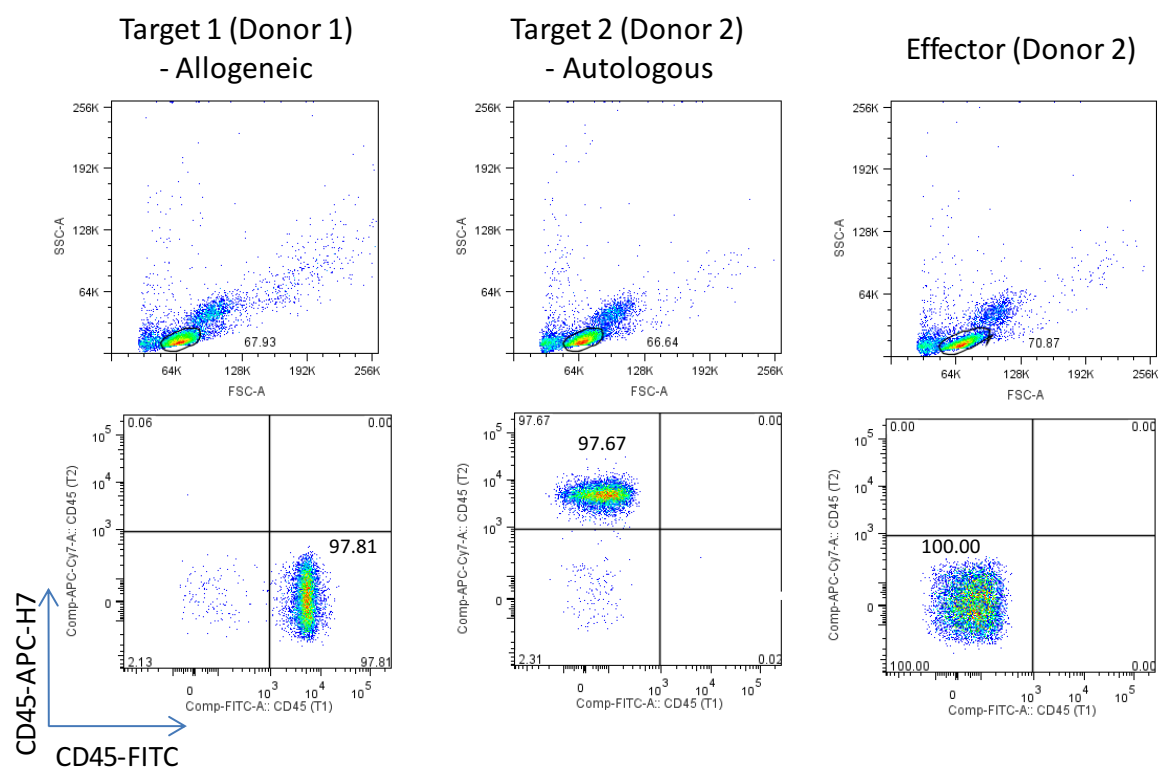


Figure 4-15 Control samples for extended one-way MLR killing assay at day 0

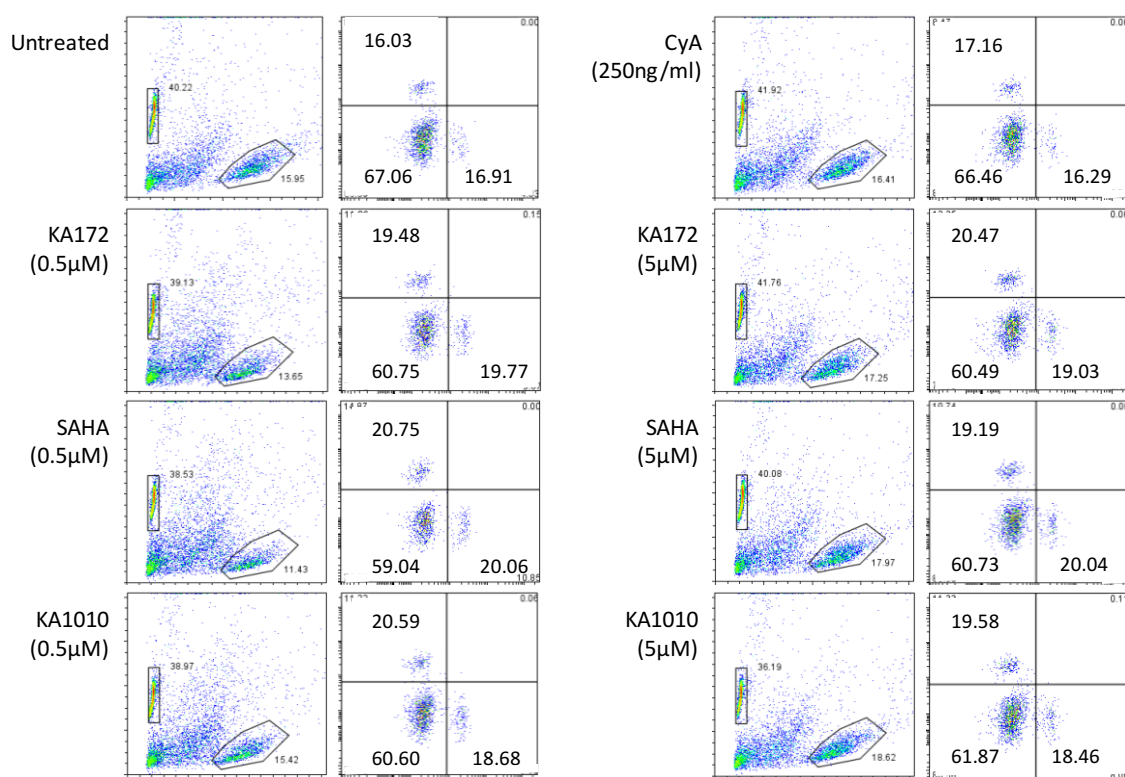


Figure 4-16 Effect of compound on killing assay at 24 hours

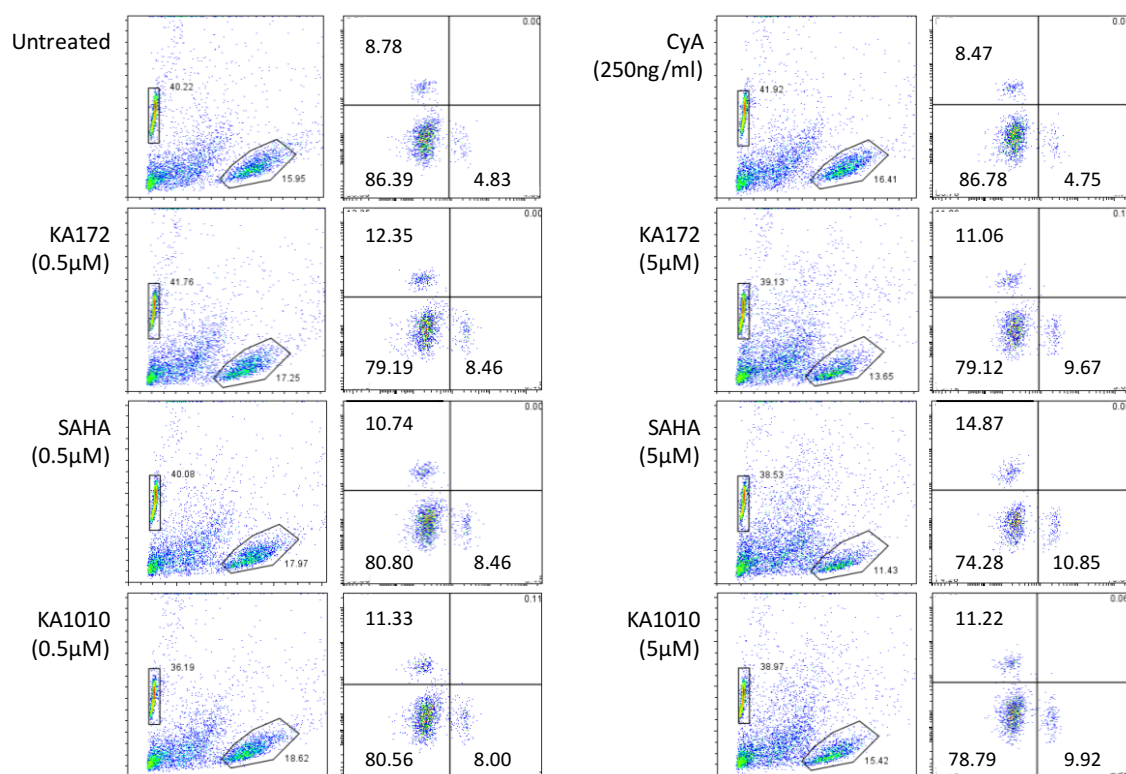


Figure 4-17 Effect of compound on killing assay at 48 hours

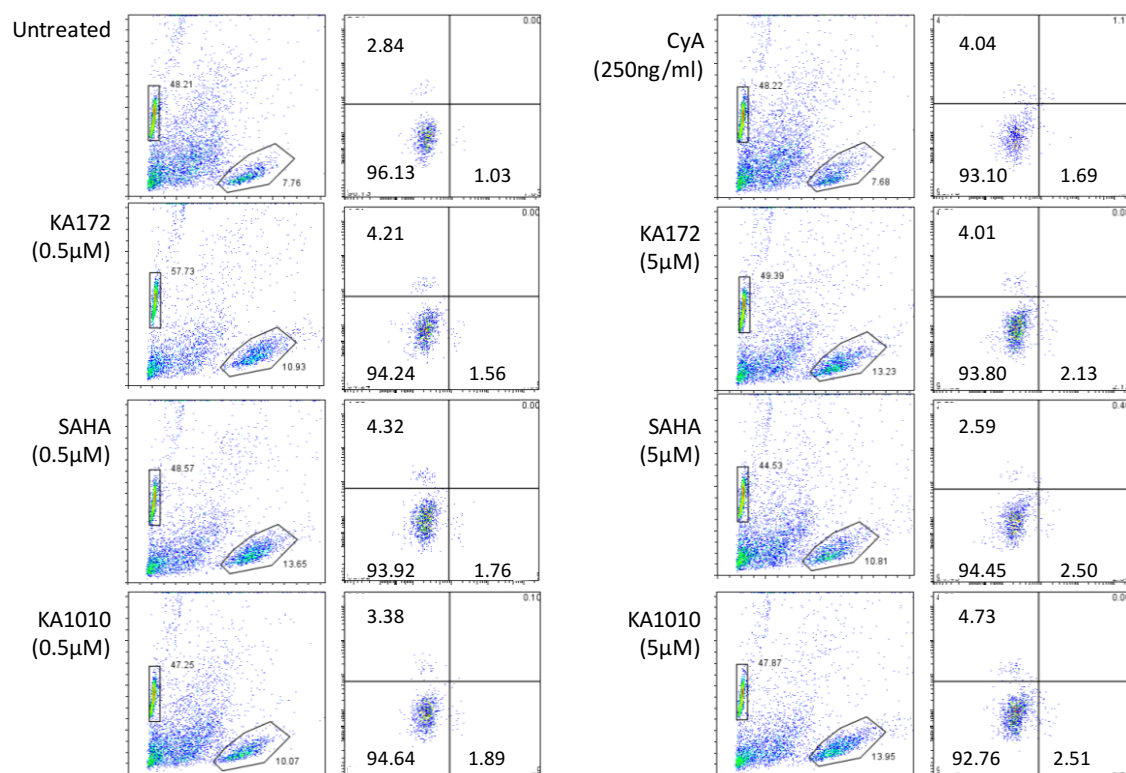


Figure 4-18 Effect of compound on killing assay at 72 hours

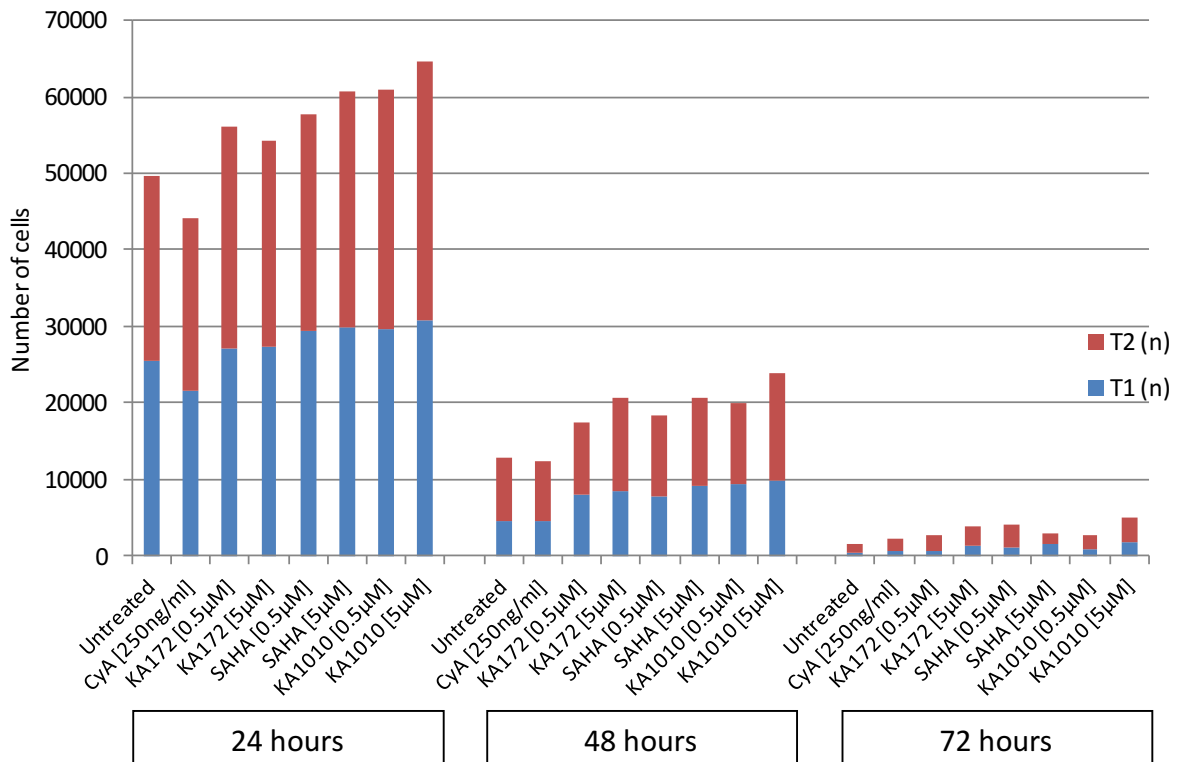


Figure 4-19 Effect of treatment on absolute cell number

Over the course of the treatment period, there was a steady decline in cell numbers (Figure 4-19). After 24 hours the untreated allogeneic population reduced by 29.5% and by 98.9% at 72 hours representing only 400 detectable cells. The addition of CyA at a dose of 250ng/ml had no demonstrable effect over control on preserving target cells in the first 48 hours and only a minimal effect at 72 hours, resulting in a modest 62% increase in cell number over target (equivalent to 646 cells).

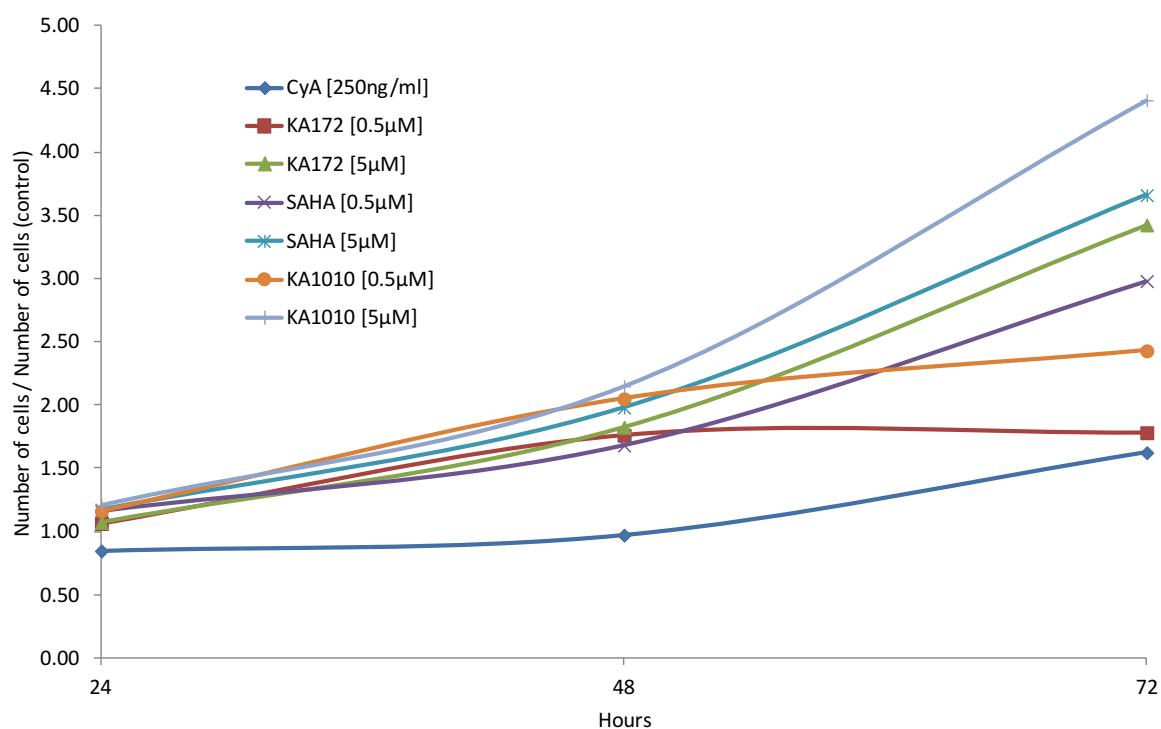


Figure 4-20 Relative effect of compound on allogeneic target population in extended killing assay

The addition however of HDACi did have a measurable effect on cell numbers. At 24 hours this effect was modest (Figure 4-20) and in keeping with previous data. By 48 hours there were a considerably greater proportion of allogeneic cells surviving (in the region of 1.7 – 2.25 times greater than control). In the case of KA1010 [0.5µM] and KA172 [0.5µM], the protective effect plateaued. For the other compounds assessed (KA172 [5 µM], SAHA [0.5µM], [5µM] and KA1010 [5µM]) there was further proportional preservation of the allogeneic population, with the higher doses proving most effective. By 72 hours, SAHA [5µM] treatment resulted in a 3.66-fold increase and KA1010 [5µM] a 4.41-fold increase.

Though these results demonstrated a degree of allogeneic preservation in the presence of HDAC inhibition, there still remained a number of persistent targets in the untreated control assay. This was notable as it would be expected that this untreated allogeneic population

would be completely absent after 48-72 hours of MLR. Therefore, this assay was adapted by priming the effector cells first with both autologous and allogeneic cells prior to assessing the effect of MLR with and without compounds of interest (Figure 4-21). This was achieved by culturing the effector population with irradiated targets at days 0, 7 and 14, prior to commencing the MLR proper at day 21. Target populations were then assessed at 24 hours and 72 hours (days 22 and 24 of the assay respectively).

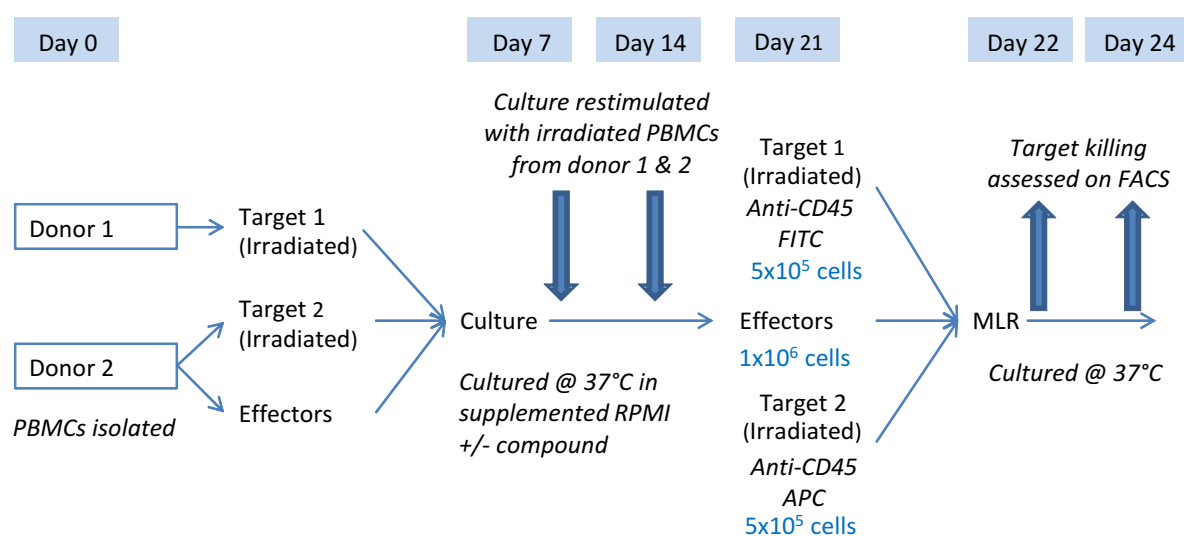


Figure 4-21 Schematic representation of modified primed MLR

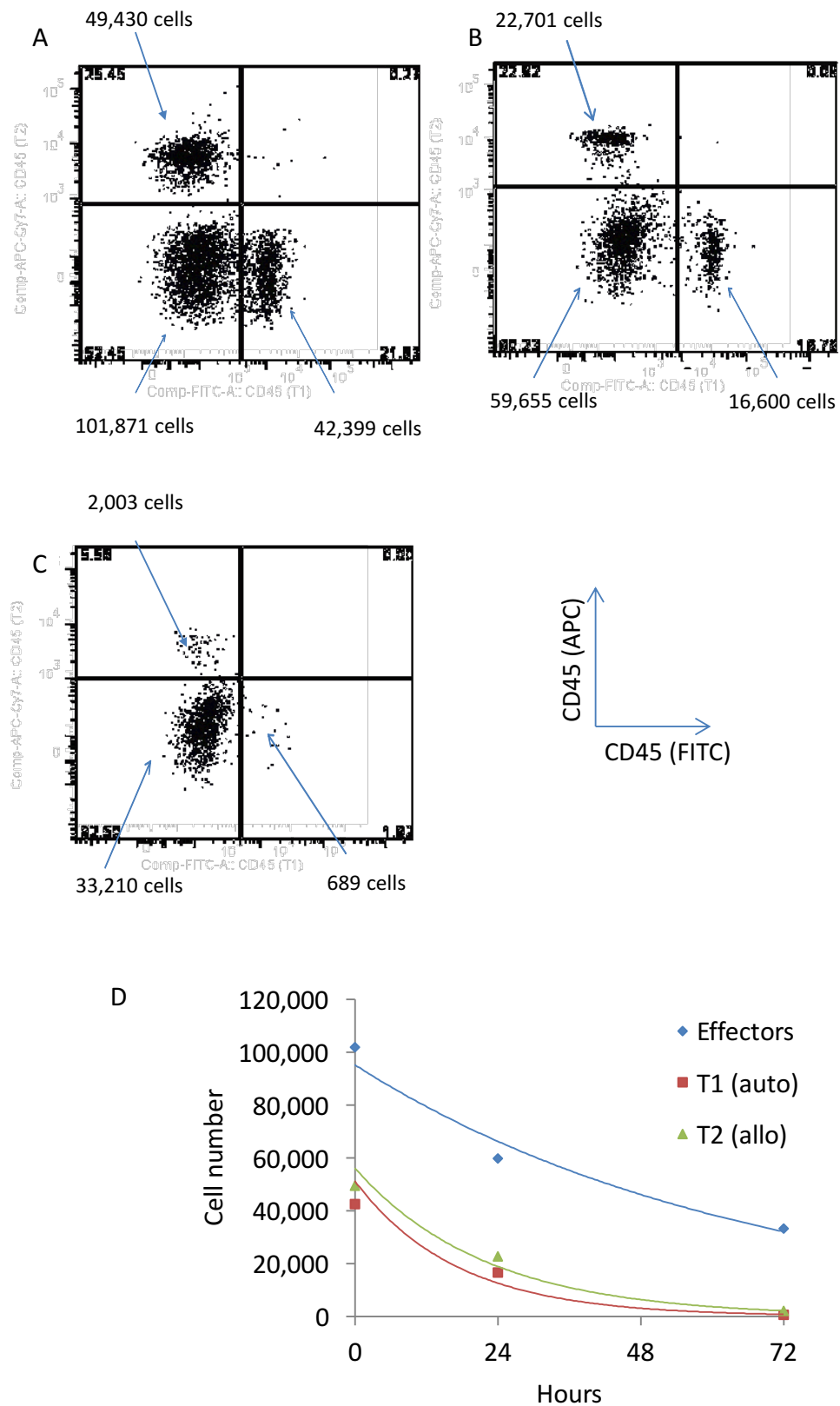
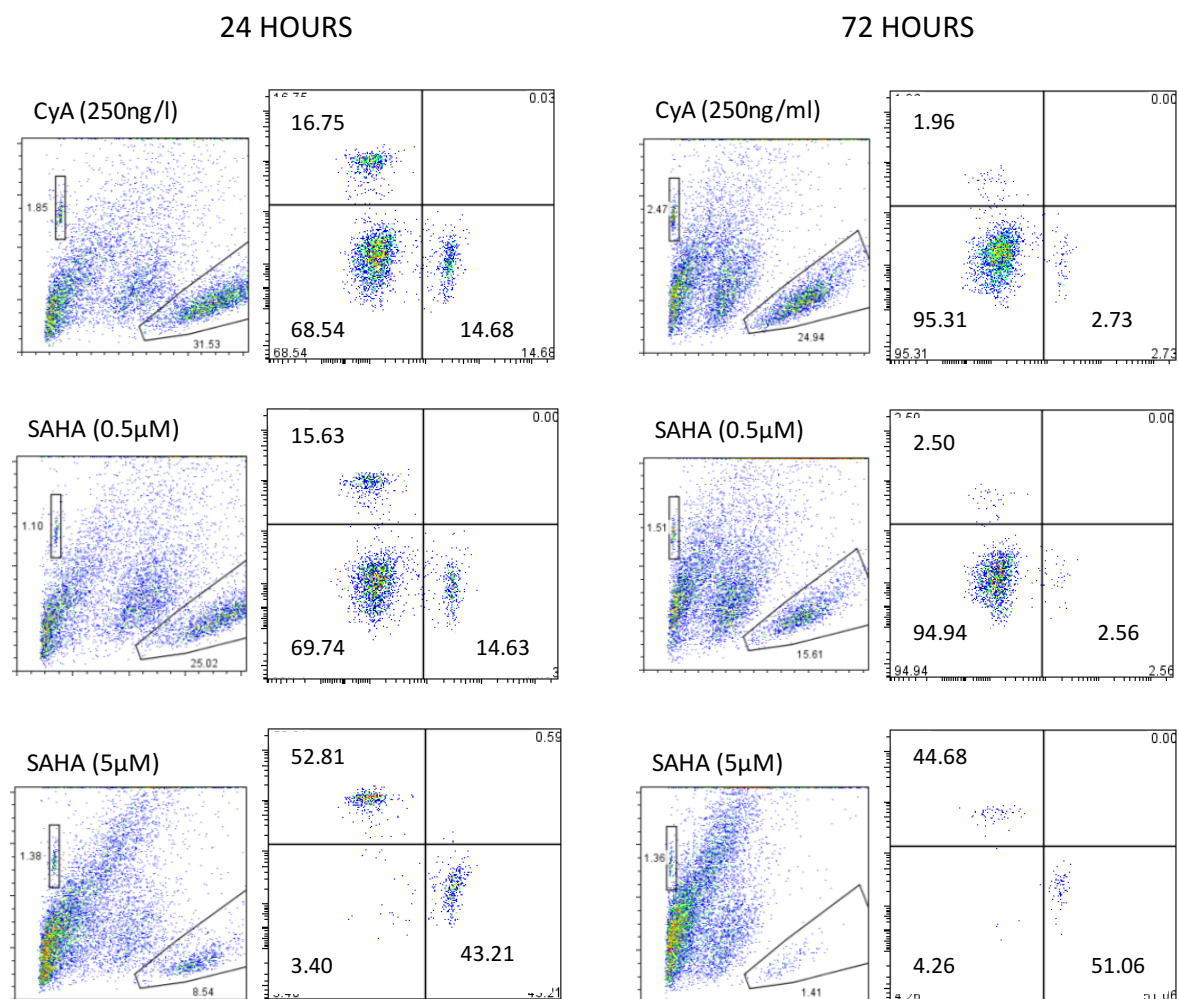


Figure 4-22 MLR control samples at A) 0 hours, B) 24 hours and C) 72 hours. D) Rate of cell attrition over experimental period

Once again the staining strategy identified distinct populations and calculated cell numbers were consistent with estimated planned numbers as per experimental protocol (Figure 4-22 A-C). As with previous assays, the allogeneic target population though at lower numbers compared to autologous targets, and declined at similar rates (Figure 4-22 D). Comparing the absolute reduction in cell numbers also demonstrates a similar effect on both allogeneic (98.4%) and autologous (95.9%) populations, with a 67.4% reduction in effector numbers over the assessment period.



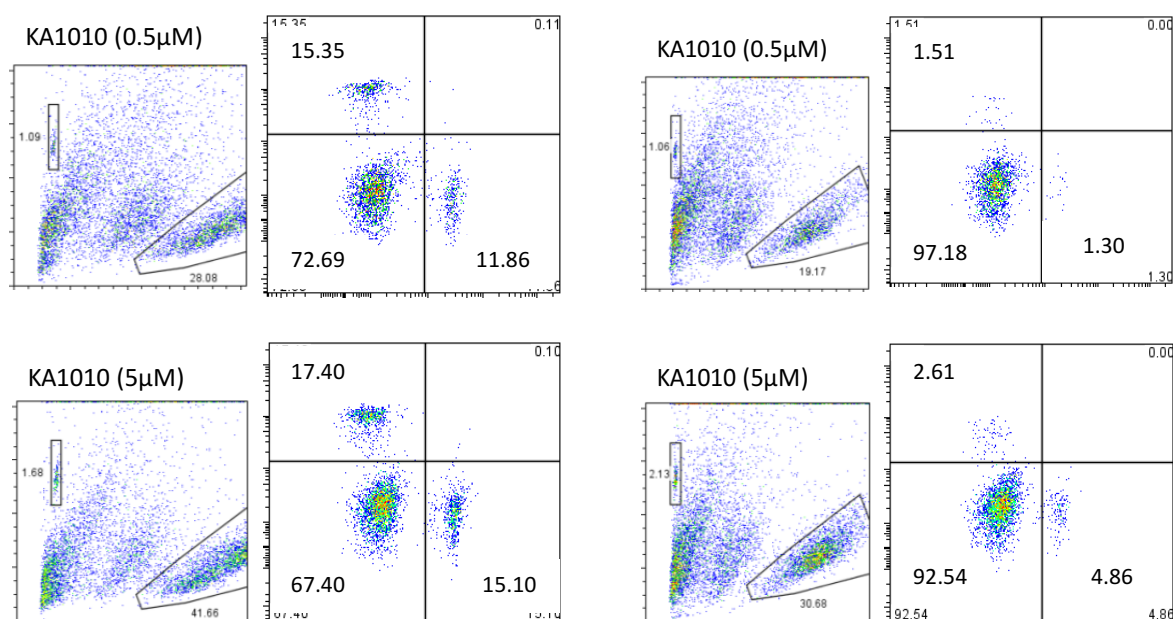


Figure 4-23 Flow cytometry raw data for treated primed killing assay

The effect of CyA and HDACi on primed targets followed the general trend seen in the extended killing assay previously. Review of the raw data (Figure 4-23) suggests SAHA at the higher dose of 5μM has a significantly toxic effect on cell survival with a negligible number of effector cells surviving, however, experimental error cannot be ruled out and it is noteworthy that target cell numbers were consistent with prior data. With regards to the other compounds tested, only KA1010 [5μM] appeared to have any beneficial advantage in terms of preserving the T1 allogeneic population over the autologous population. However when absolute cell numbers are assessed rather than comparative proportions, greater effects become evident. As with previous data KA1010 [5μM] had the greatest effect at 72 hours with a 7-fold greater number of allogeneic cells surviving compared to control. SAHA [5μM] also had a notable effect, resulting in a 5.5-fold increase. The other compounds tested (CyA [250ng/ml], SAHA [0.5μM] and KA1010 [0.5μM]) had a far more modest effect with an approximately 2 to 3-fold increase in allogeneic survival over control.

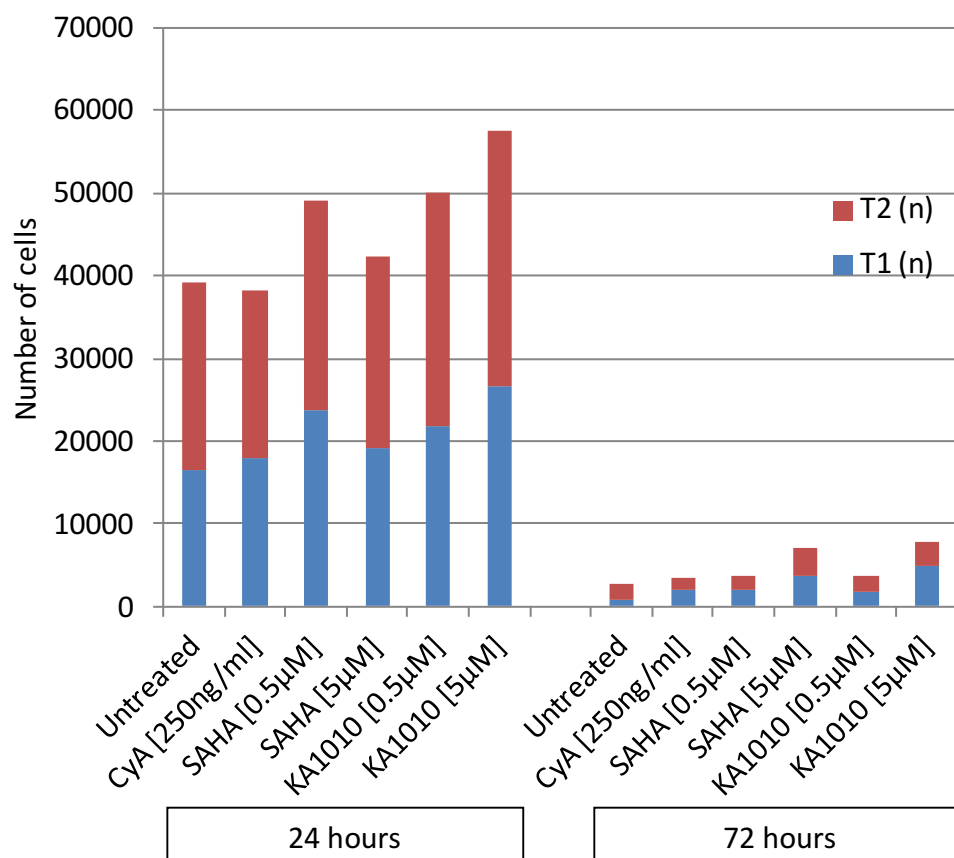


Figure 4-24 Comparative data demonstrating effect of compound on cell number in primed killing assay

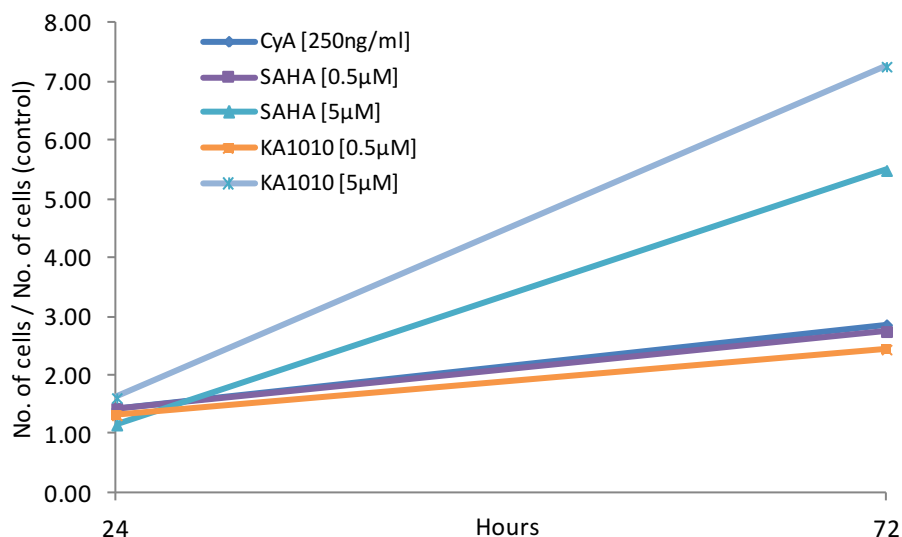


Figure 4-25 Relative effect of compound on allogeneic target population in primed killing assay

Considered in its entirety, this MLR killing assay data supports the notion that HDAC inhibition has a positive effect in the context of suppression of allogeneic responses *in vitro*. Both pan-HDAC inhibition (through SAHA and KA172) and HDAC-6 specific inhibition (through KA1010) proved more effective than the well established immunosuppressant Cyclosporin (Figure 4-24, Figure 4-25).

However, it should be noted there was a deficiency in these assays with regard to allogeneic survival. It was expected, particularly in the extended assay and the primed assay that there would be almost, if not complete killing of the allogeneic T1 population with significant preservation of the autologous T2 population. The reasons for this not occurring are unclear.

4.5 MLR - ELISA

In order to quantify an allogeneic response in the MLR setting and to, therefore, better assess effect of an immunomodulatory compound, an assay based on IFN- γ production was developed. This involved measuring supernatant IFN- γ utilising an established ELISA kit. The basis for this assay relied upon proportional IFN- γ production as a marker of alloresponse secondary to proliferating CD4⁺ T helper cells. Interestingly in the context of this study, evidence suggests that IFN- γ production induces T_{reg} promotion (Eljaafari, Li, & Miossec 2009) (this will be commented upon further in the Discussion).

4.5.1 Assay development

To standardise, extrapolate and quantify IFN- γ production from the ELISA data, it was first necessary to establish a standard curve. This was achieved using serial dilution of a known concentration of IFN- γ . A typical standard curve (Figure 4-26) was then plotted and the quadratic equation of the trend line used to interpret measured ELISA data of the MLRs.

A	IFN-g (pg/ml)	Reading 1	Reading 2	Abs
	2000	1.868729	2.04204	1.955385
	1000	1.125734	1.197155	1.161445
	500	0.766742	0.792622	0.779682
	250	0.6066	0.52418	0.56539
	125	0.438903	0.37193	0.405416
	62.5	0.32438	0.339284	0.331832
	31.25	0.303043	0.274441	0.288742
	0	0.27787	0.237559	0.257714

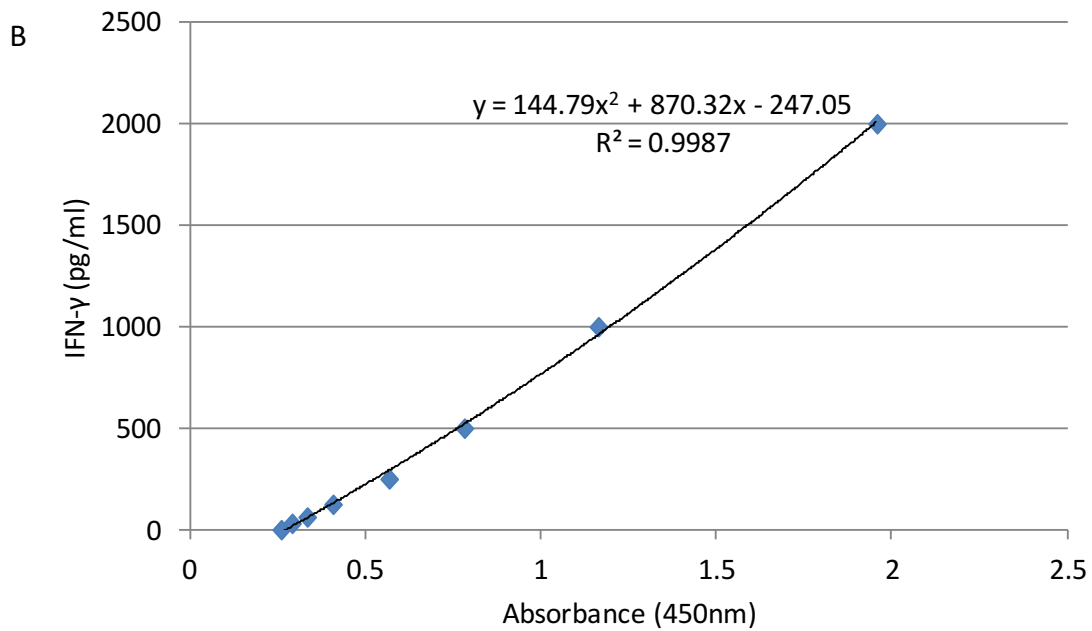


Figure 4-26 Typical standard curve for ELISA. (A) Raw data used to plot a standard curve (B). An R2 value close to 1 is desirable

The first stage involved in developing this assay was to assess the differential response in terms of IFN- γ production between a one-way and a two-way MLR. This was performed using frozen PBMCs from allogeneic donors, with autologous culture acting as control. To achieve a one-way MLR, one of the populations was irradiated with either 10 or 20 Gray, in order to maintain cell viability, but prevent division. A lower than standard dose of radiation was used to determine as to whether or not there was an effect of irradiation on IFN γ production. Cells were cultured for three days at 37°C. The expected outcomes were as follows:

10 Gray	20 Gray	Expected outcome
A + A	A + A	<i>Negative control</i> - No MLR (background IFN- γ production only)
B + B	B + B	
A + B	A + B	2-way MLR (IFN- γ production ++)
A ^I + B ^I	A ^I + B ^I	<i>Negative control</i> - Both sides irradiated (background IFN- γ production only)
A + B ^I	A + B ^I	
		1-way MLR (IFN- γ production +)
A ^I + B	A ^I + B	

Table 4-1 Expected outcomes for IFN- γ production in one-way and two-way MLR (A = donor A, B = donor B)

Unfortunately, in reality these conditions resulted in negligible IFN- γ production. Therefore, the assay was repeated using freshly isolated PBMCs from allogeneic donors and a positive control incorporated using mitogen stimulation (in the form of LPS or Con A) of the autologous culture. PBMCs were isolated from three allogeneic donors in order to increase diversity and reduce the risk of HLA matching. An alternative media was used (AIM-V + 10% FCS) and all cultures were incubated at 37°C for 24 hours. Cells irradiated for one-way MLR were treated with 20 Gray.

Background autologous IFN- γ production ranged in the order of 4-80 pg/ml (Figure 4-27).

There was no significant allogeneic response with IFN- γ levels of 0-73 pg/ml. However, the

mitogen-stimulated positive control did result in a heterogeneous degree of production with values ranging from 30-900 pg/ml. Though the allogeneic MLR disappointingly did not result in an anticipated rise in IFN- γ production, the results of the positive control added validity to the proposed assay. It was therefore decided to increase the time period to three days over which incubation was performed. In addition, a T cell-specific mitogen in the form of Phorbol Myristate Acetate (PMA) and Ionomycin was adopted in an effort to produce a more 'uniform' positive control.

No other conditions were changed from the previous experiment.

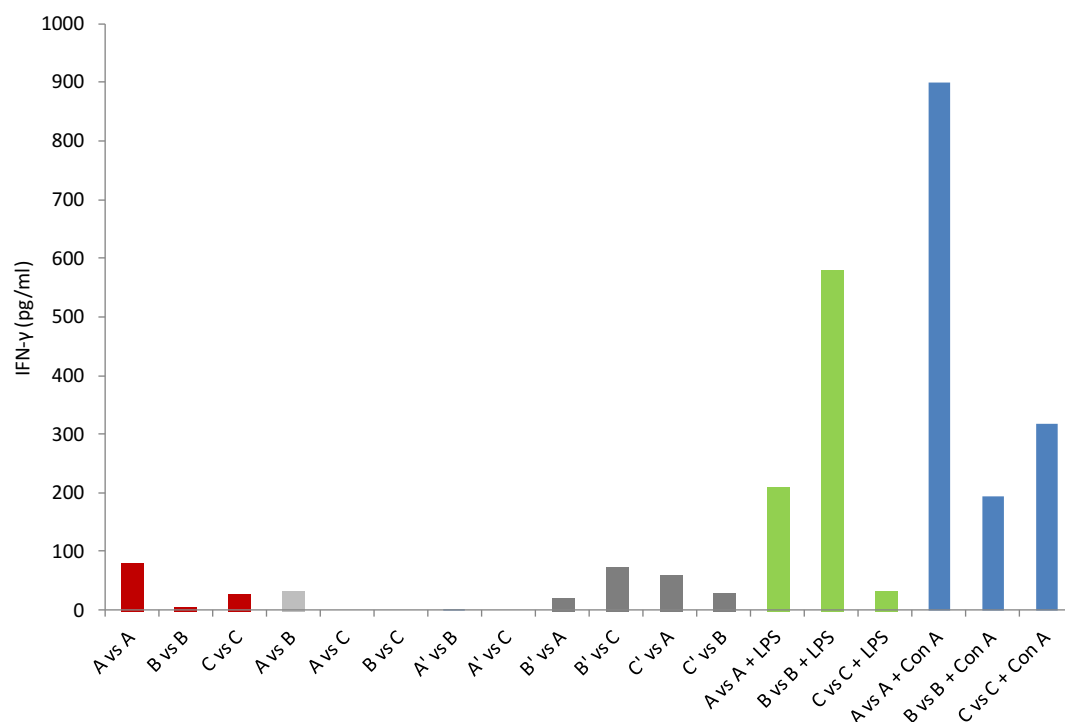


Figure 4-27 IFN- γ production following 24 hour allogeneic MLR (' represents irradiated population)

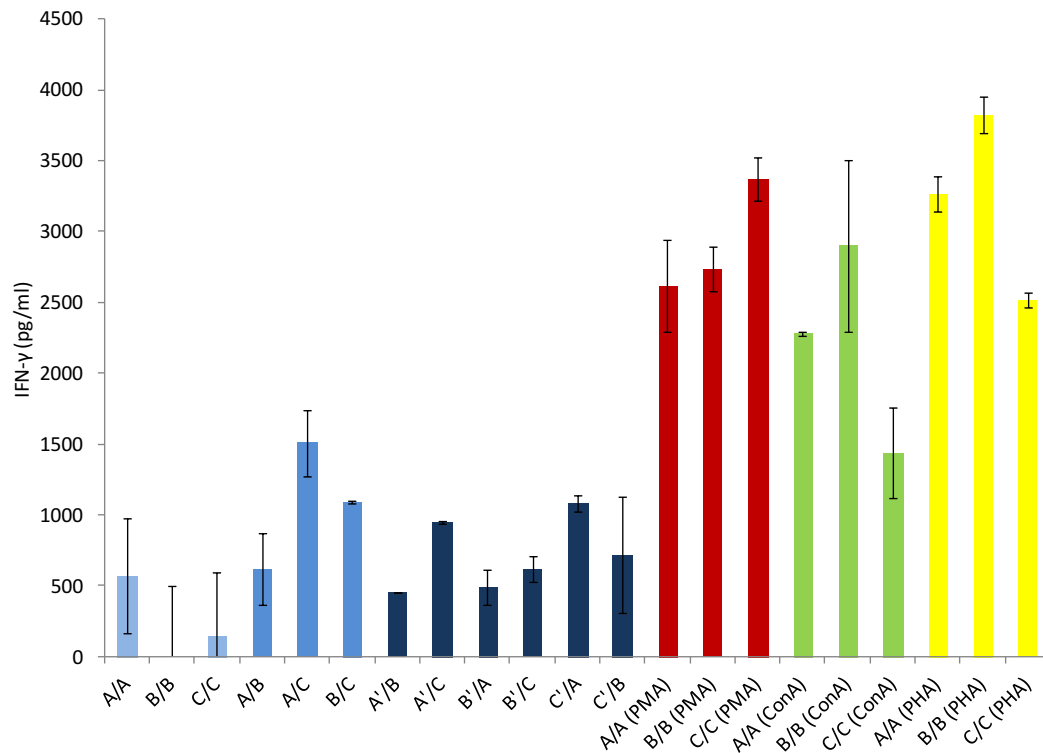


Figure 4-28 IFN- γ production following 72 hours allogeneic MLR (' represents irradiated population)

Under these modified conditions the background autologous IFN- γ production ranged from 0 – 570 pg/ml (mean 360 pg/ml) (Figure 4-28). This is a higher value than in previous experiments and suggests a longer incubation period provides more interpretable results. In general, the unstimulated allogeneic assays resulted in a trend towards increased IFN- γ production over autologous. These values ranged from 617 to 1509 pg/ml with a mean value of 1073 pg/ml (Figure 4-29) in the two-way MLR whereby neither population was irradiated. There was less IFN- γ production from the one-way group with a mean of 719 pg/ml (range 449-1084 pg/ml), which was still greater than autologous.

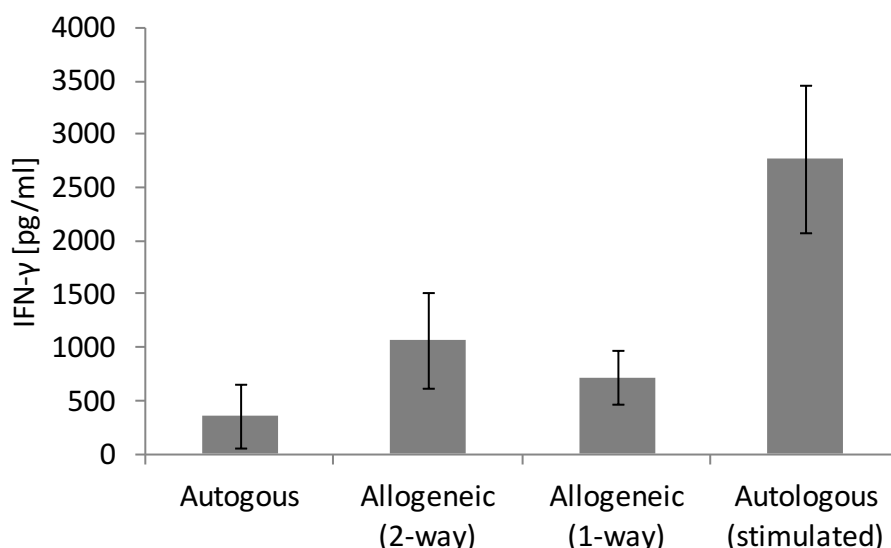


Figure 4-29 Mean IFN-γ production in 72-hour MLR

Mitogen stimulation resulted in a considerable increase in IFN-γ production with a mean value of 2772 pg/ml (range 1438-3822 pg/ml). Of the stimulants used, PMA + Ionomycin treatment resulted in the most consistent effect with a mean IFN-γ production of 2909 pg/ml (SEM \pm 409 pg/ml).

4.5.2 Effect of KA1010, SAHA and CyA on IFN-γ production

Overall this data provided sufficient evidence, that under these conditions, the assay provided the basis for an *in vitro* model of allotransplantation. Therefore, the next step was to assess the effect of immunosuppression on IFN-γ production. Autologous PBMCs were cultured as negative controls and PMA + Ionomycin was again used to stimulate a positive control. On this occasion, the autologous populations used in the control samples were either irradiated or non-irradiated (i.e., there was no one-way MLR control) in order to assess the effect irradiation has on IFN-γ production. For the compounds of interest assessed, culture media was supplemented with CyA at 250ng/ml or KA1010 at a range of

doses from 0.01 μ M to 3 μ M. Again, effects on a both one-way and two-way MLR were measured.

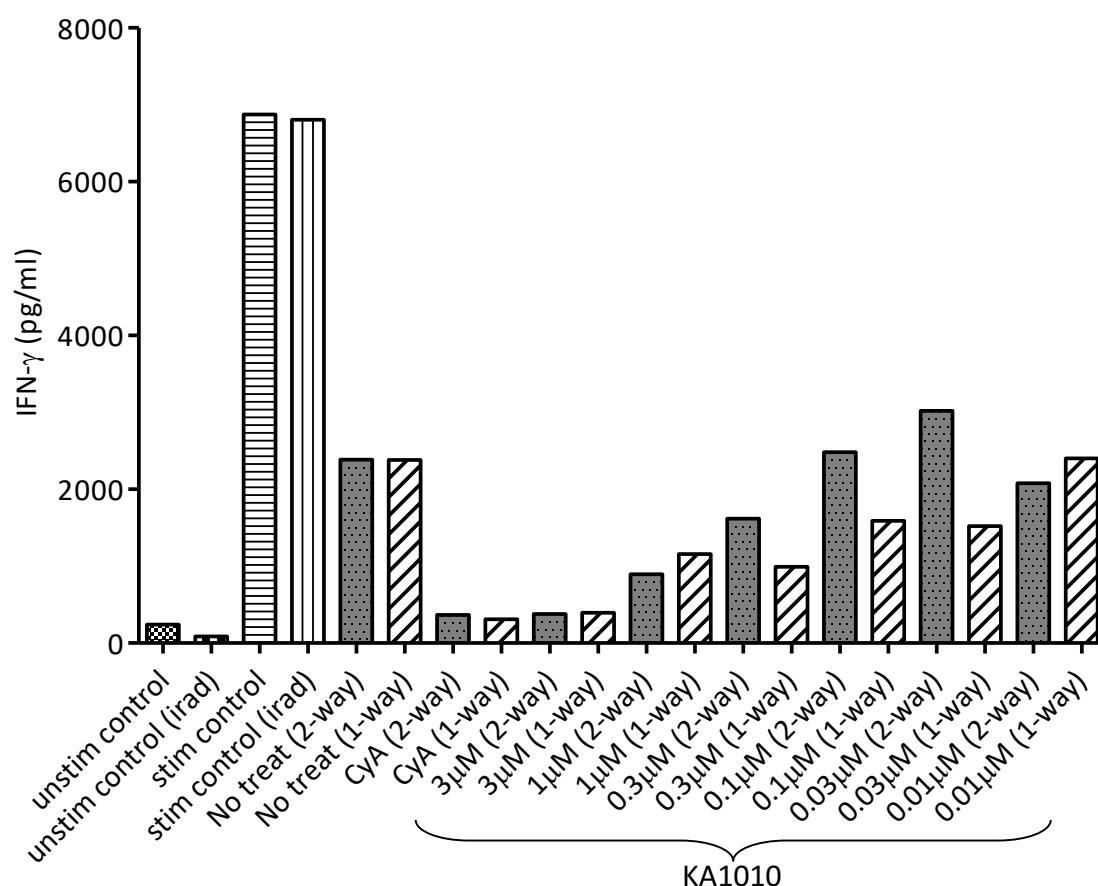


Figure 4-30 Effect of compound on IFN- γ production in 72 hour MLR

The stimulated positive control samples resulted in almost 7000 pg/ml of IFN- γ production and there was no significant effect of irradiation on this figure (Figure 4-30). The negative control (unstimulated single donor) population, also produced anticipated results with less than 300 pg/ml of IFN- γ production. There was a significant increase in IFN- γ production of the untreated MLR over negative control with levels of around 2400 pg/ml. Again, irradiation had no detectable effect.

With regards to HDAC-6 inhibition through KA1010, there was an apparent dose-dependent effect, particularly in the two-way MLR samples. At the low doses of 0.01 and 0.03 μ M, IFN- γ production was approximately 3000 pg/ml (both one and two-way MLR). At concentrations of 0.1 μ M and greater there was a dose dependent reduction in IFN- γ production, such that treatment with 3 μ M resulted in levels of just 12% (377 pg/ml) of that produced at 0.01 μ M.

Treatment with CyA, at a concentration of 250ng/ml resulted in IFN- γ levels (300-400 pg/ml), comparable to that seen with the higher dose of KA1010.

In this model, the effect of irradiating cells had no significant outcome on IFN- γ production in the control samples. As this model is designed to reflect allogeneic transplantation *in vitro*, and in reality, both donor and recipient graft present antigen in a two-way fashion it was decided that subsequent MLRs would not require an irradiated population. Therefore, further allogeneic assays from this point would comprise of a two-way MLR (unless otherwise stated).

In order to assess if this apparent dose-response was unique to KA1010, assays were repeated by treating a two-way MLR with CyA, SAHA and KA1010 at differing doses. All experimental conditions were similar to those described above.

The untreated control response produced a supernatant IFN- γ production of approximately 800pg/ml (Figure 4-31). The addition of compound of interest to the culture medium resulted in a notable (and statistically significant) reduction of IFN- γ production (one-way ANOVA $p < 0.0001$, $F = 16.44$). At a comparable dose of 0.3 μ M, CyA reduced IFN- γ release by 47%. Of the HDAC inhibitors, SAHA reduced the level by 50% and KA1010 by 67%. At a

higher dose of 1 μ M, all compounds resulted in further significant reduction in IFN- γ , again with the HDAC6-selective KA1010 having the greatest effect, reducing production by 91%.

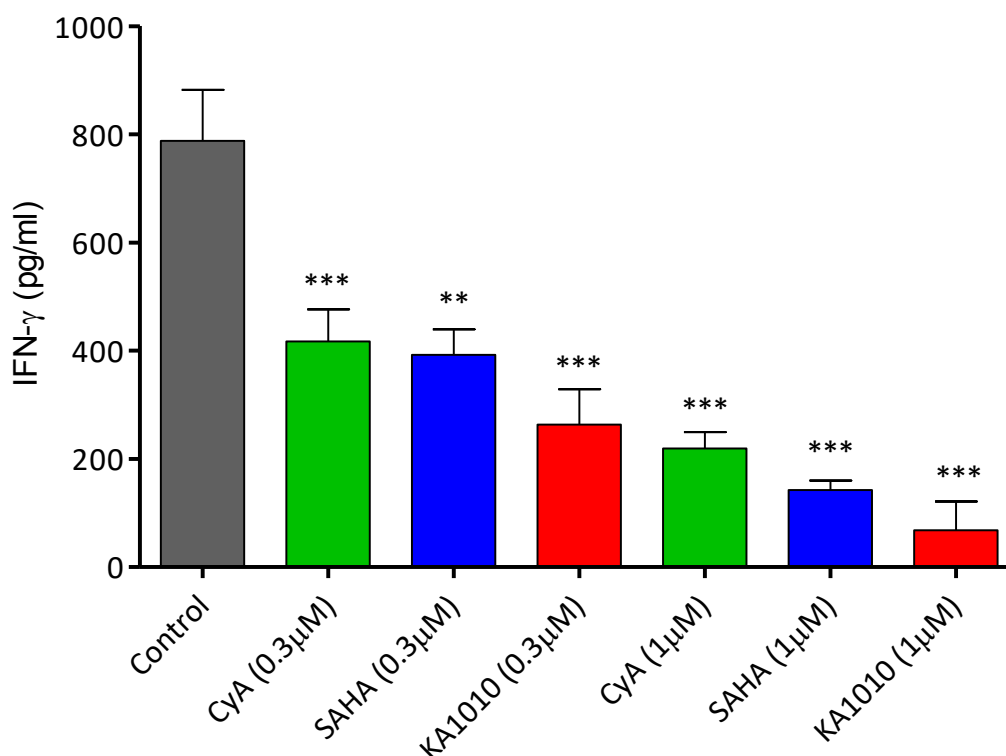


Figure 4-31 Culturing allogeneic PBMCs for five days in the presence of CyA or HDACi results in a statistically significant reduction in IFN- γ production compared to an untreated MLR

4.5.3 Dose response curves for KA1010, SAHA and CyA

In order to further demonstrate, the dose dependent effect of CyA and HDACi, a series of dose response MLRs were undertaken. This was performed using the compounds (and doses) in Table 4-2.

Compound	Dose (Range) - μ M
CyA	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10
SAHA	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10
KA1010	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10

Table 4-2 Compounds and doses used to produce dose response curves

As with previous MLR assays, fresh PBMCs were isolated from allogeneic donors and cultured for 72 hours. The negative control consisted of autologous cell culture only. An allogeneic assay stimulated using PMA & Ionomycin was used as the positive control.

Dose-response curves were produced by plotting IFN- γ production against the log of the concentration of compound (Figure 4-32). From these curves, it was then possible to calculate the half maximal inhibitory concentration (IC_{50}) for each compound. This quantitative value reflects the potency of the compound in question. It was also possible to calculate the Hill slope from this data. As these compounds have an inhibitory effect, this number will always be negative (Goutelle et al. 2008). The Hill slope, though not an absolute measure of efficiency does provide an indication as to compound function. Calculated from the gradient of the dose response curve, the further the value is from 1, suggests greater allosteric binding of the compound (Prinz 2010).

In this assay, KA172 proved excessively potent with considerable impairment of IFN- γ production at very low doses. It was therefore excluded from further analysis (data not shown).

In these experiments, both CyA and SAHA had relatively low Hill slope values (0.35 and 0.31 respectively) compared to KA1010 which had a value of 0.89. This likely reflects the specificity of HDAC6 inhibition, compared to the heterogeneity of binding sites and differing affinities (Shah & Haylett 2000) of CyA and SAHA.

With regards to the dose response curves specifically (Figure 4-32), all compounds produced sigmoid curves, demonstrating a degree of predictability in their dosing. At the higher doses of CyA (3 and 10 μ M), negligible IFN- γ was detected, suggesting there may be toxicity at these concentrations. CyA demonstrated an IC₅₀ of 625nM (consistent with published data of ~600nM (Leonessa et al. 2002)). The HDACis were however considerably more potent compared to CyA with IC₅₀'s of 82.0nM and 13.4nM for KA1010 and SAHA respectively. Once again the potentially toxic effect of SAHA was demonstrated with no IFN- γ production at the higher dosages (> 3 μ M).

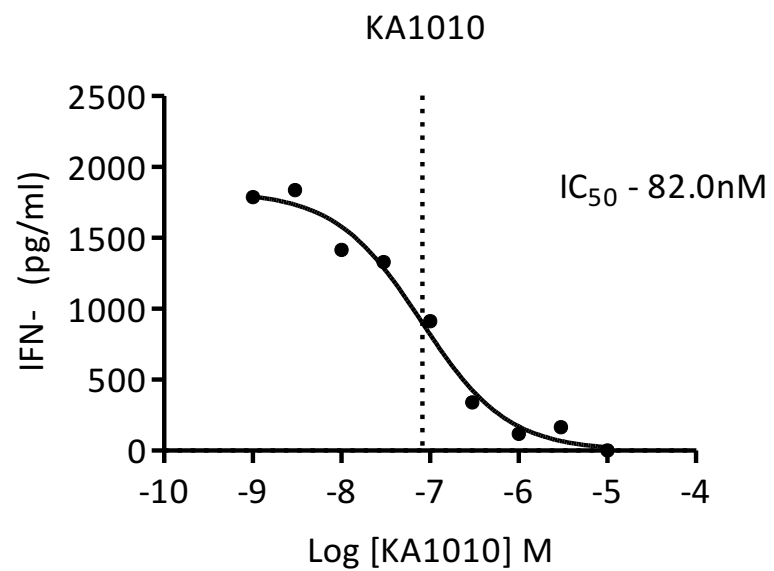
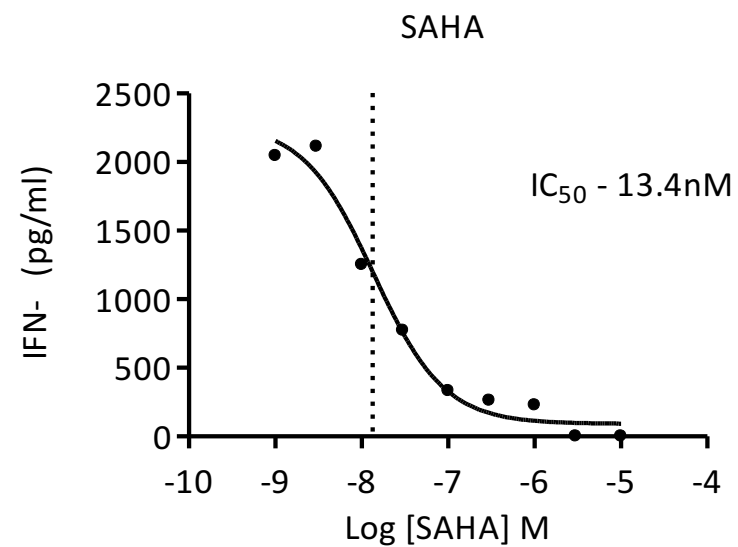
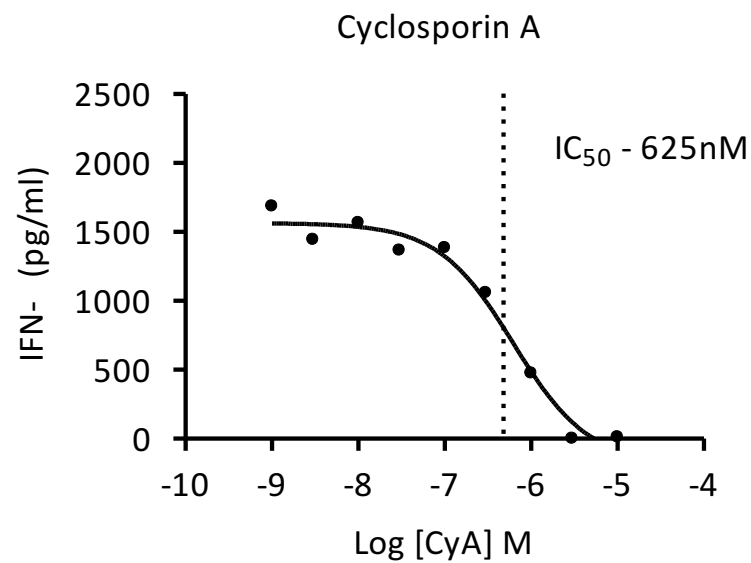


Figure 4-32 Dose response curves for CyA, SAHA and KA1010 on two-way MLR

4.6 MLR – Activation Markers

Within the activation process of T cells, several surface molecules are expressed *de novo* (Figure 4-33). Evaluation of the expression of these markers can describe the activation status of the lymphocyte. The effects or biological activities of a factor (such as a novel compound, in this case, HDACi) on a T cell are often studied by the expression of these antigens. In this series of experiments, the effect of CyA and HDACi on the following activation markers is assessed:

- CD69 (Very early expression)
 - earliest inducible cell surface glycoprotein acquired during lymphoid activation
 - involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, including natural killer (NK) cells, and platelets
- CD25 (Early expression)
 - α -chain of the IL-2 receptor
 - not involved in signal transduction, but is part of the high affinity IL2-R, expressed on activated cells
- HLA-DR (Mid-late expression)
 - MHC Class II surface receptor, high levels of which expressed on APCs
 - T cells also express HLA-DR molecules upon activation, although with slower kinetics (when compared to APCs)

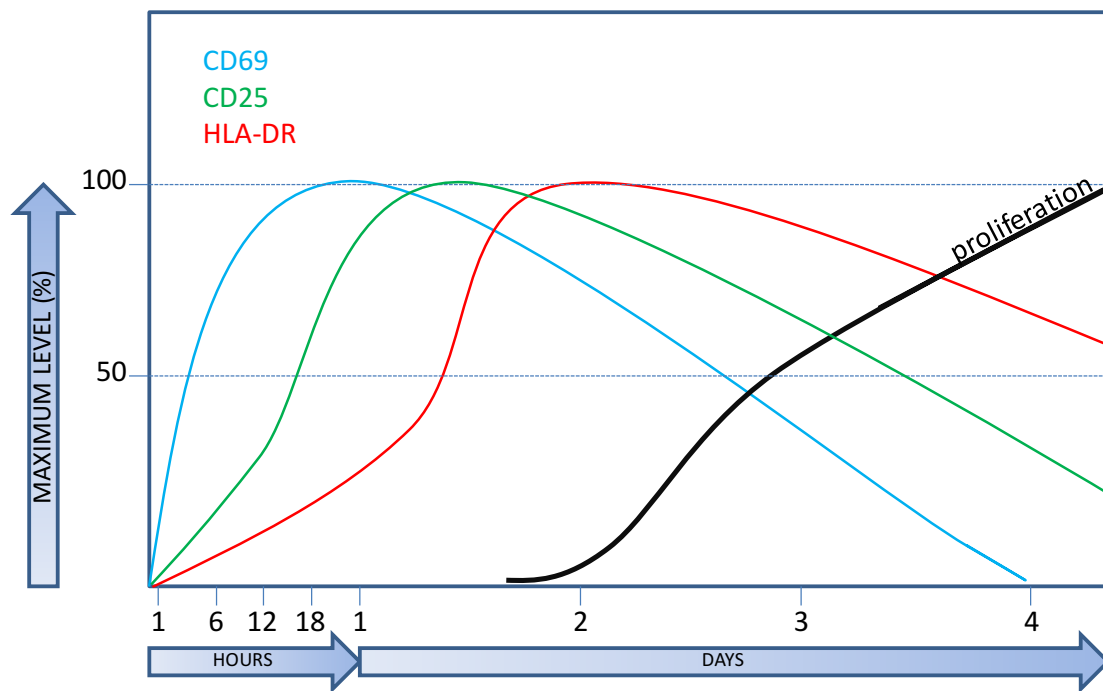


Figure 4-33 Typical pattern of T cell activation markers

Once again, this assay was based on a typical two-way MLR assay, using freshly isolated PBMCs from healthy volunteers. Cultures were incubated at 37°C for three days. RPMI media supplemented with 10% FCS. Negative control consisted of an autologous cell culture, and the positive control, an allogeneic culture, stimulated using PHA at a concentration of 5µg/ml. Activation marker expression was assessed at 0,24,48 and 72 hours. The flow cytometry gating strategy (Figure 4-34) involved, first of all, isolating the CD4⁺ population through negative selection (by staining for CD3⁺CD8⁺ cells) and then subsequent staining for individual activation markers.

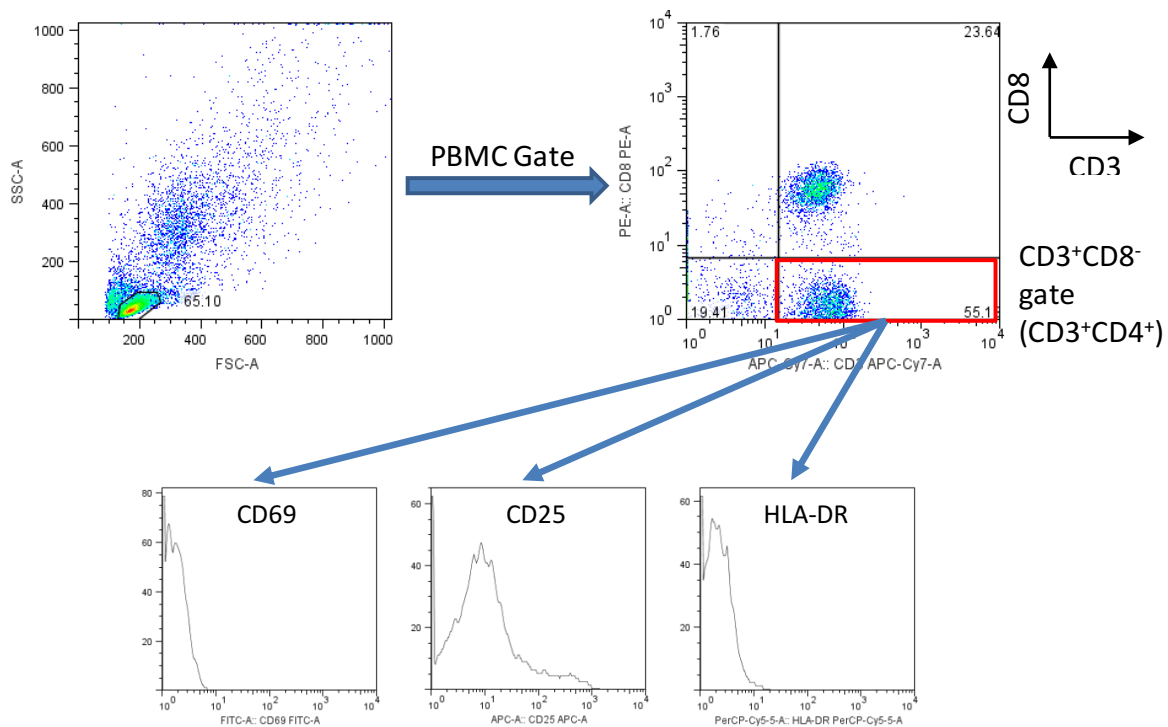


Figure 4-34 Gating strategy used in assessing activation markers

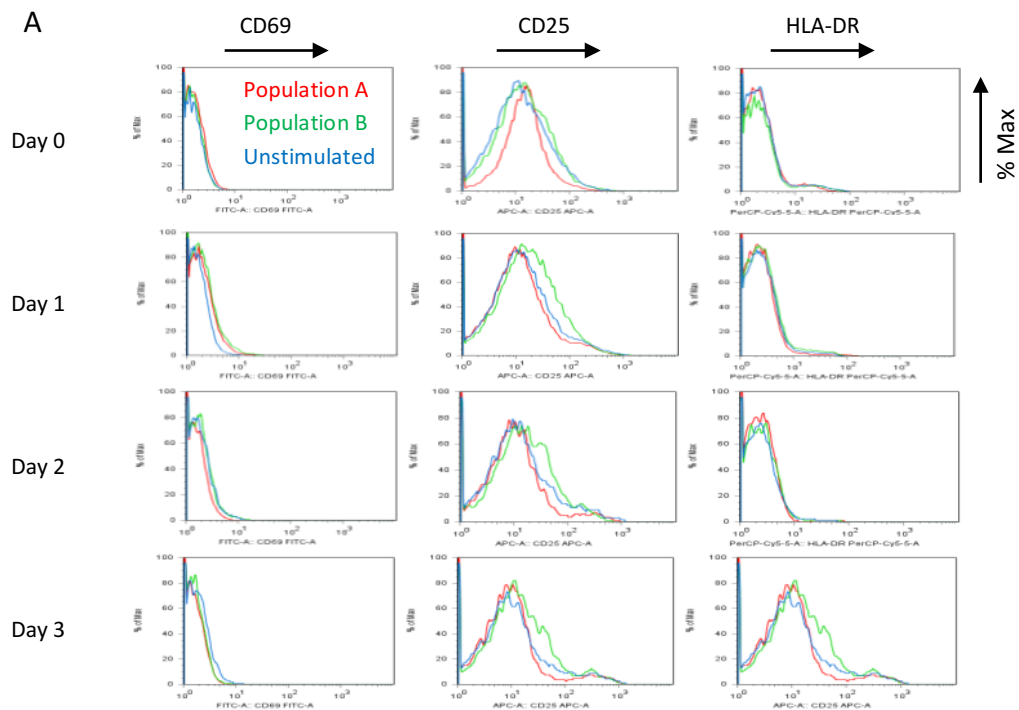
In the initial control assay, CD69 expression is relatively low (Figure 4-35 A). CD25 expression is greater than CD69 and levels are potentially falling having peaked around day 2. The expression of HLA-DR was expected to be higher at day 3, though a moderate increase is noted. This is not a truly dynamic study and it is therefore difficult to establish whether or not this is pre or post-maximal expression.

In the unstimulated control samples, a simple allogeneic culture was not sufficient to elicit any significant response in terms of T cell marker activation (Figure 4-35 A). However, mitogen stimulation of the assay (using PHA) did invoke a notable response (Figure 4-35 B). This was most pronounced in terms of both CD69 and CD25 activation but also notable in terms of HLA-DR expression.

CD69 demonstrated an early response with maximum stimulated expression (as expected) at day one compared to unstimulated. Increased CD69 expression was maintained over the

assessed time period. In contrast, CD25 expression increased over the time period up to the end of the assay, and the effect of stimulation on CD25 expression may have been more significant over a longer time course (published data suggests CD25 expression returns to baseline around day 5 following activation). Stimulation had a modest effect on HLA-DR expression. At day 3, there was a notable peak emerging, consistent with increased late expression compared to CD69 and CD25.

These findings were, on the whole, expected and consistent with published data on T cell activation markers.



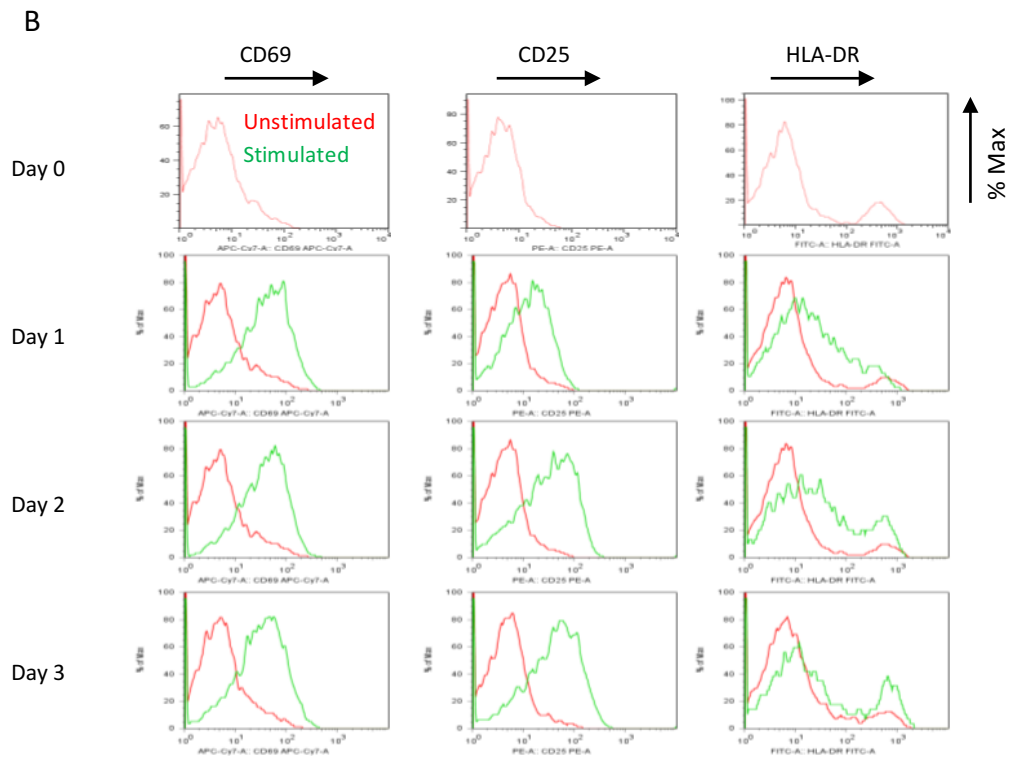


Figure 4-35 Control samples for two-way MLR, assessing activation markers. Unstimulated autologous vs. allogeneic samples (A). Unstimulated allogeneic vs. stimulated allogeneic

(B)

With effective controls established, it was next possible to assess the effects of CyA and HDACi on activation markers in a two-way MLR.

Effect of CyA

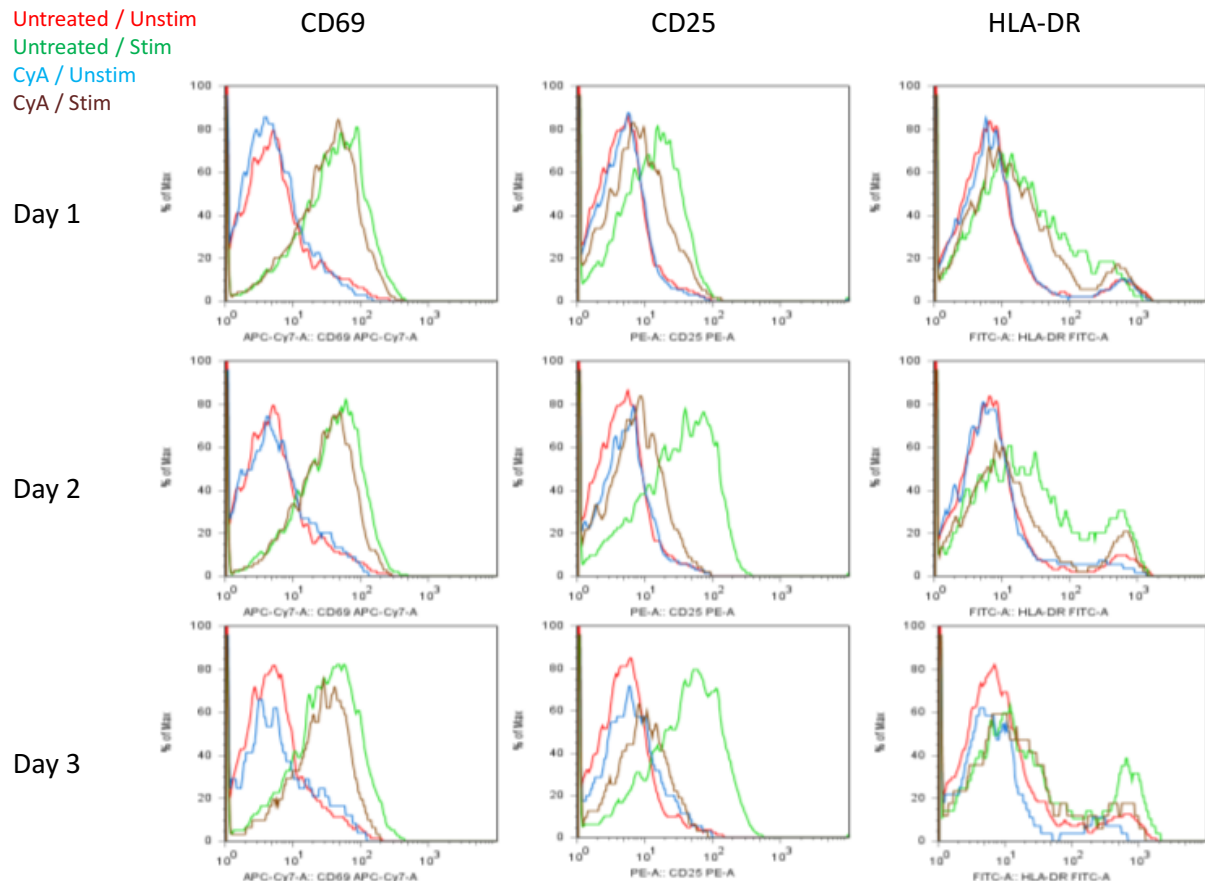


Figure 4-36 Effect of CyA on activation markers in a two-way MLR

At a concentration of 5 μ M, CyA (Figure 4-36) had a small effect on CD69 expression over three days in a stimulated assay.

However, there was a notable effect on CD25 expression. At 24 hours a slight reduction in CD25 expression was observed and this effect was further exacerbated over time, such that at 72 hours the CyA treated, stimulated assay produced levels comparable to the

unstimulated assay. Again this was not an entirely unexpected effect as the T cell (including T_{reg}) depleting effects of CyA are well documented (McMurchy et al. 2011).

Treatment of the assay with CyA also had an effect on HLA-DR expression. The baseline, unstimulated levels of expression remained consistent over the time period. With the untreated stimulated assay, a late, high signal second peak was observed, which became more evident over time. The effect of CyA on this peak was to restrict expression to baseline, unstimulated levels.

Effect of SAHA

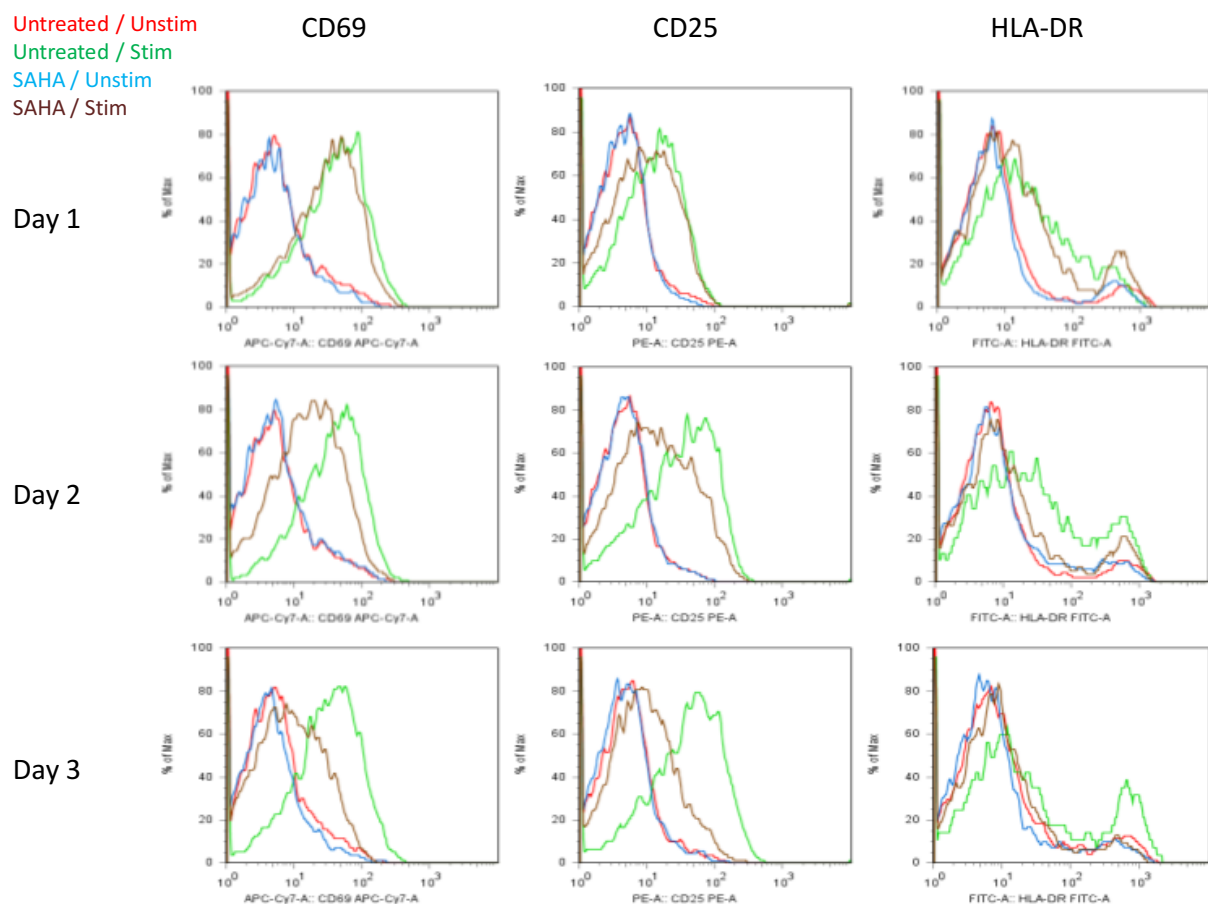


Figure 4-37 Effect of SAHA on activation markers in a two-way MLR

The effect of SAHA (at a concentration of 100nM) was assessed.

Treatment with SAHA did demonstrate an effect on CD69 expression (in contrast to CyA) when compared to an untreated, stimulated population (Figure 4-37). This effect was progressive over time, with expression in the stimulated population maintaining consistency compared to a steady return to baseline levels in the treated population.

A similar pattern of effect was also noted on CD25 expression. However, compared to CyA (and particularly at day 2), there was a degree of preservation of CD25 expression in the treated, stimulated population. This likely represents the alternative mechanism of action demonstrated by HDACis and suggests a degree of IL-2 preservation compared to CyA.

With regards to HLA-DR expression, SAHA treatment resulted in an early peak at day 1, followed by a steady decline in expression over time, the reasons for this are unclear. The stimulated, untreated assays demonstrated the reverse of this with a steady increase in HLA-DR expression over time.

Effect of KA1010

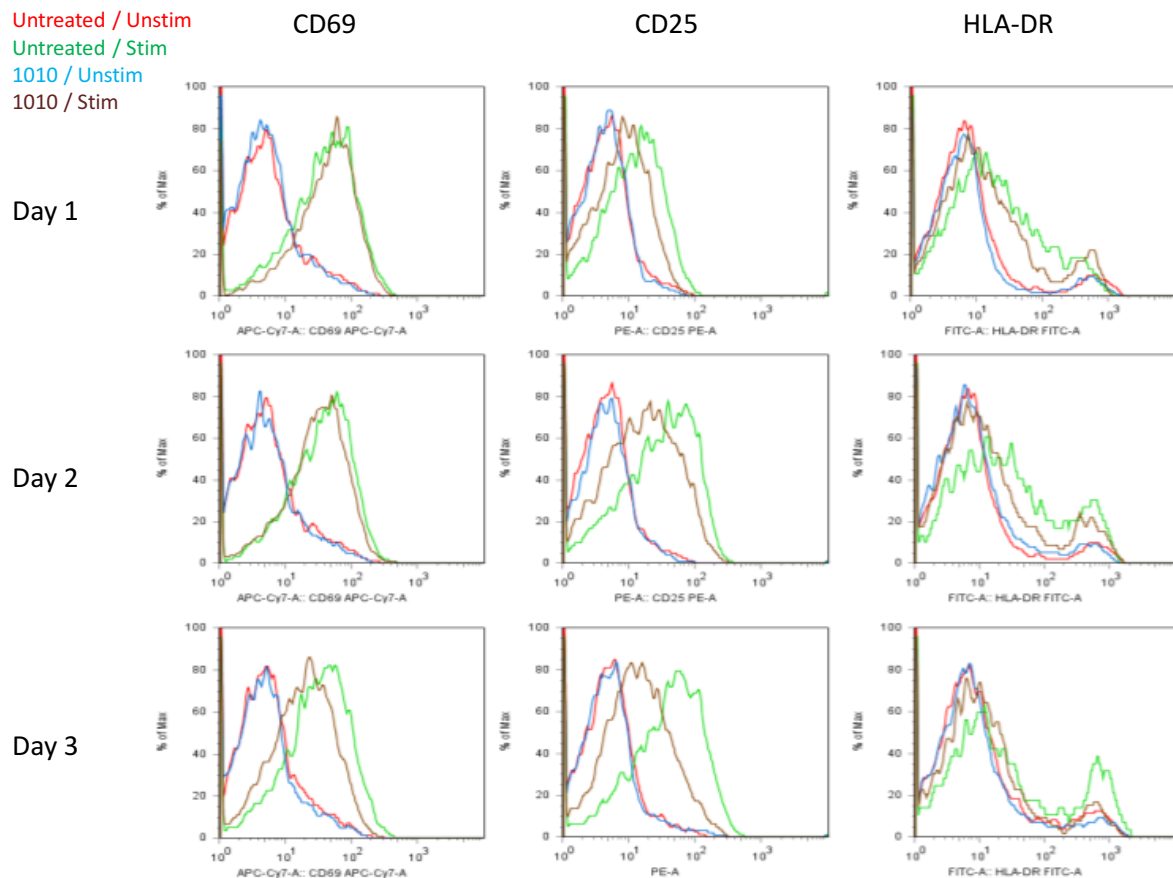


Figure 4-38 Effect of KA1010 on activation markers in a two-way MLR

The effect of KA1010 (at a concentration of 1000nM) was assessed (Figure 4-38).

As with CyA, CD69 expression was largely unaffected by the presence of KA1010. There was only a small shift toward CD69 downregulation at Day 3. However, compared to CyA there was a degree of preservation of CD25 expression over time with the greatest effect at day 3 (similar to SAHA), and this possibly reflects the HDACi-mediated preservation / enhancement of the the T_{reg} phenotype. Again the pattern of HLA-DR expression was similar amongst the HDACis with a notable peak in expression detected at day 3.

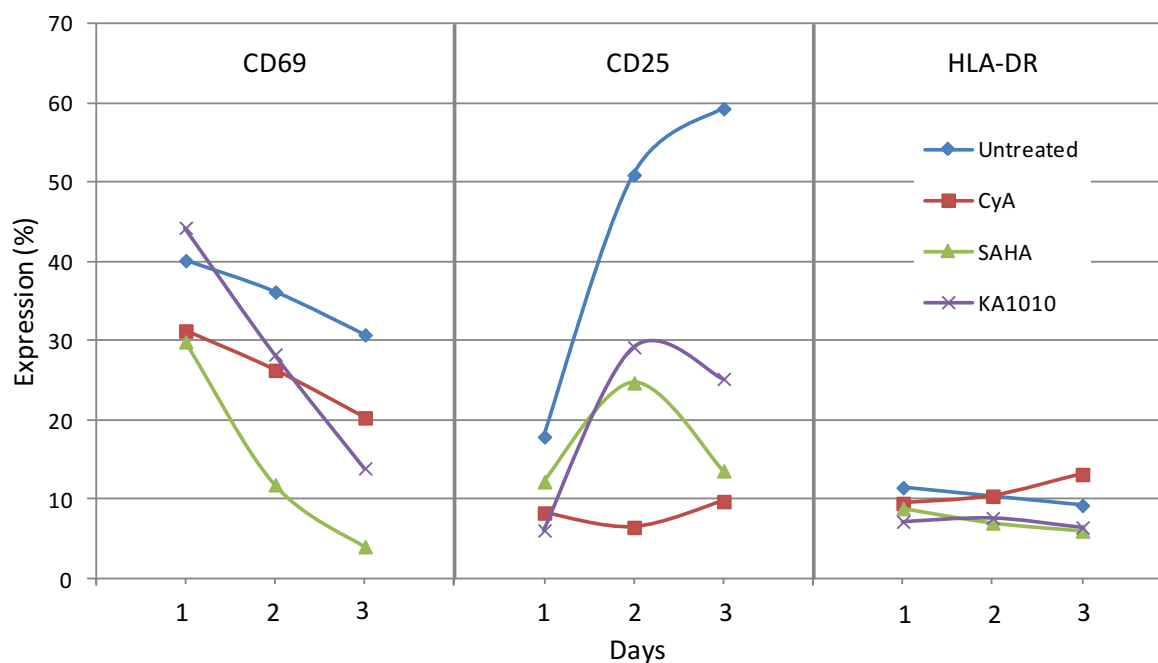


Figure 4-39 Comparative effects of CyA and HDACi on activation markers in a two-way MLR

By plotting the percentage change in expression compared to the unstimulated cells a clearer picture emerges (Figure 4-39). In general, the expression of CD69 is in gradual decline, though the steepness of decline in the HDACi treated samples is somewhat greater compared to untreated (stimulated) and CyA samples, suggesting a more rapid and complete effect. An even greater effect is demonstrated on CD25 expression. All treatments used, resulted in a significant reduction in CD25 expression. The effect of CyA was to prevent any significant increase in expression. However, HDACi treatment did permit a transient rise in CD25 expression at day 2, though not to the extent of the stimulated sample. This observation almost certainly reflects the differing mechanism of action between CyA and HDACi. None of the compounds assessed had any significantly differential effect on HLA-DR expression compared to control.

4.7 Effect of HDACi6 treatment on cytokine production

A body of evidence exists, that demonstrates one of the potentially beneficial effects of HDACis in terms of transplantation is a reduction in the release of pro-inflammatory cytokines. One such study involving experimental models of allogeneic haematopoietic cell transplantation (allo-HCT) (Reddy et al. 2004) demonstrated such an effect with SAHA through induction of indoleamine-2,3-dioxygenase (IDO). The likely mechanism of this is through the activation of signal transducer and activator of transcription 3 (STAT-3) (Reddy et al. 2008; Sun et al. 2009). More recently, SAHA has been demonstrated to reduce proinflammatory cytokines in the plasma of patients undergoing SAHA treatment in a GVHD phase 1/2 trial (Choi & Jeong 2005) as well as increase T_{reg} cell number.

However, the effect of targeted HDAC inhibition on cytokine production in the setting of an allogeneic response has not been extensively studied, though one recent study has reported that Tubastatin A (HDAC6 inhibitor) dampened the inflammatory response in a murine model of lethal sepsis (Li et al. 2015).

Therefore to assess the effect of HDAC6 inhibition on cytokine production in a transplant model, ELISA-based assays were performed on plasma recovered from two-way MLRs. The MLRs had been treated with either CyA, SAHA or KA1010. The panel of cytokines selected were based on those typically associated with an alloresponse and are listed in the table below (Table 4-3).

Cytokine	Produced by	Role in allogeneic response	Effect on T _{reg} s
Tumour necrosis factor alpha (TNFα)	Macrophages, CD4 ⁺ lymphocytes, NK cells,	Involved in proliferative	Reduces FoxP3 levels(Nie et al.

PRO-INFLAMMATORY	mast cells, eosinophils	response and development of cytotoxic T cells (Lukacs et al. 1993)	2013;Valencia et al. 2006) Reduces suppressive capacity of T _{regs} (Wang et al. 2008)
MMP-9 PRO-INFLAMMATORY	Monocytes, macrophages, endothelial cells	Reduces surface expression of CD25 on responding T-cells (Ding et al. 2009)	Reduces Foxp3 expression
IL-10 ANTI-INFLAMMATORY	Monocytes, Macrophages	Inhibitor of IFN γ , TNF α , IL-1 β , IL-6 and IL-8 production Downregulates HLA-DR expression	Produced by T _{regs} (Maynard et al. 2007)
IL-1a PRO-INFLAMMATORY	Macrophages, neutrophils, epithelial cells and endothelial cells	Stimulates thymocyte proliferation by inducing IL-2 release. Involved in inflammatory response as endogenous pyrogens	Unclear
IL-17 PRO-INFLAMMATORY	T cells (Th17), fibroblasts	associated with allograft rejection (Min et al. 2009) by stimulating secretory inflammatory cytokines such as IL6	Unclear, but may cause conversion of T _{reg} phenotype (to Th17)

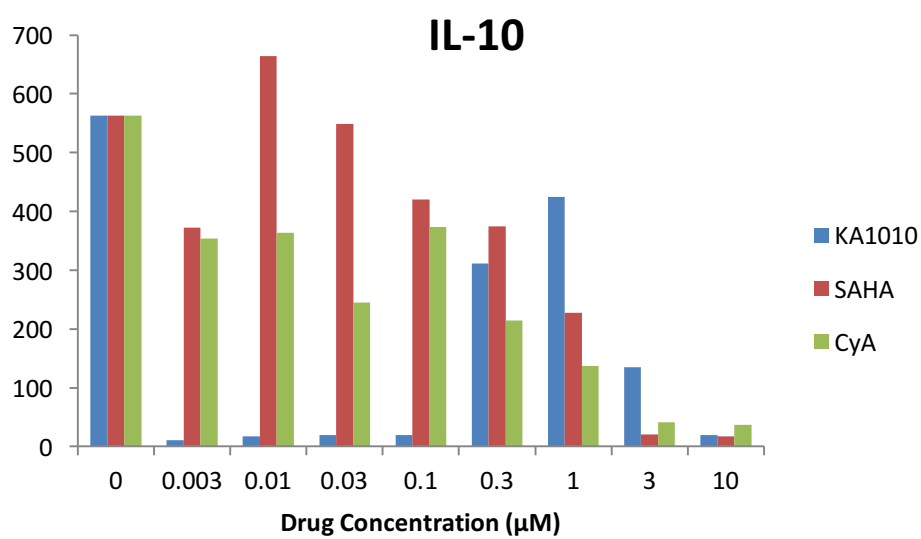
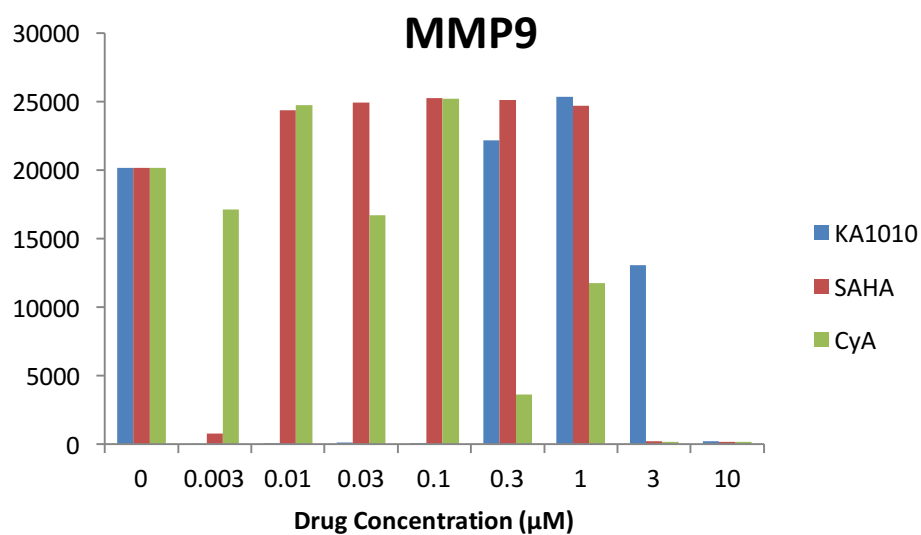
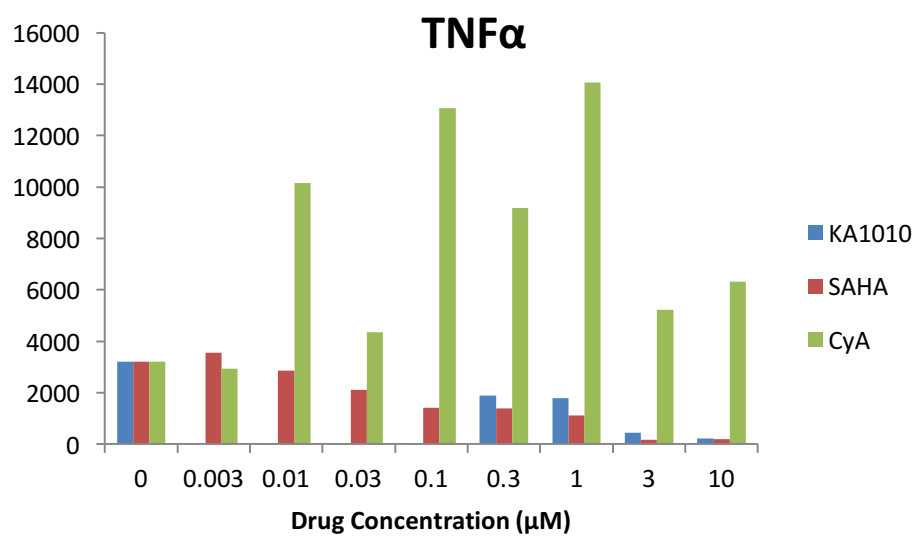
		and TNF α	
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Table 4-3 Cytokines involved in alloimmune response

The assay involved a bead-based multiple analyte detection platform whereby antibody coated beads are incubated with treated culture supernatants. Target analytes are then captured by specific antibodies on each bead. Biotin-conjugated paired antibodies are added, followed by Streptavidin-PE in order to facilitate detection. Flow cytometry is then used to differentiate specific populations, based on bead size and fluorescence.

The effects of the following compounds (and concentrations) in a human MLR were assessed:

- CyA 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 μ M
- SAHA 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 μ M
- KA1010 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 μ M



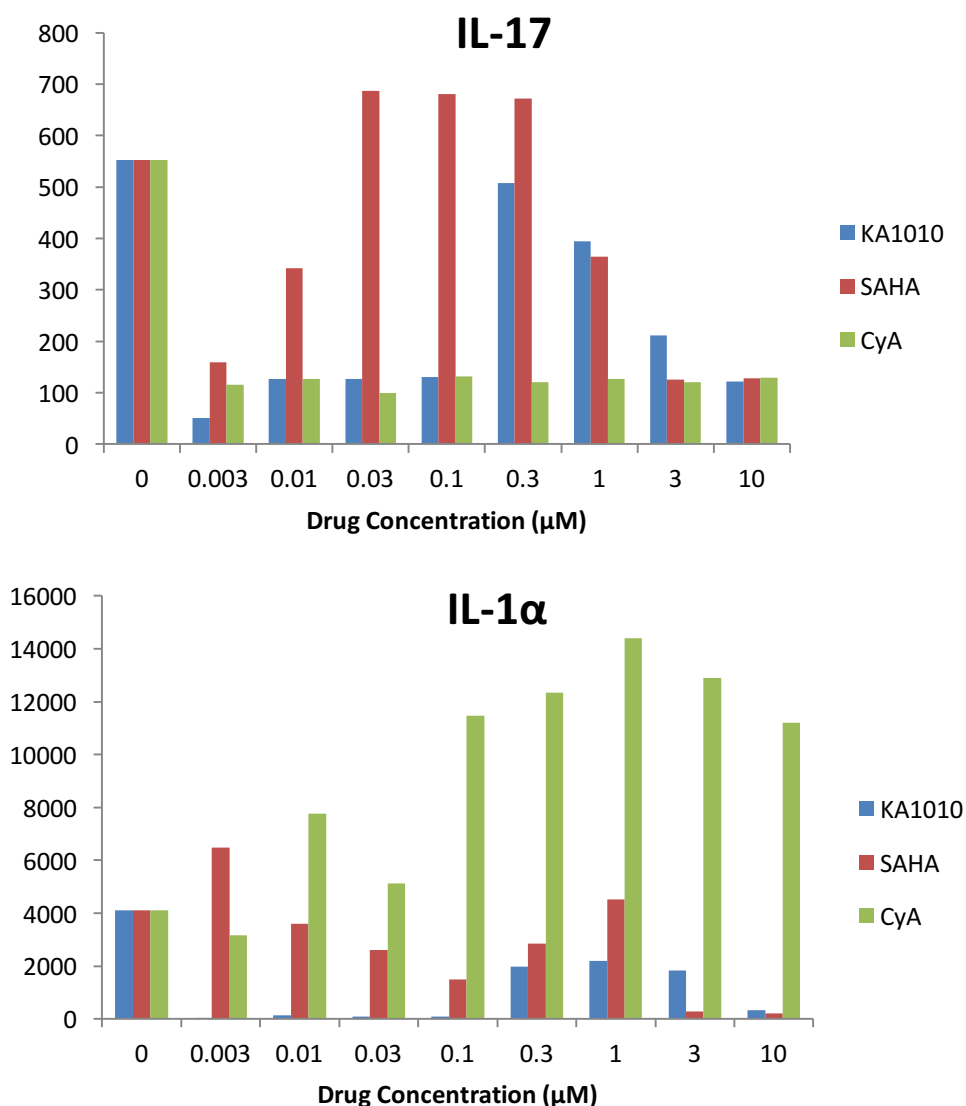


Figure 4-40 Two-way MLR cytokine release following immunomodulatory treatment

The results of these assays were somewhat variable (Figure 4-40) with very few providing any consistent trends from which to draw a meaningful conclusion.

The data from the TNFα analysis would suggest that treatment with the HDACi compounds had the effect of reducing production of this cytokine when compared with CyA. Though interpretation of this data could certainly not be considered reliable, this would appear consistent with published data relating to the effects of CyA (Bunikowski et al. 2001) and the pan-HDACi Trichostatin A (TsA) (Grabiec et al. 2010).

A similar trend was also observed in the analysis of IL-1 α , particularly at the higher range of doses of compounds used. At the higher doses of CyA (>0.1 μ M) there is appreciably more IL-1 α production (>10,000). This finding goes against previously published data (Won, Sauder, & McKenzie 1994) which has demonstrated a 3-4 fold inhibitory effect with a dose of 20 μ M CyA. The effect of HDACi was inconsistent, though KA1010 treatment at all doses resulted in reduced IL-1 α expression, below the 4000pg/ml observed under control conditions. These findings were similar to those described by Joosten et al. (Joosten et al. 2011) when they treated synovial cells with the class I and II HDAC inhibitor, ITF2357 (givinostat).

IL-10 expression did have a slightly more predictable response to treatment. There was generally a dose dependent effect seen with CyA and SAHA and this was quite profound at the higher doses (both treatments resulted in IL-10 production of <50pg/ml at doses of 3 μ M and above, compared to 563pg/ml production in the untreated control). Previous studies examining the effect of HDACi on IL-10 production have demonstrated that treatment with TSA (Class I & II inhibitor) and Panobinostat (pan-HDACi) increased IL-12 production with a concomitant reduction in IL-10 production (Wang et al. 2011). Further studies (Cheng et al. 2014), looking specifically at the role of HDAC6 in IL-10 production in murine APCs, found that pan-HDAC and Class II HDAC inhibitors down regulate IL-10 production by these cells. It is somewhat surprising therefore that KA1010 did not appear to have more of an effect.

Finally, IL-17 production was again significantly reduced with the treatment of CyA. At all concentrations, CyA reduced IL-17 production to 100-150 pg/ml compared to 550 pg/ml in the untreated control. It has previously been reported that the treatment of T cells with CyA

in normal and rheumatoid arthritis patients reduced IL-17 production at the protein and mRNA level. It has been postulated that this effect is due to a similar response

The effect of HDACi on IL-17 production was less consistent. SAHA had the least effect in terms IL-17 suppression with several mid-range concentrations resulting in production greater than baseline control. Again this was in contradiction to previously published reports of SAHA causing significant reduction in IL-17 expression at both gene and protein levels in murine cardiac allograft models (Zhang et al. 2012).

Overall, the outcomes of this multiplex assay were generally disappointing, with significant variation observed among individual cytokines, making interpretation difficult. There were however, a small number of results which could be cautiously interpreted as described above. Principally these were the effect of HDACi on TNF α and IL-1 α suppression, the dose-dependent suppression of IL-10 by CyA and SAHA and the uniform suppression of IL-17 by CyA.

Chapter 5 - Results

5 Ex vivo effects of histone deacetylase inhibition on allotransplantation

5.1 Introduction

The previous two chapters reveal a potential role of HDACi in terms of *in vitro* models of transplantation and the MLR is a useful surrogate tool. However to further assess the immunosuppressive effects of KA1010, an *in vivo* model was required.

The utilisation of murine models has had a pivotal role in transplantation, and the discovery and development of numerous related aspects from evolving surgical techniques to testing new and novel immunosuppressive treatments.

Since the advent of modern transplantation and early research by the likes of Billingham and Medawar, the murine skin graft model has become a mainstay of *in vivo* research. Though it has limited transferability in terms of reproducing complex human alloimmune processes, it does provide a rapid and simple platform on which to assess potentially immunosuppressive, novel compounds such as KA1010.

This chapter details the results of untreated syngeneic and allogeneic skin transplant in mice and defines the changes observed during the rejection process. Beyond this, the effect of treating murine recipients with various KA1010 regimens is observed both macroscopically and histologically.

The effects of treatment on several physiological parameters such as body weight and splenic index are also assessed, looking for any potential short or longer term side effects KA1010 treatment may manifest *in vivo*.

Finally, to support the hypothesis, that KA1010 mediates its immunosuppressive effect through the upregulation of regulatory T cells, the effects of treatment on T cell subset expression is analysed through flow cytometry

5.2 The Skin Allograft Model

In order to further assess the immunosuppressive effects of KA1010, an *in vivo* murine model of transplantation was adopted. This was based on an established model using allogeneic skin grafts in mice, to investigate the immune mediated response of acute allograft rejection (Lagodzinski, Gorski, & Wasik 1990; Yun et al. 2003). This model has several advantages in that the procedure itself is quick and relatively simple, requiring only a short period of time to develop the surgical techniques required. Secondly, skin is a highly immunogenic tissue, and the process of skin graft rejection is well studied and provides a robust and reproducible immunological reaction. The difficulty with this model, however, can be in determining the point at which skin rejection has occurred *in vivo*. In order to overcome this, a quantitative scoring system based on digital photography, developed by Schwoebel et al. (Schwoebel et al. 2005) was used (Table 5-1). In addition, histological analysis was also undertaken to determine further the extent (or lack of) rejection microscopically.

Score	Graft rejection
0	Intact graft
1	First clear signs of graft rejection
2	>25%

3	>50%
4	>75%
5	Complete rejection (>95%)

Table 5-1 Quantitative assessment scoring system for mouse skin transplant model

(adapted from Schwoebel et al.)

5.2.1 Syngeneic and Allogeneic controls

A standard protocol based on methods described by Garrod and Cahalan was adapted (Garrod & Cahalan 2008) – see Materials & Methods. This model utilised two widely available immunocompetent inbred strains of mice; C57BL (or C57 black 6) and BALB/c. These strains of mice are commonly available and have a robust history of use in transplant related research. A syngeneic model was used as the positive control (would expect 100% survival) and an untreated allogeneic model as the negative control (Figure 5-1).

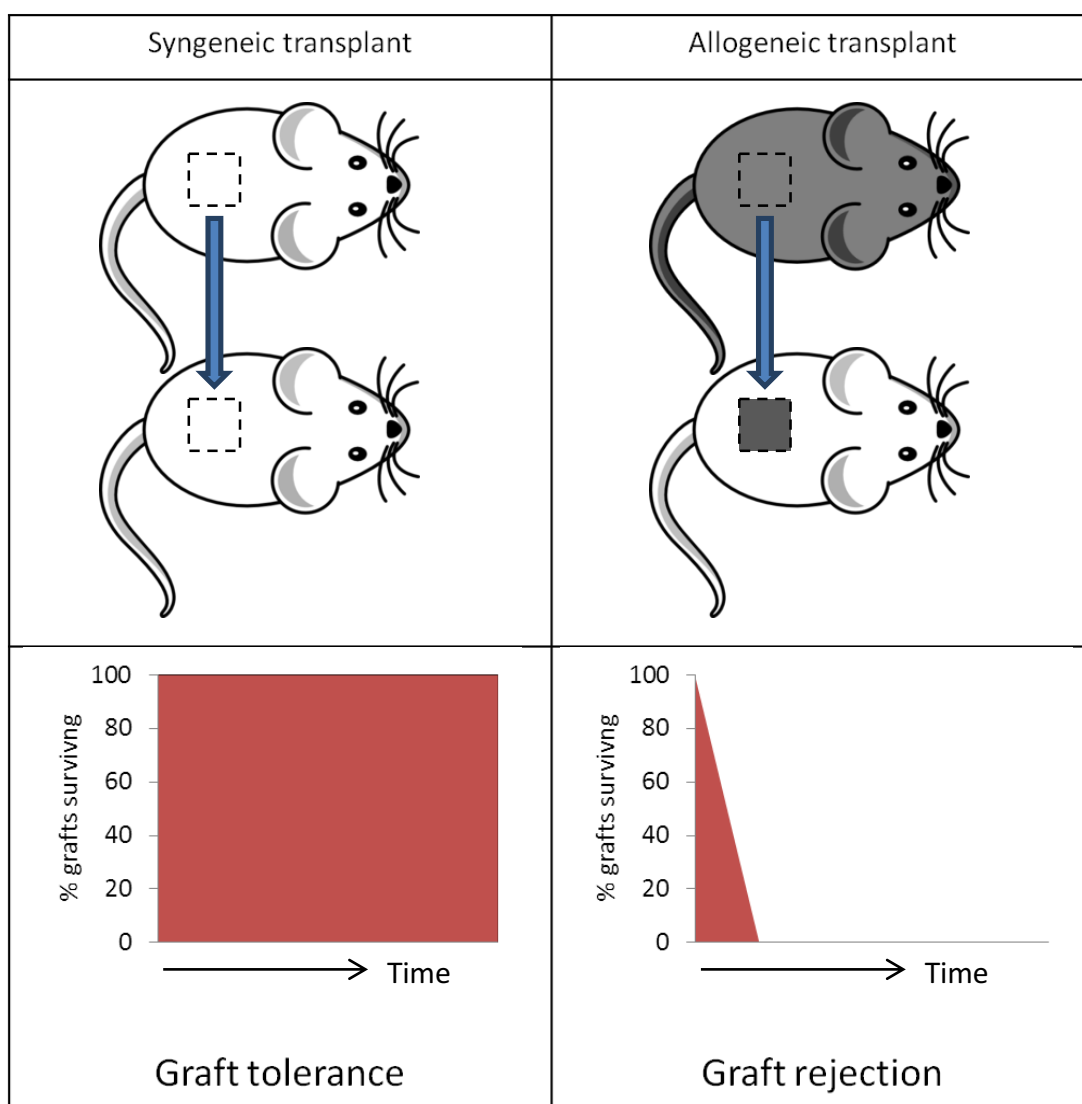


Figure 5-1 Expected outcome for syngeneic and allogeneic murine skin grafts

In summary, the skin graft model consisted of harvesting skin samples from both the ear and the dorsum of donor mice. The donor skin samples were then prepared by removing excess connective tissue and fat. Recipient graft sites were prepared by excising an area of skin on the dorsal surface. Donor grafts were then placed on the recipient and secured using sutures. A dressing was applied to the graft site which remained in situ until the 5th post-operative day.

Controls were established using syngeneic and allogeneic grafts in untreated recipients.

Syngeneic control

Donor skin was harvested from black C57/BL strain mice and grafted onto the dorsum of albino C57/BL strain recipients (Figure 5-2 A).

Syngeneic transplant

A



C57BL mouse



C57BL albino mouse

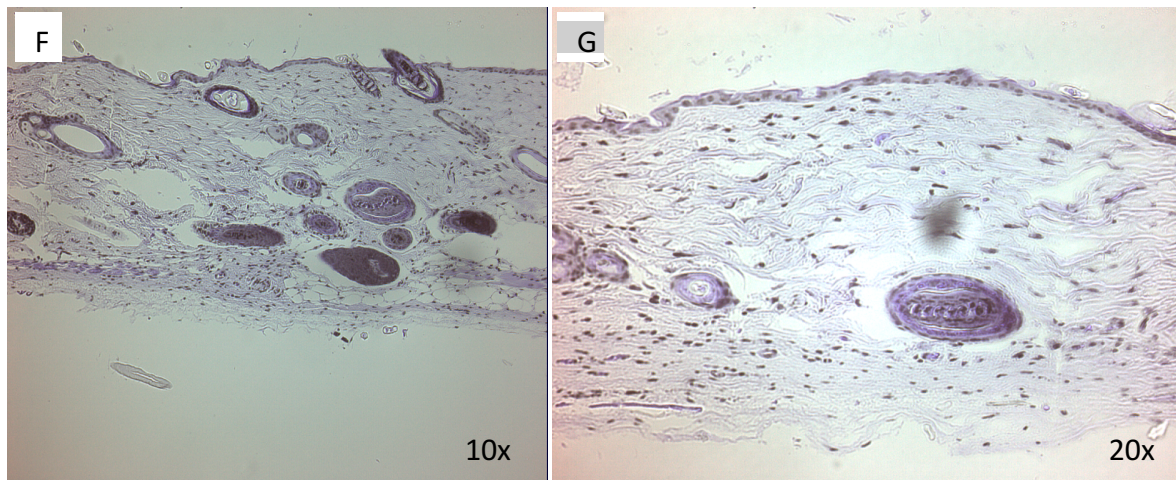


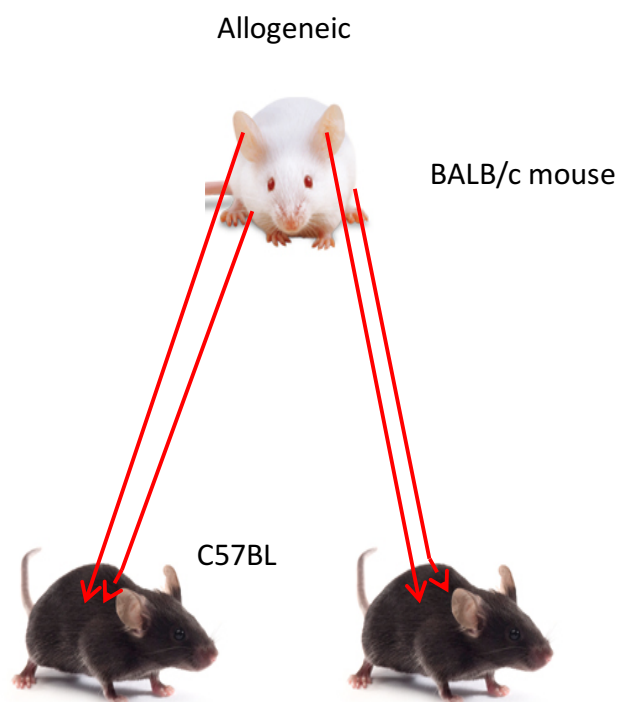
Figure 5-2 Syngeneic murine skin graft. A) Schematic representation of donor and recipient graft sites. B-E) Representative photographs demonstrating healing graft with no macroscopic evidence of rejection. F&G) Histological appearances of syngeneic graft

After 28 days (without any form of immunosuppressive treatment) there was no macroscopic evidence of rejection (Figure 5-2 B-E). Once the observation period had been completed the recipient subject was sacrificed and microscopic analysis of the graft site and adjacent skin / soft tissue undertaken. Microscopic analysis also demonstrated no evidence of histological rejection (Figure 5-2 F, G).

Allogeneic control

Donor skin was harvested from white BALB/c strain mice and grafted onto the dorsum of black C57/BL strain recipients (Figure 5-3 A).

A



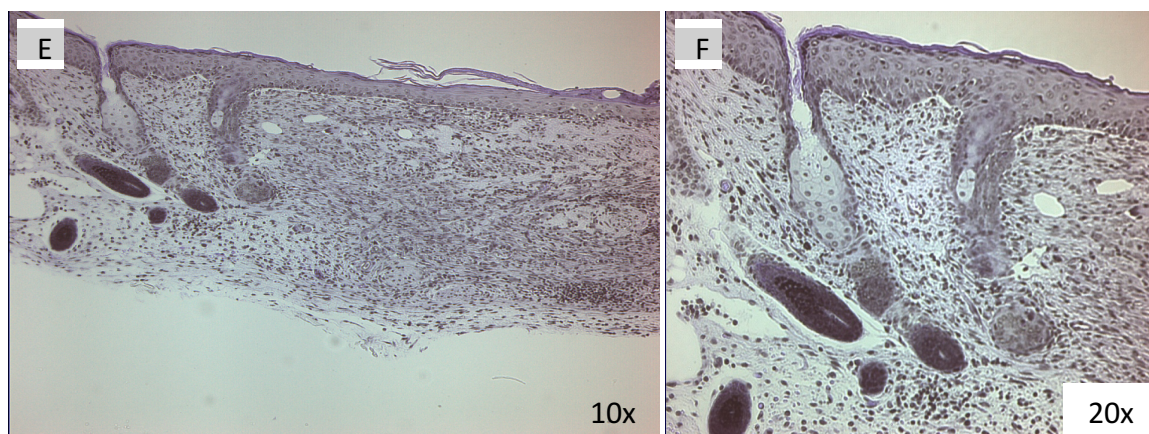


Figure 5-3 Allogeneic murine skin graft. A) Schematic representation of donor and recipient graft sites. B-D) Representative photographs demonstrating graft dehiscence and scarring. F&G) Histological appearances of allogeneic graft (day 14)

After 7-21 days, all allogeneic grafts had undergone a complete rejection process (n=6) which resulted macroscopically in graft dehiscence and graft site scarring (Figure 5-3 B-D). Histological appearances manifested as epidermal ulceration and lifting of the graft epidermis, dermal lymphocyte infiltration and fibroplasia with destruction of hair follicles (Figure 5-3 E, F).

5.2.2 Effect of KA1010 treatment on allogeneic model

In order to assess the *in vivo* effect of KA1010, the same allogeneic model was adopted. Recipients were stratified into a number of groups and treated accordingly (Table 5-2) for up to 70 days. Macroscopic rejection was analysed using the method previously described (Schwoebel et al. 2005). Rejection was considered to have occurred at >50% graft loss. Following the treatment period, the graft site was excised, and formalin fixed paraffin embedded sections were stained with Haematoxylin & Eosin and histological assessment made.

Arm	1	2	3
Treatment	No Treatment	KA1010 [160mg/kg] <i>ip od</i>	KA1010 [80mg/kg] <i>ip od</i>
Number	6	6	6

Table 5-2 Treatment groups for skin model of allogeneic transplantation

Macroscopic graft survival analysis (Figure 5-4) demonstrates that no significant rejection was noted among untreated syngeneic graft recipients (C57BL/6 donor skin, grafted onto C57BL/6 albino recipient). In this series of untreated allogeneic graft recipients, rejection occurred from day five and all subjects had rejected by day 18.

Assessment of allogeneic recipients treated with single-agent KA1010 at a once-daily dose of 160mg/kg (edisylate salt) [160mg/kg of edisylate salt is equivalent to 100mg/kg of free base] via the intra-peritoneal injection (*ip*) route, resulted in significant graft survival, with 77% of recipients maintaining a viable graft at the end of the treatment period. Graft survival reduced to 50% in allogeneic recipients receiving the lower dose of KA1010 [80mg/kg of edisylate salt - equivalent to 50mg/kg of free base]. Log-rank analysis of the survival curves (Mantel-Cox) demonstrates a significant difference between the curves with a p value < 0.0001.

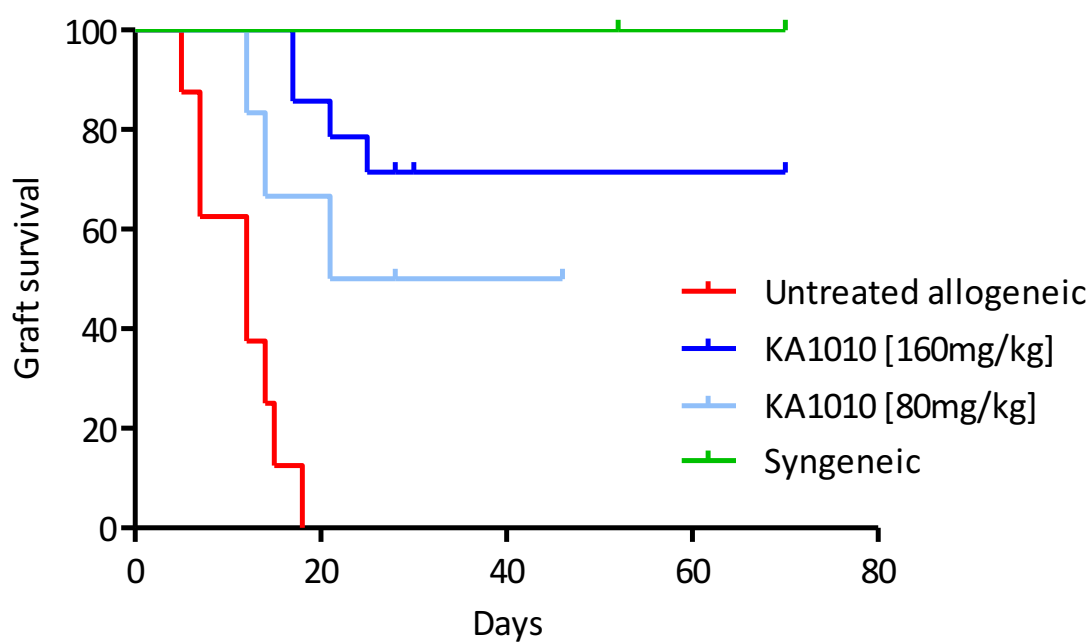


Figure 5-4 Kaplan-Meier survival curves demonstrating the macroscopic effect of KA1010 on murine skin graft model

5.3 Effect of KA1010 on subject weight

In addition to treating mice with the single agent HDACi (KA1010), the effect of treatment with Rapamycin (both in isolation and in combination with KA1010) was also assessed (

Table 5-3).

Skin donated from BALB/c mouse grafted onto gender matched C57/Bl6 mouse						
Arm	1	2	3	4	5	6
Treatment	No	KA1010	KA1010	KA1010	KA1010	Rapamycin
	Treatment	[160mg/kg]	[160mg/kg]	[80mg/kg]	[80mg/kg]	[50mg/kg]
		<i>ip od</i>	<i>ip od</i>	<i>ip od</i>	+ Rapamycin [50mg/kg] <i>ip od</i>	<i>ip od</i>
Number	6	6	6	4	4	4
Duration	28 days	28 days	70 days	28 days	28 days	28 days

Table 5-3 Treatment groups used to assess physiological effects of KA1010 and Rapamycin

Treating mice with the higher dose of KA1010 (160mg/kg) long-term (70 days) had a negative impact on weight gain over time (Figure 5-5). Treating mice with the lower dose of KA1010 (80mg/kg) had no significant effect on protracted weight gain.

All subjects undergoing the skin graft procedure lost an initial 5-6% in weight over the first 24-48 hours post-procedure. Untreated mice and those treated with KA1010 at a dose of 80mg/kg gained weight at a steady rate over the study period, such that by day 14 both groups had gained approximately 10% compared to their initial weight. Beyond this period the rate of weight gain in the group treated with KA1010 (80mg/kg) remained positive but

at a rate less than the untreated group. Stopping treatment at 28 days had little impact on the rate of weight gain (compared to the effect seen in the group treated with KA1010 at a dose of 160 mg/kg for 28 days).

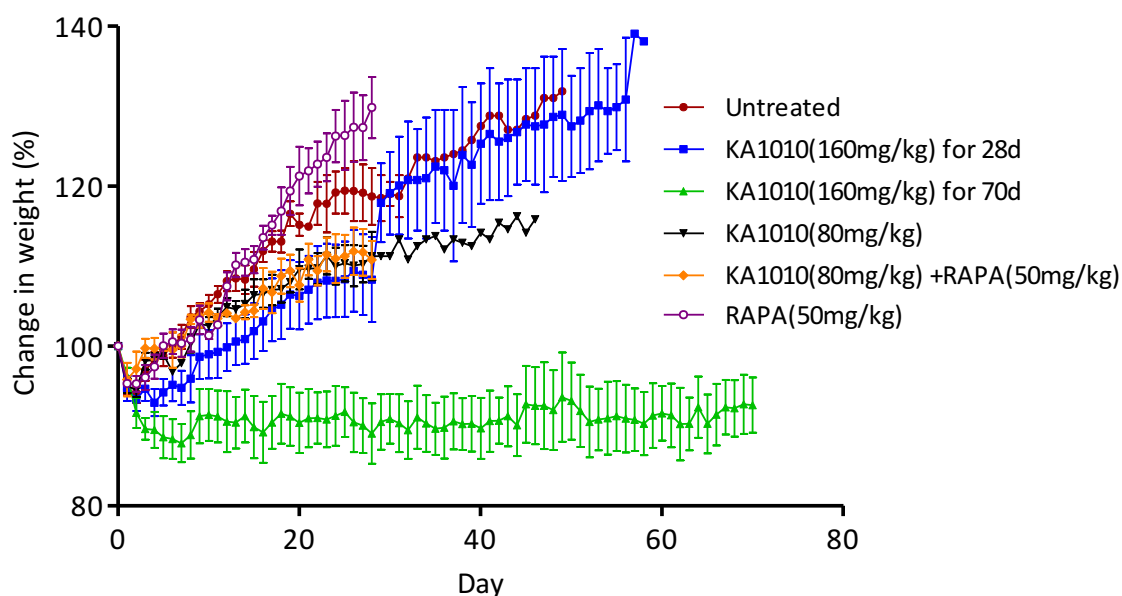


Figure 5-5 Effect of KA1010 and Rapamycin on weight gain in mice undergoing skin grafting

Treatment with the higher dose of KA1010 (160mg/kg) long term (for a period of 70 days) resulted in weight loss to approximately 90% of original weight. A steady weight was subsequently maintained at this level throughout the treatment period. However, data for those mice treated for a shorter period (28 days) would appear to contradict this as they returned to initial weight after 10 days. They continued to gain weight steadily throughout the treatment period so that by day 28 they were approximately 110% of their initial body weight. The reasons for this disparity are unexplained. After stopping treatment with the higher dose HDACi, there was a dramatic increase in weight gain such that subjects gained approximately 10% of body weight over a very short period of time (24-48 hours), again the reasons for this are unclear.

The effect of treating mice with Rapamycin had a slightly different effect compared to KA1010. In those subjects treated with Rapamycin alone (at a dose of 50mg/kg) steady weight gain was observed throughout the treatment period, such that at 28 days this group had a mean body weight which was approximately 10% greater than the untreated control group. Interestingly, when Rapamycin (50mg/kg) was used in combination with KA1010 (80mg/kg) as treatment, the weight gain profile was almost identical to that observed in the single agent KA1010 (80mg/kg) group.

5.4 Effect of KA1010 on lymphoid organs

Calculating the splenic index is an established method of assessing the effects of a compound on lymphoid organs and potential immunotoxicity (Yu et al. 2007). It is calculated by the following equation:

$$\text{Weight of spleen (mg) / body weight (g)}$$

This results in a relative index and allows comparisons to be made between different treatment groups; however detailed immunotoxic effects do need to be assessed using histopathological techniques to assess lymphoid tissue in detail.

Previous studies assessing the potential role of HDACis as an anti-tumour agent in mouse models of acute lymphoblastic leukaemia (Einsiedel et al. 2006), found that SAHA reduced spleen weight non-significantly. This data assesses the effects of KA1010 and Rapamycin on the splenic index of mice undergoing allogeneic skin transplant.

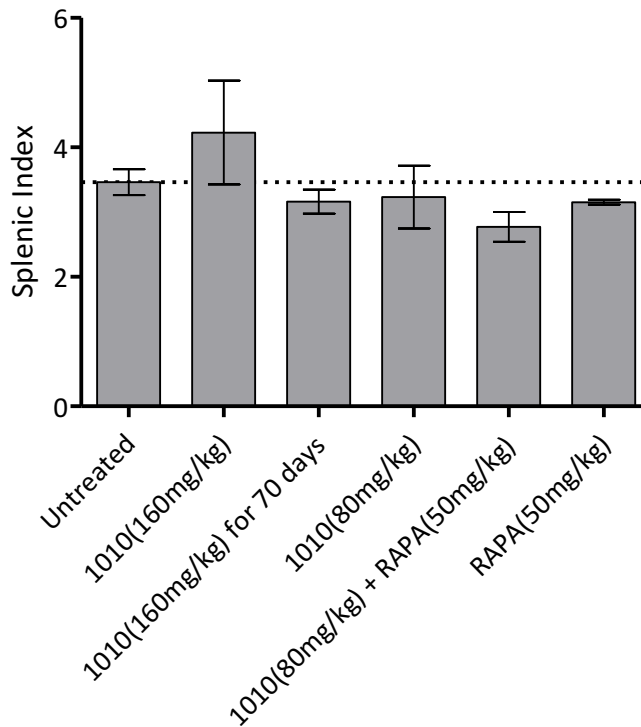


Figure 5-6 Effect of KA1010 and Rapamycin on mice splenic index. Treatment period is for 28 days unless stated (n=8 for each group)

A normal splenic index would be expected to be approximately 3.7 (Grass et al. 1999). In this series, the untreated control group had a mean splenic index of 3.5 (SEM 0.2, IQR 3.0-3.9). Treatment with KA1010 (Figure 5-6) at a dose of 160mg/kg for 28 days resulted in a non-significant increase (unpaired t-test $p=0.37$) in splenic index to 4.2 (SEM 0.8, IQR 2.9-5.3). If this treatment period was extended to 70 days (same dose), the splenic index fell back to a value of 3.2 (SEM 0.2, IQR 2.7-3.6). The effect of reducing the dose of KA1010 to 80mg/kg over 28 days had less of an effect compared to the higher dose treatment with a splenic index of 3.2 (SEM 0.5, IQR 2.5-4.2). Again this dose was not significant ($p=0.43$) when compared to a dose of 160mg/kg.

The effect of treatment with Rapamycin in combination with low dose KA1010 (80mg/kg) for 28 days resulted in the most dramatic change (though not quite statistically significant, $p=0.08$). Under these conditions a reduction in splenic index compared to control was observed with a value of 2.8 (SEM 0.2, IQR 1.8-3.6). Treatment for 28 days with Rapamycin alone (50mg/kg) resulted in a reduction of the splenic index to 3.2 (SEM 0.04, IQR 3.1-3.2). This reduction was not statistically significant ($p=0.39$).

5.5 Effect of KA1010 and Rapamycin treatment on CD4 expression in murine allogeneic skin transplant model

To determine the effect of HDAC6i on the T_{reg} population, the levels of $CD4^+$ T cells and $CD4^+FoxP3^+$ regulatory T cells in harvested splenic tissue were compared, from treated and untreated subjects.

Lymphocytes were first isolated from the splenic tissue and stained for $CD4^+$ expression. The proportion of $CD4^+$ cells identified were calculated using flow cytometry (Figure 5-7).

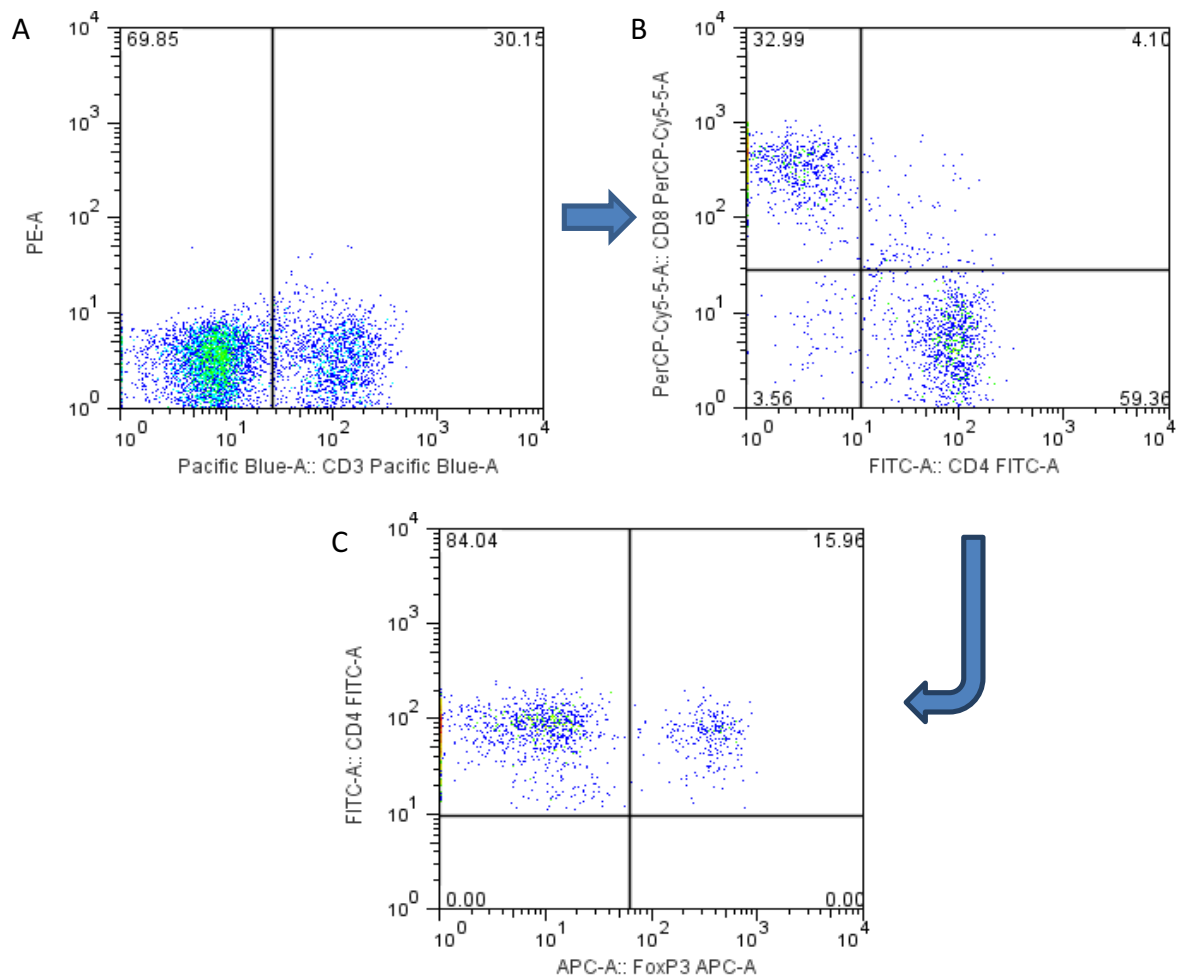


Figure 5-7 Example of gating strategy employed to identify CD4⁺ T cells and regulatory (FoxP3⁺) T cells. A) Isolation of CD3⁺ T cells. B) Isolation of CD4⁺ T cells. C) Identification of FoxP3⁺ T cells

Treating mice undergoing skin transplantation with KA1010 (at a dose of 160mg/kg) for 28 days resulted in a non-significant ($p=0.09$) reduction in CD4⁺ expression from 57.7% (SEM ± 1.9) to 53.3% (SEM ± 1.4) (Figure 5-8). If the treatment period was extended to 70 days, however, a significant reduction in CD4⁺ T cells to 46.9% of the lymphocyte population ($p=0.0009$) was observed. KA1010 treatment at a dose of 80mg/kg, had a negligible effect on CD4⁺ expression (reducing expression to 55.9%, SEM ± 2.4). However, treatment with this dose in combination with Rapamycin (50mg/kg) did result in a significant reduction

($p=0.003$) in CD4⁺ expression to 48.1% (± 1.4). This reduction is most likely due, primarily to the effect of Rapamycin in this combination, as Rapamycin alone has a similar effect (as opposed to the negligible effect of KA1010 at 80mg/kg) in reducing CD4⁺ expression to 51.3% (± 0.9 , $p=0.01$), though the difference is not statistically significant.

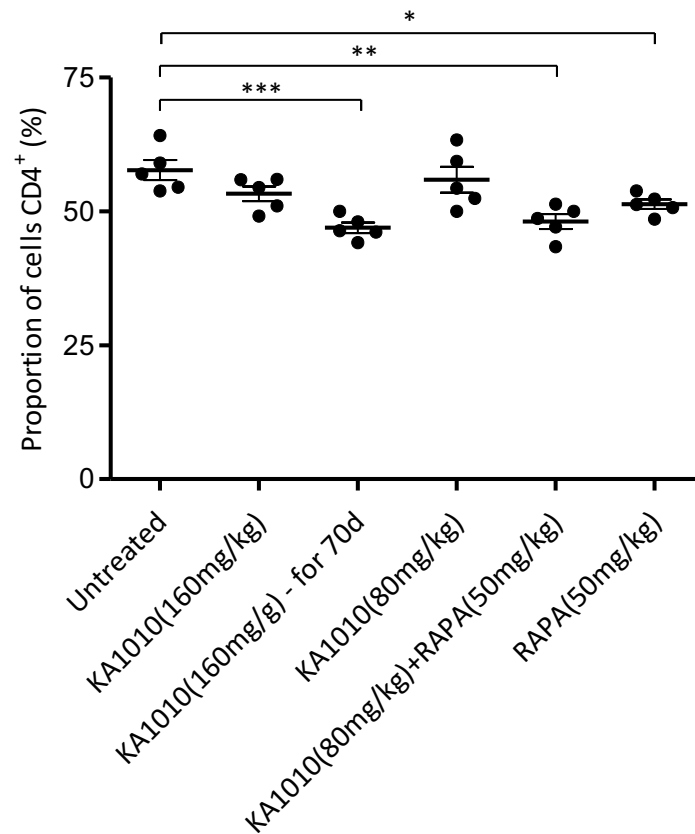


Figure 5-8 CD4 expression following HDAC6i and Rapamycin treatment. Treatment for 28 days unless stated (n=5 for each group)

5.6 Effect of KA1010 and Rapamycin treatment on T_{reg} expression in murine allogeneic skin transplant model

The findings observed with respect to CD4⁺ expression were in general converse to those seen in terms of regulatory T cell expression. Once again, lymphocytes were isolated from splenic tissue of mice undergoing identical treatment conditions. Positive selection of CD4⁺ T cells was undertaken and these cells were then stained for FoxP3 transcription factor in order to identify the T_{reg} subpopulation (Figure 5-7).

Within the untreated population, 18.5% (+/- 0.7) of the CD4⁺ T cells expressed FoxP3 (Figure 5-9). Treatment with KA1010 (160mg/kg) resulted in a significant increase in FoxP3 expression to 21.2% (+/- 0.6, p=0.02). As with CD4⁺ expression, the apparent effect of KA1010 at this dose over an extended period (70 days) was amplified, resulting in FoxP3 expression proportionally increasing to 25.7% (+/- 0.7, p=0.0002).

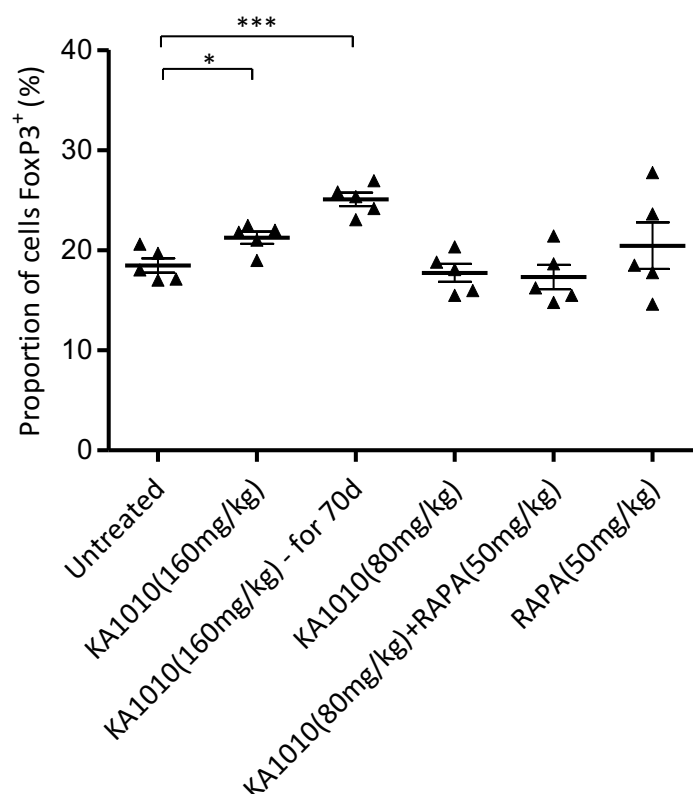


Figure 5-9 FoxP3 expression among the CD4⁺ population, following HDAC6i and Rapamycin treatment. Treatment for 28 days unless stated (n=5 for each group)

KA1010 at the lower dose of 80mg/kg had no effect with the total proportion of FoxP3⁺ cells representing 17.7% (+/-0.9, p=0.5) of the CD4⁺ population. The addition of Rapamycin (50mg/kg) to this treatment regimen had little effect (17.3% +/-1.2, p=0.4). Treatment with Rapamycin (50mg/kg) alone also had a limited effect in terms of FoxP3 expression, though the mean expression was slightly greater than control at 20.1% (+/-2.3, p=0.4).

In order to facilitate comparisons between the effects of compounds on T cell morphology the relative proportions of FoxP3⁺ cells to CD4⁺ cells was calculated (Figure 5-10).

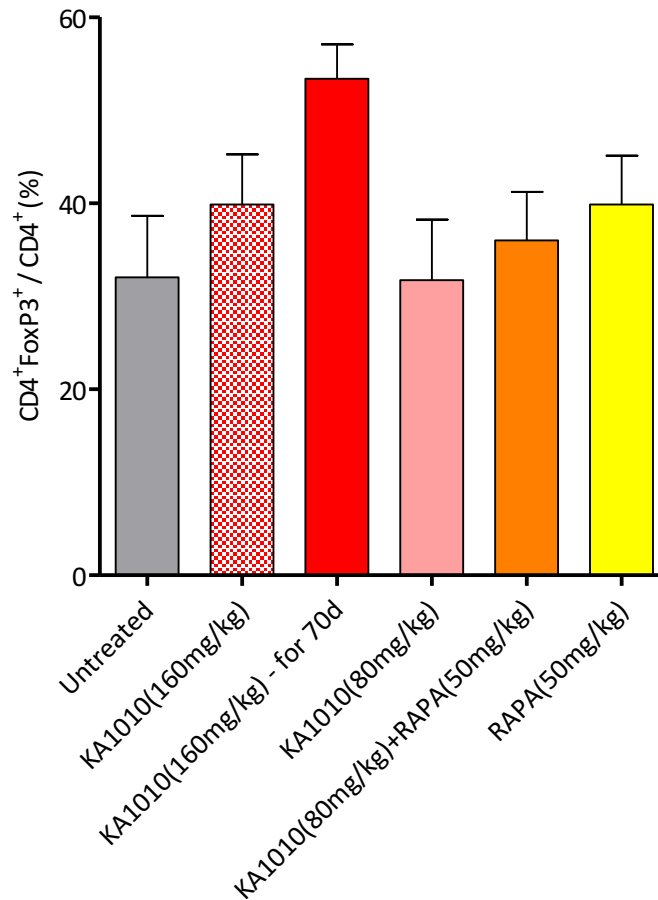


Figure 5-10 Effect of treatment on proportion of T_{reg}:CD4⁺ T cells expression

By combining this data, to compare the T_{eff}:T_{reg} ratio, it can be concluded that the most effective treatment regimen, achieving the greatest proportion of T_{reg} numbers was treatment with KA1010 (at a dose of 160mg/kg) for a 70 day period (statistically comparing treatment regimens with control using Dunnett's multiple comparison test demonstrated this as the only significant regimen with p<0.05).

Published data suggests, the frequency of T_{regs} in lymphoid tissue of C57 strain mice of a similar age to be in the vicinity of 15-20% (Zhao et al. 2007) and this would be consistent with the data above. It has also been shown that higher numbers of FoxP3 cells are directly

proportional to improved graft outcomes (Brown et al. 2007), which would again be consistent with this data.

Chapter 6

6 Discussion

The aim of this study was to assess the potential immunosuppressive role of the histone deacetylase inhibitor group of compounds as pharmaceutical agents for use in transplantation. In addition to this, identifying a specific compound from this family, with desirable characteristics which could be evaluated further, with the goal of taking it forward for additional development and even clinical trial has also been achieved.

Through Western blot analysis we have identified the ubiquitous presence of a number of HDACs among T cell sub-populations, including HDAC6, the specific target of KA1010. This is consistent with other studies which have demonstrated the presence of all eleven HDACs in resting T cells (Keedy et al. 2009). The presence of HDAC within the CD4⁺CD25⁺ subset can be assumed, through multiple studies demonstrating an effect of HDACi on FoxP3 acetylation, however, no evidence can be found which has previously delineated this specifically through Western blot protein analysis. These results partially address this by demonstrating the presence of HDACs 2, 3, 4, 6 and 9 within this important and relevant sub-group.

One of the major dose-limiting factors of current immunosuppression regimens relates to off-target toxicity profiles, notably nephrotoxicity. Clinically approved pan-HDACis such as SAHA, are reasonably well tolerated agents, with generally better side effect profiles compared to CNIs (Wagner et al. 2010). They are of course not without some deleterious effects.

The main application of pan-HDAC inhibitors to date has been in an oncological setting, where the broad target profile and comparatively high dosages administered have been generally accepted, despite these side effects. Nausea, fatigue, cardiac conductive abnormalities and thrombocytopenia (de Ruijter et al. 2003) have all been associated with the clinical use of pan-HDAC inhibitors such as SAHA and sodium valproate, though in general these effects have been found to be reversible. It is unsurprising that given their broad range of targets, a wide and varied side effect profile among different pan-HDACi compounds occurs. However, as the availability of potential HDAC-specific drugs with improved targeting increases, through engineering and continued discovery of naturally occurring compounds, the side effect profiles would also be expected to lessen.

Despite being a relatively recently discovered class of compounds, the side effect profiles of pan-HDACis are fairly well documented, however the toxicity profiles of HDACi at a cellular level, in general, are poorly understood. This may be because their role to date has been limited to the oncological environment and an effect which may be deemed covetable in this setting would possibly be seen as detrimental in an alternative clinical setting, such as transplantation, where therapeutic dosages are likely to be different. With this in mind, we undertook a cell viability assay, in order to ensure that any potential compounds we took further forward would not have unacceptable toxicity that would preclude them from any potential clinical application. The basic toxicity studies undertaken in this study, assessed the effect of treatment on PBMCs and were found to support the theory that targeted HDAC inhibition is potentially safer. In our study we found evidence that SAHA causes greater toxicity at a dose of 2 μ M compared with HDAC specific inhibition in the form of KA1010 at doses of 10 μ M.

Once it had been established that HDAC inhibitors are generally safe up to dosages in the μM range *in vitro*, it was then possible to establish which agents from a panel of both traditional and novel compounds had potential immunosuppressive properties, through simple proliferation assays involving Jurkat cells and PBMCs. A panel of ten HDACi compounds were supplied by Karus. As investigators, in order to reduce interpretive bias, the only information supplied at this stage was the molecular weight and therapeutic range for each compound.

The effect of HDACi on Jurkat cell proliferation was variable and no consistent pattern was noted. Though this series of experiments provided few conclusions in terms of proliferative effect, it did however provide a degree of validation to the toxicity assays previously undertaken. A better examination on the proliferative effects of HDAC inhibition was undertaken using CFSE-dilution in PBMCs. As well as three blinded HDACis, SAHA, a pan-HDACi which has undergone the most research in this group of compounds, was taken forward for further analysis and was there on consistently utilised as a comparator throughout the *in vitro* sections of this study. These proliferation studies, revealed a dose-dependent inhibitory effect of specific HDAC inhibition superior to that seen with SAHA ($p=0.002$, at the lower dose of 1nM this inhibitory effect represented a 4.7% and 6.8% reduction over control for SAHA and KA1010, respectively. At the higher dose of 1000 nM, these figures increase to 9.0% and 22.6% respectively). The presence of IL-2 had a minimal effect. Although no published data exists to quantitatively compare these findings too, the inhibitory effect is consistent with a similar trend demonstrated following the treatment of CD4^+ T cells with a comparable dose range of the selective-HDACi (Class I & II), TSA (Moreira, Scheipers, & Sorensen 2003). It has been postulated that this effect was mediated through

suppression of NF- κ B protein and IL-2 gene expression levels (Licciardi & Karagiannis 2012).

The possible cellular mechanisms by which HDACis may exert their effect will be discussed in further detail later in this chapter.

In a number of assays performed throughout this study, the effect of supplementing culture media with IL-2 was considered. IL-2 is of course, a key cytokine in lymphocyte physiology, particularly in T cell differentiation and T_{reg} function. The rationale for supplementing media in many of the assays performed therefore was a concern that lack of IL-2 might hamper a T-cell mediated response. In reality, very few effects of IL-2 supplementation were noted.

Harnessing the immunomodulatory functions of regulatory T cells is an ever increasing area of interest to many research groups. A number of strategies have been tried in order to exploit the immunomodulatory function of this cellular immune compartment by promoting their function, expansion, or adoptive transfer. One such strategy of particular relevance is the effect of HDACis on T_{regs}.

The potential role of HDACi therapy in FoxP3 homeostasis has been discussed briefly in chapter 1. During the course of this project, a number of groups have also studied this link and the body of evidence supporting a possible role for HDACis in bolstering the regulatory T cell population as an immunotherapy modality is gaining momentum. Many of these studies were in the context of inflammation, and the evidence for a role in transplantation has generally been limited.

In two murine models of colitis (one induced by treating with dextran sodium sulphate and the other induced through the adoptive transfer of T cell dependent CD45RB^{hi}), the pan-HDACis TSA and SAHA were both reported to have prevented the development of colitis (de Zoeten et al. 2010; Tao et al. 2007). However, when the mice were treated with the potent

class I HDACi, MS275 there was no significant effect and the subjects proceeded to develop worsening colitis suggesting the protective effect of HDACi is not mediated by this particular class. By depleting mice of T_{regs} or using a strain of mice who have a FoxP3 mutation (Scurfy mice), the authors were able to demonstrate that the favourable effects of the pan-HDACis were regulatory T cell dependent, as these mice succumbed to colitis despite previously effective treatment.

A similar effect has subsequently been demonstrated several times *in vitro* using pan-HDACis (in the nanomolar range) in rhesus macaque and human regulatory T cells (Akimova et al. 2010; Johnson et al. 2008). When HDACis of other structural class such as the short chain fatty acid (valproic acid) have been tested however, their effect on T_{reg} function only becomes apparent at the micro- to millimolar ranges. Interestingly, and of significant relevance to this study, it was also found that the use HDACi compounds specific to class I (such as the benzamides) had very little effect on T_{reg} function, even at high dosage, suggesting the apparent effect is mediated by class II activity.

In this study, in order to demonstrate an HDACi effect of T_{regs} we adopted and modified an assay based on Sakaguchi's original T_{reg} suppression experiments. Following optimisation of the assay, we were able to demonstrate the suppressive capabilities of regulatory T cells, through increasing ratios of T_{regs} to effector T cells. The addition of HDACi (four compounds assessed – SAHA, KA185, KA142 and KA1010, at nanomolar concentrations) resulted in a modest though relevant inhibition of responder cell proliferation from around 75% in the control group to 86-90% in the treatment groups (at a T_{reg} to responder ratio of 1:1). All of the HDACis tested inhibit class II, supporting the notion that this is a class-specific effect and that the treatment of T_{regs} does indeed potentiate their suppressive ability.

Given that HDACi have an apparent effect on regulatory T cells, the final experiment undertaken in this part of the study considering basic T cell physiology, was to consider the impact on markers of lymphocyte subsets (CD3, CD4, CD8 and CD19). There is very little evidence that this effect of HDACis has been assessed previously. In our assessment of the flow cytometry data, we found that the pan-HDACi, SAHA had a profound dose-dependent effect resulting in a large increase in the CD4 and CD19 populations compared to control (1078.9% and 181% respectively) and a decrease in the CD3 and CD8 populations (69.2% and 88.5% respectively), when treated at a dose of 1000 μ M. The reasons for such a sizeable shift in phenotype are unclear, and if these results are not aberrant, it may be postulated that the changes observed are due to the non-specific nature of this compound or a related toxic effect. The effect of KA1010 on lymphocyte phenotype was also interesting. At the lower dose range (1-100 μ M) very little effect was observed. However at the higher dose (of 1000 μ M) there was a subtle shift resulting in a reduction in CD8 expression by 17.0% and a corresponding increase in CD4 expression by 31.1%. Unfortunately, the CD4 population was not scrutinised further, but given that HDAC6 inhibition appears to enhance the T_{reg} population, there is a question as to whether this result may be due to an expansion of this subtype.

Following on from these initial studies on the *in vitro* effects of HDACi on lymphocyte physiology it was concluded, that the agent with the greatest potential in terms of immunosuppressive effect was the HDAC6i, KA1010. In addition to the evidence presented here, there has been significant research into both the selective inhibition (in terms of class of HDAC) and specific inhibition of individual HDACs. Of particular interest, and with relevance to transplantation, were findings reported by Professor Wayne Hancock's

laboratory in Philadelphia. Here they found that the HDAC6 protein was expressed at levels a number of fold higher in T_{regs}, compared to conventional T cells (de Zoeten et al. 2011). Around the same time, they were also able to show that treating mice with an alternative HDAC6 inhibitor doubled the expression of FoxP3 in regulatory T cells as well as a number of T_{reg} associated genes (CTLA-4, IL-10, PD-1 and GITR) and down-regulated IL-2 and IFN- γ gene expression. However, this effect was mediated by Tubacin, a selective HDAC6i with limited clinical relevance due to its non-drug-like structure, difficult synthesis and high lipophilicity (Butler et al. 2010) making it more a useful tool for research, rather than an agent with potential application in transplantation. As a result however, the Tubacin molecule has undergone subsequent bioengineering and a new HDAC6i, Tubastatin A has been developed (though less selective for HDAC6 and therefore requires higher dosing). Treatment of murine T_{regs} *in vitro* with this drug also demonstrated an increased suppressive activity of T_{regs} on a T cell proliferation assay. Similar assays using conventional T cell responders from mice lacking HDAC6 (HDAC6 knockout or HDAC6^{-/-} mice) demonstrated a similar result, suggesting the effect was not dependent on HDAC6 expression in T con cells. Tubastatin A is currently undergoing testing as a therapy for neurodegenerative disorders.

Research using HDAC6^{-/-} mice has provided useful information into the possible mechanisms by which HDAC6i agents have an immunosuppressive effect. These mice undergo normal lymphocytic development though do have higher than normal levels of hyperacetylated α -tubulin in several tissue types (Zhang et al. 2008). Assessment of their regulatory T cells reveals an effector-memory phenotype (T_{REM}, with typically reduced expression of CD44 and CD62L and increased CD103 expression). A feature of T_{REM} cells,

relevant to the transplant setting, is that they are capable of activation, expansion and memory in response to localised inflammation (Kleinewietfeld et al. 2005).

More recently, another selective HDAC6i, ACY-738, has been tested in NZB/W mice in the treatment of SLE. It was found to increase the proportion of regulatory T cells, while also alleviating some of the hallmarks of SLE (unpublished data). Further studies, using ACY-738 in conjunction with a sirtuin 1 inhibitor, found there was an increase in the suppressive ability of T_{regs} (Beier et al. 2012).

Given our initial findings that HDACi are generally safer compounds with an inhibitory effect on proliferation, and the evidence above, KA1010 presented as a viable agent to take forward into *in vitro* and *in vivo* models of transplantation. The first of these undertaken was a one-way MLR. These assays were originally used in order to test HLA matching and assess whether or not recipient lymphocytes would react to donor alloantigens. Though now superseded by other more technological methods clinically, this assay still provides a valid, if not basic platform to assess the effects of HDACi treatment.

The first assay in this series assessed the effect of cyclosporin, SAHA (pan-HDACi) and KA1010 (HDAC6i) treatment on allogeneic responders. We found that at a dose of 200ng/ml (equivalent to the standard clinical range), CyA had a moderate inhibitory effect reducing proliferation of the parent population by 18.5%. Two doses of the HDACi compounds were compared. At the lower dose of 0.5µM, neither had a particularly marked effect, though SAHA treatment did result in a statistically significant reduction in proliferation of 14%. At the higher dose of 5µM however, both compounds had a marked, statistically significant effect. Treatment with SAHA elicited the greatest response with a 47% reduction in proliferation, however, as has become increasingly evident in this study, the lack of

specificity associated with this compound resulted in considerable toxicity and much of this effect was likely due to cell apoptosis. Though the effect of KA1010 treatment was not quite as profound at 31% it was still statistically highly significant. Closer examination of the flow cytometry plots reveals further evidence of a toxic effect with high dose pan-HDACi treatment. Analysis demonstrates a living population in the KA1010 treated sample of 65-70%, consistent with the control, as compared to 20-25% cells surviving SAHA treatment.

In order to confirm that the effect demonstrated by these compounds was indeed a dampening of the alloresponse, and that autologous effector cells were remaining largely unaffected, a novel 'killing' assay was developed, based on the one-way MLR. This involved culturing both autologous and allogeneic targets, with the premise that the allogeneic population would be affected preferentially, and the autologous population would be relatively spared. Though the results of this assay were not as conclusive as hoped, for reasons that remain unclear, they do still support the hypothesis. Rather unexpectedly there was a significant lack of autologous survival over time, represented by a precipitous fall in cell number. However we were able to demonstrate that HDACi treatment resulted in a relative degree of allogeneic preservation with a 3.7-fold increase and 4.4-fold increase in allogeneic cell number over autologous (SAHA and KA1010 at a dose of 5 μ M respectively) at 72 hours. The effect of SAHA toxicity at this dose was not assessed in this assay.

Despite the apparent shortfalls of this novel assay, it did provide valid data on demonstrating a relative immunosuppressive effect by HDACi compounds. No similar assay has been reported and the results warrant its further development in terms of methodology.

A one-way MLR essentially represents the recipient response to donor antigen, but does not take into account the reciprocal effect of recipient antigen presentation (as in the indirect pathway). This assay may be partially representative of chronic rejection, but of course in reality, antigen presentation and recognition occurs bilaterally, particularly in the earlier stages following allotransplantation. It could be argued therefore, that an *in vitro* model of transplantation is better represented by a two-way MLR.

Freshly-isolated PBMCs from HLA-mismatched donors were used and IFN- γ release was adopted as the measure of alloreactivity in this assay. This important pro-inflammatory cytokine has a complex role in the alloresponse, usually in association with other cytokines (IL-1 β , TNF α). It is released primarily by activated T cells and NK cells as part of the effector response during allorecognition and results in induction of MHC expression in a number of tissues. It has both advantageous and disadvantageous roles in the transplant setting. For example, it has a pivotal role in the cellular immune response by stimulating macrophages to produce further pro-inflammatory cytokine responses. It also upregulates the expression of certain chemokines and adhesion molecules as well as enhancing MHC class II antigen presentation, resulting in increased effector cell recruitment (Ferrara et al. 2009).

Conversely, a number of *in vivo* transplant models using subjects deficient in IFN- γ demonstrate a protective role toward the graft. For example, islet transplants in mice lacking IFN- γ receptors undergo a rapid rejection process (Steiger et al. 1998) which has been suggested, is due to the inhibitory effect of IFN- γ on the proliferation of lymphocytes and cytotoxic T cell generation (Hassan et al. 1999; Konieczny et al. 1998). More recently, evidence has emerged suggesting IFN- γ may have a role in the promotion of T_{reg} function and / or expansion (Eljaafari, Li, & Miossec 2009). These effects of IFN- γ in the setting of

transplantation extend beyond the scope of this study and are made even more complex due to altered roles in acute and chronic rejection. They do however pose further questions regarding the role of HDACis (and their effect on IFN- γ production) and represent an area for further study. In the context of this study, IFN- γ release provides a sensitive and reliable measure of alloreactivity.

In a two-way MLR, we found that CyA, SAHA and KA1010 all had a statistically significant dose-dependent inhibitory effect. At the lower dose range of 0.3 μ M, IFN- γ release was reduced by 47%, 50% and 67% (CyA, SAHA and KA1010 respectively). At the higher dose range of 1 μ M the level of inhibition rose to 72%, 82% and 91% respectively.

These findings are in keeping with published data on the effect of HDACi on IFN- γ , primarily in autoimmune disease. In one such model of diabetic disease using rat islet cells, the effect of treatment with SAHA or TSA resulted in reduced IFN- γ mediated β -cell destruction and insulin production (Larsen et al. 2007). In another model of GVHD in murine bone marrow transplantation, treatment with pan-HDACi suppressed the release of a number of pro-inflammatory cytokines, including IFN- γ (as well as IL-1 and TNF- α) (Reddy et al. 2008a). A further study, using the synthetic pan-HDACi, ITF2357 at a dose of 25nM, found IFN- γ release was reduced by 50% (Leoni et al. 2005).

Current research suggests HDACs have a potential role in transplantation through both the innate and adaptive immune systems. As many of these roles are HDAC-specific, it is likely therefore that tailoring HDAC-specific inhibitors to modify relevant pathways is not only covetable but also increasingly plausible in the transplant setting.

For instance, HDAC5 has been shown to be upregulated during the differentiation of monocytes to macrophages (Baek et al. 2009). HDAC3 has been shown to negatively regulate myeloid cell differentiation (Ueki, Zhang, & Hayman 2008) and the function of dendritic cells and macrophages have been shown to be influenced by the production of HDAC regulated inflammatory mediators.

In general, the innate immune system is primarily regulated by Class I HDACs via regulation of the production of inflammatory cytokines. Class IIa HDACs have a pivotal role in the adaptive immune system, principally through the regulation of T cell function. Interestingly, it is likely that HDAC6 has a role in both pathways.

The role of HDACs in innate immunity

As part of the innate immune response, HDACs have been demonstrated to have both positive and negative regulatory effects over TLR pathways. The specific role of HDAC expression (and the specific HDACs involved) in encoding for pro-inflammatory cytokines such as IL6, IL12, TNF and IFN- β (Bode et al. 2007; Brogdon et al. 2007; Roger et al. 2011) is poorly understood, though it has been suggested that deacetylation of receptors in the TLR pathway are inherently implicated.

HDACs may have a positive effect on TLR signalling through the regulation of transcription factors, though this is somewhat controversial. In order for TLR-induced production of IFN, certain members of the interferon regulatory factor (IRF) transcription factor family are required (Gabriele & Ozato 2007). A number of HDACs have been demonstrated to be involved in multiple cell-specific TLR pathways, a full description of which goes beyond the remit of this thesis. Of relevance however to the allogeneic response is the TLR-mediated recruitment of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in

which it has been shown (through HDAC inhibition) that HDACs have a positive effect (Bode, et al. 2007; Roger et al. 2011). However, this is controversial and questioned by several groups.

It is more likely that the predominant role of HDAC regulation in TLR pathways is a negative one. Class I HDACs are most likely to invoke a negative regulatory response, through histone-dependent pathways resulting in transcription factor suppression. HDAC1 is capable of suppressing inflammatory gene promoter activity of several inflammatory cytokines (eg IL-12, COX2 and IFN- β). HDAC2 can impart a similar cytokine effect through the sequestration of specific transcriptional activators (Shakespeare, Halili, Irvine, Fairlie, & Sweet 2011). HDAC1, 2 and 3 are also capable of deacetylating NF- κ B (normally activated by TLR-ligation) allowing the binding of I κ B α (an inhibitory subunit) and switching off NF- κ B dependent responses (Chen & Greene 2003).

The role of HDACs with regard to interferon release is of particular relevance to this study and has been mentioned briefly. The exact mechanism by which Class I HDACs are involved is not clearly understood, though in contrast to their often transcriptional regression role, it would appear that they are necessary for the expression of IFN-stimulated genes (ISGs). The signal transducer and activator of transcription (STAT), regulates both type I IFN signalling (IFN- α and IFN- β) and type II (IFN- γ) which subsequently regulates the expression of ISGs (Tang et al. 2007). It is likely that class I HDACs are involved in the acetylation of STAT but conclusive evidence on their precise involvement remains elusive.

In addition to the role of Class I HDACs in the innate pathway, there is also increasing, putative evidence of Class IIa involvement (principally HDAC7) though this would appear to

be a myeloid-specific role in terms of chemokine regulation and unrelated to lymphocytes (Barneda-Zahonero et al. 2013).

Recent data has implicated a possible role of HDAC6 in the innate system through the regulation of macrophage responses. Macrophages are complex cells with both anti-inflammatory and pro-inflammatory roles depending upon their subtype (M1 or M2). In a study involving tubacin treated macrophages stimulated with LPS it was found that activation and production of pro-inflammatory cytokines was significantly reduced (Yan et al. 2014). It is likely that the role of HDAC6 in microtubule acetylation is responsible for this effect.

The role of HDACs in adaptive immunity

Class II HDACs have an inherent role in the development and function of T cells.

HDAC7 has an important role in cytotoxic T lymphocyte (CTL) function through the regulation of expression of adhesion molecules, cytokines and cytokine receptors (Navarro et al. 2011; Sweet et al. 2012). In activated T cells, the nuclear export of HDAC4 is critical in the expression of IL-5.

Of particular interest in the context of this study is the role HDAC9 has in T_{reg} homeostasis through its interaction with FoxP3. Increased numbers of T_{regs} have been demonstrated in HDAC9 knockout mice (de Zoeten, Wang, Sai, Dillmann, & Hancock 2010). As the results described above and others have demonstrated, pan-HDAC inhibition results in increased numbers and function of T_{regs} and this effect, at least in part, may well be due to an inhibitory effect on HDAC9. Unfortunately however, the clinical manipulation of this

mechanism is likely to remain elusive due to a current lack of HDAC9-specific inhibitor molecule.

Pivotal to this study is the role of HDAC6 and its effect on T_{regs} . The exact mechanism by which HDAC6 inhibition exerts an enhancing effect on T_{regs} is not well understood. It is likely its effect is not mediated through direct interaction with FoxP3 (as is the case with HDAC9), but rather through HSP90 and HSF1 as part of a multifaceted deacetylase-dependent and independent effect (these effects have been described in the Introduction).

In terms of evidence of a deacetylase-dependent role, HSP-90 in T_{regs} from HDAC6^{-/-} mice have been shown to be hyperacetylated and several associated HSF1 regulated genes upregulated (Hancock et al. 2012). The same study also demonstrated that in HDAC6^{-/-} T_{regs} , another heat-shock protein, HSP-70 forms a complex with FoxP3 (as a chaperone protein) which results in increased T_{reg} function and survival (Figure 6-1). In addition, they also demonstrated that inhibiting HSP-70 results in defective function and reduced survival and that upregulating HSP-70 has the opposite effect under cell stress conditions. Further evidence supporting this mechanism of action can be demonstrated through the effects of HSP90 inhibitors resulting in similar T_{reg} effects.

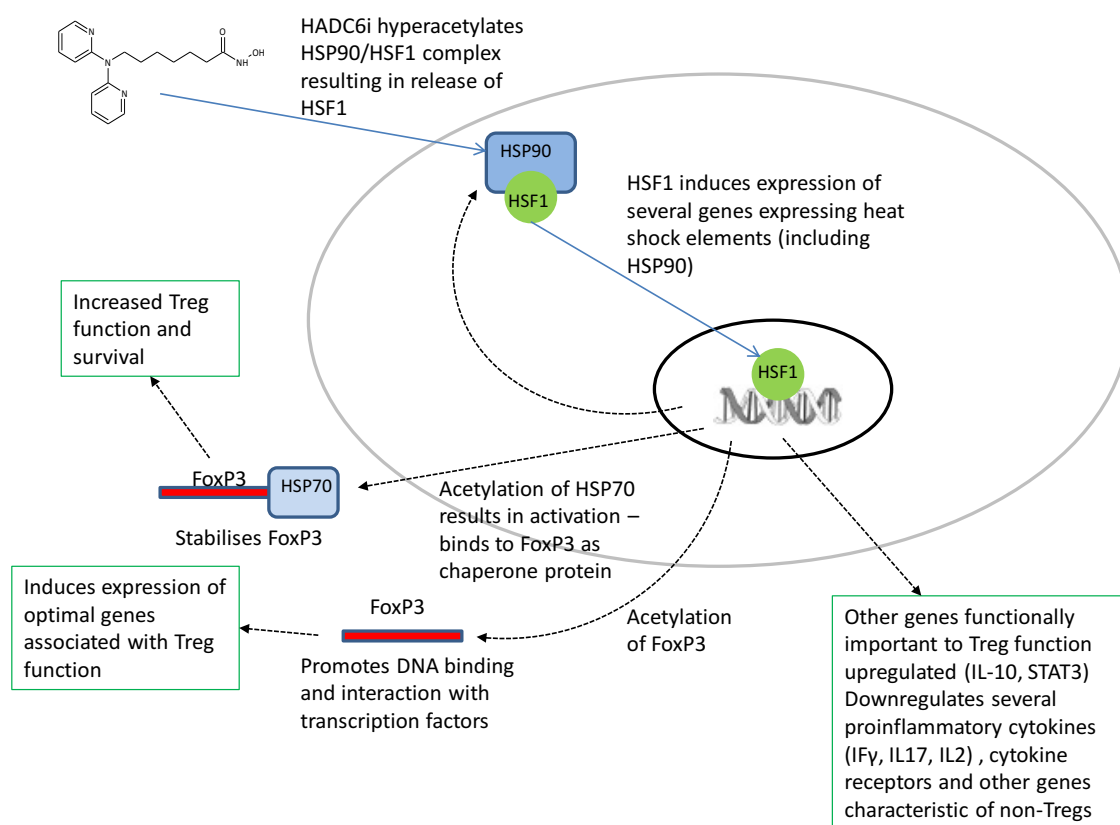


Figure 6-1 Potential mechanisms by which HDAC6i may potentiate regulatory T cells

We further demonstrated a predictable, dose-dependent effect of HDACi on the two-way MLR by producing dose response curves. In this *in vitro* model we found an IC₅₀ for CyA of 625nM, which was comparable to the published data. The effect of HDACi treatment was considerably more potent with an IC₅₀ of 82nM and 13nM for KA1010 and SAHA respectively. We also assessed the specificity of these compounds by calculating the Hill slope value for each (0.89 for KA1010, 0.31 for SAHA and 0.35 for CyA). This value is a marker of efficiency in terms of binding and almost certainly is a reflection of the specificity of the tested compounds with a more targeted effect of KA1010.

As has been described, we have demonstrated several HDACi-mediated effects on cellular proliferation. Therefore, as part of the two-way MLR we considered if CyA or HDACi altered the expression of resting CD4⁺ T cell activation markers. To achieve this we evaluated a standard panel of surface markers, namely CD69, CD25 and HLA-DR. We demonstrated a slightly superior effect of HDACi over CyA in reducing CD69 expression. In terms of CD25 expression, all HDACi permitted a transient rise at day two (as would be expected) with CyA treatment inhibiting any significant expression at all. None of the compounds had any effect on expression of HLA-DR. These observations, particularly those seen in CD25 expression likely reflect the different mechanisms of action for CyA (via its effect on IL-2) and HDACi (modulation of T_{regs}).

The final group of experiments presented here examine the effect of KA1010 on an *in vivo* murine skin graft model of transplantation. First described by Medawar in 1955, this highly immunogenic model provides a well established platform with which to assess the effect of MHC-disparate donor antigen presentation on recipient T cell responses. The procedure itself is relatively quick and straightforward to perform and is highly reproducible. The nature of the model allows assessment at both the macroscopic level through continued visual assessment and at a histological level through well characterised responses.

In this study, we found that the untreated allogeneic graft was macro- and microscopically rejected in 7-21 days while the syngeneic graft demonstrated negligible evidence of rejection up to the end of the assessed time period (up to 70 days). At a dose of 160mg/kg, treatment with KA1010 resulted in graft survival of 77% at 70 days and at the lower dose of 80mg/kg graft survival of 50%.

In terms of systemic effects, KA1010 was generally well tolerated. The effect of sustained treatment (daily *ip* injection for 70 days) was to prevent normal weight gain and maintain it at around 80% birth weight. However interpretation of this observation requires caution, as mice treated with KA1010 for a shorter period of time (28days) gained weight at a rate similar to control subjects. The reason for the disparity observed is not easily explained. There were no other statistically significant effects of HDACi treatment on weight.

Rapamycin (Sirolimus) is a naturally-derived macrolide with both immunosuppressive and anti-proliferative effects. The initial enthusiasm for rapamycin as an alternative *de novo* immunosuppressant has waned over recent years due to its side effect profile and its potential role in transplantation remains uncertain. It has been studied extensively, in particular as a possible adjunct or even alternative to current calcineurin-inhibitors though the potential advantage in terms of reduced nephrotoxicity and risk of malignancy, compared to CNIs have often been offset by other side effects such as impaired wound healing, hyperlipidaemia and thrombocytopenia.

Rapamycin shares many structural similarities to Tacrolimus, but rather than blocking the calcineurin pathway, it inhibits B and T cell proliferation by blocking the cytokine stimulated mTOR pathway. Interestingly, a number of studies have demonstrated a positive effect of rapamycin on T_{reg} expansion (Battaglia, Stabilini, & Tresoldi 2012; Gao et al. 2007; Segundo et al. 2006). As the potential effect of HDACi therapy is to mediate an immunosuppressive effect through upregulation of regulatory T cells, via an alternative mechanism, the potential effect of combining these two agents, but at lower dosage (in theory reducing potential side effects) was assessed.

In terms of systemic effects, Rapamycin at a dose of 50mg/kg either as a single agent or in combination with low dose KA1010 had no significant effect on weight or splenic index as compared to control. In order to assess the potential *in vivo* effects of KA1010 and rapamycin, CD4⁺ and CD4⁺CD25⁺ expression was evaluated. We found that rapamycin alone resulted in a significant reduction in CD4⁺ expression from 57.7% to 51.3% (representing an 11.1% reduction), though low dose KA1010 (80mg/kg) had a negligible effect. Rapamycin treatment in combination with low dose KA1010 resulted in a slightly greater effect (to 48.1%) but this apparent synergistic effect was not significantly superior to rapamycin alone. The effect on CD4⁺CD25⁺ expression was essentially reciprocal, though not significant. Rapamycin treatment alone resulted in a marginal increase in expression from 18.5% to 20.1%. Low dose KA1010 and combination treatment had no effect.

The role of rapamycin, in conjunction with specific HDAC6i, requires further investigation. In this study, we found that rapamycin treatment reduced the proportion of CD4⁺ T cells but had a minimal effect on T_{reg} numbers *in vivo*. Due to the plasticity between various T cell subsets, there is a differential response between T_{eff} cells and T_{regs} to rapamycin. These data are consistent with the previously published observations demonstrating a relative increased suppressive effect on effector T-cells over regulatory populations (Zeiser et al. 2008), (Keating et al. 2013).

The most significant effect on CD4⁺ and CD4⁺CD25⁺ expression resulted from KA1010 treatment at a dose of 160mg/kg. Treatment for 70 days significantly impaired CD4⁺ expression by 16.2% and had a corresponding positive effect on CD4⁺CD25⁺, increasing expression by 18.0%.

These observations support the hypothesis that KA1010 enhances the immunomodulatory effect of regulatory T cells and as a result proliferation of the effector T cell population is impaired. These findings are consistent with the limited published data assessing HDAC6-specific inhibition (as already described - HDAC6^{-/-} knockout mice and in mice treated with HDAC6i in models of inflammation and autoimmunity (Beier et al. 2011; Beier et al. 2012; de Zoeten et al. 2010).

6.1 Summary

Enormous advancements have been made over the last 50 years in terms of immunosuppression and the agents currently available today have evolved significantly resulting in improved graft survival. However current immunosuppressive regimens, based on the ELITE-Symphony study (Ekberg et al. 2007) are far from perfect and little has changed over the last twenty years in terms of side effects and graft survival rates. As the evolution of transplantation continues and the boundaries of what is possible are pushed further (for example composite tissue transplantation), it is likely that newer, cleaner and more efficient immunosuppressants are going to be needed to keep pace. One such group of compounds which has shown potential in this respect are the histone deacetylase inhibitors. Though their clinical role has been limited to haematological malignancies at present, pre-clinically they have shown great potential in the management of a number of other malignancies and inflammatory conditions.

As a result of this interest in HDACi, discussions with Karus therapeutics led to the development of an academic-industrial partnership which has resulted in this study. They were able to provide us with a panel of optimised, novel engineered molecules with specific

targets, upon which we were able to perform a number of step-wise investigations. Firstly, we assessed simple effect and toxicity at a cellular level and considered mechanistic elements of an apparent inhibitory function. We then used robust *in vitro* models of alloreactivity, many of which have been used in the development of current immunosuppressants, and compared the effects of our candidate novel compound with appropriate well characterised compounds. This revealed a potent and predictable immunomodulatory effect for KA1010 with apparently reduced toxic effects compared to less specific HDACi. Finally we used a murine model of transplantation to demonstrate the immunosuppressive effectiveness and tolerability of HDAC6i treatment.

In attempting to elucidate a mechanism of action, we have considered a number of likely contributory factors. The likely mechanism of action of KA1010 in terms of its clinical application is almost certainly a combination of both acetylase dependent and independent effects. In this study, we found that there was a significant effect on the function and number of regulatory T cells and it is likely this is mediated through the HSP90/HSF1 pathway described above.

Overall, this study adds weight to the argument that HDACi analogues have clinical utility, with potentially favourable toxicity profiles compared with existing immunosuppressive agents. The availability for safer treatment regimens has the potential to enhance organ survival and permit broader application of organ replacement for patients with organ failure.

6.2 Limitations of the study

The primary limitations in this study involved the *in vivo* model used. There are obvious limitations to this murine model and translating the above findings into the clinical setting is

somewhat limited by the differences in human and mouse physiology, genetics and immunology. The graft itself is essentially non-vascularised and therefore, ischaemia may contribute significantly to the immune response. In addition to this, as well as tissue-specific antigens the skin also contains Langerhans cells (specific antigen presenting cells within the skin) which may skew the immune response by activating host T cells. One further consideration is graft size. It has been suggested that larger grafts contain a larger number of APCs and, therefore, may invoke altered host responses (Sun et al. 1996). However, further detailed research comparing pancreatic islet, cardiac and skin transplant rejection found that numbers of APCs or tissue specific antigens had little effect on the degree of the host T cell response. Rather, it has been suggested that factors such as graft micro-environment and size have more of an important role in rejection than number of APCs (Jones et al. 2001). Therefore, a standardised protocol was used when grafting to ensure skin samples were similar and minimise this potential factor.

Further limitations of this study concern the established immunosuppressants used as comparison to the novel compounds tested. Though CyA is a very well characterised drug, its use in modern immunosuppression is dwindling. In hindsight comparing effects with Tacrolimus would have been favoured. Fortunately both CyA and Tacrolimus have similar mechanisms of action and the findings therefore remain valid.

Finally, the novel MLR killing assay has shortcomings which we were unable to overcome. For reasons unknown, the autologous population underwent significant inhibition when it would have been expected to be better preserved. Fortunately a differential effect was demonstrated. With some modifications and assay development, this could become a useful tool.

6.3 Further studies

Throughout this study, the targeted inhibition of specific HDACs has been central. Of particular interest in the context of transplantation is the potential role of an HDAC9i molecule. Internationally, many groups are focussing their efforts on harnessing the inhibitory effects of regulatory T cells for a number of therapeutic applications. From the studies reviewed here, the role of HDAC9 (through HDAC9 knockout studies) in T_{reg} regulation may be of significant benefit. Unfortunately, no compound is yet available which can target HDAC9 specifically. If one were to be developed, it may represent a potential therapeutic drug which could be effective in its own right or in combination with HDAC6 targeting.

The potential role of Rapamycin has been briefly assessed in this project, but was only considered at the *in vivo* stage. Further assessment of its potential use as a combination drug with HDAC6i may reveal a possible novel role.

Though the skin graft model adopted for this study was useful in the initial assessment of our novel compound, the next stage would be to examine its effects in a vascularised model. Previous studies have demonstrated a difference in rejection mechanism between vascularised and non-vascularised models, particularly in terms of routes of migration of donor dendritic cells (in the skin graft model, donor DCs tend to migrate via lymphatic drainage before vascular connections have been established). A large number of vascularised graft models would be appropriate. In terms of this project, a murine sub-capsular foetal pancreatic islets model is planned as well as a rat cardiac allograft model.

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Publications / Presentations related to this study

Publications

- Ellis, J., Inston, N., Ready, A., Jenkinson, E., Drayson, M., Shuttleworth, S. & Cobbold, M. (2016) Inhibition of Histone Deacetylase 6 reveals a potent Immunosuppressant effect in an in vitro model of transplantation – Published in Transplantation (Aug 2016)
(see Appendix 1)

Oral Presentations

- A Novel Histone Deacetylase-6 Inhibitor Reveals a Potent Immunosuppressant Effect – *World Transplant Congress, San Francisco, USA* (July 2014)
- Inhibition of Histone Deacetylase 6 reveals a potent immunosuppressive effect in an in vitro model of transplantation - *16th Congress of the European Society for Organ Transplantation, Vienna, Austria* (September 2013)
- Histone Deacetylase Inhibition reveals a potent immunosuppressant effect in an in vitro model of Transplantation – *15th Congress of the European Society for Organ Transplantation, Glasgow, UK* (September 2011)
- Histone Deacetylase-6 (HDAC6) plays a critical role in Dendritic Cell maturation and reveals a new mechanism for HDAC-targeted immunosuppression - *15th Congress of the European Society for Organ Transplantation, Glasgow, UK* (September 2011)

- Immunosuppressive effect of novel histone deacetylase inhibitors and their potential role in renal transplantation – *European Society for Surgical Research 45th Annual Congress, Geneva, Switzerland* (June 2010)

Poster Presentations

- Histone Deacetylase Inhibition reveals a potent immunosuppressant effect in an in vitro model of Transplantation - *XII TTS Basic Science Symposium and II ESOT Basic Science Meeting, Boston, USA* (June 2011)

7 Appendix 1

Paper published in Transplantation

Ellis, J., Neil, D., Inston, N., Jenkinson, E., Drayson, M., Hampson, P., Shuttleworth, S., Ready, A. & Cobbold, M. (2016) Inhibition of Histone Deacetylase 6 Reveals a Potent Immunosuppressant Effect in Models of Transplantation. *Transplantation*, vol. 100(8):1667-74. doi: 10.1097/TP.0000000000001208.

