# Investigation of T-cell suppression in the acute myeloid leukaemia microenvironment

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# INVESTIGATION OF T-CELL SUPPRESSION IN THE ACUTE MYELOID LEUKAEMIA MICROENVIRONMENT

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## Declaration

I hereby declare that the work presented in this thesis is my own, except where stated otherwise.

## Abstract

Allogeneic haematopoietic stem cell transplantation (HSCT) is a curative treatment for acute myeloid leukaemia (AML) in which donor-derived anti-leukaemia reactive T cells have an important role in disease control. However, relapse rates are high. AML is known to create an immunosuppressive microenvironment that likely limits the efficacy of allogeneic HSCT, and perhaps will also constrain other novel immunotherapeutic approaches being developed for the treatment of AML. Previous *in vitro* studies have investigated suppression of T cells by AML cells or supernatants from AML cell cultures. In this study, an *in vitro* model was established in which T cells were cultured in the presence of AML cells with the HS5 stromal cell line to better recapitulate the AML microenvironment.

The expression of activation markers, proliferation and cytokine production by $CD4_+$ and $CD8_+$ T cells from healthy individuals stimulated non-specifically with $\alpha$CD3/CD28 antibody coated-beads were significantly suppressed in the presence of AML cells with stromal cells. Inhibition was observed both in the context of immortalised AML cell lines and primary AML cells. A finding with therapeutic implications was that the AML microenvironment suppressed the activity of T cells from allogeneic HSCT patients and two types of leukaemia-reactive T cells that are being championed as potential novel immunotherapeutic strategies. The ability of $V\delta2_+$ $\gamma\delta$ T cells to lyse zoledronic acid sensitised AML cells was suppressed in the presence of stromal cells. Evaluation of $\alpha$CD123 CAR T-cell recognition of AML cells in the presence of stromal cells showed that both cytolytic activity and cytokine production were significantly suppressed in the AML microenvironment. Investigation of the mechanistic basis for suppression indicated roles for both soluble factors and contact between T cells and AML cells. Neither immune checkpoint blockade with anti-PD-1 or anti-PD-L1 antibodies or the immunomodulatory drug lenalidomide counteracted suppression. Analysis of the gene expression profile of CD8+ T cells stimulated with $\alpha$CD3/CD28 antibody coated-beads in the presence of AML cells with HS5 cells showed only subtle changes indicative of diminished overall activity. Of interest, responses to stimulation with $\alpha$CD3 antibody coated-beads were not suppressed in the AML microenvironment, suggesting that inhibition may target the CD28 pathway. Consequently, naive and quiescent memory T cells that express CD28 might be more susceptible to suppression in the AML microenvironment.

In summary, an *in-vitro* co-culture model has been established that recapitulates inhibition of T-cell activity in the AML microenvironment. The platform can be used to investigate the important problem of how to circumvent suppression and thereby improve efficacy of current and new immunotherapeutic strategies that are being developed.

# Table of Contents

# Table of Figures

# Table of Tables

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# Abbreviations

| Abbreviation | Meaning |
|---|---|
| ALL | Acute Lymphoblastic Leukaemia |
| AML | Acute Myeloid Leukaemia |
| APC | Antigen Presenting Cell |
| ATP | Adenosine Triphosphate |
| Bcl | B-cell Lymphoma |
| BiTE | Bispecific T-cell Engager |
| BM | Bone Marrow |
| BMSC | Bone Marrow Stromal Cell |
| CAR | Chimeric Antigen Receptor |
| CD | Cluster of Differentiation |
| CDR | Complementarity Determining Regions |
| CEBPA | CCAAT Enhancer Binding Protein Alpha |
| CFSE | Carboxyfluorescein Succinimidyl Ester |
| CIK | Cytokine Induced Killer |
| CK | Complex Karyotype |
| CLIP | Class II Associated Invariant Chain Peptide |
| CLL | Chronic Lymphocytic Leukaemia |
| CMV | Cytomegalovirus |
| CTLA-4 | Cytotoxic T-Lymphocyte Associated Protein 4 |
| DC | Dendritic Cell |
| DLI | Donor Lymphocyte Infusion |
| ELN | European LeukaemiaNet |
| FACS | Fluorescence Activated Cell Sorting |
| FLT3 | Fms-related Tyrosine Kinase 3 |
| FLT3-ITD | Fms-related tyrosine kinase 3 – Internal Tandem Duplication |
| FoxP3 | Forkhead Box P3 |
| G-CSF | Granulocyte Colony-Stimulating Factor |
| GM-CSF | Granulocyte Macrophage Colony-Stimulating Factor |
| GvHD | Graft versus Host Disease |
| GvL | Graft versus Leukaemia |
| HLA | Human Leukocyte Antigens |
| HSC | Haematopoietic Stem Cell |

| | |
|---|---|
| HSCT | Haematopoietic Stem-cell Transplant |
| iC9 | Inducible Caspase 9 Suicide Gene |
| IDO | Indoleamine 2,3-Dioxygenase |
| IFN-γ | Interferon - gamma |
| IKK | Inhibitor of NFκB Kinase |
| IL-10 | Interleukin 10 |
| IL-12 | Interleukin 12 |
| IL-15 | Interleukin 15 |
| IL-2 | Interleukin 2 |
| IL-3 | Interleukin 3 |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| IRF | Interferon Regulatory Factor |
| KLRG-1 | Killer Cell Lectin-like Receptor Subfamily G Member 1 |
| LAA | Leukaemia Associated Antigen |
| LSA | Leukaemia Specific Antigen |
| MAC | Myeloablative Conditioning |
| MAGE-A1 | Melanoma-associated antigen 1 |
| MAPK | Mitogen Activated Protein Kinase |
| Mb | megabase |
| MDSC | Myeloid Derived Suppressor Cell |
| MFI | Median Fluorescence Intensity |
| mHag | Minor histocompatibility antigen |
| MHC | Major Histocompatibility Complex |
| MIP-1α | Macrophage Inflammatory Protein 1 alpha |
| MIP-1ß | Macrophage Inflammatory Protein 1 beta |
| mP3 | Membrane Proteinase 3 |
| MSC | Mesenchymal Stromal Cell |
| NAD | Nicotinamide Adenine Dinucleotide |
| NFκB | Nuclear Factor kappa B |
| NFAT | Nuclear Factor of Activated Tcells |
| NHS-BT | National Health Service Blood and Transplant |
| NK | Natural Killer |

| | |
|---|---|
| NPM1 | Nucleophosmin 1 |
| PAMP | Pathogen-associated molecular patterns |
| PBMC | Peripheral Blood Mononuclear Cells |
| PD-1 | Programmed Death Protein 1 |
| PD-L1 | Programmed Death Ligand 1 |
| PKC | Protein Kinase C |
| PMA | Phorbol 12-myristate 13-acetate |
| PSMA | Prostate Specific Membrane Antigen |
| PTLD | Post-Transplant Lymphoproliferative Disorder |
| RAG | Recombination Activating Genes |
| RHAMM | Receptor for Hyaluronic Acid Mediated Mobility |
| RIC | Reduced Intensity Conditioning |
| RNA | Ribonucleic acid |
| SCF | Stem-cell Factor |
| scFv | Single-chain Variable Fragment |
| SOCS1 | Suppressor of Cytokine Signalling 1 |
| TAA | Tumour Associated Antigen |
| TCR | T-cell receptor |
| Teff | Effector T cells |
| TGF-ß | Transforming Growth Factor - beta |
| Th | T-helper |
| TIGIT | T-cell Immunoreceptor with Ig and ITIM Domains |
| TIM-3 | T-cell Immunoglobulin and Mucin-domain Containing Protein 3 |
| TNF-$\alpha$ | Tumour Necrosis Factor - alpha |
| TNFR1 | TNF receptor 1 |
| TRADD | TNFR1-associated Death Domain Protein |
| Treg | Regulatory T cells |
| TRM | Transplant Related Mortalities |
| TSA | Tumour Specific Antigen |
| VC | Vehicle Control |
| WT1 | Wilms' Tumour antigen 1 |
| ZA | Zoledronic Acid |

# Chapter 1 Introduction

## 1.1 Cancer immunology

### 1.1.1 Introduction to T-cell mediated immunity

The immune system is divided into two distinct arms, known as the innate and adaptive immune responses, that work synergistically to protect the host. Innate immunity is the first line of defence, as it is immediate, and relies on limited numbers of germline encoded receptors and proteins for the recognition and elimination of pathogens. Common motifs of pathogens such as lipopolysaccharides or unmethylated CpG DNA, also collectively known as pathogen-associated molecular patterns (PAMPs), are recognised by pattern recognition receptors and pathogens eliminated through the complement pathways or anti-microbial enzymes or peptides[1, 2]. Innate immunity does not generate long-term immunological memory, whereas adaptive immunity is capable of forming memory cells that provides long-term specific immunity[1]. Adaptive immune responses are initiated by dendritic cells that present antigens, engage co-stimulatory molecules and provide a third cytokine-mediated signal that primes naive T cells over 2-3 days, inducing their proliferation, differentiation, and cytokine production[2]. Activated T cells can differentiate into the memory phenotype which are long-lived cells that rapidly respond upon re-exposure to the same antigen[1].

T cells are central to adaptive immunity and play an important role in the elimination of foreign pathogens and tumours. T-cell receptors (TCR) recognise protein fragments, known as peptides, that are bound to major histocompatibility complex (MHC) molecules, called human leukocyte antigens (HLA) in humans, on the surface of an antigen presenting cell (APC). The name T-cell refers to the thymus where these cells mature. During the maturation process, T cells undergo positive and negative thymic selection. The positive stage selects for T cells that are capable of interacting with self-MHC, which promotes T-cell survival[3]. Negative selection eliminates T cells that bind to self-MHC/peptide complexes with high avidity, which underpins the concept of immune tolerance[4]. After thymic selection, T cells circulate throughout the blood and are termed ‘naïve’ because they have not yet encountered their cognate antigen.

There are two MHC subtypes: MHC class I and MHC class II. MHC class I molecules bind short peptides with a length of 8-10 amino acids, that are derived from proteasome cleavage of cytosolic proteins, and present them to $CD8_+$ T cells. MHC class II molecules bind slightly longer peptides, with a length of 13-17 amino acids, that are derived from exogenous proteins internalised into endosomes, and present them to $CD4_+$ T cells. Naïve T cells can only recognise peptides presented by dendritic cells, whereas primed T cells can subsequently recognise peptides presented by macrophages and B cells as well. To fully activate naïve T cells, a second signal known as co-stimulation via CD28 is required, that triggers entry into the $G_1$ phase of the cell cycle[5]. The ligands for CD28 are CD80 (B7.1) and CD86 (B7.2), which are part of the B7 family. Expression of these co-stimulatory molecules by dendritic cells, together with their provision of a third signal, such as IL-12, ensures that naive T cells are only fully activated under appropriate conditions[6]. Studies have shown the importance of IL-12 in providing the third signal to naïve $CD8_+$ T cells as the presence of IL-12 is needed for clonal expansion, development of effector functions, and the generation of memory phenotype[7, 8, 9].

### 1.1.2 T-cell subsets

T cells that express $\alpha\beta$ form of the TCR are the most abundant type and are composed of two main classes that are distinguished based on expression of the cell-surface proteins CD8 or CD4. Another subset of T cells express the $\gamma\delta$ TCR. They typically comprise 0.5% - 5% of the T-cell population and have both innate and adaptive features[10]. The TCRs of both αβ and γδ T cells are formed by somatic rearrangement of V,D and J gene segments, also known as VDJ recombination, and is of fundamental importance to the diverse repertoires of antigen receptors as the TCR diversity derives from the multiple combinations of possible VDJ gene segments[11]. The TCR $\beta$ and TCR $\delta$ are assembled from V, D, and J segments, whilst TCR $\alpha$ and TCR $\gamma$ are assembled from V and J segments[11]. The VDJ recombination occurs only in developing lymphocytes and is initiated by the recombination activating genes-1 and -2 (RAG-1 and RAG-2 respectively)[11, 12, 13]. Importantly, the ability to initiate VDJ rearrangement is critical to the development of mature lymphocytes as either RAG-1 or RAG-2 deficient mice have been reported to have no mature B and T cells leading to immunodeficiency[13, 14]. The site of contact with peptide

for TCR is the complementarity-determining regions (CDR) 3 with an estimated size of the TCR repertoire to be up to $10^{16}$ [15, 16].

#### 1.1.2.1 CD8+ T cells

$CD8^+$ T cells, also known as cytotoxic T cells, are intrinsic to the adaptive immune response against intracellular antigens expressed by virus infected cells or transformed tumour cells. As mentioned previously, $CD8^+$ T cells recognise peptides presented by MHC class I molecules, which can be found on all nucleated cells. When $CD8^+$ T cells encounter antigen and are activated, they deploy several mechanisms to kill target cells. They can secrete cytokines such as TNF-$\alpha$ and IFN-$\gamma$ [17, 18]. TNF-$\alpha$ is a pro-inflammatory cytokine which has been shown to mediate NF$\kappa$B activation by engagement with TNF receptor 1 (TNFR1)[19]. The engagement of TNF-$\alpha$ to TNFR1 leads to the recruitment of TNFR1-associated death domain protein (TRADD), which then forms a complex with serine-threonine kinase receptor-interacting protein and TNF-receptor-associated factor 2[20]. The complex then recruits and activates IKK leading to the phosphorylation of inhibitory protein I$\kappa$B and activation of NF$\kappa$B[21]. In addition to being a pro-inflammatory cytokine, engagement of TNF-$\alpha$ to TNFR1 can also induce apoptosis through the association of TRADD and Fas-associated protein with death domain[22]. Another pro-inflammatory cytokine is IFN-$\gamma$. IFN-$\gamma$ signals through JAK/STAT pathway and activates transcription factor IRF-1, which has been shown to play an important role in the induction of both Class I and Class II MHC which allows the presentation of endogenous peptides to $CD8^+$ T cells and to promote $CD4^+$ T-cell activation respectively[23, 24]. Furthermore, IRF-1 is able to induce genes such as protein kinase R and iNOS that have anti-viral and anti-bacterial effects respectively[25, 26].

$CD8^+$ T cells are also able to release cytotoxic agents, such as perforin and granzyme B, in addition to cytokines. Perforin is a protein that forms pores in the target cell membrane, allowing proteases such as granzyme B to enter and results in the apoptosis of target cells[27, 28]. Granzyme b induced apoptosis can be mediated through 2 different pathways, caspase dependent and caspase independent pathways. The caspase dependent pathway of apoptosis is achieved by the activation of caspase 3 via granzyme b[29]. Caspase independent pathway of apoptosis is achieved by the cleavage of BH3 interacting-domain death agonist leading to mitochondrial

perturbation and thus apoptosis[30]. Cytotoxic granules containing perforin and granzymes are released along the point of direct contact between T cells and target cells known as the immune synapse, which avoids bystander damage. $CD8_+$ T cells are also able to induce cell death via the Fas / Fas ligand (FasL) interaction. $CD8_+$ T cells that express FasL on their surface bind to Fas expressing target cells, which activates the caspase cascade resulting in apoptosis of the target cell[31].

#### 1.1.2.2 $CD4_+$ T cells

$CD4_+$ T cells are known as T-helper cells because they assist promotion of antibody production by B cells, activation of $CD8_+$ T-cell responses, and recruitment of innate immune cells to sites of infection[32]. $CD4_+$ T cells mediate their helper function via the production of cytokines and chemokines[32]. They can be subdivided into at least six subtypes that include Th1, Th2, Th9, Th17, Tfh, and regulatory T cells (Treg) (Figure 1-1)[33]. The combination of cytokines in the environment in which naïve $CD4_+$ T cells are primed determines the helper T-cell subtype they become, and the differentiation state can subsequently be altered if the cytokine environment changes[33]. Th1, Th2, and Treg subtypes are known to play important roles in cancer immunology. Th1 cell differentiation occurs when naïve $CD4_+$ T cells are stimulated in the presence of IL-12, type I IFNs or IFN-γ, which leads to upregulation of the T-bet transcription factor expression[34, 35]. Th2 differentiation requires the presence of IL-4 and IL-2, which promote activation of Stat6 and Stat5 respectively, resulting in upregulation of the GATA-3 transcription factor[36, 37]. The immunosuppressive cytokine TGF-β induces Treg differentiation from naïve $CD4_+$ T cells by activation of Smad3, that combines with NFAT activation induced by stimulation via the TCR to result in upregulation of the Foxp3 transcription factor[38]. The Th1 immune response is able to elicit tumour destruction, which is mediated by the secrete of IFN-γ and TNF-α, and the production of IL-2 that supports $CD8_+$ T cells[39]. However, the Th2 immune response has been shown to be immunosuppressive and favours tumour growth by promoting tumour associated macrophages to differentiate to the M2 phenotype[39]. Tregs also favour tumour survival by inhibiting effector T-cell function via mechanisms that include secretion of the immunosuppressive cytokines TGF-ß and IL-10[39].

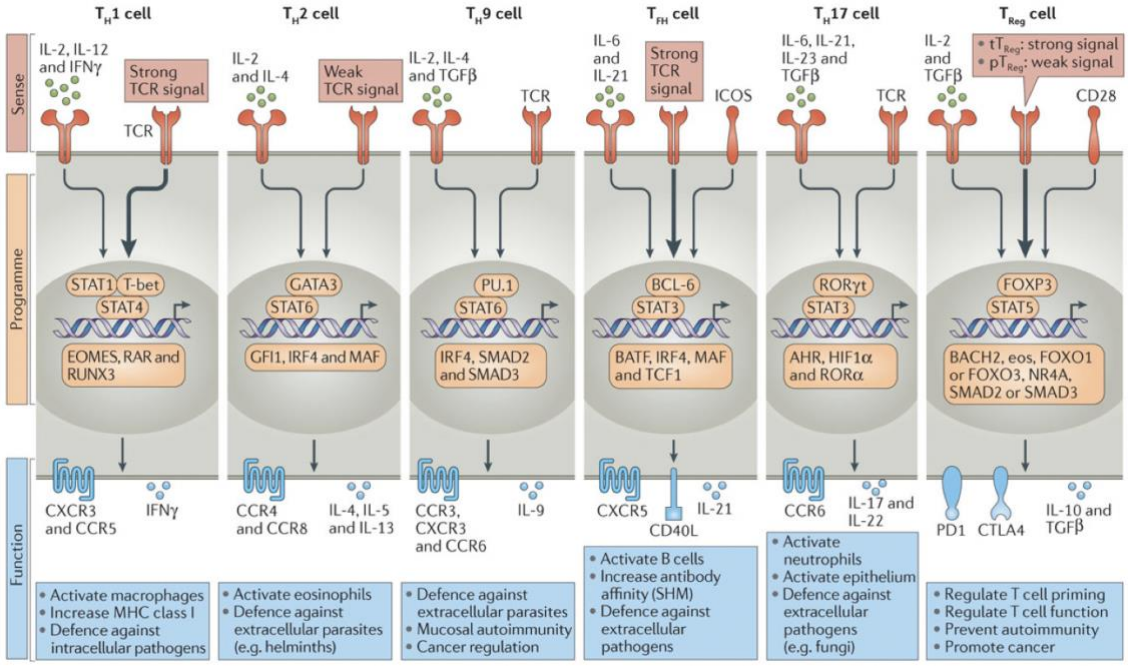



**Figure 1-1 Summary of the CD4 T-helper cells**

Differentiation pathways of naïve CD4+ T cells in the presence of different cytokines, together with the functions, cytokines produced and the transcription factors for the Th1, Th2, Th9, Tfh, Th17, and Treg subtypes are shown. (adapted from[33])

### 1.1.2.3 γδ T cells

In contrast to αβ T cells, γδ T cells are not restricted to peptides bound and presented by MHC molecules[10]. The activation of γδ T cells does not require MHC class I or MHC class II molecules and a broad range of non-peptide antigens are recognised[40], including exogenous molecules and autoantigens, such as mycobacterial heat-shock proteins[41], phycoerythrin[42], phosphoantigens[43], and MHC class I chain-related A[44]. The ability for γδ T cells to recognise both exogenous and endogenous antigens underlies the capacity to respond to both foreign pathogens and stressed-self. Furthermore, γδ T cells are considered as rapid responders because adoptively transferred γδ T cells into immunodeficient mice were able to mount an immune response against bacterial infection within one day[45].

Similar to CD4+ T cells, there are several different subsets of γδ T cells. In humans, Vγ9Vδ2+ (Vδ2+) cells are the main subset in peripheral blood, typically comprising >60% of all γδ T cells[46]. It has been shown that Vδ2+ T cells have functions similar to those of CD8+ T cells; namely

cytokine secretion, such as TNF-α and IFN-γ, and cytotoxic functions. The Vδ2+ subset is activated by phosphoantigen that is bounded by the intracellular domain of butyrophilin 3A1[47, 48]. In addition, the use of aminobisphosphonates, which inhibits a downstream enzyme in the mevalonate pathway, causes the accumulation of isopentenyl pyrophosphate thus sensitising cells to the recognition by Vδ2+ T cells[49]. When Vδ2+ T cells encounter phosphoantigen, and are activated, they deploy several mechanisms to kill target cells similar to that of CD8+ T cells. They can secrete pro-inflammatory cytokines (such as TNF-α and IFN-γ) and the release of perforin/granzyme b[50]. In addition to phosphoantigens, Rincon-Orozco et al reported that Vδ2+ T cells could also be directly activated through binding of natural killer cell activating receptor,NKG2D, to its ligand, MHC class I related protein A[44]. Furthermore, Vδ2+ T cells have been shown to display cytolytic activity against leukaemia cells and several other tumour cell lines[51, 52, 53, 54]. Lastly, studies have shown the role of γδ T cells in cancer surveillance as mice lacking γδ T cells are highly susceptible to cancer development[55].

### 1.1.3 The concept of cancer as altered-self

Cancer arises from normal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissue. Six hallmarks of cancer have been defined (Figure 1-2) and are referred to factors that promote and sustain the uncontrolled proliferation of cancer cells[56]. In some cancer types, the transformation event that leads to uncontrolled cell proliferation is initiated by an oncogenic virus, but the driver for the majority of cancers is acquisition of genetic mutations[57].

There are multiple mechanisms operating to direct the immune response to foreign pathogens and prevent autoimmune responses against normal self-tissue. These include the negative selection of T cells during their development in the thymus (termed central tolerance), immunosuppressive Tregs (contributors to peripheral tolerance) and control of T cell priming by dendritic cells to ensure activation only occurs when three signals combine. Immune tolerance of self, however, hinders recognition of most cancers. Only tumours driven by oncogenic viruses express foreign pathogenic antigens that are easily recognised by the immune system. Tumours driven by oncogenic mutations are known as 'altered-self'; they are not overtly foreign and

therefore difficult for the adaptive arm of the immune response to detect[57]. Furthermore, cancer cells do not produce danger signals, such as PAMPs, to alert the innate arm of the immune response and activate dendritic cells to provide co-stimulation and the third signal required for T-cell priming. Despite these challenges, there is evidence of an effective immune response to cancer.

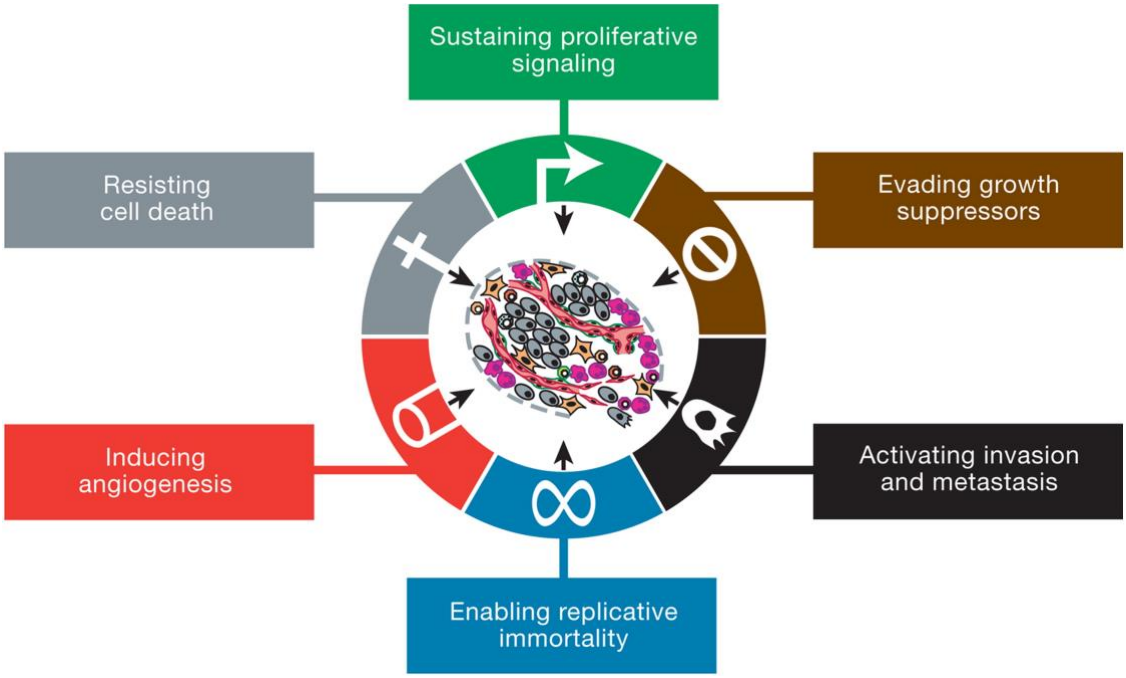



**Figure 1-2 The Six Hallmarks of Cancer**

The acquired capabilities of cancer in six essential alterations in cell physiology that dictate malignant growth. Each of the physiological changes depicts the successful breaching of anti-tumour defence mechanisms of immune system. (adapted from[56])

### 1.1.4 Cancer immunosurveillance

The idea that the immune system can recognise and eliminate cancer cells was originally proposed by Burnet and Thomas in 1957[58]. There has subsequently been substantial advancements in our understanding of the molecular basis for cancer immune surveillance[59]. Animal models have provided evidences of adaptive immune response specific for tumours that can eradicate disease. Boon et al have initially shown that mice immunised with variant teratocarcinoma cells generated by mutagens were able to reject teratocarcinoma cells with the same variant or partially with the wild type teratocarcinoma cells[60]. Subsequent study by Van Pel et al showed that the protective effect observed was a direct result of cytotoxic T-cell responses,

as co-culturing of the wild type tumour cells with spleen cells from mice that has rejected tumour cell variants resulted in the lysis of target cells[61]. The study also demonstrated the cytolytic activity was higher on the tumour cell variants used for immunisation in mice compared to wild type tumour cells, thus indicating antigen specific T-cell response[61]. Additional evidence of adaptive immune response in the clearance of disease is shown when tumours generated in RAG2$^{-/-}$ immunodeficient mice developed more quickly compared to those generated in wild-type mice, indicating suppression of tumour development by the adaptive arm of the immune system[13].

Although the concept of immunosurveillance was first developed in animal models, there are evidences for anti-tumour responses mediated by the immune system in humans as well. The anti-tumour immune response can be best observed in immunosuppressed patients with either primary or acquired immunodeficiencies as heightened risks of tumour development, such as lymphoma or lymphoproliferative disorders (collectively known as posttransplant lymphoproliferative disorder, PTLD)[62], skin cancer[63], and AML[64, 65]. Importantly, studies have also shown that the reduction in immunosuppressants improves the survival of PTLD patients[66] and reduces the incidence rate AML development[64], indicating immune-mediated anti-tumour protection. Several studies have shown that the immune-mediated anti-tumour effects are mediated by T cells, as T-cell infiltration to the tumour sites are associated with favourable prognosis in several different cancers, such as melanoma[67] and colorectal cancers[68]. Several cases of spontaneous tumour regression has been coupled to massive lymphocyte infiltration at the site of tumour cells were also reported[69, 70]. Furthermore, Ferrandini et al have shown evidence for T-cell clonal expansion at the tumour site, indicating antigen-specific T-cell response that plays a key role in immunosurveillance[70].

Tumour antigens that are able to elicit antigen-specific T-cell responses are known as either tumour-specific or tumour-associated antigens. Tumour-specific antigens (TSA) typically arise from point mutations or gene rearrangements during tumourigenesis, such that the binding of peptides and TCR can be sensitive to single amino acid substitutions and thus able to elicit T-cell responses.[1, 71, 72] Tumour associated antigen (TAA) are antigens that are overexpressed in tumour cells compared to normal cells, however, T cells that have been activated by TAA have been shown to be anergic, thus contributing to the immune tolerance of self[1, 73].

### 1.1.5 Cancer immunoediting

Immunosurveillance represents only one aspect of the relationship between the immune system and cancer. Despite evidence that cancer cells are recognised and eliminated by the immune system, tumours can still develop in immunocompetent individuals[74]. A contributing factor is the ability of cancer to evade the immune system. This phenomenon is another of the hallmarks of cancer (Figure 1-3)[56].

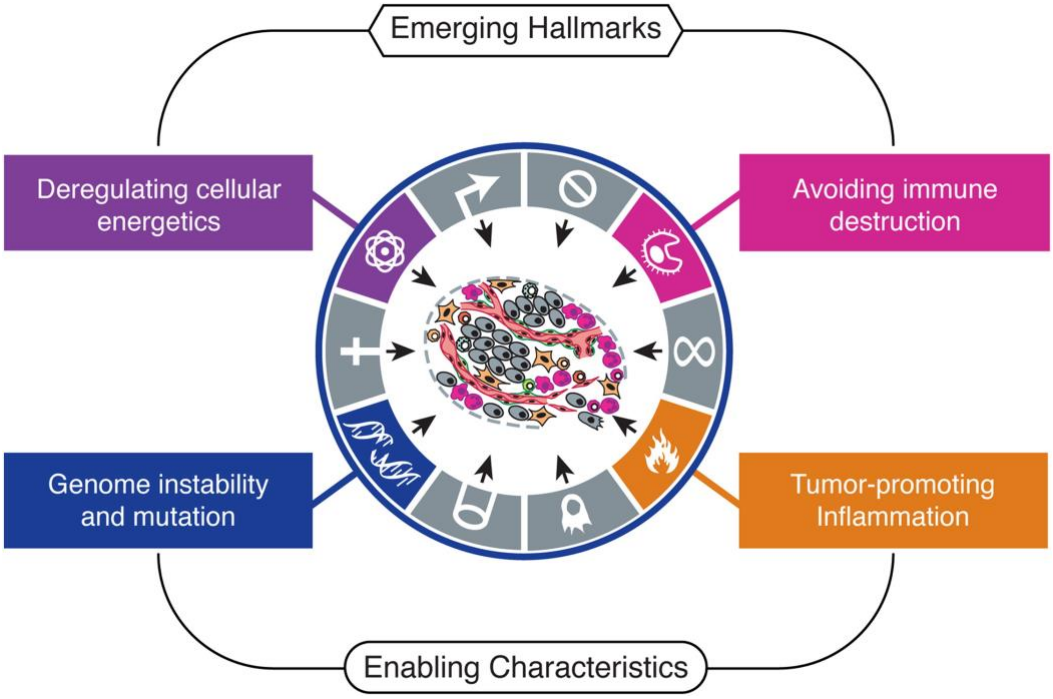



**Figure 1-3 Emerging Hallmarks of Cancer Involved in the Pathogenesis of Cancer**

Two emerging hallmarks of cancer involved in the pathogenesis of cancers. The modification of cellular metabolism effectively supports proliferation of cancer cells and the evasion of immunological destruction prevents cancer cells being killed by immune cells, such as T cells. (Adapted from[56])

There is evidence that immunosurveillance imposes selective pressure on cancer cells that favours variants with reduced immunogenicity. Seminal work has been reported by Schreiber and colleagues who studied tumour transfer between immunocompetent and RAG2$^{-/-}$ immunodeficient mice[75]. Tumours injected into either immunocompetent or RAG2$^{-/-}$ mice were found to grow at similar kinetics when subsequently transferred into RAG2$^{-/-}$ mice. Tumours injected into

immunocompetent mice and subsequently transferred to naïve syngeneic immunocompetent mice likewise continued to grow in all recipients. In contrast, RAG2$^{-/-}$-derived tumours were frequently rejected when transferred to immunocompetent mice, which indicates that tumours passaged through immunodeficient animals had become more immunogenic due to lack of immunosurveillance[75]. Sculpting of the immunogenicity of tumours by the immune system is termed cancer immunoediting[74, 75]. The process of immunoediting occurs in three phases, known as the three Es of immunoediting: elimination, equilibrium and escape. These are described schematically in Figure 1-4..

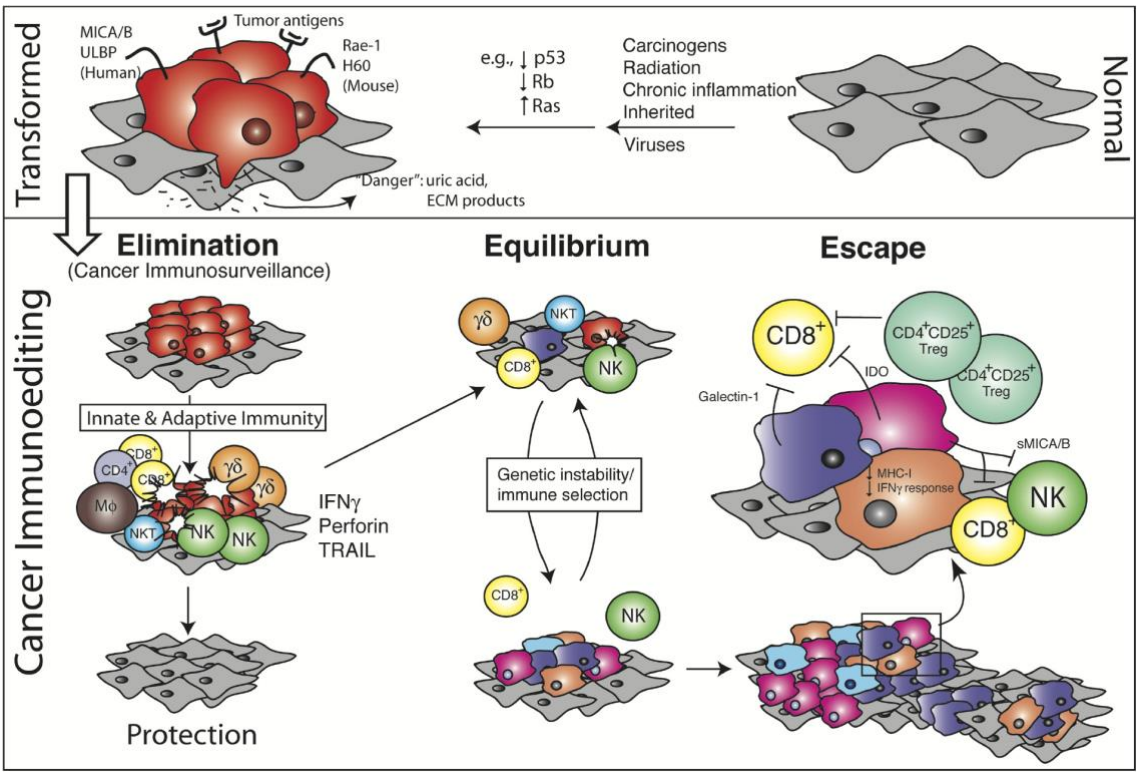



**Figure 1-4 The Three Phases of Cancer Immunoediting**

Normal cells (grey) undergoes transformation to become tumour cells (red). During early stages of tumourigenesis, tumour cells can express tumour specific markers and generate "danger" signals that initiates the immunoediting process. The immunoediting process is comprised of three phases, elimination, equilibrium, and escape. (Adapted from[59])

The elimination phase is the initial stage of effective immunosurveillance, where the immune system recognises and successfully eliminates all cells that have undergone malignant transformation. The process of elimination begins when the innate immune system is triggered

by proliferation of the cancer cells. Growth of a tumour mass causes local tissue damage due to the remodelling associated with angiogenesis[76] or invasion and metastasis, which are hallmarks of cancer (Figure 1-2)[56, 77]. Cancer cells and local damaged tissue produce proinflammatory cytokines and chemokines, including IL-6 and MIP-1$\alpha/\beta$, that recruit and activate innate immune cells such as macrophages, dendritic cells, $\gamma\delta$ T cells or NK cells to the tumour site[78]. The innate immune response includes production of IFN-$\gamma$, which acts as a positive feedback loop to recruit more innate immune cells to the tumour site and also activates IFN-$\gamma$ dependent killing of tumour cells by macrophages and NK cells[1, 57]. Tumour cell debris produced as a consequence of tumour death can be taken up by dendritic cells. They migrate to adjacent lymph nodes where tumour antigen can be presented to naive CD4 and CD8 T cells. After priming, these T cells circulate and can directly recognise and destroy any antigen-bearing tumour cells that they encounter[1, 57].

The equilibrium phase occurs when some tumour cells manage to avoid the immune response and a balance is established between elimination by the immune system and survival of some cancer cells. The cancer is held in check but not completely eliminated. The molecular basis for this phase is poorly understood and there is only anecdotal evidence for its existence[79]. An example is a report describing transfer of melanoma from an organ transplant donor to the recipient, despite the donor being considered tumour-free for 16 years[80]. The cancer was presumably held in check in the immunocompetent donor, but not eliminated, and emerged in the pharmacologically immunosuppressed allograft recipient. During the equilibrium phase, genetic mutations and epigenetic changes are accrued by the surviving cancer cells due to the inherent genetic instability of transformed cells. Darwinian selective pressure favours new tumour variants that evade immune recognition and those that promote tumour growth and metastasis[79, 81].

The final escape phase is where tumour variants sculpted by the immune response are resistant to immune detection, grow and become clinically apparent. Details of immune evasion mechanisms utilised by tumour cells will be described in section 1.4.

## 1.2 Acute myeloid leukaemia

### 1.2.1 Biology

Acute myeloid leukaemia (AML) is a haematological malignancy that accounts for 80% of acute leukaemias in adults[82]. The age-adjusted incidence is approximately 4 new cases per 100,000 person/year[83]. Although both adults and children can develop AML, it is predominantly a disease of older adults. Median age at diagnosis is 67 and incidence rate increases incrementally with age[83, 84]. AML has a poor prognosis, with a 5-year survival rate of only 25.9%[83]. Advances in chemotherapy regimens, supportive care, and allogeneic haematopoietic stem cell transplantation (HSCT) have improved the survival rates for younger AML patients (age < 60), with cure rates of 40% now achieved[83]. However, prognosis for patients over the age of 60 remains dismal, with cure rates of only 5 to 15% of patients reported[83, 85].

AML originates in the bone marrow and is characterised by the abnormal growth of immature myeloid progenitor cells that are unable to differentiate into mature cell types of the myeloid lineage[83]. AML can arise from two distinct routes. The majority of cases arise *de novo*[86, 87]. Alternatively, disease can progress from previous myelodysplastic syndrome (MDS), when it is known as secondary AML, or as a consequence of prior therapy such as radiation[88].

The accumulation of AML cells in the bone marrow disrupts normal haematopoiesis, leading to multi-lineage cytopenia[83]. Symptoms of the disease include tiredness due to lack of red blood cells, bruising and bleeding caused by low platelet count and opportunistic infections as a result of low white blood cell count[89]. Diagnosis of AML can be based on the percentage of immature precursor cells, also known as blasts, present in the bone marrow or blood, with a threshold of 20%. Diagnosis of AML cases associated with presence of the recurrent clonal cytogenetic abnormalities t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22), and t(15;17)(q22;q12) is made irrespective of the blast percentage[86].

#### 1.2.1.1 AML microenvironment

The bone marrow (BM) is a unique environmental niche in which a range of growth factors and cytokines are produced that sustains haematopoietic stem cells (HSCs) and orchestrates their differentiation to produce the various cells types of the haematopoietic lineage. Furthermore, the bone marrow contains HSC, endothelial cells, and mesenchymal stromal cells, also known as bone marrow stromal cells (BMSC). Importantly, BMSC is capable of differentiating into several different cell types, such as adipocytes, fibroblasts, osteoblasts, and chondrocytes, which contribute to the establishment of the bone marrow microenvironment to support haematopoiesis[90]. The BM can be categorised into two distinct marrows, the yellow marrow, where haematopoiesis does not occur, and the red marrow, where haematopoiesis occurs[90]. The yellow marrow consists primarily of adipocytes and it has been shown to negatively regulate normal haematopoiesis as the frequency of progenitor cells are reduced in adipocyte rich BM[91]. Endothelial cells, fibroblasts, osteoblasts, and osteoclasts all resides in the red marrow. Endothelial cells are found in the blood rich areas of bone marrow, such as the sinusoids and arterioles, as it has been shown to facilitate the exchange of molecules and movement of cells between the blood and the surrounding bone marrow[92]. Fibroblasts and osteoblasts are known to synthesise structural components, such as collagen and extracellular matrix, and bone tissues for the vascularisation and angiogenesis of the bone marrow[90, 93]. Lastly, chondrocytes are located at the periosteum of bone marrow and produces cartilage[94].

In the context of AML, the crosstalk with other cell types present in the bone marrow and the soluble factors that they produce are important features that favours AML cell growth and prevents apoptosis[90]. Importantly, AML can be found in the perivascular region of the bone marrow[95, 96], which is an hypoxic area with an oxygen concentration of 1%[97]. Cells such as endothelial cells and BMSCs, including adipocytes, osteoblasts, and fibroblasts, are all found within the perivascular region and have been shown to be manipulated by the AML cells, which contributes to the reshaping of the BM microenvironment that supports the survival and the proliferation of AML cells[90]. Previous report have shown that IL-1 secreted by AML cells was able to induce endothelial cells to produce growth factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF), to support AML cell proliferation[98]. The IL-1 and TNF-$\alpha$ secreted by AML cells were also found to induce endothelial cell activation, which

promotes AML cells adhesion to the endothelium and result in leukaemic cell dissemination[99]. Furthermore, the adhesion of AML cells to endothelial cells have also been found to be able to prevent chemotherapy induced apoptosis[100].

Of particular importance is the interaction between AML cells and BMSCs, such as adipocytes, osteoblasts, and fibroblasts, as many different soluble factors involving chemokines, cytokines, growth factors, and integrin-mediated signals have been shown to be used by AML cells to accelerate expansion [101, 102]. The intricate communications that occur between AML cells and the bone marrow microenvironment are summarised in Figure 1-5.

Shafat et al have shown that AML cells alter the metabolic processes within adipocytes resulting in lipolysis to release fatty acids that can be used as a fuel source for AML cells to promote survival and proliferation[103]. Furthermore, Moschoi et al demonstrated that AML cells are able to increase their mitochondrial mass via the uptake of mitochondria from stromal cells within the BM, thus conferring to a survival advantage as increased total adenosine triphosphate (ATP) within the AML cells were detected[104]. Osteoblasts have been shown to produce GM-CSF and were able to induce the release of IL-1 and IL-8 by AML cells, which promotes proliferation of AML cells[105]. The presence of IL-8 has been shown to not only promote angiogenesis within the bone marrow, but also induced migration of BMSCs, resulting in increased BMSCs observed in the BM of AML patients compared to healthy BM[106]. Furthermore, IL-8 has been shown to directly enhance endothelial cell proliferation, which creates a positive feedback loop that further supports AML cell growth and prevents apoptosis[107]. Lastly, fibroblasts have been shown to produce several cytokines that favour AML tumourigenesis, such as stem-cell factor (SCF)[108], IL-6, and GM-CSF[109]. Studies have shown that IL-6, a pro-inflammatory cytokine, promotes AML tumourigenesis and survival through the activation of STAT3 pathway leading to the induction of anti-apoptotic protein, Bcl-2[110, 111, 112]. Furthermore, it was shown that SCF acts synergistically with GM-CSF to promote AML cell growth and proliferation[113].

As well as growth factors and cytokines, the chemokine CXCL12 has been shown to be constitutively expressed by BMSCs[114]. It has been shown that the interaction between CXCL12 and its receptor, CXCR4, promotes AML cell survival and prevents apoptosis through the

activation of PI3K/Akt signalling pathway[114]. Furthermore, CXCR4 and very late antigen (VLA)-4, an adhesion molecule, has been shown to mediate the transendothelial migration of AML cells beneath the BMSCs, also known as pseudoemperipolesis, to the BM, which indicates an important role of BMSCs for AML cells homing within the BM microenvironment[115]. Lastly, the adhesion of VLA-4 on AML cells to the stromal fibronectin has been shown to have a protective effect for AML cells against spontaneous or drug-induced apoptosis[116].

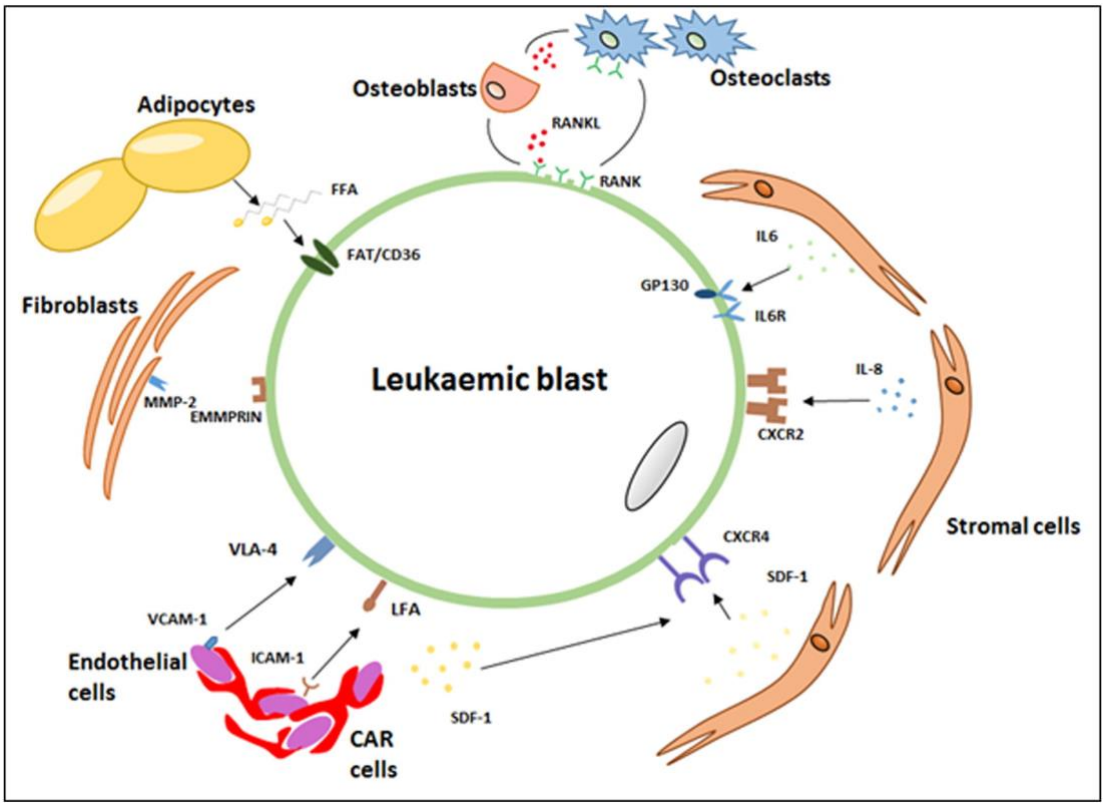



**Figure 1-5 The complex interaction between cells of BM and AML**

A complex and diverse group of cells within the bone marrow, ranging from endothelial cells to the different types of BMSCs, such as adipocytes, osteoblasts, and fibroblasts, have been implicated in the maintenance of AML cells. The growth factors and cytokines produced by the cells of BM have been shown to promote AML cell survival and proliferation. AML cells have also been shown to alter cells within the BM to promote its growth. (Adapted from[90])

#### 1.2.1.2 Disease classification and genetic landscape of AML

AML is a genetically heterogeneous disease. Cytogenetic studies have identified chromosome structural abnormalities within AML blasts that harbours more than 3 aberrations, also known as complex karyotype (CK). Complex karyotype is detected in 10%-12% of all AML patients and is

associated with adverse prognosis[117]. The most frequent lost chromosome segment is 5q, which is detected in about 80% of CK-AML patients.[117, 118] However, almost 60% of patients have a normal karyotype (CN-AML)[119]. Work undertaken by The Cancer Genome Atlas Research Network to evaluate AML heterogeneity by studying the coding sequences of 200 adults with *de novo* AML found 2315 somatic single-nucleotide variants and 270 small insertion and deletions[120]. Some of these mutations affect the epigenetic regulation of gene transcription, such as *DNMT3A*, *IDH1,* and *IDH2*[121, 122]. The most common recurrent mutations occur in the NPM1, CCAAT/enhancer-binding protein-α (CEBPA), and FLT3 genes[123, 124]. The recurrent mutated genes found in AML have been organised into nine functional categories, that are depicted in Figure 1-6. Nearly all AML cases have at least one mutation affecting a biological process that is known to be implicated in the pathogenesis of AML, such as activated signalling (59%), DNA methylation (46%), chromatin modifiers (30.5%), NPM1 (27%), transcription factors (22%), tumour transcription factor fusions (18%), myeloid suppressors (16.5%), spliceosome (13.5%), and cohesion complex (13%)[120, 125].

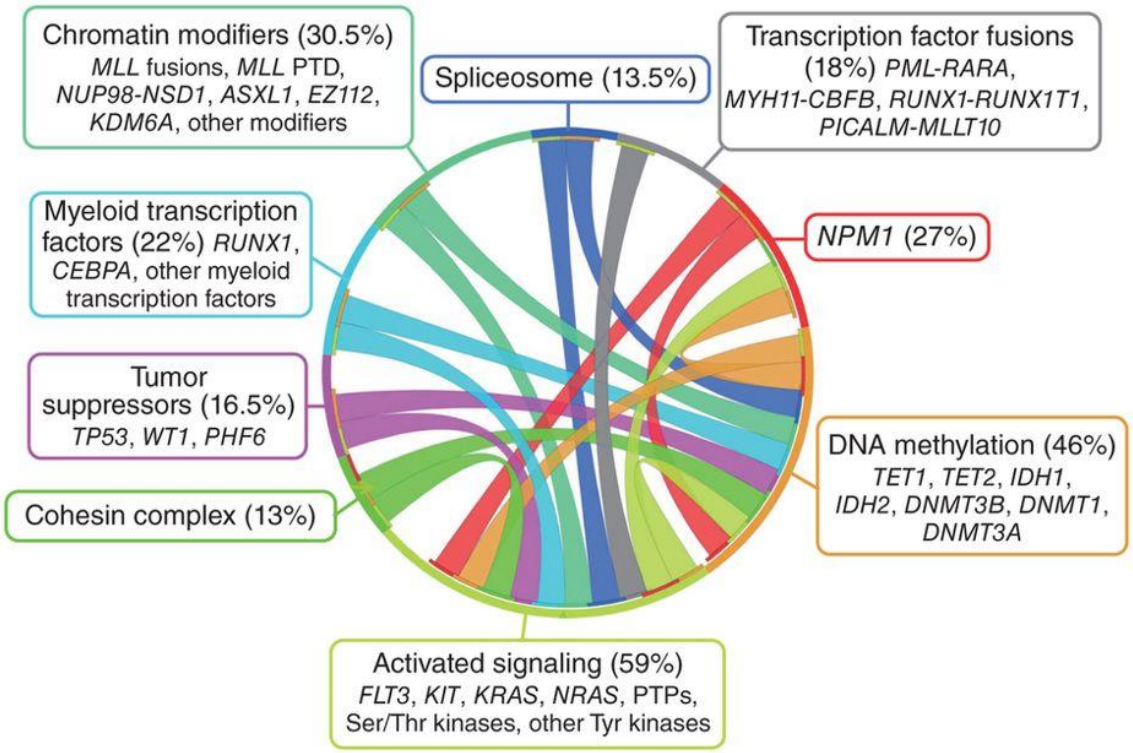



**Figure 1-6: Overview of the functional categories of mutated genes that leads to pathogenesis of AML**

The figure depicts the heterogeneity of AML. The ribbons connecting the distinct categories reflect the associations of the mutations with the different pathways and mutually exclusive alterations that may exist are not connected by ribbons. (Adapted from[125])

Knowledge of the cytogenetic and molecular genetic abnormalities in AML patients has provided insights into the underlying molecular defects, which may be used as prognostic factors[124]. The current AML classification system, World Health Organization classification of AML, divides AML based on either the cytogenetic or molecular genetic irregularities observed, but it does not provide information regarding prognostic factors. Recently, the European LeukaemiaNet (ELN) introduced a risk group classification that incorporates the observed molecular and genetic abnormalities in CEBPA, FLT3, and NPM1 to provide prognostic and therapeutic indications. There are three risk group categories: favourable, intermediate, and adverse, shown in *Table* 1-1.[85, 120]

| Risk category* | Genetic abnormality |
|---|---|
| Favorable | t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* |
| | inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* |
| | Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low}$† |
| | Biallelic mutated *CEBPA* |
| Intermediate | Mutated *NPM1* and *FLT3*-ITD$^{high}$† |
| | Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low}$† (without adverse-risk genetic lesions) |
| | t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*‡ |
| | Cytogenetic abnormalities not classified as favorable or adverse |
| Adverse | t(6;9)(p23;q34.1); *DEK-NUP214* |
| | t(v;11q23.3); *KMT2A* rearranged |
| | t(9;22)(q34.1;q11.2); *BCR-ABL1* |
| | inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* |
| | −5 or del(5q); −7; −17/abn(17p) |
| | Complex karyotype,§ monosomal karyotype‖ |
| | Wild-type *NPM1* and *FLT3*-ITD$^{high}$† |
| | Mutated *RUNX1*¶ |
| | Mutated *ASXL1*¶ |
| | Mutated *TP53*# |

**Table 1-1 Risk group classification of AML based on genetics**

Clinical characteristics and outcome of AML disease based on genetic abnormality. (Adapted from[85])

Compared to other cancer types, AML has one of the lowest somatic mutation frequencies at 0.37/Megabase (Mb), and contrasts dramatically with melanoma at 100/Mb. [126] It is speculated high somatic mutation frequencies are more likely to give rise to novel neoantigens that can be recognised by T cells[127]. The somatic mutation frequencies observed in exomes of different cancers and likelihood of neoantigen formation is shown in ***Figure*** 1-7. Given that AML has one of the lowest somatic mutation frequencies and consequently potential for neoantigen formation is low, there is limited scope for recognition of AML cells by T cells.

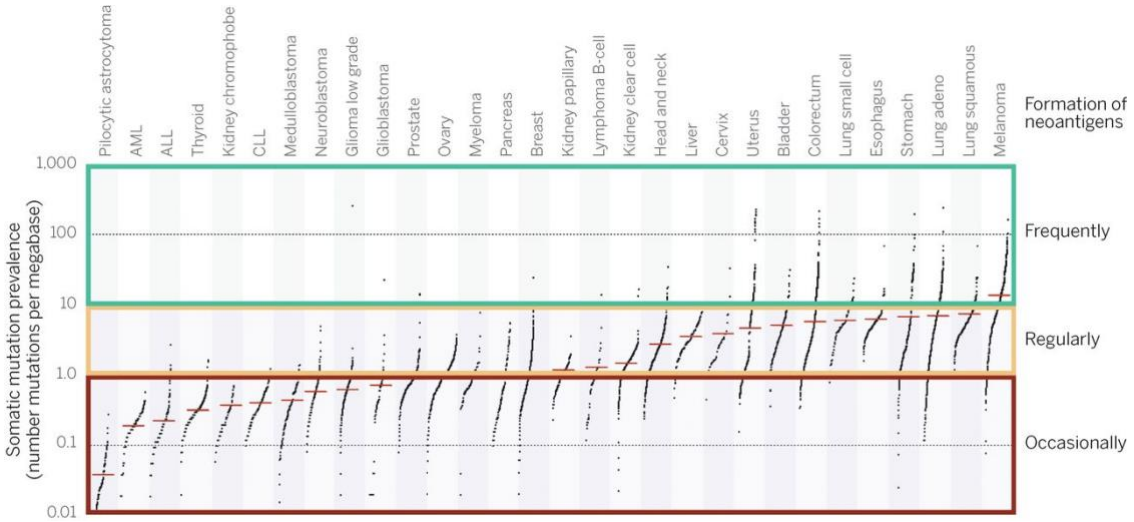



**Figure 1-7: Somatic mutation frequencies and the estimate of neoantigen repertoire in cancer**

The figure shows the number of somatic mutations across different tumours. The likelihood of the formation of neoantigens for the different cancers based on the somatic mutation prevalence and the frequency of neoantigen observed is indicated. (Adapted from[127])

### 1.2.2 Current treatments and outcomes

Despite recent advances in the understanding of AML genetics and biology, current AML treatments still rely heavily on intensive chemotherapy and allogeneic haematopoietic stem cell transplantation (HSCT). Advances in the standard drug dose and safer HSCT procedures have improved remission rates and reduced treatment-related toxicities[128]. The standard of care in AML uses cytarabine and anthracycline in a 7+3 combination, where a 7-day continuous infusion of

100 or 200mg/$m^2$ of cytarabine is administered on days 1 to 7 and daunorubicin (an anthracycline) at 60mg/$m^2$ on days 1 to 3. Clinical trials by the Eastern Cooperative Oncology Group and the European trials have compared the benefits of doubling the daily dose of daunorubicin from 45mg/$m^2$ to 90mg/$m^2$ [129]. The higher dose of daunorubicin was associated with improved complete remission rates and better overall survival benefits were observed in all cytogenetic groups[130].

Chemotherapy achieves a complete remission rate of 80% in younger patients, however the 5-year overall survival is only approximately 40% and the majority of AML patients will relapse within 3 years[85, 128]. After induction therapy, the post-remission consolidation therapy for patients that do not undergo allogeneic HSCT is high dose cytarabine twice daily at 3g/$m^2$ on days 1, 3, and 5[131]. In addition, patients may receive targeted therapy for specific AML subgroups, such as the protein kinase inhibitor dasatinib for core-binding factor AML[132] or the multi-kinase inhibitor sorafenib for FLT3-ITD AML[133].

Although most patients enter remission after induction therapy and despite receiving post-remission therapy, also known as consolidation therapy, the risk of relapse remains high[134, 135, 136]. Patients with favourable AML receives chemotherapeutic consolidation, however, allogeneic haematopoietic stem cell transplant (Allo-HSCT) is the preferred consolidation therapy for patients with intermediate and poor-risk AMLs[137]. In a 2017 study by Passweg et al, based on data collected from 683 centres in 50 countries, it was found that the use of allogeneic HSCT were the standard practise for myeloid malignancies and that 39% of all allogeneic HSCT were performed for the treatment of AML, as not only is allo-HSCT used as consolidation therapy, it is also the only curative treatment available for patients that have relapsed[138].

HSCT was originally meant to be a rescue therapy for patients that have undergone intensive chemotherapy or radiotherapy that results in myeloablation for the reconstitution of the bone marrow compartment, as stem cells have the ability to self-renew and generate progenitor cells[139]. Haematopoiesis can be branched into two main progenitors, common lymphoid progenitors, which can be further developed into T cells, B cells, dendritic cells or NK cell, and common myeloid progenitors, which can be further developed into myeloblasts, including monocytes, erythroblast, or megakaryocytes[139, 140].

Prior to infusion of allo-HSCT, patients receive myeloablative conditioning (MAC) regimen, using alkylating agents such as cyclophosphamide or fludarabine, to sufficiently suppress the immune system to prevent host rejections of the haematopoietic stem cell[141]. However, toxicity and severe graft versus host disease (GvHD) is often associated with MAC[142]. Importantly, as the median age at diagnosis for AML is 67 years old, reduced intensity conditioning (RIC) have been developed to reduce non-relapse mortality in older or medically unfit patients that cannot tolerate toxicity of MAC[143]. RIC regimen consists of total body irradiation or alkylating agent that is reduced by at least 30% used by MAC[143]. The success of the allo-HSCT is determined by the engraftment of HSCs, which allows haematopoiesis to occur and give rise to the different immune cells. A study by Shimoni et al have compared the outcome of different conditioning regimen for allo-HSCT in AML patients, whereby the intensity of the conditioning regimen was found to have no effect on engraftment, of donor or recipient origin, after 3 months of receiving allo-HSCT[144]. Furthermore, transplant related mortalities (TRM) has been shown to be higher in MAC than RIC, 22% and 8% respectively, with most incidents related to increased lethal risk of GvHD[144]. Despite TRM, the overall benefits of allo-HSCT for patients with AML is the strong graft versus leukaemia (GvL) effect exerted by allogeneic lymphocytes[145]. A trend of lowered relapse rate were also observed in patients that received MAC compared to RIC, 32% and 53% respectively, however, the intensity of the conditioning regimen received by the patient was not predictive of the outcome[144].

The therapeutic benefits of allogeneic HSCT will be further discussed in section 1.3.1.

## 1.3 T-cell Recognition of AML

Although T-cell responses to AML cells are not easily generated because cancer cells are altered self (not overtly foreign) and the low somatic mutation frequency of AML limits the potential neoantigen repertoire, several lines of evidence indicate a role for the immune system in the elimination of AML cells. Compelling evidence has come from studies of relapse rates after HSCT. AML patients who receive a syngeneic transplant have an increased relative risk of relapse of 2.58 compared to recipients of an allogeneic transplant[146]. Relapse rates are lower in patients that develop GvHD after allogeneic HSCT[146]. Inclusion of T-cell depletion during the transplant procedure to prevent GvHD is associated with increased risk of disease relapse.[147] These observations all point to a beneficial role of the donor derived T-cell response in the recognition and elimination of AML cells, which is known as the graft versus leukaemia effect (GvL)[146, 147]. The GvL response is mediated by donor T-cell recognition of minor histocompatibility antigens (mHag), leukaemia-associated antigens (LAA) and leukaemia-specific antigens (LSA).

mHag are peptides of cellular proteins bound to the MHC molecules for presentation to T cells.[1] The haematopoietic lineage specific mHag HA-1 and HA-2 play an important role in the GvL effect after allogeneic HSCT[148]. Marijt et al. reported that successful induction of long-lasting disease remission in patient / donor pairs that were mismatched for HA-1 or HA-2 was associated with large expansions of $CD8_+$ T cells specific for HA-1 or HA-2, and that these T cells recognised and lysed the patient AML blasts[148].

In addition to the mHags recognised by alloreactive T cells, anti-leukaemic activity has been reported in the syngeneic transplant setting which indicates that the GvL effect also includes responses specifically against AML cells[149]. These are mediated by T cells that recognise LAA and LSA. The LAA are self-proteins that are overexpressed on leukaemic cells compared to normal cells. Wilms' Tumour antigen 1 (WT1), receptor for hyaluronic acid mediated motility (RHAMM), and IL-3 receptor (CD123) are examples of LAA[150, 151]. The LSA are expressed exclusively by leukaemic cells[150]. They typically arise from mutational changes that contribute to the pathogenesis of AML, such as FLT3-ITD and $NPM1_{mut}$ [152, 153]. Evidence that priming of LAA and LSA reactive T cells occurs in AML patients comes from studies that show *ex vivo* stimulation

with antigens such as RHAMM and $NPM1_{mut}$ stimulates patient $CD8_+$ T cells to release IFNγ, granzyme B and lyse AML cells[154, 155]. Furthermore, presence of T cells specific for antigens such as $NPM1_{mut}$ in patients correlates with longer survival compared to patients in whom responses cannot be detected, which suggests that they have an important role in controlling disease[156].

### 1.3.1 Current immunotherapeutic strategies for AML

When first introduced over 50 years ago, the purpose of HSCT was as a rescue therapy to reconstitute the bone marrow compartment after administration of otherwise lethal doses of intensive radiotherapy and chemotherapy. Retrospective reviews of clinical outcomes subsequently revealed the evidence described in section 1.3 that shows allogeneic HSCT has an immunotherapeutic role, mediated by the GvL effect. This plays a particularly important part in disease control after allogeneic HSCT with the reduced intensity conditioning regimens used to treat AML. Restricted use of chemotherapy alone allows treatment of older patients with co-morbidities that preclude use of high intensity regimens, but disease is less likely to be completely eradicated. Consequently, there is greater reliance on the GvL effect to eliminate residual disease.

Despite the immunotherapeutic properties of the treatment, approximately 40% of patients experience AML relapse after allogeneic HSCT[157]. Relapse is typically treated by chemotherapy to reduce the number of leukaemia blasts, followed by immunotherapy using donor lymphocyte infusions (DLI). Studies in the early 1990s performed soon after the introduction of therapeutic DLI showed durable remission could be induced, but only 15% of patients had a complete response[158]. A study by Schmid et al. reported the overall 2-year survival of patients that received therapeutic DLI following AML relapse after allogeneic HSCT was 21% compared to 9% for those that did not receive DLI[159]. This study also reported that therapeutic DLI was most effective in the context of low leukaemia burden at relapse and favourable cytogenetics of the disease[159].

In addition to the administration of therapeutic DLI post-relapse, some patients receive prophylactic DLI to treat falling donor chimerism in an attempt to prevent graft loss. Subsequent retrospective outcome studies have shown that patients given pre-emptive DLI after allogeneic

HSCT have reduced incidence of disease relapse and improved overall survival rates, which suggests that the procedure reinforces the GvL effect[160, 161, 162].

### 1.3.2 Novel T-cell immunotherapies for AML

Given the evidence that T-cell mediated immune recognition of AML cells plays an important role in the curative effects of allogeneic HSCT, other novel immunotherapeutic strategies are being developed that attempt to harness the anti-leukaemic activity of T cells.

#### 1.3.2.1 Leukaemia vaccines

Knowledge of the LAA and LSA recognised by AML reactive T cells has facilitated attempts to develop peptide-based vaccines to stimulate anti-leukaemic activity. One candidate antigen that has been widely studied is WT1; a transcription factor that is overexpressed by AML cells[163]. Several studies have shown that cytotoxic T cells responses can be elicited using antigenic peptide epitopes from the WT1 sequence[164, 165, 166]. Vaccination of patients with these peptides has been shown to promote expansion of WT1 specific $CD8_+$ T cells and 43% of patients that received the vaccination resulted in either reduced number of leukaemic cells within BM or undetectable WT1 expression levels[167, 168]. In addition to conventional vaccination methods, the administration of WT1 mRNA electroporated dendritic cells were administered to patients, with 50% of patients to be clinical responders, with an increased WT1-specific $CD8_+$ T cells, additionally, patients that were in partial remission were brought to complete remission[169]. However, results from clinical trials have been variable and indicate limited efficacy. In a trial reported by Brayer et al., all patients relapsed within a year of treatment[170]. Two other trials reported relapse rates of 44% and 68%[171, 172].

Other candidate antigens, such as PR1 and RHAMM-R3, have also been explored. Qazilbash et al have shown that 35 of 66 patients that have received PR1 peptide vaccination had an increase in PR1-specific $CD8_+$T cells[173]. Furthermore, the clinical response for PR1 peptide vaccination correlates with the PR1-specific T-cell response, with 12 of the 35 patients with immune response having a clinical response: 8 complete remission, 1 partial remission and 3 haematological improvements[173]. Similar to PR1 and WT1, clinical response for RHAMM-R3 peptide vaccine

correlates with peptide-specific immune response, whereby 33% of patients that achieved clinical response, reduced AML cell counts, also exhibit RHAMM-R3-specific T cell response[154].

Peptide-based vaccines require knowledge of the antigenic epitopes and tailoring of the peptides to the patient HLA type. An alternative approach is vaccination with the patient's own leukaemic cells, which does not require detailed knowledge of the antigens recognised. One strategy evaluated by Chan et al uses a self-inactivating lentiviral vector encoding the CD80 costimulatory molecule and IL-2 to transduce AML blasts to confer expression of CD80 and secretion of IL-2 with the aim of enhancing T-cell responses[174]. The *in vitro* stimulation of allogeneic T cells with CD80/IL-2 modified AML cells enhanced proliferation by 33% compared to stimulation with unmodified AML cells[174]. Success was also observed in the context of $CD8_+$ T cells from allogeneic HSCT patients. At least four-fold *in vitro* expansion was achieved and increased levels of IFN-$\gamma$, TNF-$\alpha$, and IL-2 produced when stimulated with CD80/IL-2 modified AML cells compared to unmodified AML cells[174]. Of particular importance, the study also showed that CD80/IL-2 modified AML cells stimulated expansion of AML-specific T cells that were subsequently capable of recognising and responding to unmodified AML cells[174].

Another whole-cell vaccine strategy for AML is with irradiated tumour cells engineered to secrete GM-CSF to promote anti-tumour and GvL effects, as this strategy has already shown efficacy against other cancers such as melanoma[175, 176]. Ho et al have shown that at the time of study, 60% of patients that have received GM-CSF-secreting leukaemia cell vaccine remained in complete remission with a median follow up of 25.5 months and a two-year overall survival of 88 ± 12%[176]. Furthermore, it was revealed that the vaccination was able to elicit significant accumulations of dendritic cells, macrophages, $CD4_+$ and $CD8_+$ T cells, thus indicating an immune response[176].

#### 1.3.2.2 Bispecific T-cell engager antibodies

Bispecific T-cell engager (BiTE) antibody is a new class of immunotherapeutic molecule. BiTE consists of two antibody-derived single-chain variable fragment (scFv) with one specificity of the two scFv is directed against the CD3 of T cells and the other is directed against tumour antigen[177]. An example of BiTE that has been used clinically in the context of haematological malignancy is

the CD3/CD19 BiTE for B-cell acute lymphoblastic leukaemia, whereby depletion of B cells and expansion of both $CD4^+$ and $CD8^+$ T cells were observed[178]. The use of BiTE antibody can also be extended to AML patients, with CD33 as the tumour target, which is overexpressed on AML cells[179]. Pre-clinical studies showed that CD3/CD33 BiTE antibody (AMG330) is able to recruit T cells and lyse both $CD33^+$ AML cell lines and $CD33^+$ primary AML cells.[180, 181] Furthermore, it was found that patient derived T cells can be engaged by AMG330 and effectively lyse autologous AML cells, with a specific lysis of 50%-93% of targets, leading to increased activation marker expression and proliferation[180].

#### 1.3.2.3 TCR gene modified T cells

Transduction of T cells with TCR genes to confer specificity for tumour antigens has been extensively studied in the context of WT1. Tsuji et al. demonstrated that T cells transduced with a TCR specific for a WT1 peptide presented by HLA-A24 could be expanded *in vitro* by stimulation with WT1 peptide-loaded $HLA\text{-}A24^+$ APCs. The modified T cells exhibited cytotoxicity and cytokine production in response to $HLA\text{-}A24^+$ $WT1^+$ leukaemia cells, but not $HLA\text{-}A24^-$ $WT1^+$ leukaemia cells[182]. An *in vivo* study using a humanised mouse model showed that T cells transduced with a WT1 TCR were able to eliminate human leukaemia cells in 15 out of 17 mice (12%)[183]. These studies lent support to an evaluation in AML patients, and encouraging results have recently been published by Chapuis et al.[184] Post-transplant AML patients that received WT1 TCR T cells had a 100% relapse-free survival at median of 44 months, compared to 54% relapse-free survival for patients with similar disease risk profiles that did not receive the infusion[184].

#### 1.3.2.4 Chimeric antigen receptor (CAR) T cells

CAR T cells are also a genetically modified product. In this case, specificity for tumour antigen is conferred by transduction with a construct encoding a scFv linked to signalling domains of CD3ζ and costimulatory molecules such as CD28 and/or 4-1BB (Figure 1-8)[185]. An advantage of CAR T cells is that antigen recognition is MHC-independent, but a limitation is that only antigens expressed on the surface of tumours can be easily targeted[186].

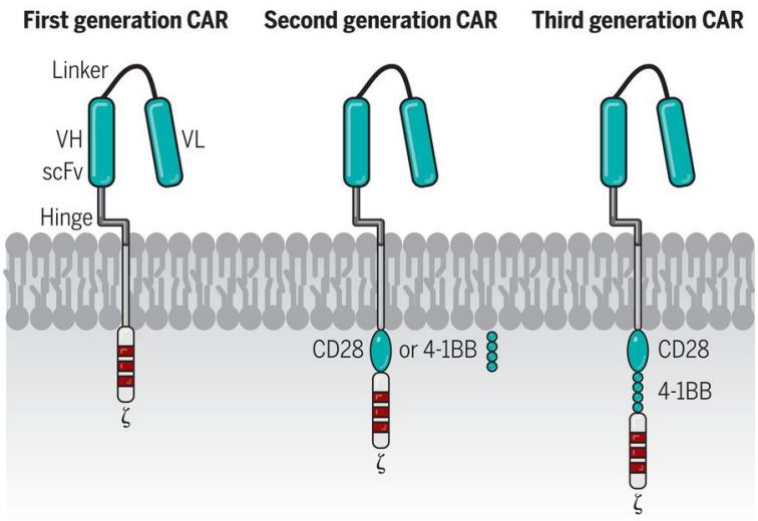



**Figure 1-8: Skeleton structure of CAR T-cell constructs**

CAR T-cell constructs are composed of an extracellular portion that is derived from an antibody specific for the antigen of interest and intracellular signalling molecules derived from T-cell signalling proteins. First generation CAR T-cell constructs only contain CD3ζ. Second generation CAR T-cell constructs contain one co-stimulatory endodomain linked to CD3ζ. Third generation CAR T-cell constructs contain two co-stimulatory endodomains linked to CD3ζ. (Adapted from[185])

CAR T-cell therapy is showing particular promise for the treatment of B-cell malignancies. Clinical trials using second-generation CAR T cells targeting CD19 have shown that 63% of complete remission of B-cell malignancies are achieved[187]. However, relapse can occur after αCD19 CAR T-cell therapy, and it has been associated with loss of CD19 expression[188]. This represents an example of tumour immuno-editing under selective pressure to escape recognition by αCD19 CAR T cells. Treatment of other haematological malignancies is also being explored, such as multiple myeloma with a BCMA targeted CAR[189] and acute lymphoblastic leukaemia with a αCD22 targeted CAR[190]. Both CD123 and CD33 can be overexpressed on the surface of AML cells in comparison to normal haematopoietic stem cells[179, 191], and therefore represent potential candidates for targeting CAR T cells to treat AML. Findings from *in vitro* and *in vivo* studies have shown potent anti-leukaemic activity against $CD123^+$ or $CD33^+$ AML blasts. Tettamanti et al have shown that co-culturing of αCD123 CAR T-cell with either $CD123^+$ AML cell lines or $CD123^+$ primary AML cells resulted in the lysis of both AML cell lines or primary AML cells *in vitro*, with a

mean lysis of 60% and 59% respectively[192]. Similarly, αCD33 CAR T-cell efficiently lysed both CD33+ AML cell lines and CD33+ primary AML cells in an *in vitro* co-culture, with a mean lysis of 66% and 61% respectively[193]. Moreover, patient derived αCD123 CAR T cells were able to lyse 50% of autologous primary AML cells in two out of the three samples *in vitro*[194]. *In vivo* cytolytic assessment of αCD123 CAR T cells also exhibit anti-leukaemic effects as significant increase in survival were observed for mice treated with αCD123 CAR T-cell compared to αCD19 CAR T-cell treated, 100 days and 60 days respectively[194].

A concern with targeting CD123 or CD33 is that these molecules are also expressed by normal haematopoietic cells, albeit at a lower level, so there is a risk of 'on target - off-tumour' toxicity. This is a problem facing exploitation of most tumour associated antigens. To limit the potential damage caused by recognition of normal cells, CAR T cells are being equipped with strategies for their elimination, if toxicity develops. One approach involves including a transgene-encoded cell surface polypeptide containing the epitope recognised by a cytolytic anti-EGFR monoclonal antibody (Cetuximab), which can be administered for the clearance of CAR T cells, if required[195]. Another approach being developed is the incorporation of an inducible caspase 9 suicide gene (iC9) that, upon activation, has been shown to eliminate 90% of CAR T cells[196].

#### 1.3.2.5 Vγ9Vδ2 T cells

Awareness of the anti-tumour reactivity of γδ T cells (described in section 1.1.2.3) has encouraged interest in their potential application in tumour immunotherapy, with a particular focus on the Vγ9Vδ2 T-cell subset. Studies have shown that Vγ9Vδ2 T cells can be readily expanded by stimulation with aminobisphosphonate drugs, such as zoledronic acid[53, 197]. Small scale clinical trials in a variety of cancer types, such as head and neck, prostate, and lung, have been conducted based on treatment with phosphoantigens or aminobisphosphonates plus IL-2 to promote *in vivo* expansion or adoptive transfer of *ex vivo* expanded cells[198, 199].

Several observations suggest potential utility of γδ T-cell therapy for AML. An *in vitro* study by Gertner-Dardenne et al showed that *ex vivo* expanded patient derived Vγ9Vδ2 T cells were able to recognise and kill autologous AML blasts in a perforin/granzyme-dependent manner[54]. They

also used a humanised mouse model to show that Vγ9Vδ2 T cells homed to the bone marrow where leukemic cells had engrafted and survival was improved[54] Clinical observations are also encouraging. An association between improved survival of AML patients after allogeneic HSCT with enhanced γδ T-cell recovery has been reported[200]. *In vivo* activation of γδ T cells by treatment with zoledronic acid plus IL-2 has been shown to promote activation and expansion of γδ T cells and two AML patients exhibiting partial disease remission[201]. Furthermore, some AML patients achieved complete remission after infusion of γδ T cells followed by administration of zoledronic acid plus IL-2[202].

## 1.4 Immune escape by AML

Although evidence suggests that T-cell responses to AML can control the disease and T-cell immunotherapies are being developed to exploit the phenomenon, AML has mechanisms for escaping T-cell responses that may limit efficacy. Immune escape is likely to be one of the factors contributing to the relapse rate of approximately 40% after allogeneic HSCT, and the variable efficacy of immunotherapies such as therapeutic DLI[158, 159] and leukaemia vaccination[157, 170, 171]. AML is known to exploit a wide range of mechanisms for escaping recognition by T cells. Mechanisms in the AML microenvironment that are known to suppress T-cell function are presented schematically in Figure 1-9.

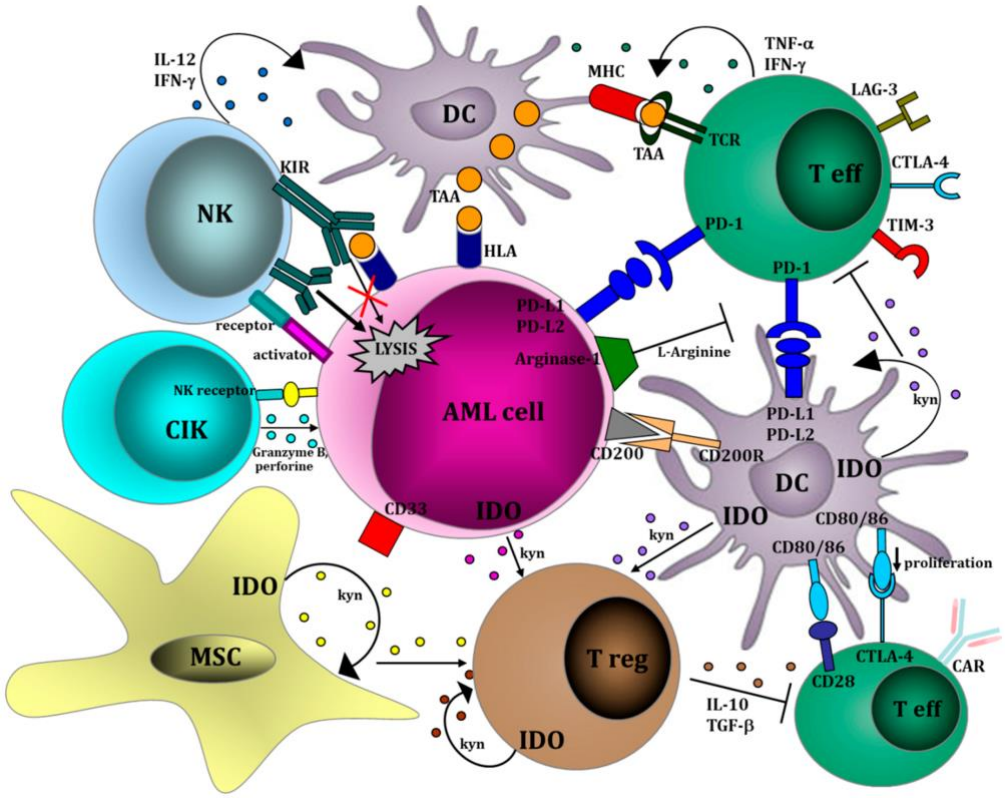



**Figure 1-9 Summary of mechanisms in the AML microenvironment that suppress T-cell function and support immune escape**

The figure depicts the complex network of cellular and molecular interactions that contribute to the immunosuppressive AML microenvironment. AML cells interacts with a variety of cells, such as mesenchymal stromal cell (MSC), natural killer (NK) cells, cytokine induced killer (CIK) cells, Effector T cells (Teff), Tregs, and DCs. (Adapted from[203])

### 1.4.1 Impaired recognition of AML cells

HLA-mismatched allo-HSCT where loss of the mismatched HLA-haplotype by AML blasts, due to acquired uniparental disomy of chromosome 6, has been reported in 30% of the patients that have relapsed[204]. Of note, alloreactivity of T cells that is critical to the therapeutic effects of GvL can be mediated by HLA type II mismatches[205]. Therefore, the acquired loss of mismatched HLA-haplotype represents a mechanism that drives immune escape by AML blasts from the alloreactive T-cell response. In addition to the loss of HLA molecule, factors that affect the ability of antigen presentation by HLA-class II molecule were also found to be a mechanism of immune escape, as high expression of class II associated invariant chain peptide (CLIP), a peptide that must be released prior to the binding of antigen to the HLA-class II antigen groove, were associated with poor clinical outcome[206]. Furthermore, absence of CLIP on AML blasts were able to stimulate autologous $CD4_+$ T cells compared to $CLIP_+$ AML blasts.[206] As well as loss or downregulation of HLA molecules, low expression of co-stimulatory molecule CD80 by AML blasts have been reported, which may contribute to T-cell anergy, indicating the selection for escape from T-cell response[207, 208, 209].

The importance of immune escape as a contributing factor to relapse of AML after allogeneic HSCT was highlighted by two recent reports[210, 211]. Comparison of AML samples before and after relapse found no evidence that acquisition of novel oncogenic changes was a key factor driving disease progression. Instead, relapse was associated with acquisition of gene expression profiles likely to adversely affect immune function, such as reduced expression of HLA class II molecules and upregulation of inhibitory checkpoint molecules[210, 211].

### 1.4.2 Immune suppression by AML cells

Two studies published in 2001 reported that AML cells secrete soluble factors that suppress proliferation and cytokine production by T cells from healthy individuals[212, 213]. Buggins et al. showed that supernatant from AML cells decreased IL-2 and IFN-$\gamma$ production by T cells, reflecting inhibition of NF-$\kappa$B and NFATc nuclear translocation, and blocked T-cell proliferation by preventing entry into S-phase of the cell cycle[212]. Studies performed indicated that the

immunosuppression observed was not mediated by nitric oxide, vascular endothelial growth factor, prostaglandins, gangliosides, TGF-β or IL-10[212, 213].

#### 1.4.2.1 Enzyme-mediated immunosuppression

AML cells are known to produce several enzymes that have immunosuppressive properties. The most extensively studied is indoleamine 2 3-dioxygenase (IDO). Levels of IDO in plasma were found to be significantly higher in some AML patients compared to healthy controls[214, 215]. IDO is a key enzyme that catalyses tryptophan degradation along the kynurenine pathway. Tryptophan is an important amino acid that is required by cells for protein synthesis and metabolic functions, such as nicotinamide adenine dinucleotide (NAD+) activity[216]. Tryptophan starvation caused by IDO consumption inhibits T-cell activation and proliferation[217, 218, 219]. Frumento et al have shown a dose-dependent inhibition of T-cell proliferation by tryptophan-derived catabolites, kynurenine, picolinic acid, and quinolinic acid, through blocking cell cycle in the G1 phase[219]. In addition to the direct effects of IDO on effector T-cell activity, Curti et al have shown that IDO expression by AML cells promotes reprogramming to immunosuppressive $CD4^+$ Treg cells[220]. Co-culture of T cells with IDO-expressing AML cells resulted in increased expression of the Treg phenotypic markers CD25 and CTLA-4, which was not seen with $IDO^-$ AML cells. Furthermore, these IDO-induced $CD4^+$ $CD25^+$ T cells were demonstrated to exhibit regulatory activity, as proliferation and IL-2 production by effector T cells was not observed when they were co-cultured with IDO-induced $CD4^+$ $CD25^+$ T cells[220].

Another enzyme with immunosuppressive properties that can be produced by AML cells is arginase II[221]. AML patients have raised plasma concentrations of arginase II compared to healthy individuals[221]. Similar to IDO, arginase II depletes an essential amino acid, in this case arginine, that adversely affects several aspects of T-cell function. Arginine starvation during activation of T cells leads to cell cycle arrest in the $G_0$ – $G_1$ phase as a consequence of the inability of T cells to upregulate cyclin D3 and cyclin-dependent kinase 4[222]. Depletion of arginine also downregulates expression of the TCR by modulating expression of CD3ζ[223]. In addition to suppressing T-cell proliferation, it has also been reported that arginase produced by AML cells polarises surrounding

monocytes toward a suppressive M2-like / myeloid-derived suppressor cell (MDSC)-like phenotype[221].

Adenosine has immunosuppressive properties and is known to play a role in tumour immune escape[224]. Production of adenosine is catalysed by the sequential enzymatic activities of CD39, which is an ectonucleoside triphosphate diphosphohydrolase-1 that metabolises adenosine triphosphate to adenosine monophosphate, followed by CD73, which is an ectonucleotidase that converts adenosine monophosphate to adenosine[225]. Binding of extracellular adenosine to adenosine receptors expressed by anti-tumour T cells has been shown to inhibit their function[226]. Evidence that extracellular adenosine may contribute to immune escape by AML comes from a report that CD39 is expressed by AML cells and that *in vitro* proliferation of $CD8^+$ T cells in the presence of AML cells could be enhanced by treatment with an ecto-ATPase inhibitor[227]. It has been shown that mesenchymal stromal cells in the bone marrow of AML patients express CD73; thus representing a potential source of the second enzyme in the pathway for adenosine production[228].

#### 1.4.2.2 Immune checkpoint ligands

Exploitation of the immune checkpoint pathway is a mechanism of immune escape that many different types of cancers, including AML cells, are known to utilise to suppress T-cell responses[229]. Immune checkpoints comprise a plethora of inhibitory and stimulatory pathways (Figure 1-10) that are critical for controlling T-cell responses and maintaining self-tolerance[229].

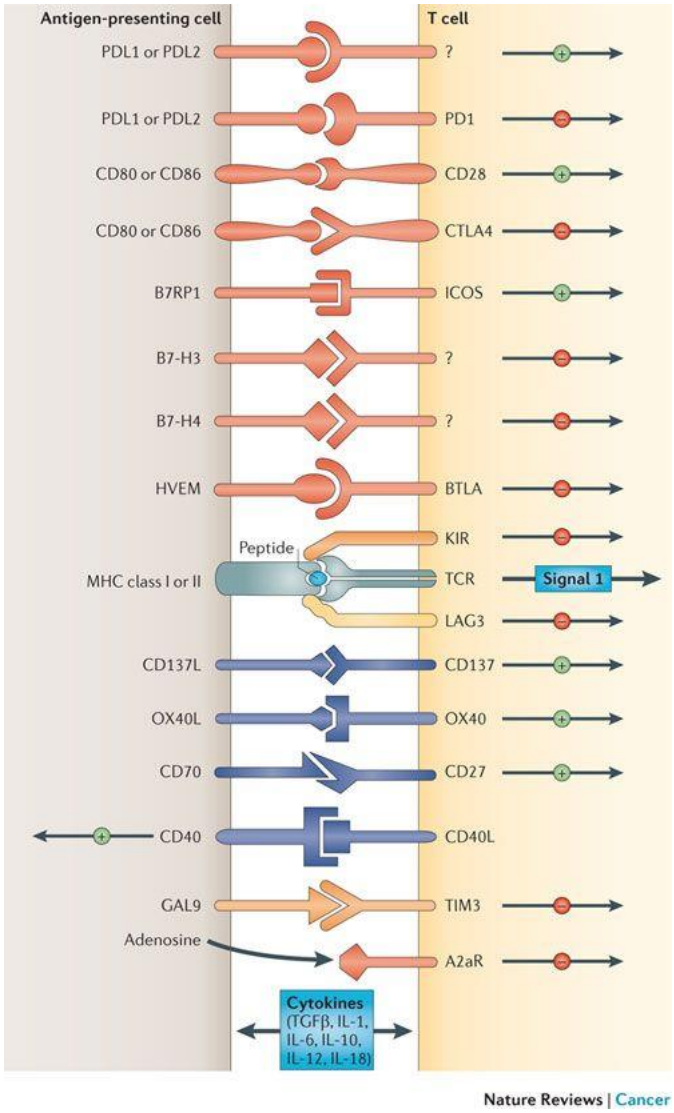



**Figure 1-10: Immune checkpoint molecules that regulates T-cell responses**

Schematic shows the various ligand-receptor interactions between APCs and T cells that regulate T-cell responses. Some ligands deliver inhibitory signals (orange circle with -) and others deliver co-stimulatory signal (Green circle with +). Pairs of co-stimulatory and inhibitory receptors bind to the same ligands, such as CD28 and CTLA-4 to CD80/CD86. (Adapted from[229])

Several studies have demonstrated that expression of the inhibitory immune checkpoint ligands PD-L1 and B7-H3 are sometimes up-regulated on patient AML cells[210, 230, 231, 232, 233]. The observations that expression of PD-L1 on AML cells is associated with relapse after allogeneic HSCT and can be induced by *in vitro* exposure to IFN-γ[230, 231] suggest that up-regulation by AML cells is a response to escape immune recognition.

For immune checkpoint ligands to exert suppressive activity, the corresponding inhibitory receptors must be expressed by T cells. There is evidence that this is the case in AML patients. Enhanced PD-1 expression by T cells from AML patients is well described and associated with suppressed function[232, 234, 235]. Several recent studies have reported comprehensive profiling of T cells in AML patients that shows co-expression of multiple inhibitory checkpoint molecules, including PD-1, CTLA-4, TIM-3, TIGIT and KLRG-1 on T cells collected from patients at the time of AML relapse after allogeneic HSCT but not patients in long-term complete remission[233, 236, 237]. Importantly, the profiles indicative of exhaustion were found more frequently on T cells present in the bone marrow of these patients, and *in vitro* analysis showed that their function was impaired[233, 237].

#### 1.4.2.3 Presence of immunosuppressive cells

Tregs play an important role in down-regulating immune responses and maintaining self-tolerance, but their activity is counter-productive in the context of diminishing anti-tumour T cell responses. Multiple immunosuppressive mechanisms are used by Tregs including production of inhibitory cytokines such as IL-10 or TGF-β, metabolic disruption by IL-2 consumption or intracellular transfer of cyclic AMP, and inhibition of DC maturation and function[238]. It has been found that patients with AML have abnormally high levels of Tregs within their peripheral blood and bone marrow compared to healthy individuals, and these cells have superior suppressive functions.[239] Furthermore, presence of excessive Tregs correlates with poor treatment outcome of AML patients[240]. The factors driving expansion of the Treg population in AML patients are not completely understood but reprogramming of effector T cells to so-called induced Tregs mediated by IDO likely plays a role (see section 1.4.2.1).

MDSCs are a heterogenous population of immature myeloid cells with potent ability to inhibit T-cell function via mechanisms that include production of reactive oxygen and nitrogen species, arginase and IDO[241]. Patients with AML have increased levels of MDSCs compared to healthy individuals, and these cells can suppress proliferation of autologous T cells and induce a switch from a Th1 to a Th2 phenotype[242]. Factors driving expansion of the MDSC population likely includes polarisation from monocytes mediated by arginase (see section 1.4.2.1) and acquisition of an MDSC-like phenotype by AML cells themselves[243].

#### 1.4.2.4 T-cell abnormality

In addition to the immune evasion strategies utilised by AML cells described above, there is also evidence that T cells in AML patients themselves become abnormal[244]. Le Dieu et al. reported an increase in the absolute numbers of T cells in the peripheral blood of AML patients compared to healthy age-matched individuals, with the $CD8^+$ T-cell population increased more than the $CD4^+$ T cells. Gene expression profiling indicated activation, but not typical of the normal signalling associated with T-cell activation. Instead, there was differential regulation of genes associated with actin cytoskeletal formation. This manifests as impaired ability to form immune synapses with autologous AML cells and recruit phosphotyrosine signalling molecules to the synapse. T-cell dysfunction may therefore contribute to failure of the immune response to eradicate AML cells[244].

## 1.5 Treatments to overcome immune suppression in AML

Attempts are being made to circumvent immune suppression by targeting the pathways exploited by AML for immune escape.

### 1.5.1 Immune checkpoint blockade

The use of check-point inhibitors to block the interaction between immune checkpoint receptors and their ligands aims to restore T-cell function. Although there has been notable clinical success in the context of treatment of solid tumours such as melanoma, studies so far indicate limited efficacy for AML[245]. Several *in vitro* studies have reported that immune checkpoint blockade using antibodies can augment the activity of T cells from AML patients[231, 246, 247, 248]. *In vivo* studies using mouse models of AML have shown that immune checkpoint blockade improves survival, although most animals eventually succumbed to disease[249, 250, 251]. A recent study demonstrated the co-expression of Tim-3 and PD-1 by $CD8_+$ T cells in AML patients has led to the testing of dual blockade with anti-Tim-3 and anti-PD-1 antibodies[250]. An additive effect on reversing T-cell exhaustion, restoring anti-tumour immunity and reducing tumour burden in animal models was observed[250]. Mixed results have been obtained in AML patients treated with immune checkpoint inhibitors. Davids et al reported use of an anti-CTLA-4 monoclonal antibody, ipilimumab, to restore the GvL effect in AML patients that had relapsed after allo-HSCT; only 23% had a complete response[252]. Berger et al reported use of anti-PD-1 monoclonal antibody, pidilizumab, to modulate T-cell response and only one of the eight patients enrolled in the study resulted in a reduced percentage of AML blasts in peripheral blood whereby disease progressed after 61 weeks[253]. Albring et al reported the use of anti-PD-1 monoclonal antibody, nivolumab, to restore GvL effects in AML patients that had relapsed after allo-HSCT; only 1 out of 3 patients had complete response[254].

### 1.5.2 Immunomodulatory drugs

Several immunomodulatory agents derived from the parent compound thalidomide are already approved for treatment of haematological malignancies. These drugs have a multitude of effects on the immune response, including cytokine modulation, up-regulation of co-stimulatory and down-regulation of inhibitory molecules on T cells, inhibition of Tregs and repair of abnormal

synapse formation[255]. Studies using lenalidomide as a single agent to treat AML have reported relatively low response rates. Lancet et al reported the case of a patient whom was treated with lenalidomide for deletion 5q AML induced complete cytogenic remission;[256] however, Sekeres et al[257] and Blum et al[258] both observed a complete response rate of only 14% and 16% respectively. In addition, lenalidomide has also been used to treat AML without deletion 5q; however, Fehniger et al observed a compete response rate of only 30% with a median time of only 30 days[259]. Since the use of lenalidomide as a single agent has been shown to have relatively low response rates, lenalidomide has also been studied in combination with other agents such as 5-azacitidine or cytarabine. Visani et al found that treatment with low dose lenalidomide and cytarabine produced a complete response rate of 39.2%, which suggests that combination therapy is more favourable compared to lenalidomide as a single agent[260].

### 1.5.3 Epigenetic drugs

The DNA methyltransferase inhibitor 5-azacitidine reverses the hypermethylation that is frequently seen in cancer patients. It is used to treat elderly AML patients who are not eligible for allogeneic HSCT. Use of epigenetic agents aims to restore normal gene expression and thereby release the block on leukaemic cell differentiation. In addition, 5-azacitidine is also known to up-regulate epigenetically silenced LAA, such as MAGE-A1 and WT1, and modulate immune response[261, 262]. The inhibition of T-cell proliferation and reduced cytokine production, and expansion of Treg cells as a result of demethylation of FoxP3 promoter were previously observed in patients and *in vitro* upon treatment with 5-azacitidine[261, 262]. Furthermore, Goodyear et al. have shown induction of a MAGE-A1 specific $CD8^+$ T-cell response in AML patients treated with 5-azacitidine which suggests that capacity to induce anti-leukaemia specific T cell responses may play a role in clinical activity[261].

## 1.6 Study objective and specific aims

The immune escape mechanisms used by AML comprise a complex system of multiple pathways. Successful implementation of the T-cell mediated immunotherapies that are being developed to treat AML requires an understanding of the impact of the leukaemia microenvironment on T-cell function and use of methods to prevent inhibition.

### 1.6.1 Objective

The goal of the work described in this thesis was to test the hypothesis that the efficacy of T-cell mediated immunotherapies for AML are diminished by the environment conditioned by the disease.

### 1.6.2 Specific Aims

1. Establish an *in vitro* model to assess T-cell function in the AML microenvironment

Use the *in vitro* model to:

2. Evaluate the efficacy of T-cell mediated immunotherapies in the AML microenvironment
3. Investigate the mechanisms of inhibition of T-cell function in the AML microenvironment
4. Test strategies to augment T-cell function in the AML microenvironment

# Chapter 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Reagents, Chemicals, and Supplier Information

| Reagent | Manufacturer |
|---|---|
| 1% $O_2$, 5% $CO_2$ supplemented with $N_2$ | BOC |
| 10x Tris-Borate-EDTA (TBE) Buffer | ThermoFisher |
| 6x Loading Dye | ThermoFisher |
| Agarose | Sigma-Aldrich |
| Annexin V – FITC/Pacific Blue | Biolegend |
| Annexin V Binding Buffer | Biolegend |
| Benzonase Nuclease, Purity >90% | Merck |
| CellTrace Violet Cell Proliferation Kit | ThermoFisher |
| DEPC Treated Water | ThermoFisher |
| Dimethylsulfoxide (DMSO) | Sigma-Aldrich |
| Direct-zol™ RNA MicroPrep w/ Zymo-Spin™ IC Columns (Capped) | Zymo Research |
| Dulbecco's Modified Eagle's Medium (DMEM) | Sigma-Aldrich |
| Dulbecco's Phosphate Buffered Saline (DPBS) | Sigma-Aldrich |
| DynaBeads Human T-Activator CD3/CD28 | ThermoFisher |
| Ethanol (100%) | Merck |
| Ethylenediaminetetraacetic Acid (EDTA) | Fisher Scientific |
| EZ-PCR Mycoplasma Test Kit | Biological Industries |
| Fetal Calf Serum (FCS) | Gibco |
| Ficoll-Paque Premium | GE Healthcare |
| Fixable Viability Dye eFluor® 780 | eBioscience |
| Foxp3 Fixation / Permeabilization Concentrate and Diluent | eBioscience |
| GelRed™ Nucleic Acid Gel Stain (10,000x) | Biotium |
| Gene Ruler DNA Ladder Mix | ThermoFisher |
| GlutaMax™ 10x | Gibco |
| HEPES Solution 1M | Sigma-Aldrich |

| | |
|---|---|
| Human AB Serum | ThermoFisher |
| Human IFN gamma ELISA Ready-SET-Go! | eBiosciences |
| Hydrochloric Acid (HCl) | Sigma-Aldrich |
| Hypoxia Incubation Chamber | StemCell |
| IC Fixation Buffer | eBioscience |
| IFN gamma Human ProcartaPlex™ Simplex Kit | ThermoFisher |
| IL-4 Human ProcartaPlex™ Simplex Kit | ThermoFisher |
| Iscove's Modified Dulbecco's Medium (IMDM) | Gibco |
| LEAF™ Purified anti-human CD3 Antibody (Clone: OKT3) | Biolegend |
| LEAF™ Purified anti-human PD-1 Antibody (Clone: EH12.2H7) | Biolegend |
| LEAF™ Purified anti-human PD-L1 Antibody (Clone: 29E.2A3) | Biolegend |
| LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody (Clone: MG1-45) | Biolegend |
| LEAF™ Purified Mouse IgG2b, κ Isotype Ctrl Antibody (Clone: MPC-11) | Biolegend |
| Lenalidomide (Powder) | Sigma-Aldrich |
| Live/Dead Fixable Viability Dye eFluor 780 | eBiosciences |
| Monensin Solution (1000x) | Biolegend |
| Monopotassium Phosphate ($KH_2PO_4$) | DBH |
| Nuclease-Free water | ThermoFisher |
| Pan T cell Isolation Kit | Miltenyi Biotec |
| LS Columns | Miltenyi Biotec |
| Penicillin/Streptomycin | Sigma-Aldrich |
| Permeabilization Buffer (10x) | eBioscience |
| Potassium Chloride (KCl) | Sigma-Aldrich |
| Precision Count Beads | Biolegend |
| ProcartaPlex Human Basic Kit | ThermoFisher |
| Recombinant Human Interleukin 15 (rhIL-15) | Peprotech |
| Recombinant Human Interleukin 2 (rhIL-2) | Peprotech |
| Recombinant Human Interleukin 3 (rhIL-3) | Peprotech |
| Recombinant Human Stem Cell Factor (SCF) | Peprotech |

| | |
|---|---|
| RetroNectin® Recombinant Human Fibronectin Fragment | Takara |
| RNA 6000 Nano Kit | Agilent Technologies |
| Rosewell Park Memorial Institute media (RPMI-1640) | Sigma-Aldrich |
| Sodium Azide ($NaN_3$) | Sigma-Aldrich |
| Sodium Chloride (NaCl) | Sigma-Aldrich |
| Sodium Phosphate Dibasic ($Na_2HPO_4$) | Sigma-Aldrich |
| Synth-a-Freeze Cryopreservative Medium | Sigma-Aldrich |
| TCR γ/δ+ T Cell Isolation Kit | Miltenyi Biotec |
| TNF-α Human ProcartaPlex™ Simplex Kit | ThermoFisher |
| Tris-Acetate-EDTA (TAE) Buffer, 50x | ThermoFisher |
| Trypan Blue solution (0.4%) | Sigma-Aldrich |
| Trypsin-EDTA Solution 10x | Sigma-Aldrich |
| Tween-20 | Sigma-Aldrich |
| UltraComp eBeads™ Compensation Beads | ThermoFisher |
| Zoledronic Acid (4mg/5ml) | Tillomed Laboratories |

### 2.1.2 Peptides

The following 15mer Human Cytomegalovirus Antigen (HCMVA) peptides were purchased from JPT Peptide Technologies at >70% purity for IE-1 and >90% for pp65 in 25μg aliquots:

- PepMix™ HCMVA (IE-1) positive control pool, comprised of 120 × 15-mer sequences, with 11 amino acids overlap
    - CMV Immediate-early 1 (IE-1) Protein ID: P13202 (Swiss-Prot)

- PepMix™ HCMVA (pp65) positive control pool, comprised of 138 × 15-mer sequences, with 11 amino acids overlap.
    - CMV phosphoprotein (pp65) Protein ID: P06725 (Swiss-Prot)

### 2.1.3 Flow Cytometry Antibodies

All antibodies used were purchased from Biolegend unless otherwise stated. Clone, Fluorochrome Conjugation, and titrated volumes of antibodies used per $1 \times 10^6$ cells are listed below:

**Table 2-1 Anti-human Antibodies**

| Antigen | Clone | Conjugation | Volume (μl) |
|---|---|---|---|
| CD3 | HIT3a | APC1 | 2.5 |
| CD3 | OKT3 | PE-Cy72 | 2.5 |
| CD4 | OKT4 | FITC3 | 2.5 |
| CD4 | OKT4 | Pacific Blue™ | 2.5 |
| CD8 | SK1 | PerCP-Cy5.54 | 2.5 |
| CD25 | M-A251 | BV510™ | 2.5 |
| CD29 | TS2/16 | Alexa Fluor® 700 | 2.5 |
| CD33 | WM53 | BV421 | 2.5 |
| CD33 | P67.6 | BV605™ | 2.5 |
| CD34 | 561 | BV421™ | 2.5 |
| CD34 | 581 | BV605™ | 2.5 |
| CD34 | 581 | BV510 | 2.5 |
| CD45RA | HI100 | APC | 2.5 |
| CD69 | FN50 | PE-Cy7 | 2.5 |
| CD123 | 6H6 | PE5 | 2.5 |
| CD127 | A019D5 | PE | 2.5 |
| CD137 | 4B4-1 | PE | 2.5 |

1 Allophycocyanin
2 Phycoerythrin-Cyanin7
3 Fluorescein Isothiocyanate
4 Peridinin-chlorophyll-protein Complex: CY5.5
5 Phycoerythrin

| | | | |
|---|---|---|---|
| CD137 | 4B4-1 | PE-Dazzle™ 594 | 2.5 |
| CD154 | 24-31 | APC | 2.5 |
| CD274 (PD-L1) | 29E.2A3 | BV711™ | 2.5 |
| CD279 (PD-1) | EH12.2H7 | BV510™ | 2.5 |
| TCR Vδ2 | B6 | PE | 2.5 |
| TCR γ/δ | B1 | PerCP-Cy5.5 | 2.5 |
| Myc | polyclonal | FITC | 1 |
| HLA-A2 | BB7.2 | PE | 2.5 |
| Anti-mouse Immunoglobulin (Agilent) | | PE | 10 |
| IFNγ | B27 | PE | 5 |
| FoxP3 | 206D | FITC | 5 |

### 2.1.4 Solutions prepared in-house

#### 2.1.4.1 Cell Culture Complete Media

- Cell Culture media, RPMI-1640, DMEM and IMDM were supplemented with 10% FCS, 100U/ml penicillin and 0.1mg/ml streptomycin.

#### 2.1.4.2 MACS Buffer

- A calcium and magnesium free Dulbecco's Phosphate Buffered Saline (PBS) containing 0.5% fetal calf serum (FCS), and 2 mM EDTA

#### 2.1.4.3 FACS Buffer

- A calcium and magnesium free PBS containing 2.5% FCS and 0.1% $NaN_3$

#### 2.1.4.4 Cell Sorting Buffer

- Calcium and Magnesium free PBS supplemented with 2% FCS, 25mM HEPES, and 2mM EDTA

#### 2.1.4.5 Cell Freezing Media

- FCS supplemented with 10% DMSO

#### 2.1.4.6 ELISA Washing Buffer

- 8 g of NaCl.
- 0.2 g of KCl.
- 1.44 g of Na2HPO4.
- 0.24 g of KH2PO4.

The above reagents were dissolved to a final volume of 1 litre in distilled water and adjusted to a pH of 7.4 to make 1x PBS. The 1x PBS solution was used to make ELISA washing buffer, containing 0.05% Tween-20.

#### 2.1.4.7 Lenalidomide

- 50mg of Lenalidomide was dissolved in 1ml DMSO to obtain stock concentration of 193mM and was frozen at -20°C in 1μl aliquots.

#### 2.1.4.8 CMV Peptide Libraries

- Peptides were reconstituted in 100μl 40% DMSO to obtain a stock concentration of 0.25μg/μl and was frozen at -20°C in 1μl aliquots.

#### 2.1.4.9 RetroNectin

- RetroNectin was diluted with sterile PBS to a stock concentration of 20μg/ml and frozen at -20°C in 5μl aliquots.

## 2.2 Cell Lines

All cell lines were handled aseptically in a Class II biological safety cabinet and grown in tissue culture treated flasks. Cells were incubated at 37°C and 5% carbon dioxide.

### 2.2.1 AML cell lines

The human AML cell lines listed in Table 2-2 were kind gifts of Dr Linda Barber. All were grown as suspension cultures in complete RPMI-1640 medium and maintained by passage at 1 in 5 dilution when medium was spent.

**Table 2-2 AML cell lines**

| Cell Lines | Characteristics |
|---|---|
| U937 | Histiocystic lymphoma origin. Macrophage-like with potential for terminal monocytic differentiation[263]. |
| HL60 | acute promyelocytic leukaemia origin |
| THP-1 | acute monocytic leukaemia origin |
| NB-4 | acute promyelocytic leukaemia origin with PML-RARa rearrangement due to t(15;17) translocation[264, 265]. |
| MOLM-14 | acute myeloid leukaemia origin with monocyte-specific esterase and MLL-AF9 fusion gene due to ins(11;9)(q23;p22p23)[266]. |
| KG1 | erythroleukemia that developed into AML origin[267]. |

### 2.2.2 Human stromal cell line HS5

The human stromal cell line HS5 was derived from HPV-16 E6/E7 transformed bone marrow stroma cells[268]. HS5 cells were grown as adherent cultures in complete DMEM medium and passaged when cells reached 90% confluency. To obtain a cell suspension, HS5 cells were washed in PBS and incubated with 5ml 1x Trypsin-EDTA for 5 minutes at 37°C. When all cells were in suspension, trypsinisation buffer was neutralized with an equal volume of complete RPMI-

1640, centrifuged at 300$g_{max}$ for 5 minutes and resuspended in appropriate medium. HS5 cells were cultured in complete RPMI-1640 medium for 48 hours prior to use in AML co-culture experiments.

### 2.2.3 Packaging cell lines

#### 2.2.3.1 293T

The lentivirus packaging cell line 293T was used to produce lentivirus for CD123 CAR T-cell transduction as described in section 2.3.7.5. 293T is an adherent cell line and was maintained in complete DMEM and passaged when cells reached 80% confluency by trypsinisation, as described for HS5 cells. 293T cells were only maintained for a maximum of 10 passages before discard and thawing a new aliquot of cells.

#### 2.2.3.2 PG13 cell line

The Retrovirus packaging cell line PG13 was used to produce retrovirus for PSMA CAR T-cell transduction as described in section 2.3.7.6. PG13 is an adherent leukaemia cell line that was derived from TK- NIH/3T3 cells and based on Gibbon ape leukemia virus.[269] PG13 cells were maintained in complete IMDM supplemented with 1x GlutaMax and passaged when cells reached 80% confluency by trypsinisation, as described for HS5 cells.

## 2.3 Methods

### 2.3.1 Primary cells

#### 2.3.1.1 Primary AML cells

Primary AML cells were obtained from the bone marrow of patients at King's College Hospital who were participants in a Phase I clinical trial of vaccination using CD80/IL-2 modified AML blasts in patients with relapsed AML post allogeneic haematopoietic stem cell transplantation (HSCT) (RFUSIN2-AML1 vaccine trial, EudraCT Number 2005-000806-29). Patients that received allogeneic HSCT and experienced relapse of AML with 5% > blasts in the bone marrow aspirate were eligible for cryopreservation of AML blasts. Suspected AML relapse after allogeneic HSCT was confirmed by bone marrow aspirate and trephine biopsy of bone marrow, whereby morphologic, immunophenotypic, cytogenetic, and chimerism were assessed. The cytogenetic profiles of the patient samples are listed in Table 2-3. Up to 50ml of bone marrow aspirate was collected in EDTA-containing vacutainer tubes for isolation and cryopreservation of mononuclear cells from eligible consenting patients. Cells were isolated by Ficoll-Paque density gradient centrifugation and cryopreserved as described section2.3.1.2. Primary AML cells were thawed in assay medium containing 0.2% benzonase to prevent formation of cell clumps.

**Table 2-3 Cytogenetics of the Primary AML Samples used**

| Samples | Cytogenetics |
|---|---|
| AML011 | Unable to see result on system |
| AML017 | 46,XX [15] |
| AML038 | 46,XY [20] |
| AML047 | 48,XX,+8,+19[9]/ 48,XX,del(7)(q22q36),+8,+19[3] |
| AML048 | 46,X,-X,del(1)(q31q42),t(2;4)(p23;q12),t(6;15)(q12;q11.2),add(10)(q11.2),add(13)(p11.2),add(13)(q14.1),add(16)(q22),add(19)(p13),+mar[10] |
| AML063 | Cytogenetic test failed |
| AML065 | 43~45,XY,add(1)(q21),-7,add(11)(q21),-16,-17,-17,-18,-20,-21,-22,-22,+6~8mar[7] |
| AML066 | 46,XY,inv(3)(q21q26)[4]/46,XY,add(2)(q21),inv(3)(q21q26),del(7)(q11q36),add(11)(p13),del(11)(q21q23)[3] |
| AML067 | 45,XY,add(3)(p13),add(3)(q21),del(6)(q13q25),-7,inv(8)(p21q11),-11,del(12)(q24.1q24.3),-14, add(17)(p11),+2mar [15] |
| AML075 | 46, XY [20] |
| AML076 | 43~44,X,-Y,add(1)(q32),del(5)(q22q33),-7,add(11)(p15),add(12)(p13),-13,-16,add(20)(q11),+2mar[cp10] |

#### 2.3.1.2 Peripheral Blood Mononuclear Cells from healthy donors

Peripheral blood leukophoresis products were purchased from National Health Service Blood and Transplant (NHS-BT) in the form of leukocyte reduction chambers (commonly known as leukocyte cones). Cells were recovered as described by Néron *et al* aseptically in a class II biological safety cabinet, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation[270].

Leukocyte cones were swabbed with 70% ethanol prior to being dispensed into a 50ml tube. Cones were flushed with 20ml PBS and the eluate made up to 35ml with PBS providing an approximately 1:3 dilution, which was then gently mixed. A tube containing 15ml of Ficoll-Paque was prepared and the leukophoresis product was carefully layered onto the gradient. Tubes were centrifuged at $1000g_{max}$ with full acceleration and zero brake for 20 minutes at room temperature. The visible white PBMC layer present after completion of the centrifuge cycle was collected into a 50ml tube and washed twice with PBS at $250g_{max}$ and $200g_{max}$ respectively for the removal of platelets. The cell pellet was resuspended in complete RPMI-1640, and live cells counted by trypan blue exclusion. PBMC were cryopreserved in 1ml chilled synth-a-freeze medium per $5*10_7$ cells in cryovials and stored within a Nalgene (Nunc International) temperature controlled freezing container containing isopropanol in a -80°C freezer. The frozen vials were removed from the Nalgene container after 24 hours and transferred to -196°C gaseous phase liquid nitrogen storage in a cryostore facility.

PBMC were retrieved from the cryostore and thawed quickly at 37°C and added to the appropriate assay medium gently, dropwise initially, for washing and counting prior to use.

#### 2.3.1.3 PBMC from patients after allogeneic HSCT

PBMC were obtained from patients attending King's College Hospital and viable cells were cryopreserved as described in section 2.3.1.2 and stored in the King's College London Haemato-Oncology tissue bank. Written informed consent was obtained from all patients and use of patient samples under ethics no. REC 08/H0906/94 was approved by Newcastle and North Tyneside research ethics committee. Patients were pseudo-anonymised with code numbers beginning RC (research case).

### 2.3.2 T-cell purification

#### 2.3.2.1 Pan T-Cell Isolation

T cells were isolated from PBMC by negative selection via MACS Pan T Cell Isolation Kit (Miltenyi Biotec). PBMC were pelleted and at $300g_{max}$ for 10 minutes, and supernatant removed completely. The cell pellet was resuspended in MACS buffer (40µl per $10_7$ cells) and a biotin conjugated antibody cocktail was added to label non-T cells (10µl per $10_7$ cells). After incubating at 4°C for 5 minutes, magnetic microbeads conjugated with anti-biotin antibody were added (20µl per $10_7$ cells) and incubated for a further 10 minutes at 4°C. This solution was then applied to a LS MACS column, where all the non-T cells were retained in the column and the negatively selected T cells were eluted and collected. Cells were counted and resuspended in appropriate culture media, and purity check was performed by flow cytometry to ensure >90% purity.

#### 2.3.2.2 γδ T-cell Isolation

γδ T cells were isolated from PBMC by negative selection via MACS TCRγ/δ+ T Cell Isolation Kit (Miltenyi Biotec). PBMC were pelleted at $300g_{max}$ for 10 minutes, and supernatant removed completely. The cell pellet was resuspended in MACS buffer (80µl per $10_7$ total cells) and a biotin-antibody cocktail was added to label non-γδ T cells (20µl per $10_7$ cells). After incubation at 4°C for 10 minutes, cells were washed with MACS buffer by adding 2ml of MACS buffer per $10_7$ cells and pelleted at $300g_{max}$ for 10 minutes. The supernatant was removed completely, cells were resuspended in 80µl per $10_7$ of MACS buffer, 20µl of anti-biotin MicroBeads were added per $10_7$ cells and incubated at 4°C for 15 minutes. Cells were washed by adding 2ml of MACS buffer per $10_7$ cells and pelleted at $300g_{max}$ for 10 minutes, and supernatant removed completely. The cell pellet was resuspended in 500µl of MACS buffer. The cells were then applied to a LS MACS column, where all the non-γδ T cells were retained in the column, and the negatively selected γδ T cells were eluted and collected. Cells were counted and resuspended in appropriate culture media, and purity check was performed by flow cytometry to ensure >90% purity.

### 2.3.3 *In vitro* co-culture Assay

Human stromal HS5 cells was plated overnight in advance to obtain an adherent monolayer before the AML cells were added and cultured with the HS5 cells overnight to establish the AML microenvironment milieu. Cultures of AML cells without HS5 cells were plated out the next day

using freshly thawed AML samples. T cells were incubated with AML cells and HS5 at a ratio of 1:1:0.1 in the presence of anti-CD3/CD28 beads (DynaBeads Human T-Activator CD3/CD28; ThermoFisher) at 1:1 T cell to bead ratio, as recommended by the manufacturer (as shown in ***Figure*** 2-1). Cultures were performed using complete RPMI supplemented with 30IU/ml IL-2 and incubated at 37°C. Cells and culture supernatant were collected after 48 hours. Cells from cultures containing HS5 cells were harvested by trypsinisation, as described in section 2.2.2. Cells were labelled with fluorochrome conjugated antibodies, as described in section 2.3.4.1 and 2.3.4.2, and analysed by flow cytometry as described in section 2.3.4.8.

For cultures treated with anti-PD-1 and anti-PD-L1 blocking antibodies or lenalidomide, reagents were added at the beginning of the co-culture at a final concentration of 20μg/ml for blocking antibodies or 5-10μM for lenalidomide. Zoledronic acid (ZA) was used to sensitise the U937 AML cell line for recognition by γδ T cells. In these assays, U937 cells were treated with 1μg/ml ZA (Tillomed Laboratories) overnight prior to setting up the co-cultures.

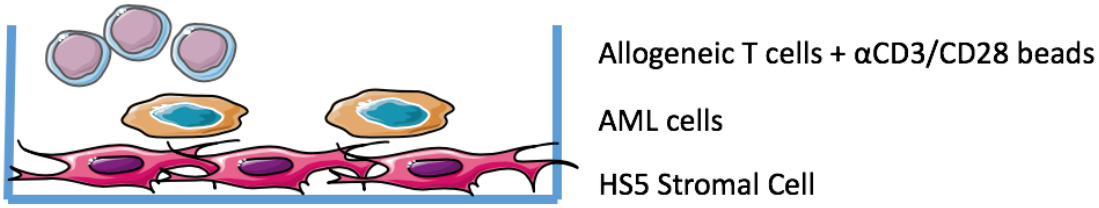



**Figure 2-1 Representation of Triple Cell Co-Culture**

HS5 Stromal cells were first co-cultured overnight in advance to achieve a monolayer. On the next day, AML cells were added to the co-culture overnight to obtain the microenvironment milieu. Finally, allogeneic T cells were added, after the establishment of the microenvironment milieu, to the co-culture and anti-CD3/CD28 beads were added to the culture to stimulate the T cells.

### 2.3.3.1 Hypoxia Incubator Chamber

Hypoxia incubator chamber is an air-tight chamber used to perform *in vitro* co-culture assay described in 2.3.3 under hypoxic conditions. Co-cultures were set up and tissue culture plates were placed and sealed within the hypoxia incubator chamber. The chamber was purged with 1%

$O_2$, 5% $CO_2$ supplemented with $N_2$ at a rate of 20 litres/min for 5 minutes and placed in a 37°C incubator. The chamber was flushed again after 1 hour of incubation and placed in a 37°C incubator for the duration of the assay.

#### 2.3.3.2 Transwell Co-Culture Assay

HS5 cells were always seeded at the bottom compartment (shown in Figure 2-2) overnight in advance to obtain a monolayer. Similar to the *in vitro* co-culture described in section 2.3.3, primary AML cells were added and cultured overnight in either the upper or bottom compartment in the presence of HS5 cells under hypoxic conditions; for cultures without HS5 cells, primary AML cells were thawed and plated in the upper compartment on the next day. The next day, T cells were added and activated with anti-CD3/CD28 beads and cultured in the absence of direct contact or in direct contact with AML cells under hypoxic conditions. Cells and culture supernatants were collected after 48 hours for analysis.

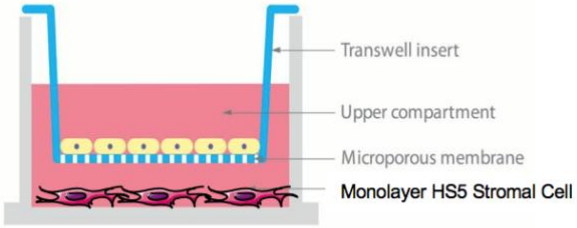



**Figure 2-2 Schematic Diagram of the Transwell Co-Culture**

Transwells were used to evaluate contact dependence of interactions between primary AML cells and T cells.

### 2.3.4 Flow Cytometry

#### 2.3.4.1 Cell Surface Phenotyping

Cell surface phenotyping was conducted using $1 \times 10^6$ to $5 \times 10^6$ cells in 100µl of PBS supplemented with 2.5% FBS and 0.1% sodium azide, also known as FACS buffer, with

fluorochrome conjugated antibodies in a FACS tube. Cells were first washed with 2ml PBS to remove serum proteins and stained with fixable viability dye eFluor 780 (eBioscience) protected from light at room temperature for 20 minutes. Cells were washed with 2ml FACS buffer twice and stained in the phenotyping cocktail (shown in Table 2-4) for 30 minutes at room temperature protected from light. After 30 minutes, cells were washed twice and resuspended in 500µl of FACS buffer.

**Table 2-4 Fluorochrome Conjugated Antibody Panel**

| | Antibody | Fluorochromes |
|---|---|---|
| T-cell Activation Panel | CD3 | PE-Cy7 |
| | CD4 | FITC |
| | CD8 | PerCP-Cy5.5 |
| | CD137 | PE |
| | CD154 | APC |
| AML cell Panel | CD33 | BV605 |
| | CD34 | BV605 |
| HS5 cell panel | CD29 | Alexa Fluor® 700 |

#### 2.3.4.2 Intracellular Phenotyping

After the last wash from cell surface staining described in section 2.3.4.1, supernatants were discarded and cells were resuspended and vortexed in 100µl of IC Fixation Buffer and incubated at room temperature protected from light for 45 minutes. Cells were washed twice with 2ml of 1x Permeabilization Buffer and centrifuged at $400g_{max}$ for 5 minutes and stained with antibodies for intracellular markers for 30 minutes at room temperature protected from light. After 30 minutes, cells were washed twice with 1x Permeabilization Buffer and resuspended in 500µl of FACS buffer.

#### 2.3.4.3 Viability Assay

This assay was performed using Annexin V conjugated to Pacific Blue or FITC. Annexin V binds to the phosphatidylserine that is present on the cell surface in the early process of apoptosis.

After the last wash from cell surface staining described in section 2.3.4.1, cells were washed with ice cold PBS twice and resuspended in 100µl Annexin V binding buffer and stained with Annexin V protected from light at room temperature for 15 minutes. 400µl Annexin V binding buffer was added to the samples to stop the reaction and used immediately for analysis by flow cytometry.

#### 2.3.4.4 Absolute Cell Counting by Flow Cytometry

Counting beads are brightly fluorescent microspheres that have a wide range of excitation and emission wavelengths. Upon completion of staining, cells were resuspended to a total volume of 500µl with FACS buffer and 50µl of Precision Count Beads™ (Biolegend) added to the sample immediately prior to analysis by flow cytometry.

Absolute cell counts were calculated as follows:

$$\text{Cell Count}\left(\frac{\text{cells}}{\mu\text{l}}\right) = \frac{\text{Cell Count} \times \text{Bead Volume}}{\text{Bead count} \times \text{Cell Volume}} \times \text{Counting bead Concentration}$$

#### 2.3.4.5 Compensation Set-up and Controls for Flow Cytometry

The compensation matrix was produced using single stained aliquots of UltraComp eBeads (Invitrogen) for cell surface and intracellular antibodies, each labelled with one of the fluorochromes used. Fluorescence minus one (FMO) controls were used for fluorescence gating.

Live/dead cells were used for viability and annexin V compensation controls. Fresh PBMC was prepared at concentration of $1 \times 10^6$ cells/ml in two FACS tubes. To obtain dead cells, one tube was placed in a 67°C water bath for 4 minutes and then placed on ice for 5 minutes. Live and dead cells were then combined and stained with fixable viability dye or Annexin V as described in section 2.3.4.3.

#### 2.3.4.6 Flow Cytometer

A flow cytometer was used to analyse cells after staining, including absolute cell counting with counting beads. Data was acquired using a BD LSRFortessa™ with FACSDiva™ software (BD). A minimum of 5,000 events were acquired for all cell types. Raw data was exported and analysed using FlowJo flow cytometry data analysis software (TreeStar Inc.)

#### 2.3.4.7 Fluorescence Activated Cell Sorting (FACS)

Fluorescence activated cell sorting allows the separation of a sub-population from a mixed-cell population. Prior to cell sorting, cells were stained for viability and cell surface markers as described in section 2.3.4.1. Cells were resuspended in 500µl of PBS supplemented with 2% FCS, 25mM HEPES, and 2mM EDTA, known as sorting buffer, and passed through a 40µm cell strainer to remove cell clumps and placed on ice. Cells were sorted using a BD FACSARIA II or BD FACSARIA III and collected into 5ml polystyrene tubes and placed on ice. Sorted cells were pelleted and RNA extracted as described in section 2.3.9.1

#### 2.3.4.8 Flow Cytometry Gating Strategy and Data Analysis

The sequential gating strategy for AML cells, HS5, and T cells is illustrated in Figure 2-3. Doublets, debris, and dead cells were excluded prior to the gating of cells based on their respective cell surface markers. **AML Cells:** used CD33/CD34$_{+}$ population. **HS5:** used CD33/CD34$_{-}$ and CD29$_{+}$ population. **T Cells:** used CD33/CD34$_{-}$, CD29$_{-}$, and CD3$_{+}$ population, with subsequent segregation into CD3+ CD8$_{+}$ (CD8 T cells) and CD3+ CD8- (CD4 T cells).

Activation marker expression by CD4 and CD8 T cells was evaluated based on both percentage of positive cells and the median fluorescence intensity (MFI) of the positive cells. Data was presented as inhibition index, calculated as follow:

$$Inhibition\ Index = \left[1 - \left(\frac{MFI\ for\ Test\ Condition}{MFI\ for\ Positive\ Control}\right)\right] \times 100\%$$

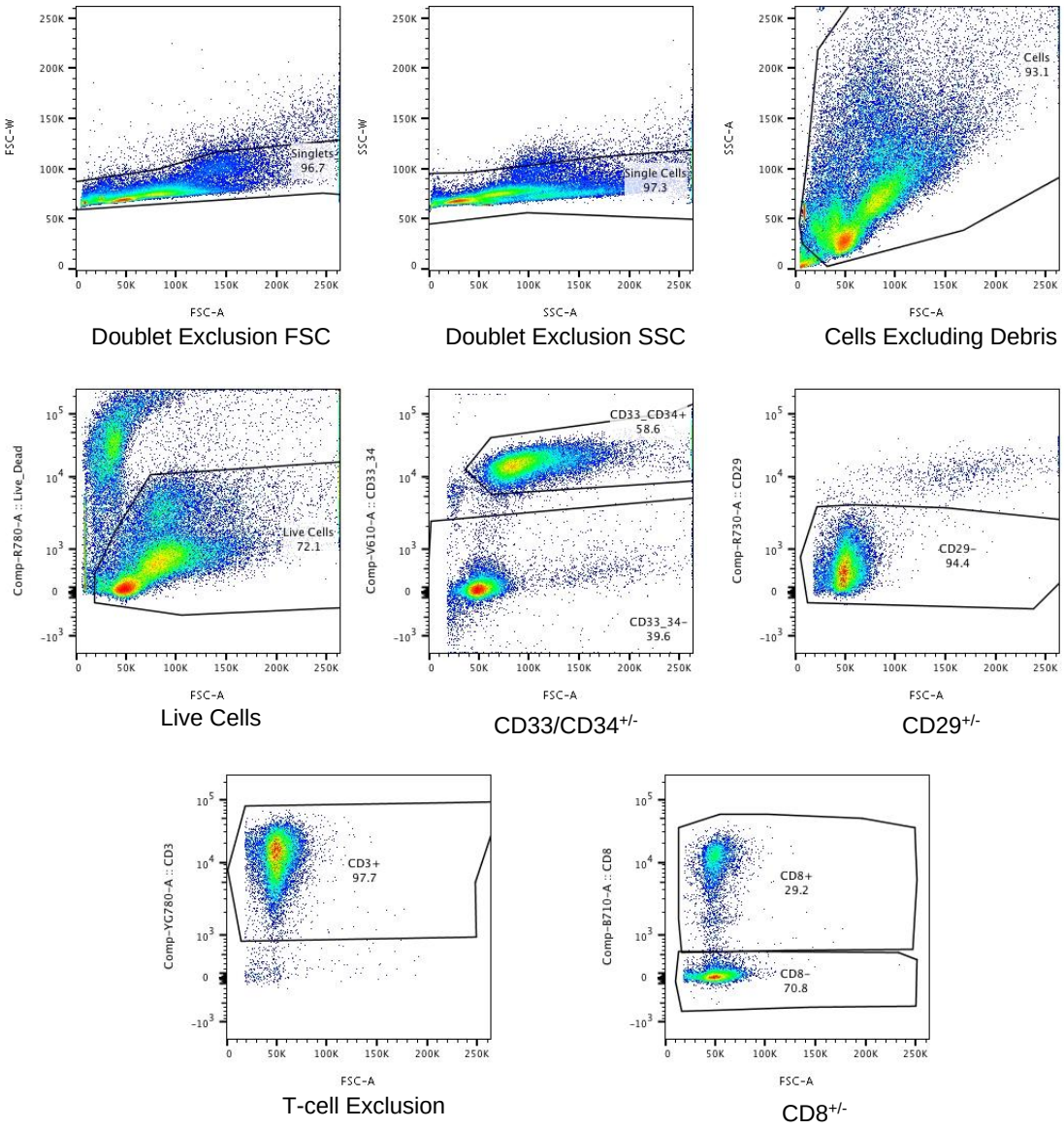



**Figure 2-3 Gating Strategy for cell types in the co-cultures**

Gating strategy for the co-culture of AML, HS5, and T cells. Gating achieved the exclusion of doublets, debris, and dead cells, followed by cell surface markers to identify the different cells within the co-culture. AML cells were gated on CD33/34+. HS5 cells were gated on CD29+ excluding AML cells. T cells were gated on CD3+ excluding AML and HS5 cells.

### 2.3.5 Cytokine Quantification

Cytokines released into the culture supernatants were quantified by ELISA or multiplex cytokine bead array using the Luminex platform. All supernatants were collected after the co-culture and stored frozen at -20°C until analysed.

#### 2.3.5.1 Cytokine Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of cytokine production were determined using uncoated ELISA kit (Invitrogen), which contains all the antibodies and reagents required for the protocol. All antibodies and reagents were diluted immediately prior to use according to the manufacturer's instructions.

Maxibinding 96 well polystyrene plates were coated with 100μl/well capture antibody and stored overnight at 4°C. Plates were subsequently washed three times in 0.05% (v/v) PBS-Tween20 and blotted dry to remove residual buffer. Plates were then blocked with 200μl/well 1x ELISA diluent for 1 hour, buffer aspirated and blotted dry prior to the addition of samples. Samples were diluted as needed with ELISA diluent to a total volume of 100μl/well to achieve optimal signal detection, and a 1:2 serial dilution was performed for the standards using ELISA diluent. Plates were kept overnight at 4°C and washed three times the next day. Plates were incubated with 100μl/well detection antibody at room temperature for 2 hours, washed three times, and incubated with 100μl/well of diluted horseradish peroxidase (HRP)-Streptavidin at room temperature protected from light for 30 minutes. Plates were then washed three times and 100μl/well of Tetramethylbenzidine substrate solution (TMB) was added and protected from light. Stop solution was added to each well to prevent further colour change after 20 minutes. Plates were read on a spectrophotometer at wavelengths of 450nm and 562nm.

#### 2.3.5.2 Cytokine Quantification by Multiplex Bead Array using the Luminex Platform

The Luminex platform is a magnetic bead-based assay that allows the quantification of multiple cytokines. Magnetic beads used in the Luminex assay are colour coded and pre-coated with cytokine specific capture antibody, which can be easily segregated and distinguished. Samples were analysed on a dual laser Luminex 200 (Luminex Corporation) machine upon conjugation of Streptavidin conjugated with the PE fluorochrome (SA-PE) to the detection antibody. The assay

was performed according to the manufacturer's instructions, with cell culture supernatants diluted as required.

### 2.3.6 γδ T cells

#### 2.3.6.1 *In vitro* Expansion of γδ T cells

Peripheral blood was collected into a sodium citrate vacutainer tube (BD) and used immediately for cell isolation and assay set up. PBMC were isolated by density-gradient separation as described in 2.3.1.2. Cells were counted and cultured at $3 \times 10^6$ cells/ml in RPMI supplemented with 10% human AB serum (Sigma), Glutamax (Gibco), Penicillin-Streptomycin (Gibco), 100U/ml IL-2 (Peprotech), 10ng/ml IL-15 (Peprotech), and 1μg/ml ZA at 37°C and 5% $CO_2$. Additional medium and cytokines, but not ZA, were added every 2 days over a period of 14 days.

#### 2.3.6.2 γδ T cells cytotoxicity assay

After 14 days of *in vitro* expansion as described in 2.3.6.1, a 6-hour cytotoxicity assay was performed to assess recognition and lysis of ZA sensitised U937 by γδ T cells. Expanded γδ cells were harvested and purified by negative selection using a MACS TCRγ/δ+ T Cell Isolation Kit, as described in section 2.3.2.2. $1 \times 10^5$ ZA sensitised U937 target cells and $1 \times 10^5$ γδ T cells were co-cultured with or without $1 \times 10^4$ HS5 in a F-bottom 96 well plate. After 6 hours, culture supernatants were collected and frozen at -20°C. Cells were harvested and stained with the cell surface antibody panel as described in section 2.3.4.1. Live cell numbers were calculated for both target cells and γδ T cells using counting beads as described in 2.3.4.4.

Percent survival of target cells was calculated as follows:

$$\%\ survival = \frac{Absolute\ \ viable\ CD33^+\ target\ cells\ cultured\ with\ \gamma\delta\ \text{T cells}}{Absolute\ \ viable\ CD33^+\ target\ cells\ cultured\ alone} \times 100$$

### 2.3.7 CAR T-cell Production

#### 2.3.7.1 CD123 CAR Lentivirus Vector Production

A 3rd generation lentiviral vector was produced as described by Dull et al via the co-transfection of 293T cells with the packaging plasmids pMDLg/pRRE (Gag-Pol; Addgene) and pRSV-Rev (Rev; Addgene), envelope plasmid pMD2.G (VSV-G; Addgene), and anti-CD123.CAR vector

(kindly provided by Dr. Georg Fey, Friedrich-Alexander-Universität Erlangen-Nürnberg)[271]. Figure 2-4 details the 3rd generation lentiviral construct.

The human renal epithelial cell line 293T was seeded at $6.6 \times 10^6$cells in 105ml of complete DMEM per HY 3-layer cell culture flask (T600 flask) for 3 days. Upon reaching 80% confluency, a mixture of transfection plasmids and transfer vector (Solution A), and PEI solution (Solution B), listed in Table 2-5, was prepared. The transfection plasmids were mixed in with PEI solution and vortexed for 20 seconds to achieve a homogenous solution. The mixture was then incubated for 15 minutes at room temperature and then topped up to 97.2ml with complete media. The media in each T600 flask was replaced with 88.65ml of the freshly made transfection mixture.

The transfection mixture was removed after 24 hours and 293T cells were gently washed with 18ml of pre-warmed Hank's Balanced Salt Solution. Each T600 flasks were topped up with 90ml of complete DMEM and incubated for a further 24 hours. Supernatants were harvested and centrifuged at 500$g_{max}$ for 5 minutes to pellet cells and filtered through a 0.45μm polyethersulfone filter to remove cell debris. Filtered supernatants were ultracentrifuged at 10,000$g_{max}$ for 16 hours at 10°C to concentrate virus. Concentrated viruses were resuspended at 1:300 of original volume and stored at -80°C in 25μl aliquots.

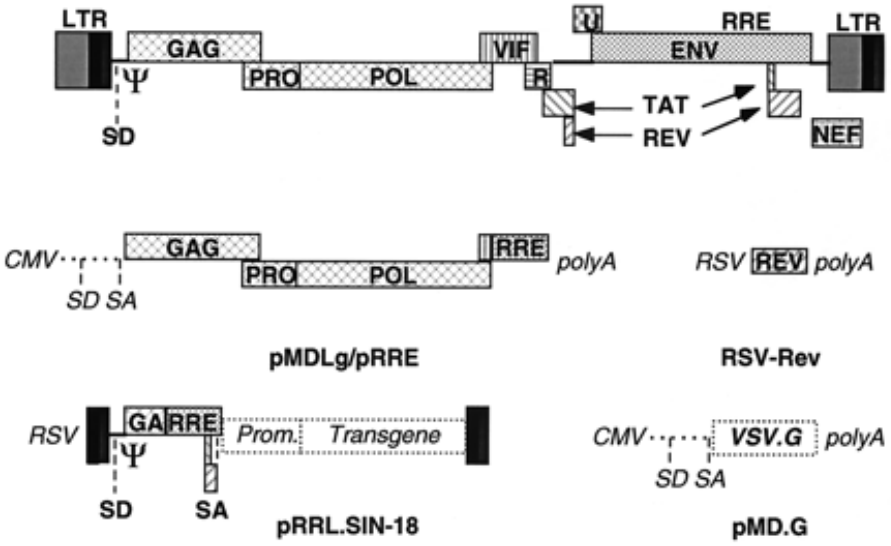



**Figure 2-4 3rd generation lentivirus Construct**

Adapted from[271]

**Table 2-5: Lentiviral vector solution mix**

| Solution A: Transfection plasmids | Total μg DNA required (per T600) |
|---|---|
| **pMDLg/pRRE (Gag-Pol)** | 12.93 |
| **pRSV-Rev (Rev)** | 19.3 |
| **pMD2.G (VSV-G)** | 7.8 |
| **Transfer Vector** | 25.85 |
| **Serum Free MEDIUM** | **Top up to 3756.16 μl** |

| Solution B: PEI mixture | total volume required (per T600) |
|---|---|
| **PEI pro** | 131.37μl |
| **serum free medium** | **Top up to 3624.79 μl** |

#### 2.3.7.2 Analysis of Lentiviral Titre

293T cells were seeded at $1 \times 10^5$ cells in 0.5ml complete DMEM in a 24 well plate overnight prior to transduction. The next day, 400μl of complete DMEM and a final concentration of 4μg/ml polybrene were added prior to virus addition. Serial dilutions of viral particles (0μl, 0.001μl, 0.01μl, 0.1μl, 1μl of concentrated virus) was added to each well in 100μl medium. After 24 hours, each well was topped up with 0.5ml of complete DMEM and cultured for a further 48 hours. Cells were harvested and functional viral titres were determined by flow cytometry analysis of transgene expression in transduced cells at each dilution.

Transgene expression of 0.5% - 20% were used in the following equation to determine viral titre:

$$Titre\left(\frac{Infection\ Unit\ (IU)}{ml}\right) = \frac{(\#\ of\ cells\ at\ transduction \times\ \%\ transduction\ efficiency)}{(vector\ volume\ \times\ dilution\ factor\ \times\ 100)}$$

#### 2.3.7.3 Preparation of RetroNectin-Coated plates

24-well plates were coated with RetroNectin at 20μg/cm2 plate area overnight at 4°C and blocked with 2% FCS in PBS at room temperature for 30 minutes. Plates were washed once with PBS and were sealed and stored at 4°C for up to one week.

#### 2.3.7.4 RetroNectin-bound virus infection method

On the day of T-cell transduction, the desired volume of diluted lentivirus solution or retrovirus stock solution at 500μl/cm2 was added to the RetroNectin-coated 24-well plates, and placed in a centrifuge pre-warmed to 32°C and centrifuged at 2,000$g_{max}$ for 2 hours to facilitate binding of virions. Plates were then washed with PBS containing 2% FCS and used immediately for CAR T-cell transduction, as described in 2.3.7.5 and 2.3.7.6.

#### 2.3.7.5 Lentivirus CAR T-cell transduction

Prior to T-cell transduction, purified T cells were stimulated with anti-CD3/CD28 beads for 48 hours in complete IMDM supplemented with 20ng/ml IL-2. After 48 hours, T cells were counted and plated at $0.75 \times 10^6$ cells/ml onto the virion coated RetroNectin plate. T cells were transduced with lentivirus at a MOI of 5 and expanded for a total of 14 days; fresh complete IMDM supplemented with 20ng/ml IL-2 was added every 2 days.

After 14 days, cells were counted and cryopreserved in 1ml chilled synth-a-freeze medium or freezing media per $1 \times 10^7$ cells in cryovials; $1 \times 10^6$ T cells were stained for expression of the transgene to assess transduction efficiency.

#### 2.3.7.6 Retrovirus CAR T cell transduction

A retroviral vector encoding a prostate-specific membrane antigen (PSMA) CAR transgene (construct J591 scFv P28z IRES; shown in Figure 2-5) was kindly provided by Dr John Maher (King's College London).

The PG13 packaging cell line was thawed and cultured in complete IMDM for a week before use. To produce the PSMA retroviral vector supernatant, the packaging cell line was passaged and plated in a 24 well plate at $1 \times 10^5$cells/well in complete IMDM for 48 hours. Supernatant was harvested after 48 hours and placed onto a RetroNectin-coated plate to facilitate retrovirus binding to the RetroNectin, as described in section 2.3.7.4. Purified T cells were stimulated with anti-CD3/CD28 beads for 48 hours in complete IMDM supplemented with 20ng/ml IL-2 and added onto the retrovirus-coated RetroNectin plates at $0.75 \times 10^6$ cells/ml. An additional 1.5ml of fresh viral supernatant was added to maximise the number of retrovirus virions present during transduction. T cells were cultured for a total of 14 days; fresh complete IMDM supplemented with

20ng/ml IL-2 was added every 2 days. Cells were counted and cryopreserved after 14 days of expansion, and $1 \times 10^6$ T cells were immediately stained for mouse IgG to assess transduction efficiency.

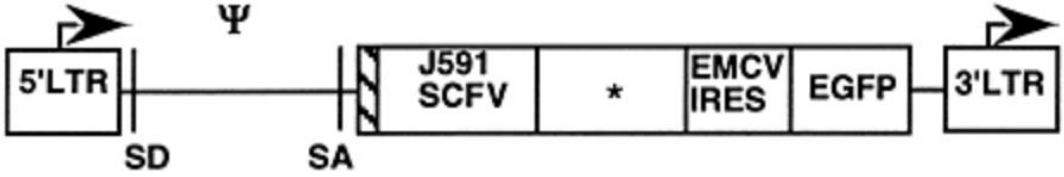



**Figure 2-5 PSMA CAR Vector Construct**

Adapted from[272]

#### 2.3.7.7 CellTrace Violet Labelling of Target Cells

Celltrace Violet (Invitrogen) is a bright fluorescence dye that stably binds to intracellular amines that is traditionally used to monitor generations of proliferating cells. Celltrace Violet was dissolved according to the manufacturer's instructions to obtain a stock concentration of 5mM, and further diluted in DMSO to a working solution of 1mM.

Target cells were washed once and resuspended in sterile PBS containing 1% FCS (PBS/FCS 1%) at $1 \times 10^6$ cells/ml and transferred to a 50ml tube covered in foil to protect from light. Target cells were stained by pipetting 1μl of diluted CellTrace working solution per $1 \times 10^6$ cells, to achieve 1μM final concentration, onto the wall of the tube and pulse vortexed to mix thoroughly. Cells were incubated at room temperature for 5 minutes and 30ml of ice-cold complete RPMI was added to the tube and incubated on ice for a further 5 minutes. Cells were pelleted and washed twice with complete RPMI-1640 at $300g_{max}$ for 10 minutes, and pellet was resuspended in appropriate medium to be used immediately.

#### 2.3.7.8 CD123 CAR T Cell Cytotoxicity Assay

CD123 CAR T cells were thawed and cultured in complete RPMI supplemented with 20ng/ml IL-2 overnight at 37°C. Next day, target cells were labelled with CellTrace Violet as per described in section 2.3.7.7; $1 \times 10^6$ labelled target cells and CD123 CAR T cells (Effector:Target Ratio of 1:1) were plated onto a 24 well plate in the presence or absence of HS5 cells, and incubated for 4

hours. Supernatants were collected for cytokine quantification, and cells were harvested and stained for viability, cell surface markers and Annexin V, a marker for apoptosis. Absolute surviving target cell count was calculated and reported as percent survival of Celltrace Violet$_+$ target cells.

The percent survival of target cells is calculated as follows:

$$\%\ survival = \frac{Absolute\ \ viable\ target\ cells\ cultured\ with\ \text{CD123 CAR T cells}}{Absolute\ \ viable\ target\ cells\ cultured\ alone} \times 100$$

### 2.3.8 *In vitro* stimulation of T cells with CMV peptide libraries

Cryopreserved patient PBMC were thawed and resuspended in complete RPMI-1640. For each culture condition, $1 \times 10^6$ PBMC were plated into a 96 well U-bottom plate. PBMC were stimulated for 18 hours with CMV peptide libraries, pp65 and IE-1, at a final concentration of 1μg/ml in 3 different culture medium conditions: fresh complete RPMI-1640, 50% primary AML cell-conditioned medium or 50% primary AML cell and HS5 cell-conditioned medium. The negative control condition was 1μl 40% DMSO only. Two positive control conditions were required, anti-CD3/CD28 beads stimulated at 1:1 PBMC to bead ratio and CMV peptide libraries stimulated PBMC; both were cultured in fresh complete RPMI-1640. Functional co-stimulatory antibodies, CD28 and CD49d, were added to all CMV peptide libraries stimulated conditions at a final concentration of 1μg/ml. Cells and culture supernatants were collected after 18 hours for cell surface phenotyping and cytokine analysis respectively.

### 2.3.9 RNA

#### 2.3.9.1 RNA Extraction

RNA was extracted using a Direct-Zol RNA MicroPrep kit (Zymo Research), whereby RNAs are selectively bound to a silica-based spin column.

CD3$_+$ CD8$_+$ sorted cells were pelleted and resuspended in 300μl Tri-Reagent and kept at -80°C until all samples had been collected and ready for RNA extraction. Frozen Tri-Reagent samples were thawed on ice completely, and an equal volume of 100% ethanol (Merck) was added to the Tri-Reagent solution and vortexed to mix thoroughly. The mixture was then transferred to the IC column in a collection tube provided, microcentrifuged at 16,000g$_{max}$ for 30 seconds, and flow-

through was discarded. The IC column was then transferred to a new collection tube, and 400μl RNA Wash Buffer was added and centrifuged to facilitate column washing. 5μl of DNase I was diluted with 35μl of DNA digestion buffer and placed directly onto the column matrix and incubated at room temperature for 15 minutes. Column was washed twice with 400μl of Direct-zol RNA PreWash and followed by 700μl of RNA Wash Buffer. The column was then carefully transferred into an RNase-Free Tube and 20μl of RNA-free water was placed directly on the column matrix to be incubated for 5 minutes at room temperature before microcentrifugation at 16,000$g_{max}$ to elute RNA. Eluted RNA was immediately stored at -80°C.

#### 2.3.9.2 RNA Quantification and Quality Check

RNA-Seq, requires good quality RNA, with little or no degradation, and free from contamination with DNA, proteins, or salts. RNA quantification and quality check was performed using an Aligent RNA 6000 Nano Kit and analysed on an Agilent 2100 Bioanalyzer. The technology utilises gels to identify ribosomal peaks and calculate the ratio of ribosomal peaks (18s/28s) to obtain RNA integrity number (RIN). Gel dye mix, RNA Nano Marker, extracted RNA samples and ladder were all loaded onto the RNA Nano Chip according to the manufacturer's instructions and the Bioanalzyer used to determine sample quantity and quality.

#### 2.3.9.3 RNA Sequencing

RNA that passed the quality check was used for sequencing analysis on a HiSeq 4000 platform with paired-end 150bp reads and a sequencing depth of 30M reads at the Novogene Bioinformatics Institute, Beijing, China.

#### 2.3.9.4 KEGG Pathway Analysis for Differentially Expressed Genes

KOBAS 3.0 software is a web-based platform that was used to identify the biological pathways that the input genes correspond to.

### 2.3.10 Mycoplasma Testing

#### 2.3.10.1 EZ-PCR Mycoplasma Test

Cell lines were routinely tested for mycoplasma contamination. The procedure is based on PCR amplification of mycoplasma 16s ribosomal RNA sequence present in the contaminated cultures.

The PCR reaction was set up based on the protocol provided with the EZ-PCR Mycoplasma Test Kit. Briefly, 10ml of supernatant was removed from a confluent cell culture and centrifuged at $300g_{max}$ for 5 minutes to pellet debris, and 1ml of resulting supernatant was transferred to a sterile 1.5ml microfuge tube and centrifuged at $16,500g_{max}$ for 10 minutes to pellet mycoplasma. After decanting the supernatant, the pellet was resuspended in 25μl of Sample Buffer solution provided in the kit and heated on a heatblock at 95°C for 3 minutes. Reaction mixtures were prepared in PCR tubes as shown in Table **2-6**. PCR was performed using a Thermal Cycler (Applied Biosystems) with the cycling conditions as detailed in Table 2-7. Template DNA provided by the kit and fresh culture media served as a positive and negative control respectively.

**Table 2-6 EZ-PCR Mycoplasma Test Reaction Mixture**

| Reagent | Volume (μl) |
|---|---|
| Molecular Biology Grade Water | 17.5 |
| Reaction Mix | 5 |
| Test Sample/Positive Control | 2.5 |
| **Total Volume** | **25** |

**Table 2-7 EZ-PCR Thermal Cycling Conditions**

| Temperature(°C) | Time (seconds) | # of Cycles |
|---|---|---|
| 94 | 30 | 1 |
| 94 | 30 | 35 |
| 60 | 120 | |
| 72 | 60 | |
| 94 | 30 | 1 |
| 60 | 120 | 1 |
| 72 | 300 | 1 |
| 4 | ∞ | ∞ |

#### 2.3.10.2 Agarose Gel Electrophoresis

Electrophoresis was used to separate the PCR products and determine the size by comparison to a standardised DNA ladder. The agarose gel was made by adding agarose powder (Sigma) to 0.5x Tris Acetate-EDTA buffer (TAE; Sigma) at 2% w/v. The agarose powder was melted and stirred in a microwave until transparent, and the vessel cooled to a temperature that was warm to the touch. GelRed™ Nucleic Acid Gel Stain (10,000x; Biotium) was added to the agarose solution to obtain a final concentration of 1x, and then poured into a casting tray with a comb inserted to create wells, which was then left to solidify for approximately 30 minutes. The comb was removed as the gel solidifies, and the casting tray was placed into a gel rig and immersed completely with 0.5x TAE buffer. 10μl of Gene Ruler DNA Ladder Mix was added to the first well to allow fragment size determination; 10μl of PCR product was mixed with 2μl of 6x loading dye thoroughly and loaded onto the gel. A current of 100 volts was applied to the gel for a duration of 1 hour to achieve fragment separation. The gel was subsequently removed from the casting tray and placed on a UV light box (UVP GelDoc-It) to be photographed. The samples were determined as either contaminated or clean when compared to the positive control band.

### 2.3.11 Statistical Analysis

Data was analysed using Graphpad Prism software version 7 (Graphpad Software). Graphs show mean ± standard error of mean. To analyse the statistical significance, a paired *t*-test was used to calculate differences between the control and treatment group, unless otherwise specified. *p* values $<0.05$ were considered statistically significant.

# Chapter 3 Assessment of Suppression of αCD3/CD28 stimulated αβ T cells in the AML microenvironment

## 3.1 Introduction

The two main objectives of this study were firstly, to determine whether the additional presence of the stromal cell line HS5, co-cultured with primary AML cells or cell lines, could enhance the immunosuppressive capabilities of the tumour and secondly, to establish a model that allows us to assess T-cell function in the AML microenvironment. Although there are plenty of evidences to suggest that T-cell responses to AML can control the disease, and immunotherapies are being developed to exploit this phenomenon, AML cells have a number of mechanisms for suppressing T-cell activity and thus limiting efficacy[273, 274, 275]. Several studies have reported that the AML microenvironment is immunosuppressive, rendering T cells dysfunctional, and thus favouring AML survival.[212, 213, 276] In addition, studies have shown that the AML bone marrow microenvironment in is hypoxic which promotes regulatory T cells suppression and interferes with T-cell activation and infiltration[277, 278, 279].

Early *in vitro* studies into the effects of AML cell line, U937, on T-cell activation and function suggested that the AML microenvironment inhibits the production of Th1 cytokines, such as IFN-γ[276]. The study by Buggins et al demonstrated that the presence of the costimulatory molecule CD80 on the U937s resulted in the increased T-cell activation, proliferation and survival; however, secretion of Th1 cytokines were low compared to dendritic cell stimulated T cells. The study also showed that PBMC stimulated in the presence of U937 cell supernatant resulted in the inhibition of IL-2 and IFN-γ production, demonstrating that the inhibitory effect was soluble factor mediated. Blocking of known Th1 inhibitors, such as IL-10 and TGF-β, did not reverse the inhibition observed and thus eliminating them as the suppressive factor. However, pre-incubation of PBMC with IL-2 for 4 hours prior to stimulation in the U937 supernatant overcame the inhibitory effect demonstrating potential for the effect to be reversed[276].

Subsequent investigations into the mechanisms of the inhibition of T cells stimulated in the presence of the AML microenvironment showed that, similar to U937 supernatant, production of IL-2 and IFN-γ were inhibited when T cells were stimulated in the presence of primary AML cell

supernatant. The analysis of primary AML cell supernatant composition showed inhibitory activity was again not due to presence of the commonly known immunosuppressive cytokines such as TGF-β or IL-10, as addition of neutralising antibodies were ineffective. However, preincubation of primary AML cells with either brefeldin A or sodium monensin, which are golgi inhibitors and thus prevent the secretion of glycoproteins, overcame the inhibitory effect. Further studies by the same group have shown that primary AML cell supernatant exerted its inhibitory effect by preventing NFATc and NFκB nuclear translocation, which was caused by the suppressed chromatin binding to $p65_{RelA}$ and $p75_{c\text{-}Rel}$ (NFκB is composed of either $p65_{RelA}$-p50 or $p75_{c\text{-}Rel}$-p50 heterodimer)[212, 280, 281]. Furthermore, T-cell proliferation was inhibited following stimulation with PMA and ionomycin in the presence of primary AML cell supernatant and the mechanism identified as being via reduced phosphorylation of pRb and p130 proteins, preventing cell cycle from entering S-phase[212, 213]. Lastly, further studies into the AML supernatant have shown that AML creates an anti-apoptotic microenvironment that delays stimulated T cells from apoptosis by preventing downregulation of Bcl-2 anti-apoptotic protein[282].

Thus, so far *in vitro* studies into the effect of AML cells on T-cell activation and function have been limited to the use of cultured AML cell lines or primary AML cell supernatants alone.[212, 276] There are several limitations to the *in vitro* model described. Firstly, the model prevents the study of the influence of AML cells on T cells upon cell:cell contact. Importantly, *ex vivo* studies have shown that autologous T cells form defective immunological synapses with AML cells[244]. Therefore, contact between AML cells and T cells may play an important role in T-cell inhibition. Secondly, primary AML cells have been shown to undergo spontaneous apoptosis when cultured alone[283, 284]. As such, to study the effects of primary AML cells and their microenvironment on T-cell function, primary AML cells are required to be sustained by either feeder cells or cytokine cocktails. Finally, AML cells do not reside alone in the body and are surrounded by other cells of the microenvironment. A model that incorporated additional cells, such as stromal cells, would be a far more physiological model.

Previous studies have shown that primary AML cells undergo apoptosis when cultured in serum-free conditions; however, the addition of growth factors such as G-CSF and GM-CSF reduces primary AML cells apoptosis. It has also been demonstrated that both AML cell lines and primary

AML cells can be differentiated *in vitro* in the presence of bone marrow stromal cells[285, 286]. The Human stromal cell line, HS5, is an immortalised stromal cell from human bone marrow that was transduced with the human papilloma virus E6/E7 genes, which prevented cell cycle arrest. HS5 cells produce significant levels of growth factors and cytokines such as G-CSF, GM-CSF, IL-6, and SCF all of which are required for AML cell survival[268, 283, 287].

It has previously been shown that the human stromal cell line, HS5, prevents primary AML cells from apoptosis[283]. This study demonstrated that direct contact of primary AML cells with HS5 prevents both spontaneous and drug-induced apoptosis (using chemotherapeutic agents such as cytarabine) compared to primary AML cells cultured alone and supplemented with IL-3 and SCF[283]. Furthermore, another study demonstrated that murine stromal cell line, MS5, prevented cytarabine induced apoptosis in both the AML cell line HL-60 and primary AML cells, which was attributed to the increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-$X_L$[287]. Consequently, the presence of stromal cell line, HS5 or MS5, was shown to have a protective effect on both AML cell lines and primary AML cells. Lastly, it has been shown that HS5 cells were able to sustain primary AML cells, as the viable cell fractions were higher in the HS5 co-culture compared to primary AML cells cultured alone[283].

The observation of the elimination of AML cells in patients that have received allo-HSCT are evidence for a T-cell mediated anti-leukaemia effect. However, the relapse free survival rate after allo-HSCT is only 40%[288]. This implies that T-cells are capable of tumour elimination, but long-term responses are ineffective in the immunosuppressive microenvironment conditioned by the AML disease. The aim of this chapter is to develop an *in vitro* co-culture model that mimics this microenvironment and combines the aforementioned *in vitro* studies, comprised of AML cell, stromal cell line HS5, and $\alpha$CD3/CD28 stimulated T cells. This allowed the assessment of T-cell function in the presence of a more physiological model of the AML microenvironment. T-cell activation and function were investigated in the presence of both AML cell lines and primary cells in both normoxic and hypoxic conditions. Five different AML cell lines and eleven different primary AML samples with different cytogenetic abnormalities, representing the heterogeneous nature of the disease, were used[212, 244, 282].

The development of such an *in vitro* model is pertinent to future works, such as T-cell based immunotherapies, as it can be utilised to assess the function of the different T-cell based immunotherapies in the presence of AML microenvironment. Therefore, in subsequent chapters, the function of different T-cell based immunotherapies will be assessed using the *in vitro* model described.

## 3.2 Results

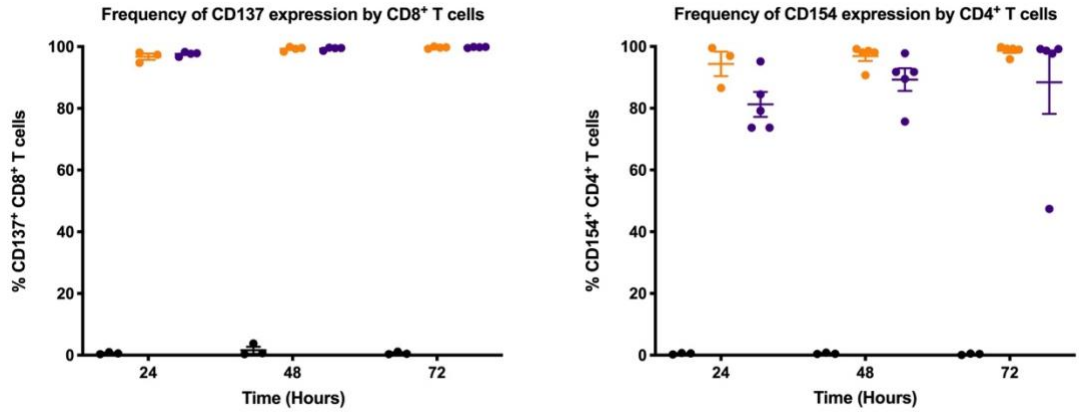
### 3.2.1 Optimization of the co-culture model

To measure T cell activation, the expression of the markers CD137 for CD8+ cytotoxic T cells and CD154 for CD4+ helper T cells were analysed by flow cytometry. CD137 is expressed primarily on activated $CD8_+$ T cells and CD154 is primarily expressed on activated $CD4_+$ T cells; furthermore, expression levels of both activation markers have been shown to be detectable after 24 hours of stimulation with maximal surface expression after 48 hours[289, 290].

T cells from 3 healthy donor PBMC were assessed for the time required to achieve the highest level of activation marker expression when stimulated with αCD3/CD28 beads at 1:1 bead to T-cell ratio alone or in the presence of HS5 cells. Figure 3-1 shows that αCD3/CD28 bead stimulation of T cells resulted in the maximum percentage of CD137 expression by $CD8_+$ T cells by 24 hours, with a mean frequency of expression of 96.8% and 97.7% for αCD3/CD28 stimulated T cells alone and αCD3/CD28 stimulated T cells in the presence of HS5 respectively. This was sustained for up to 72 hours. However, maximum percentage of CD154 expression by $CD4_+$ T cell was only achieved by 48 hours, with a mean frequency of expression of 96.9% and 89.3% for αCD3/CD28 stimulated T cells alone and αCD3/CD28 stimulated T cells in the presence of HS5 respectively. The maximum mean fluorescence intensity (MFI) of CD137 by $CD8_+$ T cells was achieved by 72 hours, with a MFI of 5593.8 and 15719.2 for αCD3/CD28 stimulated $CD8_+$ T cells alone and αCD3/CD28 stimulated $CD8_+$ T cells in the presence of HS5 respectively; interestingly, the presence of HS5 cells clearly enhanced the activation of CD8+ T cells. CD154 MFI by $CD4_+$ T cells was highest at 48 hours and declined by 72 hours, with a reduced MFI dropping from 1860.2 to 1300.3 and 1557.5 to 892.2 for αCD3/CD28 stimulated $CD4_+$ T cells alone and αCD3/CD28 stimulated $CD4_+$ T cells in the presence of HS5 respectively. Unlike the $CD8_+$ cells, $CD4_+$ T cell activation was not enhanced by the presence of HS5 cells and was marginally reduced.

The results here demonstrated that the optimal time point required for both $CD8_+$ and $CD4_+$ T cells to achieve maximum activation is 48 hours. As the HS5 cells enhance CD8 activation but marginally reduce CD4, it is important that both T cells alone and T cells + HS5s are used as positive controls for subsequent experiments.

**A**




**B**

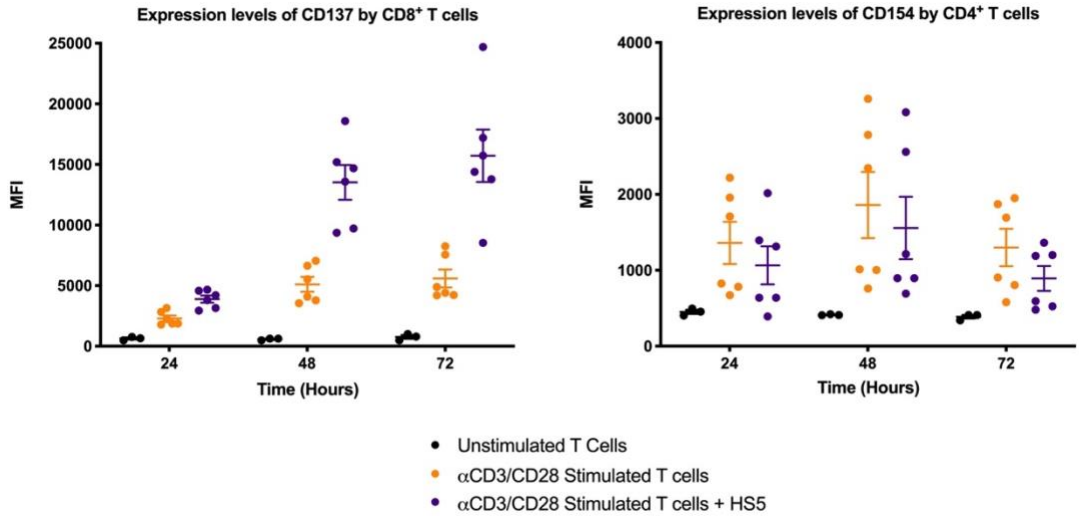



**Figure 3-1 αCD3/CD28 stimulated T cells achieved highest activation markers expression frequency and expression levels after 48 hours**

To determine the optimal time required to achieve highest activation by αCD3/CD28 stimulated T cells, selected T cells form 3 donors were cultured in 2 different conditions over a period of 72 hours and sampled every 24 hours. (A) The percentage of CD137 and CD154 positive $CD8_+$ and $CD4_+$ T cells respectively was measured by flow cytometry. Maximum frequency of CD137 and CD154 were observed in all conditions by 24 and 48 hours respectively. (B) The MFI for CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells were highest at 72 and 48 hours respectively. By 72 hours, CD154 expression levels were reduced for all conditions. Data shown represents the mean ± SEM.

### 3.2.2 Primary AML cells are sustained in the presence of stromal cell line HS5

Previous studies have shown that primary AML cells undergo spontaneous apoptosis when cultured *in vitro*; however, Garrido et al. reported that spontaneous apoptosis of primary AML cells can be circumvented by direct contact with the monolayer stromal cell line HS5[283, 284]. To reproduce and establish this model, 11 different primary AML samples were cultured in RPMI with 10% FBS for a total of 48 hours in the absence or presence of HS5 cells in both normoxic and hypoxic conditions; samples were collected after 24 and 48 hours.

Figure 3-2 shows the absolute cell count for viable primary AML cells that were cultured alone or in the presence of HS5 after 24 hours and 48 hours. The number of different primary AML samples used for normoxic and hypoxic conditions were 4 and 11 respectively. Viable AML cells were gated on by their exclusion of the fixable viability dye eFluor780 and their expression of CD33 and/or CD34. Reduced absolute primary AML cell counts were observed after just 24 hours in both the presence and absence of HS5 cells; however, under hypoxic conditions, primary AML cells were significantly reduced when cultured in the absence of HS5 compared to their presence, with mean absolute cell counts of 311410 cells/mL and 409347 cells/mL respectively ($p$=0.02). In addition, although not statistically significant, an increase in primary AML absolute cell count was seen when they were cultured in the presence compared to absence of HS5 cells under normoxic conditions; mean absolute AML cell counts 260213 cells/mL and 372256 cells/mL respectively ($p$=0.12).

After 48 hours, significant differences between the absolute cell count of primary AML cells cultured in the presence of HS5 compared to their absence under both normoxic and hypoxic conditions were observed, with a 61%($p$=0.01) and 50.5% ($p$<0.001) reduction in absolute cell count respectively. Importantly, primary AML cells cultured in the presence of HS5 were sustained, and the absolute count remained similar for both normoxic and hypoxic conditions albeit slightly higher in hypoxic conditions; mean absolute cell count of 372256 cells/mL and 366334 cells/mL after 24 and 48 hours under normoxic conditions and 409347 cells/mL and 406615 cells/mL after 24 and 48 hours under hypoxic conditions. This demonstrates that the model is suitable for co-culture of primary AML cells for up to 48 hours.

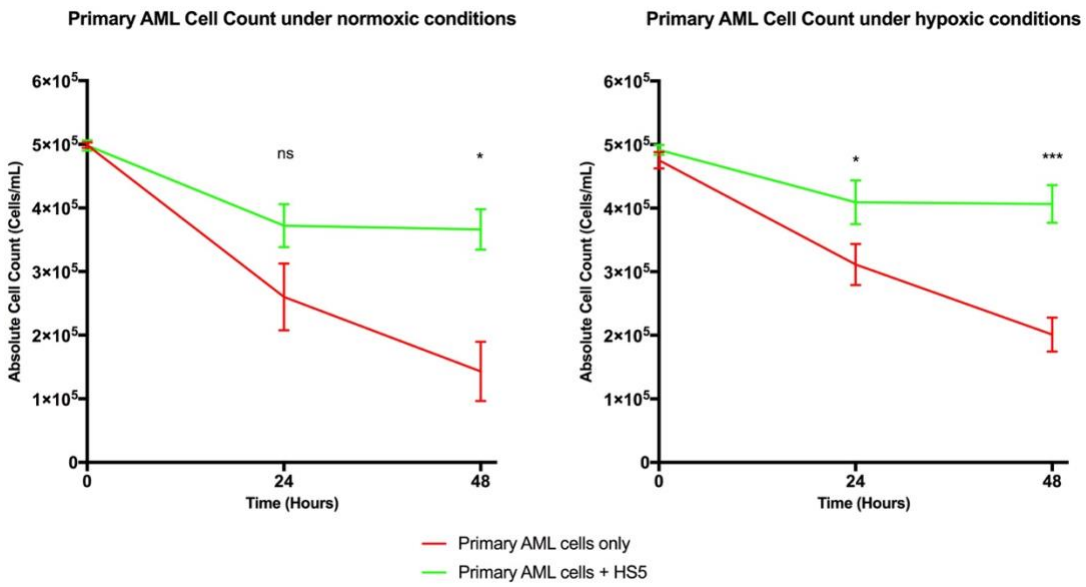



**Figure 3-2 Stromal cell line HS5 sustains primary AML cells**

Primary AML cells were sustained in the presence of stromal cell line HS5. Absolute cell count of primary AML cells was determined by flow cytometry through the use of counting beads; AML cells were gated on viable CD33+ and CD34+ cells using viability dye. Primary AML cells were co-cultured alone or on a monolayer HS5 cells at 1:0.1 primary AML cells to HS5 cells ratio for 48 hours under normoxic conditions or hypoxic conditions. Data shown represents the mean ± SEM of n=4 for normoxic and n=11 for hypoxic conditions. $^{*}p<0.05$, $^{***}p<0.001$ determined by paired t test.

### 3.2.3 Combination of AML cell lines with the stromal cell line HS5 enhances immunosuppression of T cells

To compare T-cell activation when cultured in the presence of the different AML cell lines, purified T cells from 6 healthy donors were stimulated with αCD3/CD28 beads and cultured in the presence of AML cell lines alone or with HS5 cells for 48 hours (Table 3-1 shows the different culturing conditions for the three-cell co-culture assays). Of note, 4 of the AML cell lines data sets were obtained by Arushi Ramani and Fatemeh Mosallaey. T cells were gated as per described in (Section 2.3.4.8), and Figure 3-3 shows the representative scatter plots of activation markers based on the negative controls.

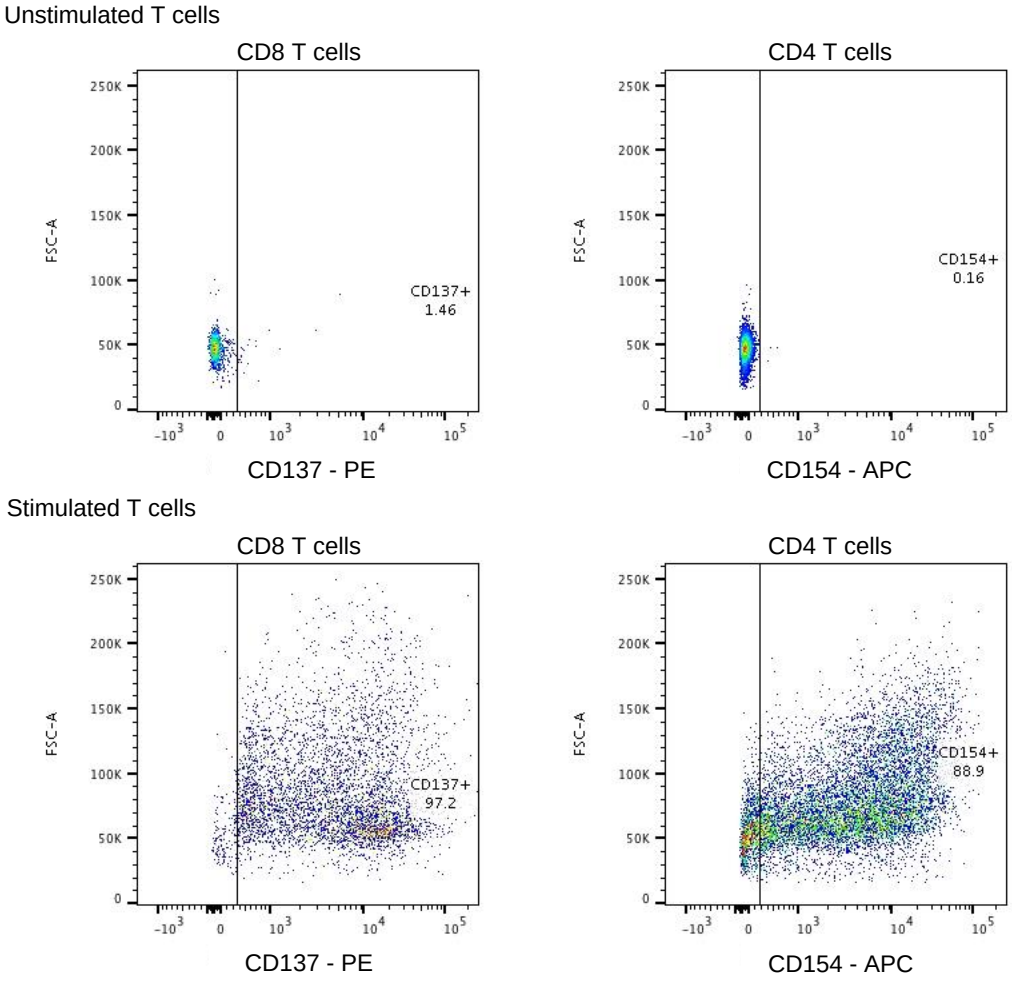



**Figure 3-3 Representative scatter plots for CD137 and CD154 Activation markers expression on CD8+ and CD4+ T cells**

T cells were gated on CD3+ viable cells upon AML (CD33+ or CD34+) and HS5 (CD29+) exclusion. CD3+ T cells were then further distinguished as CD8+ and CD4+ T cells. Gating for the activation markers expression was based on negative control, unstimulated T cells only (top panel).

Activation marker expression levels were determined by geometric median of the cells that are positive for activation marker.

The results shown here demonstrates that different AML cell lines have different inhibitory propensity but for all cell lines, the additional presence of HS5 enhances the immunosuppressive microenvironment (Figure 3-4). For some this enhancement does not reach statistical significance, however there is a clear trend. T cells co-cultured in the presence of U937 with HS5 resulted in significant reductions in percentage positive CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells compared to U937 alone from mean of 77.2% to 53.2% ($p<0.001$) and 80.9% to 40.5% ($p=0.002$) respectively. In addition, a trend was observed for decreased MFI of both CD137 by $CD8_+$ T cells and CD154 by $CD4_+$ T cells in the presence of U937 with HS5 cells compared to U937 cells alone, with mean of 2633 compared to 5946 ($p=0.09$) and mean of 1567 compared to 5486 ($p=0.06$) respectively.

Similar to U937 cells, the presence of NB4 with HS5 resulted in significant reductions in T-cell activation marker expressions compared to NB4 alone in both percentage positive CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells from mean of 77.3% to 81.9% ($p=0.04$) and mean of 61.9% to 79.5% ($p=0.02$) respectively. In addition, compared to NB4 cells alone, the MFI levels of CD154 by $CD4_+$ T cells was reduced in the presence of HS5 cells from mean of 4355 to 2118 ($p=0.04$) and a trend seen for CD137 MFI by $CD8_+$ T cells ($p=0.26$).

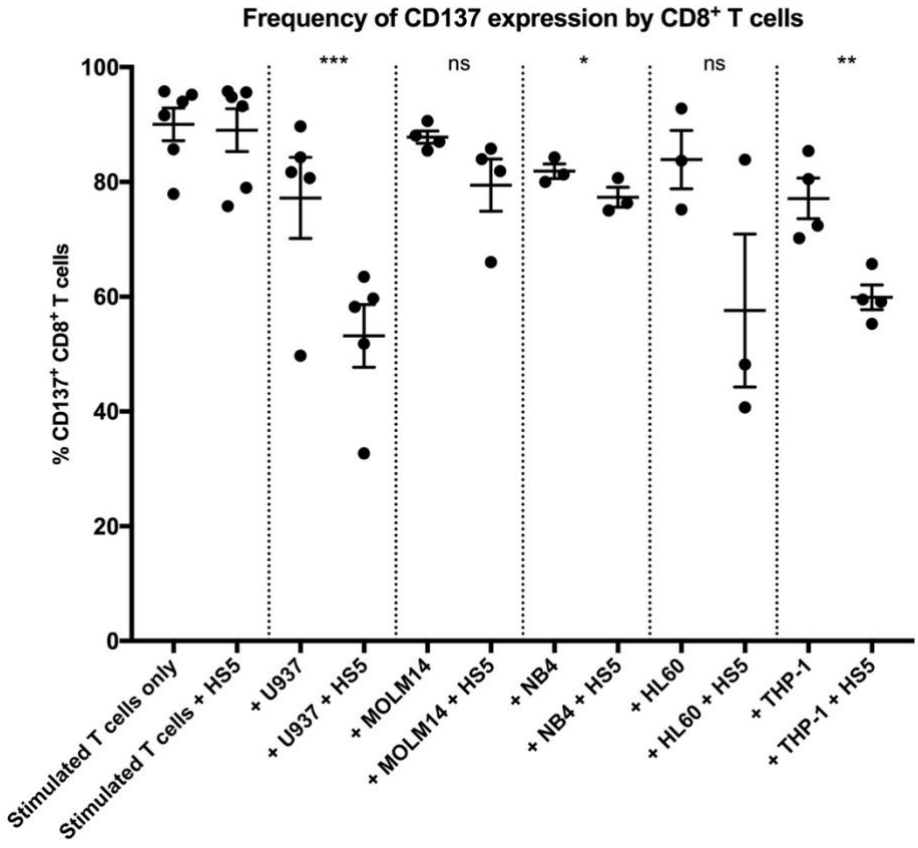
A significant reduction in CD154 % expression and MFI by $CD4_+$ T cells were observed in MOLM14 with HS5 co-cultures compared to MOLM14 alone, from mean of 22.1% to 32.4% ($p=0.001$) and mean of 1147 to 1404 ($p=0.008$) respectively. There was also a trend in both % expression and MFI of CD137 by $CD8_+$ T cells but they did not reach statistical significance ($p=0.15$) and ($p=0.26$) respectively.

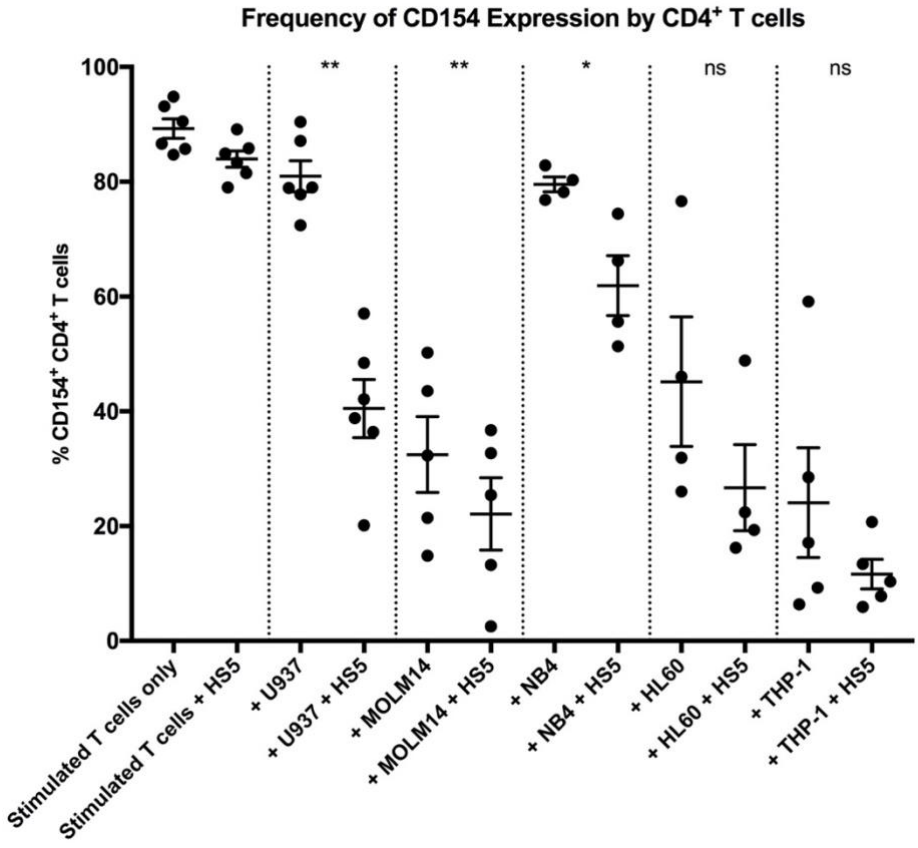
The presence of HL60 with HS5 did not reach statistical significance on decrease for three of the indices but again there was a trend for decrease in both % expression and MFI of CD154 and CD137 in the presence of HS5 cells; % expression of CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively, fell from a mean of 83.9% to 57.6% ($p=0.12$) and 45.1% to 26.7% ($p=0.054$) respectively in the presence of HS5 cells. The MFI of CD154 expression by $CD4_+$ T cells fell from

2135 to 1215 ($p$=0.19) in their presence and CD137 by $CD8_+$ from a mean of 5369 to 3033 ($p$=0.03), the one statistically significant index.

Lastly, T cells co-cultured in the presence of THP-1 with HS5 resulted in significant reduction in both percentage of CD137 expression and MFI by $CD8_+$ T cells compared to THP-1 alone, from mean of 77.1% to 59.9% ($p$=0.005) and mean of 2966 to 2015 ($p$=0.04). The presence of HS5 cells also reduced $CD4_+$ T-cell activation compared to THP-1 alone but this was just a trend and not statistically significant; % CD154 positive $CD4_+$ T cells, reduced from a mean of 24.1% to 11.6% ($p$=0.15) and MFI from 1202 to 920 ($p$=0.20).

**A**


Frequency of CD137 expression by CD8+ T cells
% CD137+ CD8+ T cells
100
80
60
40
20
0
***
ns
*
ns
**
Stimulated T cells only
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5


Frequency of CD154 Expression by CD4+ T cells
% CD154+ CD4+ T cells
100
80
60
40
20
0
**
**
*
ns
ns
Stimulated T cells only
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5

**B**

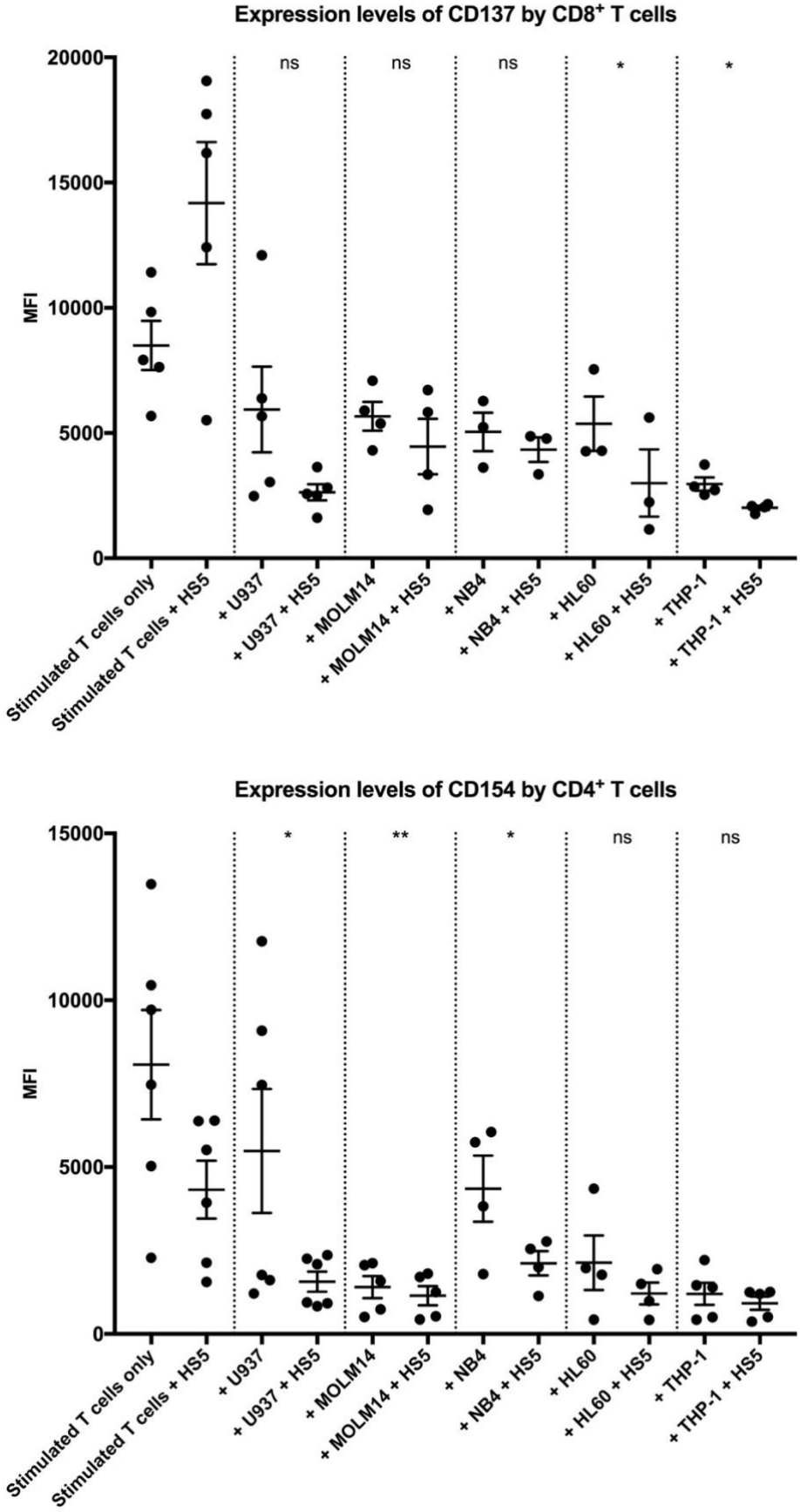



**Figure 3-4 T-cell activation markers expressions are diminished in the presence of AML cell lines and HS5**

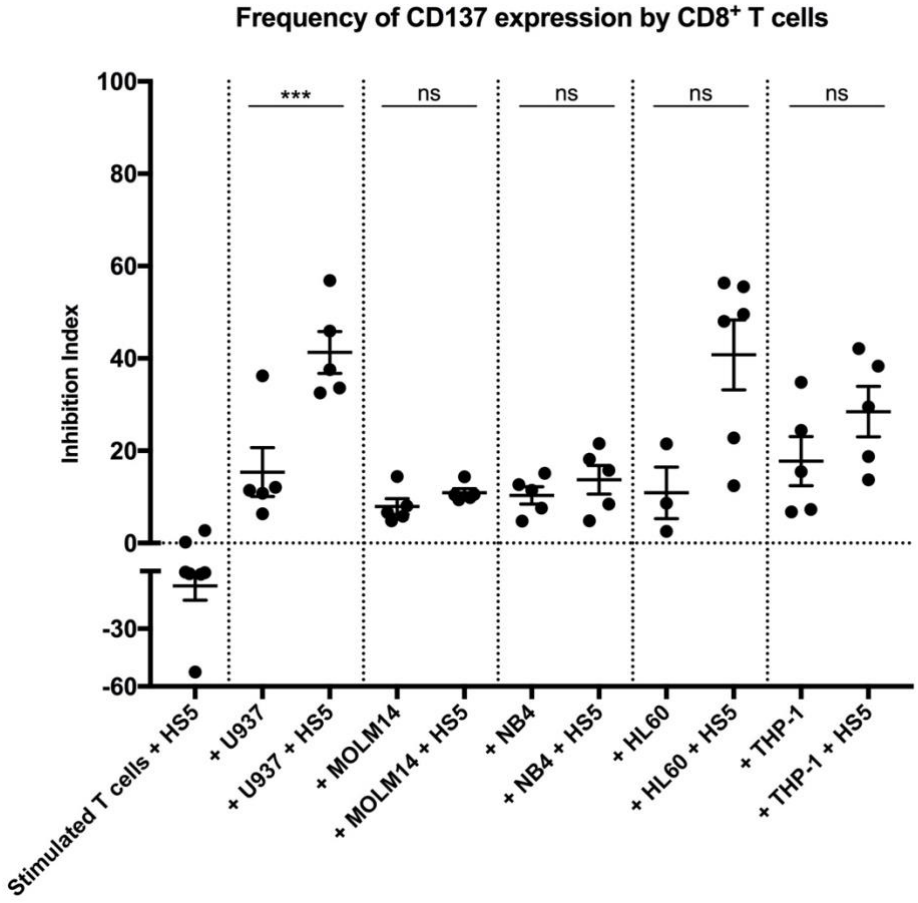
T cells stimulated with αCD3/CD28 in the presence of AML cell lines only and AML cell lines with HS5. CD137 and CD154 expression was measured at 48 hours using flow cytometry. Viable $CD8_+$ and $CD4_+$ T cells were gated on, and positivity gates were set using negative controls. (A) % frequency of CD137 expression by $CD8_+$ T cells and % frequency of CD154 expression by $CD4_+$ T cells, and (B) MFI of CD137 by $CD8_+$ T cells and MFI of CD154 by $CD4_+$ T cells. Data shown represents the mean ± standard error mean (SEM). $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$ determined by non-parametric Wilcoxon matched paired t test.

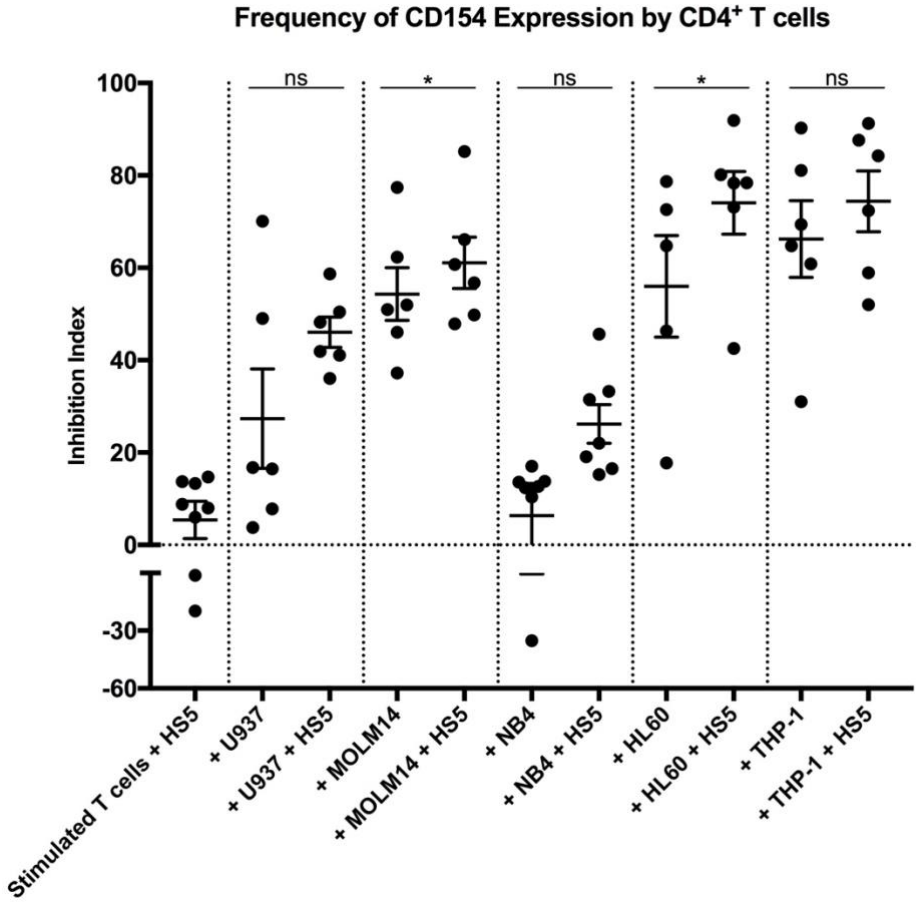
Presentation of the data in Figure 3-4 highlights a couple of limitations of the analysis as shown. Firstly, inevitably, inter-assay variations were observed whereby the extent of activation differs between the different healthy donor T cells used. This could be addressed by normalisation of the data and calculation of inhibition indexes. However, such analysis is dependent on normalisation to the correct control and the data also clearly demonstrates that HS5 cells alone have a variable effect on T-cell activation; they increase CD137 MFI and decrease CD154 MFI. Therefore, to address both of these issues the T cells + AML cells were both normalised to T cells alone but T cells + AML cells + HS5 cells were normalised to T cells + HS5 cells (Table 3-1). This enabled the data from different donors to be presented in a format that enabled better and more meaningful comparisons to be made between the different conditions. All subsequent inhibition indexes shown in this thesis were calculated using the same methods

**Table 3-1 Co-Culturing Conditions for Assessing T-cell function**

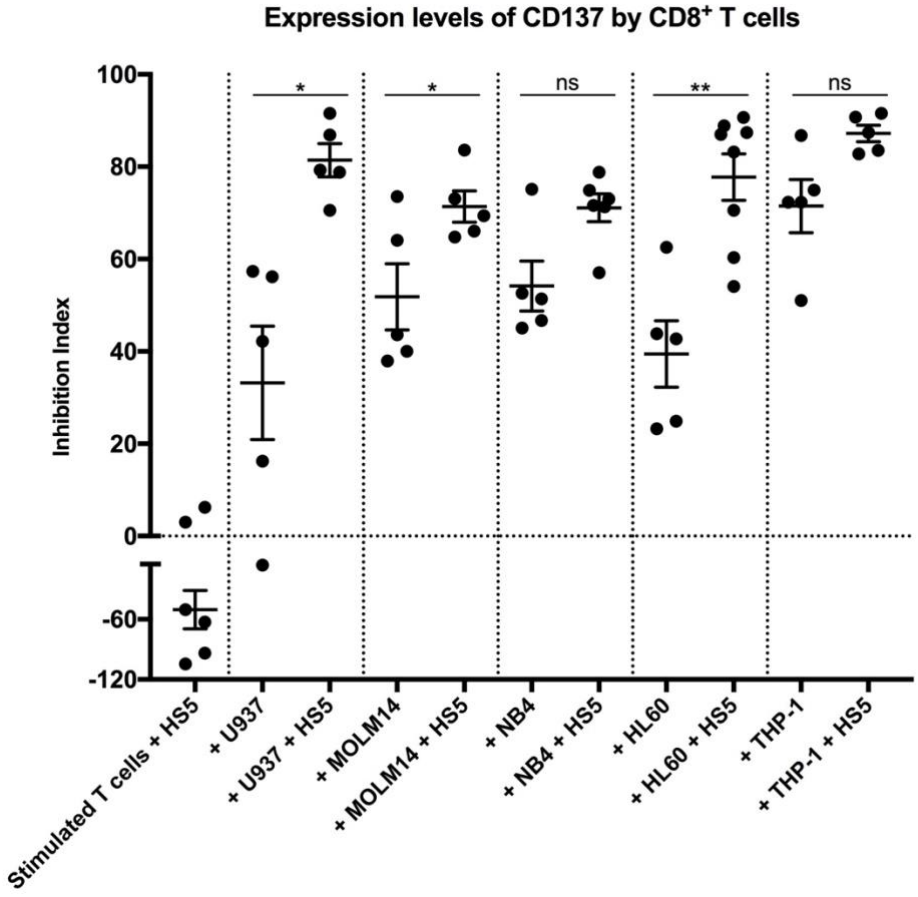
| **Conditions** | |
|---|---|
| Unstimulated T cells | Negative Control |
| **Stimulated T cells** | **Positive Controls** |
| **Stimulated T cells + AML** | **Test Conditions** |
| **Stimulated T cells + HS5** | **Positive Controls** |
| **Stimulated T cells + HS5 + AML** | **Test Conditions** |

**A**


Frequency of CD137 expression by CD8+ T cells
Inhibition Index
100
80
60
40
20
0
-30
-60
***
ns
ns
ns
ns
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5


Frequency of CD154 Expression by CD4+ T cells
Inhibition Index
100
80
60
40
20
0
-30
-60
ns
*
ns
*
ns
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5

**B**


Expression levels of CD137 by CD8+ T cells
Inhibition Index
100
80
60
40
20
0
-60
-120
*
*
ns
**
ns
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5

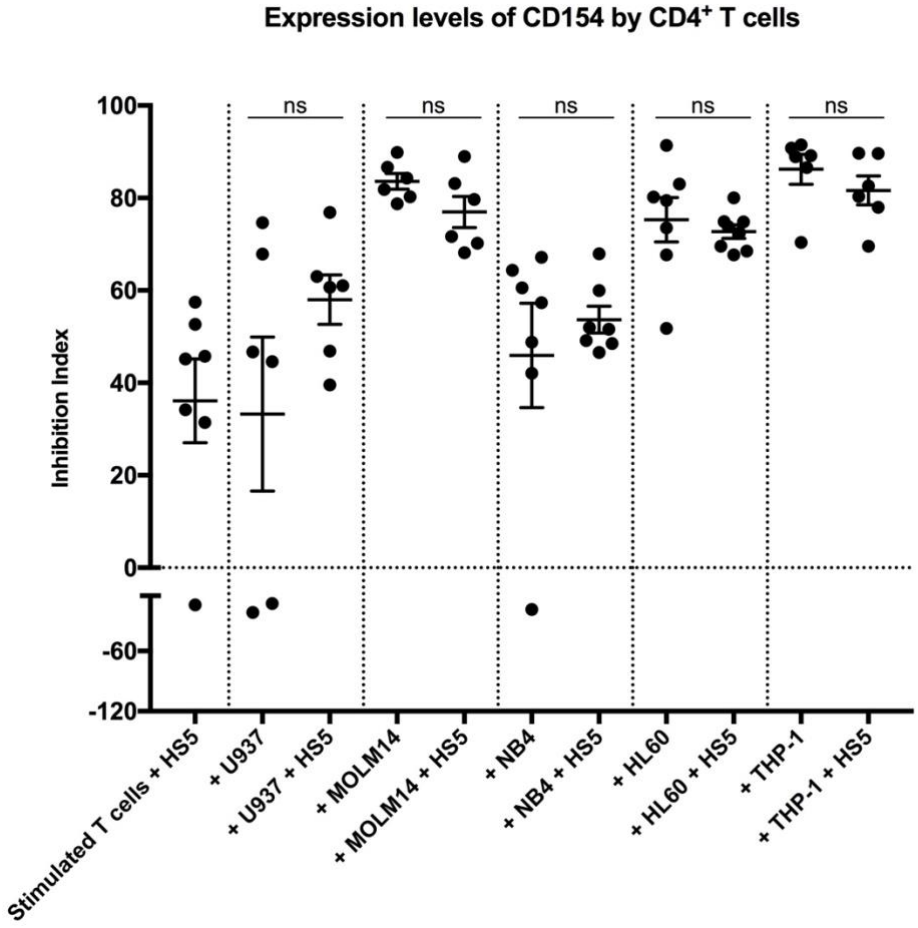

Expression levels of CD154 by CD4+ T cells
Inhibition Index
100
80
60
40
20
0
-60
-120
ns
ns
ns
ns
ns
Stimulated T cells + HS5
+ U937
+ U937 + HS5
+ MOLM14
+ MOLM14 + HS5
+ NB4
+ NB4 + HS5
+ HL60
+ HL60 + HS5
+ THP-1
+ THP-1 + HS5

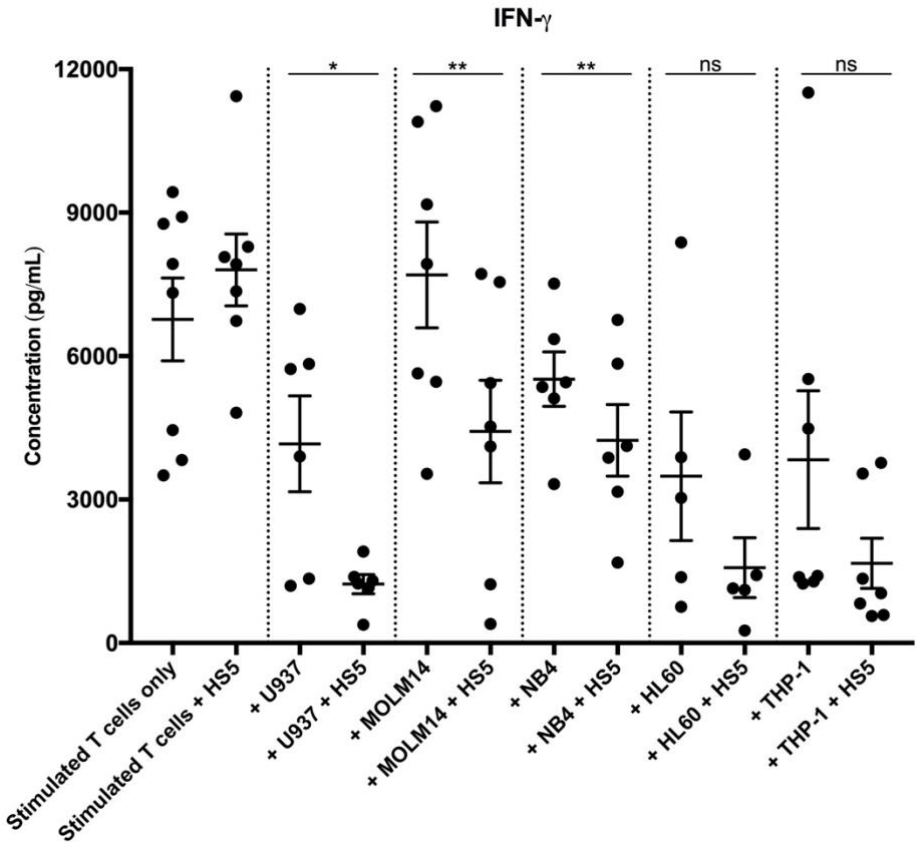
**Figure 3-5 Increased Inhibition indexes for both T-cell activation markers in the presence of AML cell lines and HS5**

T-cell activation shown as the inhibition indexes for (A) Percentage positive and (B) MFI of CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively. T cells + HS5 cells were normalised to T cells alone. T cells + AML cells were normalised to T cells alone. T cells + AML cells + HS5 cells were normalised to T cells + HS5 cells. Data shown represents the mean ± standard error mean (SEM). $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.01$ determined by non-parametric Wilcoxon matched paired t test.

As activation markers are only surrogate indicators of T-cell function, cytokine levels in the co-culture supernatants were measured to directly assess T-cell function in the absence and presence of AML cell lines with HS5s. The cytokines selected were based on those known to be secreted by the different T-cell subtypes, such as CD8 cytotoxic T cells and CD4 Th1 T helper cells are known to secrete IFN-γ and TNF-α[291, 292]. Clearly the supernatant from the co-cultures contains cytokines secreted by all three cells types but the cytokines measured were associated with T cells. As these were reduced when all three cell types were present, it is very likely that AML and HS5 cells are not producing them and that they are in fact inhibiting T cell production. Figure 3-6 shows that T cells stimulated in the presence of U937, MOLM14, and NB4 with HS5 had significantly reduced levels of IFN-γ, from a mean of 4165.6 pg/mL to 1230.9 pg/mL ($p$=0.03), 7696.6 pg/mL to 4423.2 pg/mL ($p$=0.001), and 5518.6 pg/mL to 4239.3 pg/mL ($p$=0.004) respectively compared to T cells stimulated in the presence of AML cell lines only. Although no statistically significant difference in levels of IFN-γ produced were observed in the presence of HL60 and THP-1 with HS5 ($p$=0.08 and $p$=0.20 respectively) there was a clear trend of HS5 cells reducing levels of IFN-γ. Although no statistically significant difference in levels of IFN-γ produced were observed in the presence of U937, HL60, and THP-1 ($p$=0.11, $p$=0.07, and $p$=0.12 respectively) there was a clear trend of AML cell lines reducing levels of IFN-γ, which is consistent to previous study by Buggins et al[276]. αCD3/CD28 activated T cells in the presence of U937, MOLM14, NB4, and THP-1 with HS5 had significantly reduced levels of TNF-α, from a mean of 1851.1 pg/mL to 99.4 pg/mL ($p$=0.003), 3030.7 pg/mL to 597.7 pg/mL ($p$<0.001), 2730.1 pg/mL to 391.3 pg/mL ($p$=0.009), and 2436.0 pg/mL to 330.6 pg/mL ($p$<0.001) respectively, compared to T cells stimulated in the presence of AML cell lines only. Again, no significant difference in

levels of TNF-$\alpha$ produced was observed in the presence of HL60 with HS5 but a clear trend seen ($p$=0.06). Similarly, no statistically significance in levels of TNF-$\alpha$ produced was observed in the presence of U938 but a clear trend seen ($p$=0.12).

**A**




**B**

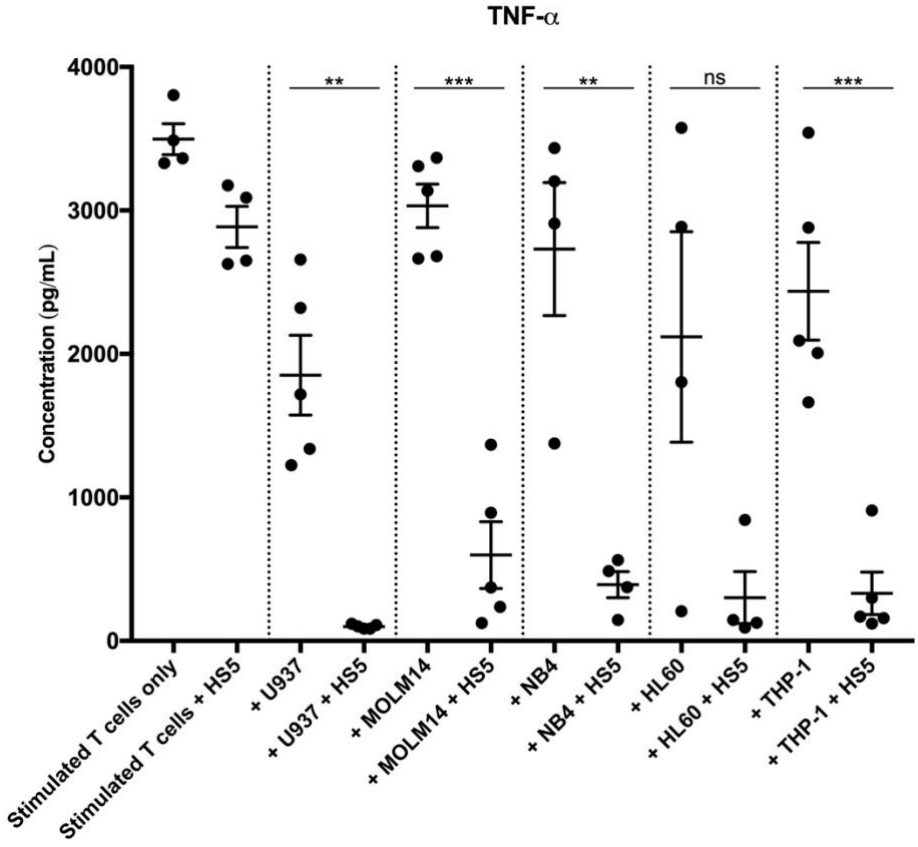



**Figure 3-6 Reduced IFN-γ and TNF-α levels in presence of AML cell lines and HS5**

Cytokines were measured using cultured supernatants. Levels of cytokines produced were analysed in the different AML cell line co-cultures. Significant differences were observed in the levels of (A) IFN-γ and (B) TNF-α when cultured in the presence of most AML cell lines and HS5 compared AML cell lines only. Cell culture supernatants were diluted as 1:25 and 1:5 for IFN-γ and TNF-α respectively. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, determined by non-parametric Wilcoxon matched paired t test.

### 3.2.4 The combination of primary AML cells and the stromal cell line HS5 enhances immunosuppression of T cells

The ability of the different AML cell lines co-cultured in the presence of HS5 cells to inhibit T-cell function was assessed in 3.2.3, and a significant reduction in activation marker expression and cytokine production was observed compared to T cells stimulated in the presence of AML cell lines alone. To assess whether primary AML cells exert similar inhibitory effect and whether the combination of primary AML cells and HS5 enhances the immunosuppression of T cells, a total of four different bone marrow AML patient samples and 3 different healthy donor T cells were used. Purified T cells stimulated with αCD3/CD28 beads at 1:1 bead to T-cell ratio were cultured in the presence of primary AML cells alone and with HS5 cells for 48 hours under normoxic conditions. Activation marker expression on T cells and cytokines released into the supernatants were assessed. Figure 3-7 shows that $CD8^+$ T cells stimulated in the presence of primary AML cells and HS5 have a significantly higher inhibition index for percentage of CD137 expression, with a mean inhibition index of 7.6%, compared to both T cells stimulated alone (mean inhibition index of -0.2%; $p$=0.02), and primary AML cells alone (mean inhibition index of 4.7%; $p$=0.03). Similarly, $CD4^+$ T cells stimulated in the presence of primary AML cells and HS5 resulted in a mean inhibition index of 24.6% for percentage expression of CD154, which is significantly higher compared to $CD4^+$ T cells stimulated in the presence of HS5 alone (mean inhibition index of 8.5%; $p$=0.02), and primary AML cells alone (mean inhibition index of 13.1%; $p$=0.004).

Consistent with the frequency of CD137 and CD154 expression by $CD8^+$ and $CD4^+$ T cells, significant increases in the inhibition indexes for the MFI of CD137 and CD154 of $CD8^+$ and $CD4^+$ T cells stimulated in the presence of primary AML cells and HS5 were observed. The inhibition index for MFI of CD137 by $CD8^+$ T cells increased from a mean of -161.3% and 31.3% for $CD8^+$

T cells stimulated in the presence of HS5 alone ($p$<0.001) and primary AML cells alone ($p$=0.003) respectively to 73.4% for $CD8_+$ T cells stimulated in the presence of both; likewise, the inhibition index for expression levels of CD154 by $CD4_+$ T cells increased from a mean of 17.8% and 61.1% for $CD4_+$ T cells stimulated in the presence of HS5 alone ($p$=0.006) and primary AML cells alone ($p$=0.02) respectively to 71.8% for $CD4_+$ stimulated in the presence of both.

Lastly, T cells stimulated in the presence of HS5 only induced higher expression frequency and expression levels of CD137 by $CD8_+$ T cells, shown as negative inhibition index of -0.2% and -161.3% respectively, which indicate an increased state of activation. However, the opposite effect was observed for $CD4_+$ T cells, such that the inhibition index for the percentage expression and MFI of CD154 by $CD4_+$ T cells were positive, mean inhibition index of 8.5% and 17.8% for expression percentage and MFI respectively, indicating a slight suppressive effect of HS5 on $CD4_+$ T cells

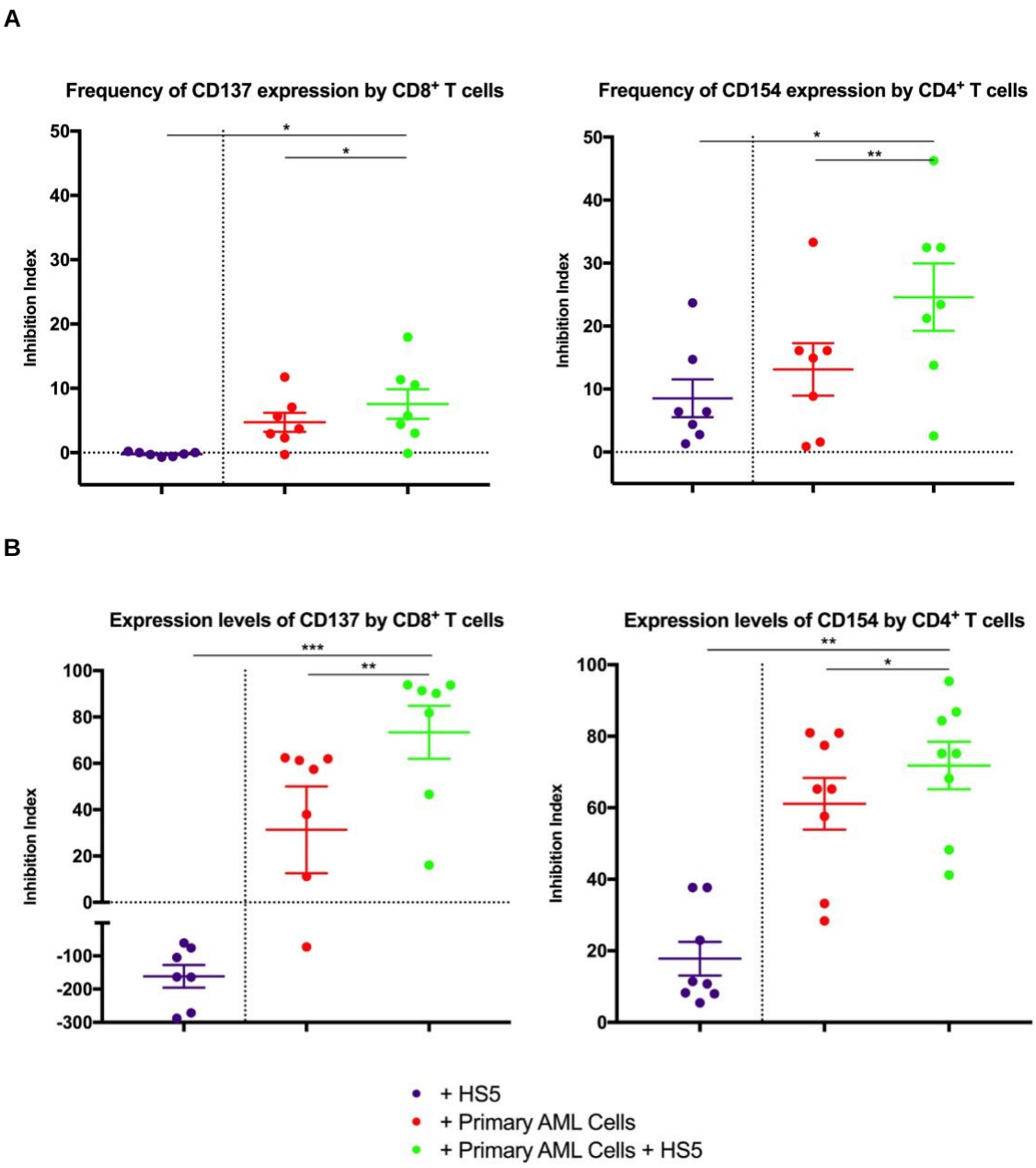



**Figure 3-7 Combination of primary AML cells and HS5 inhibits T-cell activation under normoxic conditions**

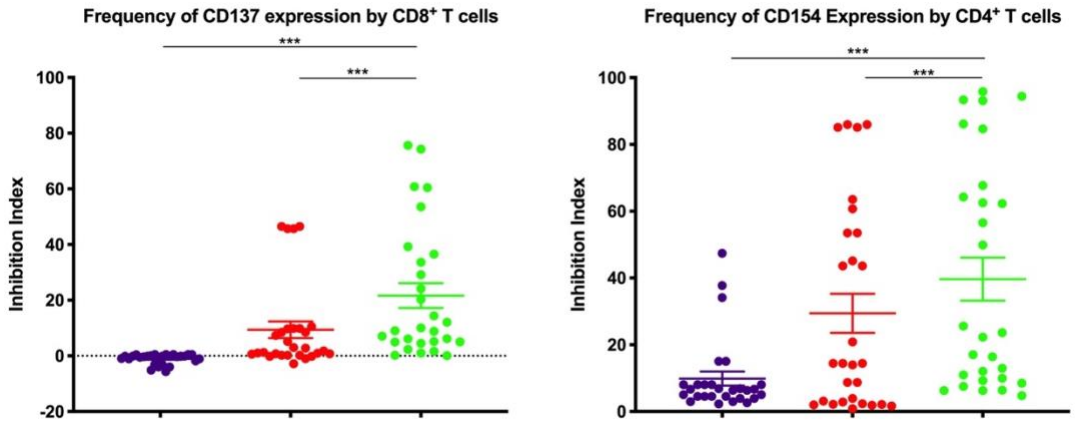
Purified T cells were stimulated with αCD3/CD28 beads at 1:1 bead to T-cell ratio for 48 hours under normoxic conditions. The ratio of T cells:primary AML cells:HS5 was 1:1:0.1. T-cell function was assessed as inhibition indexes for (A) Percentage positive for CD137 and CD154 expression by $CD8_+$ and $CD4_+$ T cells respectively, and (B) MFI of CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively. A significant increase in inhibition indexes was seen in the presence of primary AML cells and HS5 compared to HS5 or primary AML cells alone. Data shown represents the mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, determined by paired t test.

### 3.2.5 The combination of primary AML cells and stromal cell line HS5 enhances immunosuppression of T cells under hypoxic conditions

Previous experiments have already demonstrated that the presence of primary AML and HS5 cells inhibit αCD3/CD28 stimulated T-cell activation under normoxic conditions. The AML bone marrow niche is hypoxic, where it promotes the survival of AML cells[293]; therefore, to further emulate the physiological conditions in patients, experiments were repeated under hypoxic conditions; 5% $CO_2$, 1% $O_2$ supplemented with nitrogen.

A total of 11 different primary bone marrow AML samples and 8 different healthy donor T cells were used for the experiments and some AML primary samples were tested against several different healthy donor T cells. Similar to previous findings, Figure 3-8A demonstrates that the percentage of $CD137^+$ $CD8^+$ T cells and $CD154^+$ $CD4^+$ T cells were significantly lower in the presence of primary AML cells and HS5 (mean inhibition index of 21.7% and 39.7% respectively) compared to T cells stimulated in the presence of HS5 only (mean inhibition index of -1.1% and 9.9%; $p<0.001$ and $p<0.001$) or in the presence of primary AML cells alone (mean inhibition index of 9.4% and 29.4%; $p<0.001$ and $p<0.001$). Furthermore, MFI analysis showed substantially reduced levels of activation marker expression on T cells stimulated in the presence of primary AML and HS5 cells for both CD137 and CD154 compared to those stimulated with HS5 and primary AML cells alone (Figure 3-8B); the inhibition index for MFI of CD137 by $CD8^+$ T cells increased from -36.4% for T cells stimulated in the presence of HS5 only ($p<0.001$) and 29.8% for T cells stimulated in the presence of primary AML cells only ($p<0.001$) to 51.4% for T cells stimulated in the presence of primary AML cells and HS5. Similarly, the inhibition index for MFI of CD154 by $CD4^+$ T cells increased from 28.9% for T cells stimulated in the presence of HS5 alone ($p<0.001$) and 39.6% for those stimulated in the presence of primary AML cells alone ($p<0.001$) to 55.7% for T cells stimulated in the presence of primary AML and HS5 cells.

**A**




**B**

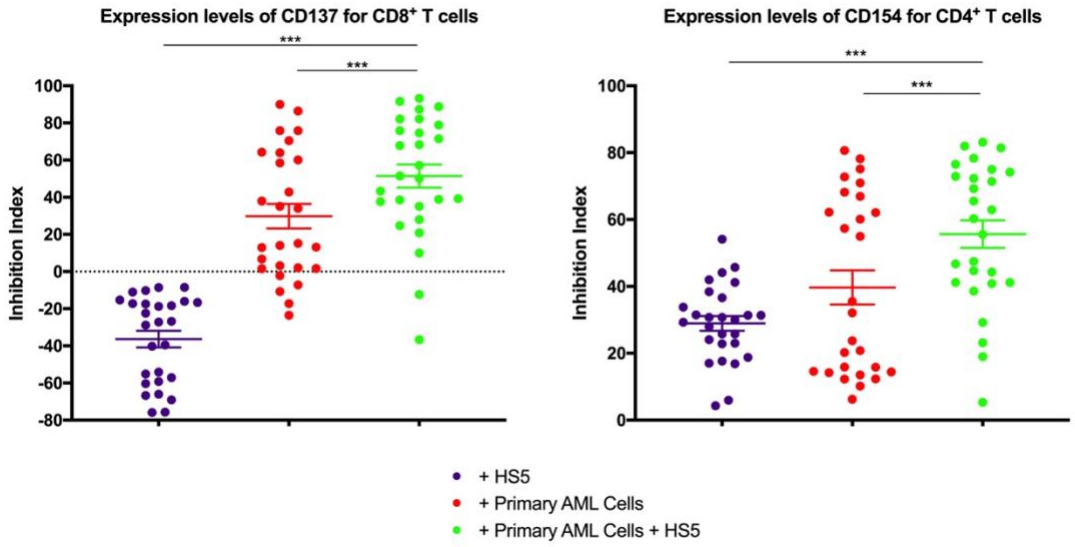



**Figure 3-8 Combination of primary AML cells and HS5 inhibits T-cell activation under hypoxic conditions**

T cells were stimulated in the presence of primary AML cells and HS5 under hypoxic conditions for 48 hours. T-cell function was assessed and shown as an inhibition index. (A) Percentage positive for CD137 and CD154 expression by $CD8_+$ and $CD4_+$ T cells respectively, and (B) MFI of CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively. A significant increase in inhibition indexes in the presence of primary AML and HS5 cells was seen compared to HS5 or primary AML cells alone. Data shown represents the mean ± SEM. $^{***}p<0.001$, determined by paired t test.

To further examine T-cell function, three different cytokines, IFN-γ, TNF-α, and IL-4, were assessed from the supernatants after 48 hours of co-culture; IL-4 was also measured for analysis of Th2 T helper cell activation as they secrete IL-4[291, 292]. Cytokine analysis allows the direct measurement of T-cell function, compared to activation markers, which indirectly measures T-cell function. Consistent with the hypothesis that the combination of primary AML with HS5 cells enhances the immunosuppression of T-cell function, Figure 3-9 demonstrates that levels of IFN-γ, TNF-α, and IL-4 were significantly reduced when αCD3/CD28 T cells were stimulated in the presence of primary AML and HS5 cells compared to primary AML cells alone; levels of IFN-γ, TNF-α, and IL-4 were reduced from a mean of 7598.9 pg/mL, 3095.3 pg/mL, and 127.8 pg/mL respectively for αCD3/CD28 stimulated T cells in the presence of primary AML cells alone to 4114.7 pg/mL ($p$<0.001), 700.4 pg/mL ($p$<0.001), and 83.7 pg/mL ($p$=0.003) respectively for those stimulated T cells in the presence of primary AML and HS5 cells. Furthermore, consistent with the CD137 activation marker expression for $CD8_+$ T cells co-cultured in the presence of HS5 only, levels of IFN-γ, but not TNF-α and IL-4, were significantly increased compared to supernatants from stimulated T cells alone, from a mean of 4466.6 pg/mL to 6005.7 pg/mL ($p$=0.007).

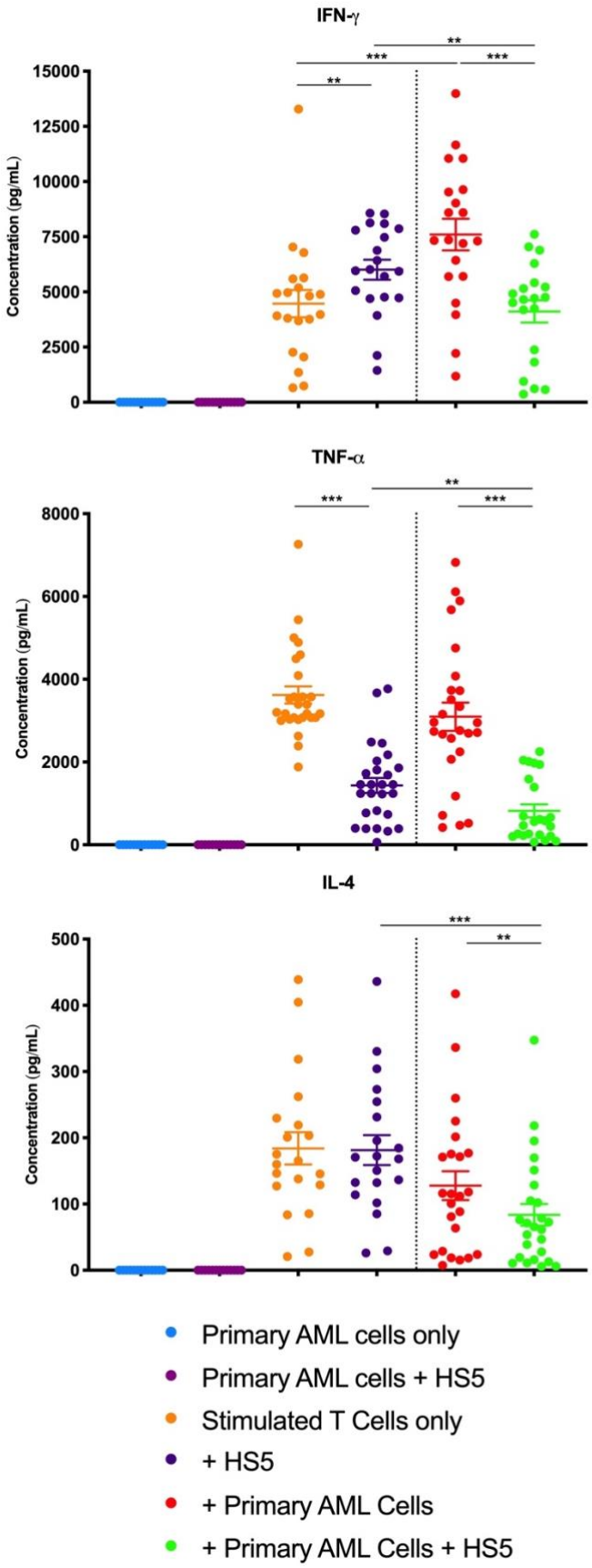



**Figure 3-9 Primary AML and HS5 cells inhibit cytokine production**

Supernatants were collected after cells were co-cultured for 48 hours. Levels of IFN-γ, TNF-α, and IL-4 were significantly reduced in the presence of primary AML cells and HS5 compared to

HS5 or primary AML cells alone. IFN-γ, TNF-α, and IL-4 were not detected in the cultures of Primary AML cells alone and Primary AML cells with HS5. Cell culture supernatants were diluted as 1:25, 1:5, and 1:5 for IFN-γ, TNF-α, and IL-4 respectively. Data shown represents the mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, determined by paired t test.

Figure 3-10 shows the absolute cell count for viable primary AML cells that were cultured alone, in the presence of HS5, in the presence of stimulated T cells, and in the presence of stimulated T cells and HS5 after 48 hours of co-culturing under hypoxic conditions. Viable AML cells were gated on by their exclusion of the fixable viability dye and their expression of CD33 and/or CD34. After 48 hours of co-culturing, a significant increase in the recovery of viable primary AML cells was observed when they were co-cultured in the presence of stimulated T cells alone compared to the absence of stimulated T cells, with a mean absolute cell counts of 295363 cells/mL and 150228 cells/mL respectively ($p<0.001$). Similarly, primary AML cells co-cultured in the presence of HS5 and the additional presence of stimulated T cells resulted in a significant increase in the recovery of viable primary AML cells compared to those cultured in the absence of stimulated T cells, with a mean absolute cell counts of 437367 cells/mL and 374001 cells/mL ($p<0.001$). The results here indicated that cytokines produced by stimulated T cells can sustain primary AML cells even in the absence of HS5.

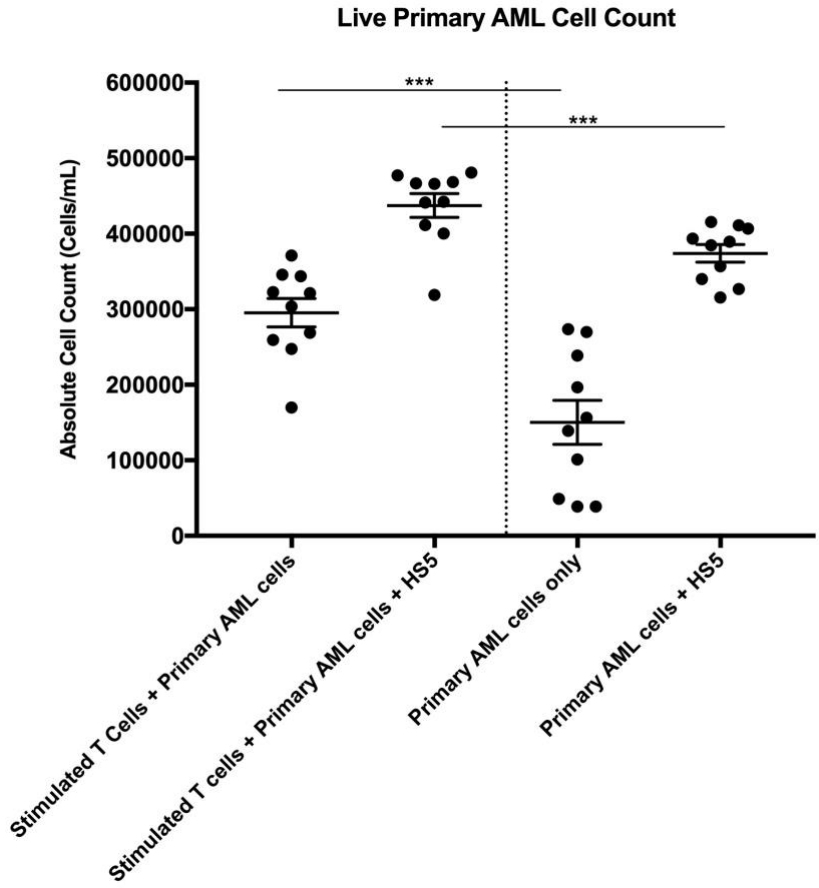



**Figure 3-10 Primary AML cells sustained in the presence of stimulated T cells**

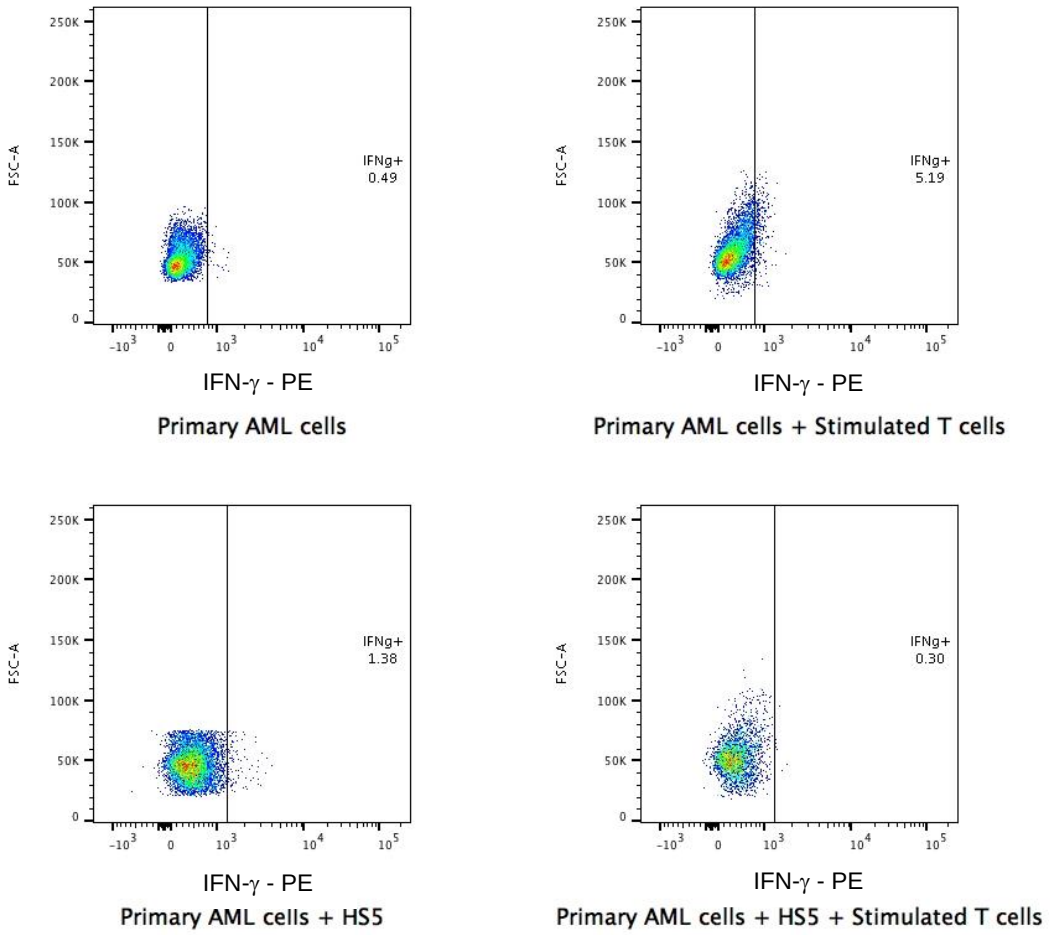
The presence of stimulated T cells significantly increased the number of viable primary AML cells. The absolute cell counts of primary AML cells were determined by flow cytometry through the use of counting beads after 48 hours of co-culturing; AML cells were selected as viable CD33 or CD34 cells using viability dye. Data shown represents the mean ± SEM. ***$p$<0.001 determined by paired t test

A significant increase in levels of IFN-γ production was observed when activated T cells were cultured in the presence of primary AML cells only compared to activated T cells alone, which is contradictory to the CD137 and CD154 activation marker expressions observed. The possibility that primary AML cells produce IFN-γ was excluded by analysis of the intracellular staining of IFN-γ of primary AML cells. Primary AML cells were cultured alone or co-cultured in the presence of HS5, αCD3/CD28 stimulated T cells, or a combination of both for 48 hours under hypoxic conditions. Intracellular staining of IFN-γ (Figure 3-11) demonstrated that primary AML cells do not produce any IFN-γ, as the percentage of cells that stained for IFN-γ were similar in all conditions, ranging from 0.49% to 5.19%. It is possible that the observation of raised IFN-γ may

be contributed by the patient T cells within the primary AML cells sample as the patient AML samples were not pure so any autologous present T cells would be stimulated by the αCD3/CD28 beads.

Cytokine analysis of the supernatant by ELISA was the preferred method as intracellular staining of cytokines required the use of either brefeldin A or sodium monensin, which are golgi inhibitors that prevent the release of glycoproteins. Therefore, treatment of brefeldin A or sodium monensin in the co-culture will prevent secretion of soluble factors that are essential to the immunosuppressive microenvironment generated by AML cells. This is pertinent to the study of the effects of the immunosuppressive microenvironment on T-cell function.

**A**



**B**

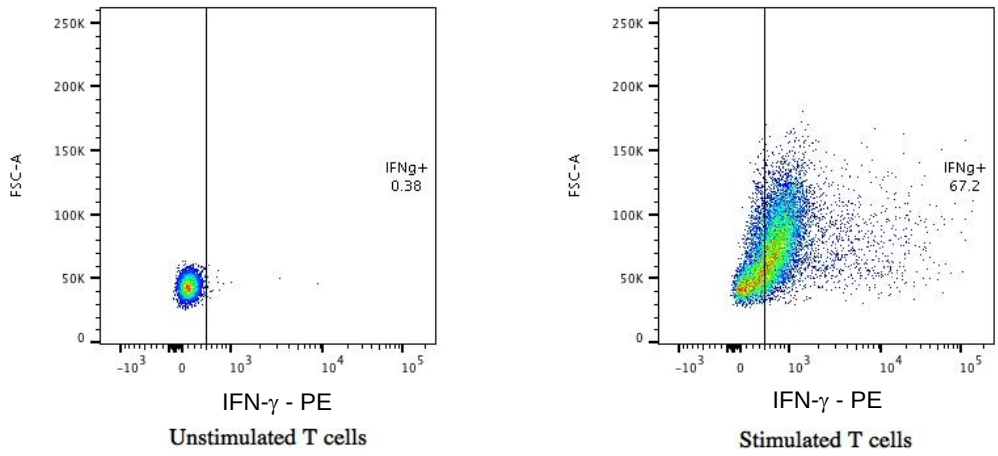



**Figure 3-11 Primary AML cells do not produce IFN-γ**

Primary AML cells were cultured under different conditions to assess their ability to produce IFN-γ. (A) Viable primary AML cells were selected for CD33 or CD34 and assessed for its IFN-γ expression under different conditions. (B) As a control, viable T cells were assessed for IFN-γ expression. The figure here are obtained using primary AML cells from one patient.

### 3.2.6 Combination of primary AML cells and HS5 reduces T-cell death under hypoxic conditions

To determine if presence of primary AML cells and HS5s affects the viability of T cells, $CD8_+$ and $CD4_+$ T-cell numbers and % viability were calculated and determined using counting beads and viability dye respectively via flow cytometry after 48 hours of co-cultures. Figure 3-12 shows that there was a 17.7% ($p$=0.12) and 26.8% ($p$<0.001) reduction in the absolute cell count for both αCD3/CD28 stimulated $CD8_+$ and $CD4_+$ T cells respectively, compared to unstimulated T cells. Furthermore, in the presence of HS5, αCD3/CD28 stimulated T cells resulted in further significant decrease in both absolute $CD8_+$ and $CD4_+$ T-cell count compared to αCD3/CD28 stimulated on its own, with a 50.8% ($p$<0.001) and 42.4% ($p$<0.001) reduction. However, T cells stimulated in the presence of primary AML cells and HS5 resulted in a 40.0% ($p$<0.001) and a 46.6% ($p$=0.02) increase for both $CD8_+$ and $CD4_+$ T-cell counts respectively compared to T cells stimulated in the presence of HS5 only. Similarly, slight improvements in $CD4_+$ absolute T-cell counts, but not $CD8_+$ absolute T-cell counts, were observed in T cells stimulated in the presence of primary AML cells only compared to T cells stimulated alone, a 14.8% increase ($p$=0.11).

In addition to the absolute T-cell counts, the percentage of viable $CD8_+$ and $CD4_+$ T cells was also assessed. Similarly to the results observed from the absolute cell count, significant reductions in the % viable $CD8_+$ and $CD4_+$ T cells were observed for $\alpha$CD3/CD28 stimulated T cells compared to unstimulated T cells, with a mean 15.9% ($p$<0.001) and 15.5% ($p$<0.001) reduction in % viability respectively; additionally, T cells stimulated with $\alpha$CD3/CD28 beads in the presence of HS5 also resulted in a significant reduction in % viable $CD8_+$ and $CD4_+$ T cells compared to T cells stimulated on its own, with a mean 11.1% ($p$<0.001) and 14.3% ($p$<0.001) reduction in % viability respectively. A significant increase in the percentage of viable $\alpha$CD3/CD28 stimulated $CD8_+$ T cells were observed when co-cultured in the presence of primary AML cells and HS5 compared to both presence of HS5 alone or primary AML cells alone, with a 6.1% ($p$=0.03) and 12.7% ($p$=0.047) increase respectively. However, a significant decrease in the percentage of viable $\alpha$CD3/CD28 stimulated $CD4_+$ T cells were observed when co-cultured in the presence of primary AML cells and HS5 and compared to both the presence of HS5 alone or primary AML cells alone, with a 12.1% ($p$=0.02) and 10.4% ($p$=0.046) reduction. Unexpectedly, a significant decrease in the % viability of both $\alpha$CD3/CD28 stimulated $CD8_+$ and $CD4_+$ T cells co-cultured in the presence of primary AML cells were observed compared to $\alpha$CD3/CD28 stimulated $CD8_+$ and $CD4_+$ T cells on its own, with a 16.5% ($p$=0.014) and 16.1% ($p$=0.005) reduction respectively. This observation may be caused by the autologous patient T cells from the primary AML cell samples undergoing apoptosis. Therefore, further experiments that allows the exclusion of autologous patient T cells will be required to allow precise analysis of the healthy donor T cell viability.

The findings here demonstrate that stimulation and activation of T cells under hypoxic conditions result in increased cell death compared to unstimulated T cells. Furthermore, this is consistent with the increased activation markers expression and levels of IFN-$\gamma$ produced by T cells stimulated in the presence of HS5 only compared to T cells stimulated alone, as a further reduction in both viable $CD8_+$ and $CD4_+$ T cells were observed. Consequently, improved absolute $CD8_+$ and $CD4_+$ T-cell count for T cells stimulated in the presence of primary AML cell and HS5 could be explained by the significant inhibition of T-cell activation, as shown in increased inhibition index and reduced levels of cytokine produced shown in section 3.2.5,

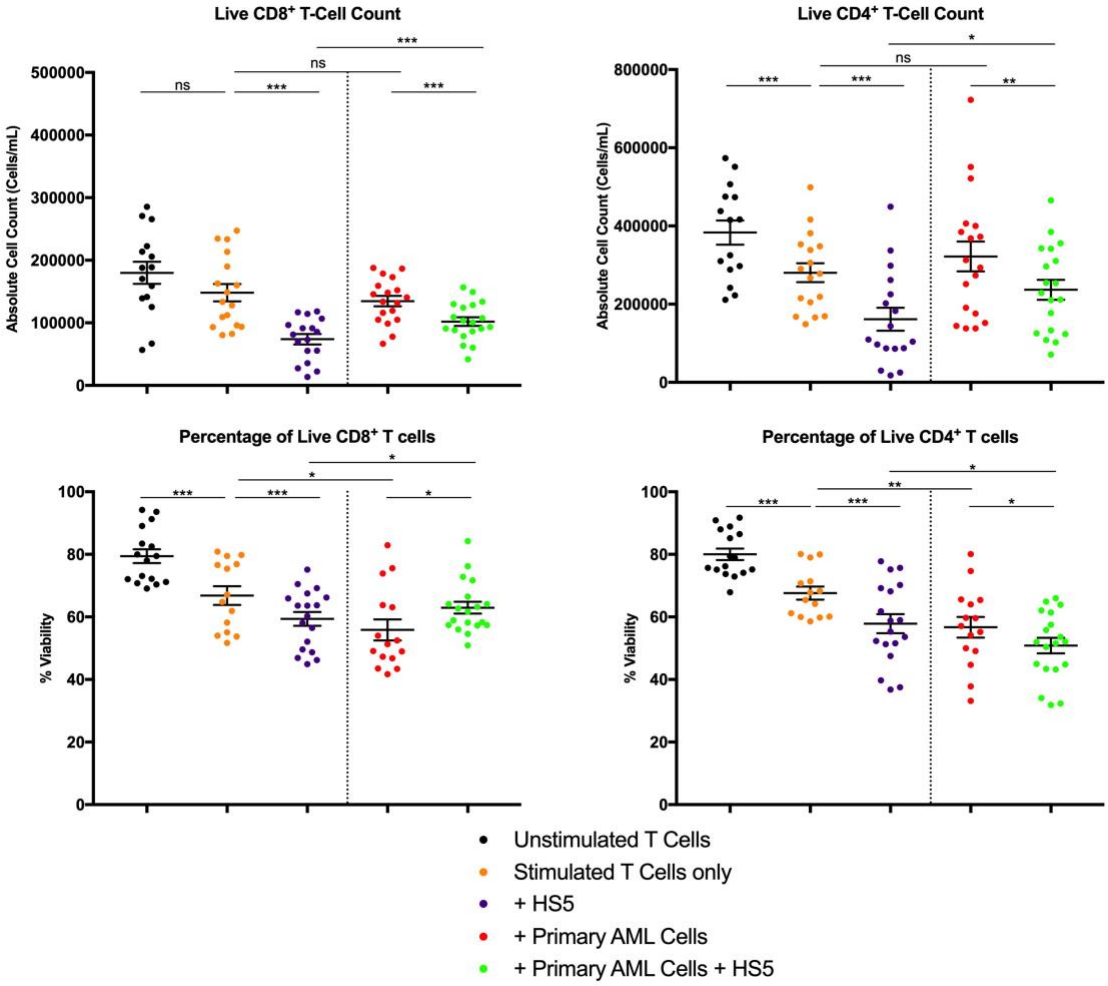



**Figure 3-12 Reduced CD8+ and CD4+ T-cell death in the presence of primary AML cells and HS5**

Absolute T-cell count was measured and calculated using counting beads. Absolute CD8+ and absolute CD4+ T-cell counts were calculated after 48 hours of culture. Stimulation of T cells resulted in a significant reduction in absolute T-cell count under hypoxic conditions. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, determined by paired t test.

### 3.2.7 A crowding effect did not inhibit T-cell activation

To determine whether media exhaustion and reduced proximity of αCD3/CD28 beads with T cells, due to the increased cell numbers, may contribute to the reduced T-cell activation marker expression observed in the presence of primary AML cells with HS5 cells, a crowding experiment was set up. Instead of AML cells, different sources of non-malignant T cells, HLA-A2$^+$ and HLA-A2$^-$, were used in a co-culture containing the same cell numbers. Table 3-2 shows the different culturing conditions used in the experiment. The possibility that the reduced T-cell activation observed was contributed by media exhaustion and reduced proximity of αCD3/CD28 beads with T cells were excluded by analysis of the activation marker expressions for HLA-A2$^-$ T cells co-cultured in the presence of HLA-A2$^+$ T cells. HLA-A2$^-$ T cells were gated on by their exclusion of both the fixable viability dye eFluor780 and the expression of HLA-A2 using a HLA-A2 antibody.

Figure 3-13 shows that in the presence of primary AML cells, the inhibition index for the percentage expression and MFI of CD137 by HLA-A2$^-$ T cells increased by 24.0% ($p$=0.04) and 52.8% ($p$=0.04) respectively compared to HLA-A2$^-$ T cells stimulated in the presence of HLA-A2$^+$ T cells. Similarly, a significant increase in the inhibition index for the percentage expression and MFI of CD137 by HLA-A2$^-$ T cells were observed, with a mean increase of 37.5% ($p$=0.03) and 69.0% ($p$=0.04) respectively, for those stimulated in the presence of primary AML and HS5 cells compared to in the presence of HLA-A2$^+$ T and HS5 cells,

Similar observations were made for the inhibition index for the percentage expression and MFI of CD154. HLA-A2$^-$ T cells stimulated in the presence of primary AML cells alone had the inhibition index of percentage expression and MFI of CD154 by HLA-A2$^-$ T cells increased by 57.1% ($p$=0.02) and 59.7% ($p$=0.04) compared to the presence of HLA-A2$^+$ T cells only. The inhibition index of percentage expression and MFI of CD154 was increased by 69.5% ($p$=0.01) and 73.5% ($p$=0.02) respectively for HLA-A2$^-$ T cells stimulated in the presence of primary AML and HS5 cells compared to HLA-A2$^-$ T cells stimulated in the presence of HLA-A2$^+$ T cells and HS5 cells.

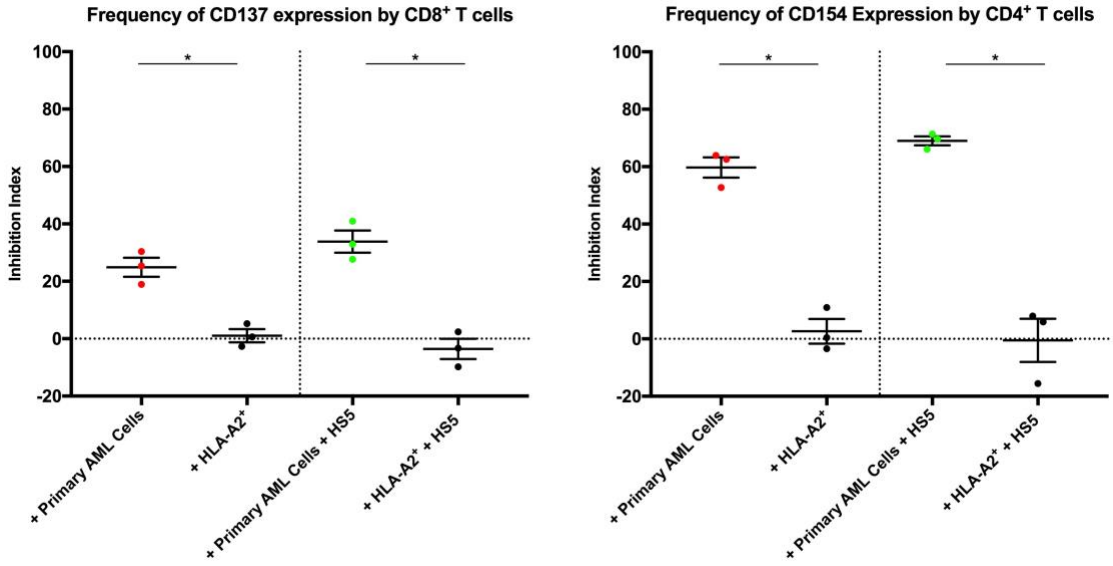
The strong inhibition of percentage expression and MFI of both activation markers observed when HLA-A2$^-$ T cells stimulated in the presence of primary AML cells compared to the presence of HLA-A2$^+$ T cells only indicated that the inhibitions observed were not a result of media exhaustion

and reduced proximity of αCD3/CD28 beads with T cells due to the increased cell numbers, but were a result of the primary AML cells.

**Table 3-2 Crowding Effect Culturing Conditions**

| **Conditions** | |
|---|---|
| Unstimulated HLA-A2- T cells | Negative Control |
| Stimulated HLA-A2- T cells | Positive Control |
| Stimulated HLA-A2- T cells + HS5 | |
| **Stimulated HLA-A2- T cells + AML** | **Test Conditions for Comparison** |
| **Stimulated HLA-A2- T cells + HLA-A2+ T cells** | |
| **Stimulated HLA-A2- T cells + AML + HS5** | **Test Conditions for Comparison** |
| **Stimulated HLA-A2- T cells + HLA-A2+ T cells + HS5** | |

**A**




**B**

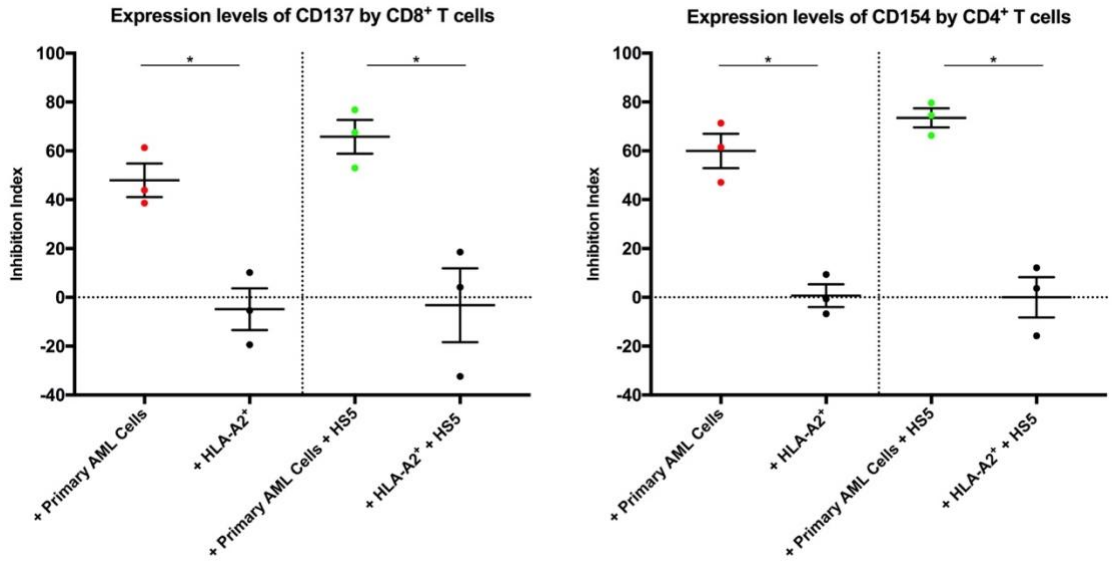



**Figure 3-13 T-cell inhibition was not caused by crowding effect**

HLA-A2- T cells were stimulated in the presence of primary AML cells or HLA-A2+ T cells under hypoxic conditions for 48 hours. HLA-A2- T-cell function, based on activation markers expression, was compared and assessed. (A) The frequency of CD137 and CD154 expression and (B) the MFI of CD137 and CD154 by CD8+ and CD4+ T cells respectively were compared between cultures with primary AML cells and HLA-A2+. Results indicate inhibitory effect observed with AML cells is not due to a crowding effect. Data shown represents the mean ± SEM and representative of 3 independent experiments. *$p$<0.05, determined by paired t test.

## 3.3 Discussion

The *in-vitro* co-culture models presented here were designed to assess T-cell function in the AML microenvironment; looking at the effects of both AML cell lines and primary AML cells in the presence of HS5 stromal cells on T cells. In order to assess T-cell function in the presence of primary AML cells, an *in-vitro* co-culture model based on that designed by Garrido et al was established, as the presence of HS5 cells were reported to sustain primary AML cells.[283] Purified $CD3_+$ T cells were stimulated with $\alpha$CD3/CD28 DynaBeads, cultured with AML cells and HS5 at 1:1:0.1 ratio and analysed after 48 hours of co-culture.

In the initial experiments, $\alpha$CD3/CD28 stimulated T cells were found to achieve the highest percentage expression of CD137 by $CD8_+$ T cells in 24 hours; however, this was not observed for CD154 expression by $CD4_+$ T cells until 48 hours of stimulation. In addition, maximum MFI of both CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively were observed by 48 hours deeming this the optimal T cell stimulation time. Further experiments demonstrated that primary AML cells were sustained in the presence of HS5 after 48 hours of co-culturing under both normoxic and hypoxic conditions; therefore, 48 hours was chosen as the time course for the model going forward.

A few reports have documented that AML cell lines produce soluble factors that inhibit secretion of Th1 cytokines, such as IFN-$\gamma$, and T-cell proliferation[213, 276]. Importantly, in these studies, AML cell lines do not require the presence of accessory cells. One study showed that the presence of the murine stromal cell line, MS5, was able to prevent apoptosis of the HL60 AML cell line when treated with the chemotherapeutic agent, cytarabine, indicating the protective effect of stromal cells on AML cells[287]. Therefore, the initial experiments presented here investigated whether the presence of the human stromal cell line HS5 could enhance AML generated immunosuppression of T cells. Initially this was done using five different AML cell lines with different cytogenetic abnormalities, U937, MOLM14, NB4, HL60, and THP-1. Two assessment methods, activation marker expression for CD137 and CD154 by $CD8_+$ and $CD4_+$ T cells respectively and cytokine production were used. CD137 is expressed primarily on activated $CD8_+$ T cells and CD154 is primarily expressed on activated $CD4_+$ T cells[294, 295, 296]. The presence of all AML cell lines with HS5 cells resulted in a marked decrease in expression of both CD137 by $CD8_+$ T cells and CD154

by $CD4_+$ T cells. For some cell lines this was statistically significant and for others a trend was seen. Increasing experimental repeats for these cell lines would almost certainly enable the data to reach statistical significance, but the results were sufficient to conclude that the presence of HS5 cells enhanced AML inhibition and moving on to use primary AML cells considered a priority.

As activation markers serve as surrogate indicators to T-cell function, T-cell associated cytokines in the co-culture supernatants were assessed. The presence of HS5 cells significantly enhanced the immunosuppressive effect of all AML cell lines, except for HL60 and THP-1, as demonstrated by a significant decrease in the levels of IFN-$\gamma$; similarly, significantly reduced levels of TNF-$\alpha$ was seen except with HL60. Although the additional presence of HS5 with HL60 and THP1 did not result in a significant reduction in the cytokines produced, a clear trend of reduced cytokine production was observed and again significance of the observations could be improved by increasing the number of independent experiments.

The enhanced immunosuppressive effects of AML cell lines with HS5 cells were further investigated to determine if similar observations could be made with primary AML cells. Previous investigations onto the inhibitory effects of primary AML cell supernatants on $\alpha$CD3/CD28 stimulated T cells have demonstrated that NFATc and NF$\kappa$B play a key role in the inhibitory process[212]. Furthermore, similar to AML cell lines, it has been shown that the presence of HS5 cells protected primary AML cells from drug-induced apoptosis, and was able to sustain primary AML cell viability[283]. To investigate the hypothesis that the presence of HS5 cells will enhance the immunosuppressive effect of primary AML cells under normoxic conditions, we repeated the above experiments using donor-derived AML cells. The results demonstrated that, consistent with previous studies, the presence of primary AML cells alone inhibited T-cell activation but that the additional presence of HS5 cells resulted in a marked increase in the inhibition index for both $CD8_+$ and $CD4_+$ T cells activation markers, indicating an enhanced immunosuppressive microenvironment.

As the bone marrow niche in which AML cells reside in is hypoxic and promotes the survival of primary AML cells[95, 97, 293], further interrogation of the enhanced immunosuppressive microenvironment by primary AML with HS5 cells was examined under hypoxic conditions (1%

$O_2$, 5% $CO_2$ supplemented with nitrogen). Similarly, to primary AML cells cultured under normoxic conditions, analysis of T cell activation markers demonstrated that the combination of primary AML cells and HS5 enhances the immunosuppression of T cells. Consistent with this, cytokine analysis showed a marked decrease in the levels of for IFN-γ, TNF-α, and IL-4 produced in the presence of primary AML and HS5 cells compared to primary AML cells alone. Importantly, the results seen were consistent as these observations were seen using 11 different AML bone marrow samples and 8 different healthy donor T cells.

The observation that the presence of HS5 cells alone induced a higher activation status for $CD8_+$ T cells compared to αCD3/CD28 stimulated T cells alone could be explained by the presence of IL-6 produced by HS5 cells, which has been shown to induce effector functions of $CD8_+$ T cells rapidly[297, 298, 299], thus resulting in a higher activation state and increased IFN-γ production. However, CD4+ T cell activation was slightly reduced in the presence of HS5 based on the MFI of CD154. An explanation to this observation may be that HS5 cells express the ligands for CD154 namely CD40 and, as studies have shown that bone marrow stromal cells can express CD40[300], binding of CD154 to CD40 is highly feasible and would result in its downregulation[301].

As mentioned earlier, it was previously thought that primary AML cells can only be sustained in the presence of stromal cell line HS5 or the addition of growth factors such as GM-CSF and G-CSF[283, 287]. However, the results here demonstrate that primary AML cells are sustained in a 48-hour co-culture model, in the presence of stimulated T cells alone, irrespective of whether HS5 cells are present. This phenomenon may be attributed to the production of GM-CSF by the activated T cells[5]. Therefore, the results presented here suggests that the HS5 cells are not alone in sustaining the primary AML cells, and that a three way process involving both HS5s and stimulated T cells exists. This creates a positive feedback loop that promotes survival of the malignant clone which in turn increases its inhibitory effect onto the very activated T cells that have promoted its survival.

Unexpectedly, the ELISA results from the supernatants of αCD3/CD28 activated T cells in the presence of primary AML cells showed a small increase in levels of IFN-γ production compared to activated T cells alone. This is contradictory both to previous studies and to the inhibition of

CD137 and CD154 activation marker expressions observed. Previous studies have used intracellular cytokine staining so that the secreting T cells can be identified using surface labelling for CD4 and CD8. However, this was not suitable for the model described here as it involves the addition of a golgi block which would not only retain the T-cell cytokine within the cell, but also any inhibitory factors within the HS5 and AML cells. However, this method was used to see whether AML cells are secreting IFN-γ themselves and thus increasing the levels produced by the activated T cells. As shown, primary AML cells did not secrete IFN-γ and thus did not contribute to this. A more likely explanation for this observation is that primary AML cells used in the experiments were not completely pure (70%< $CD33^+$ or $CD34^+$); therefore, these samples contain other cell types that could be found within the bone marrow, most notably autologous patient $CD3^+$ T cells, thus increasing the total T-cell count in the co-cultures. Since T cells were stimulated with αCD3/CD28 beads, both healthy donor T cells and patient T cells would be stimulated; hence, the increase in IFN-γ levels observed. This fits with the finding that T cell activation marker expression was inhibited as the IFN-γ increase is attributed to an increase in T cell numbers rather than the activation level of each individual T cell. Furthermore, this explanation can be further substantiated by previous experiment using pure AML cell lines, whereby presence the of AML cell lines alone did not result in an increased level of IFN-γ. Whatever the cause of the increased IFN-γ levels seen with Primary AML cells alone, the additional presence HS5 cells significantly reduced them, further emphasising the enhanced immunosuppressive microenvironment created by primary AML and stromal cells.

Enumeration of T cells under hypoxic conditions showed that $CD8^+$ T cells stimulated in the presence of primary AML cells and HS5s resulted in a reduced T-cell death demonstrated by an increased absolute cell count and percentage of viable cells when compared to $CD8^+$ T cells stimulated in the presence of HS5 alone. However, an increase in absolute $CD4^+$ T cell count but not percentage viable $CD4^+$ T cells was also observed. Unexpectedly, T cells stimulated in the presence of primary AML cells resulted in a reduced percent viability for both $CD8^+$ and $CD4^+$ T cells compared to T cells stimulated alone. Further experiments excluding patient T cells by purification of $CD33^+$ and $CD34^+$ primary AML cells or labelling of patient T cells using Carboxyfluorescein succinimidyl ester (CFSE) prior to the co-culture experiments would need to be done to establish true healthy donor T cell gating for analysis. Previous *in vitro* studies have

demonstrated that stimulation of T cells under physiological oxygen levels (5% $O_2$) results in greater cell death compared to atmospheric $O_2$ levels[302]. Consistent with these published studies, absolute T-cell count and viability for stimulated T cells alone were reduced compared to unstimulated T cells. In addition, reduced T-cell counts and percent viability was also observed in T cells stimulated in the presence of HS5 alone compared to stimulated T cells alone, indicating further cell death, which could be contributed to the increased T cell activation observed. The likely explanation for the reduced T-cell death observed in the presence of primary AML cells and HS5s is that the T cells were less activated, which results in reduced cell death.

Finally, in view of the enhanced immunosuppressive effect being in the presence of the maximum cells number (T cells plus AML and HS5 cells), the effect of media exhaustion and reduced proximity of αCD3/CD28 beads to T cells was investigated. Since T cells were stimulated with αCD3/CD28 beads at 1:1 T-cell to bead ratio, the probability of T cells being in contact with αCD3/CD28 beads were halved in the cultures containing AML cells, as the total number of cells were doubled (HS5 cells adhere to the bottom of the cell well and would not interfere). The results demonstrated that the inhibitory effect observed was not contributed to by media exhaustion or reduced αCD3/CD28 beads to cell ratio. Any effect observed in T cells would therefore be due to the intrinsic inhibitory effect of the AML cells.

In summary, this chapter describes the successful development and optimisation of a novel triple cell co-culture model to investigate the activation of T cells in the AML microenvironment. Using this model, it has been demonstrated that the presence of stromal cells enhances the immunosuppressive microenvironment created by AML cells and significantly inhibits αCD3/CD28 stimulated T-cell activation. Based on these findings, the established *in vitro* model will be used to assess T-cell function of different novel T-cell based immunotherapies in the AML microenvironment.

# Chapter 4 Assessment of suppression of antigen-specific T cells in the AML microenvironment

## 4.1 Introduction

Results presented in the previous chapter using the three-cell *in vitro* co-culture model demonstrated that the microenvironment produced by synergy between AML cell lines or primary AML cells and the stromal cell line HS5 significantly suppresses the activation and function of T cells from allogeneic healthy donors stimulated with αCD3/CD28 beads. The method of T-cell stimulation used mimics the signal 1 provided through the TCR complex and signal 2 provided by co-stimulation. Although closer to physiological relevance than the use of phorbol 12-myristate 13-acetate (PMA) and ionomycin, which stimulates T cells through phosphorylation of CD4 and CD8 via hydrolysis of phosphoinositides (second messengers) and activation of protein kinase C[303], the αCD3/CD28 stimulus is still non-specific and abnormally potent. The next objective of the study was therefore to use the *in vitro* model to assess whether antigen-specific T-cell responses, including novel T-cell based immunotherapies that are being developed to treat AML, are suppressed in the AML microenvironment.

In common with most cancers, T-cell responses against AML cells are not easily generated because, unlike foreign pathogens, cancer cells are very similar to normal self-tissue. There is, however, evidence indicating an important role for T cells in the recognition and elimination of AML cells; notably the GvL effect after allogeneic HSCT and presence of LAA and LSA-specific T cells in AML patients[146, 168]. As a result, novel T-cell based immunotherapies, such as peptide vaccination, γδ T cells and CAR T cells, are being developed that aim to improve treatment options for AML patients. Pre-clinical *in vitro* studies indicate potential efficacies of the aforementioned T-cell immunotherapies [54, 192, 194, 304], but a major limitation is that the assays used do not replicate the AML microenvironment *in vivo*.

Although allogeneic HSCT is a curative treatment for AML, approximately 40% of patients will relapse and their subsequent prognosis is dismal[85, 305]. Two recent seminal studies have revealed that relapse after allogeneic HSCT is associated with altered immune-related gene expression profiles in AML cells, which substantiates that immune escape probably has a key role in disease persistence[210, 211]. The efficacy of novel immunotherapies that are being developed for AML is

also likely to be jeopardised by the immune evasion strategies operating in the microenvironment created by the disease. It is therefore important to evaluate the activity of immunotherapies in a setting that recapitulates the immunosuppressive conditions. The aim of the work described in this chapter was to assess the activation and function of three different examples of antigen-specific T-cells in the AML and stromal cell *in vitro* co-culture model.

Donor-derived T cells are key players in the GvL effect after allogeneic HSCT[146, 273], Some of the mHags, LAAs and LSAs recognised by the T cells that mediate GvL have been identified[150], but the number of known epitopes is low and frequencies of responding T cells is also low (typically only up to 1%)[154, 261, 306]. These factors impede the assessment of the function of leukaemia-reactive T cells from allogeneic HSCT patients in the AML microenvironment. Evaluation of the response to cytomegalovirus (CMV) was therefore used as a surrogate model. Previous studies have shown that the T-cell repertoire of patients after allogeneic HSCT is often dominated by CMV-specific T cells[307, 308, 309], likely due to antigen-driven proliferation during CMV reactivation that is frequent in the early post HSCT period. Moreover, many epitopes recognised by CMV-specific T cells have been characterised[310, 311] and peptide libraries containing epitopes presented by common HLA types are commercially available. In addition to studying the activity of donor-derived T cells from allogeneic HSCT patients in the AML microenvironment, representing the current standard immunotherapy for AML, two novel T-cell based immunotherapies that are being developed were also examined; γδ T cells and genetically modified CAR T cells.

There are several lines of evidence to suggest that Vγ9Vδ2 T cells may be attractive candidates for AML immunotherapy. These cells are the predominant subset of circulating human γδ T cells, they recognise non-peptide phosphoantigens and are not HLA-restricted (see detailed description in Chapter 1 section 1.1.2.3). Vγ9Vδ2 T cells have been shown to recognise and kill AML cells *in vitro* and exhibit anti-leukaemic activity that prolongs survival in an *in vivo* xenotransplantation murine model[54]. Furthermore, the pre-treatment of AML cells with aminobisphosphonates to promote accumulation of phosphoantigens can increase susceptibility to Vγ9Vδ2 T-cell mediated cytotoxicity[312]. The association of higher numbers of circulating γδ T cells in AML patients after allogeneic HSCT with an improved disease-free survival rate of 54.4% compared to 19.1% in patients with lower numbers, suggests they may have a beneficial role in AML immune

surveillance[200]. This could potentially be exploited by treatment of AML patients with an aminobisphosphonate, combined with ex vivo expansion and adoptive transfer of Vγ9Vδ2 T cells in patients with low cell numbers. Immunotherapeutic strategies to exploit Vγ9Vδ2 T cells have begun to be evaluated in patients with various tumour types. A meta-analysis of 13 clinical trials reported significant evidence of improved survival and low toxicity grade, but overall results are somewhat inconsistent and efficacy has been modest, with only a proportion of patients exhibiting stabilization or partial remission of disease[198, 313, 314]. Cancer immune evasion may be a contributing factor to variable outcomes. Impact of the AML microenvironment on Vγ9Vδ2 T cells recognition of aminobisphosphonate-treated AML cells was therefore explored.

CAR T cells targeted to AML cells are also promising immunotherapeutic agents. The cell surface molecules CD123 and CD33 have been selected as potential targets because they are expressed by the majority of AML subtypes and overexpressed in AML cells compared to normal cells[179]. CD123 is the alpha-chain of the IL-3 receptor and CD33 is a sialoadhesin molecule that is found on myeloid cells. *In vitro* studies have shown that αCD123 CAR T cells produced using T cells from healthy individuals or patients effectively lysed $CD123^+$ AML cell lines and $CD123^+$ primary AML blasts[192, 194, 315]. Importantly, studies have demonstrated elimination of AML by αCD123 CAR T cells *in vivo* using a xenograft mouse model[194, 315]. The successful use of αCD19 CAR T-cell therapy in patients for the treatment of B-cell precursor acute lymphoblastic leukaemia (B-ALL)[187, 316] is encouraging attempts to extend utility to the treatment of AML patients. Consequently, impact of the AML microenvironment on the function of αCD123 CAR T cells was assessed using the *in vitro* co-culture model.

## 4.2 Results

### 4.2.1 Assessment of the suppression of CMV specific CD8+ T cells from allogeneic HSCT patients in the AML microenvironment

Exploitation of the CMV model of T-cell function after allogeneic HSCT was possible because samples from patients that were already known to contain high frequencies of functional CMV-specific T cells were available for study, courtesy of collaboration with another member of our laboratory, Monera Al Rukhayes. Overlapping synthetic peptide libraries of the CMV proteins pp65 and IE-1 were used to stimulate T cells because they are known to be immunodominant antigens[310, 311]. The peptide stimulation conditions used had been previously optimized by Monera Al Rukhayes and are described in Chapter 2 section 2.1.4.8. The 15-mer peptides in the libraries required processing and presentation by APC, which necessitated use of PBMC rather than purified T cells that were used in the assays described in the previous chapter. Contact between APC and T cells is needed in this assay[317, 318], therefore the immunosuppressive AML microenvironment was tested using supernatant from AML and stromal cell co-cultures to avoid cell crowding. Supernatant from cultures of primary AML cells have previously been successfully used in short duration assays to study T-cell suppression in the AML microenvironment[212]. Primary AML cells and the HS5 stromal cells were co-cultured at a 10:1 ratio for 48 hours under hypoxic conditions, supernatant was collected and used immediately because a freeze/thaw step substantially diminished suppressive activity (Andrea Pepper; personal communication). Supernatant was used at a 50% dilution with fresh culture media to mitigate the possibility of nutrient exhaustion in the supernatant. Activation of CMV-specific CD8+ T cells was measured by cell surface expression of CD137 and T-cell function was assessed by measuring the production of TNF-$\alpha$ and IFN$\gamma$, as used previously by Monera Al Rukhayes. Activation of CMV-specific CD4+ T cells was not assessed because numbers of CD4+ T cells were very low in these patients, due to treatment with the lymphocyte-depleting monoclonal antibody Campath (also known as alemtuzumab) as part of the HSCT conditioning regimen that causes profound and prolonged depletion of CD4+ T cells[319].

Samples from three allogeneic HSCT patients were tested. Pertinent clinical information for the patients is shown in Table 4-1. All had CMV reactivation early after HSCT, none were receiving immunosuppressive therapy at the time of sample collection or had relapse of AML disease. Two patients were full donor T cell chimeric (>95% CD3+ cells of donor origin) and one patient had

mixed T-cell chimerism. A total of four samples were analysed for suppression of T-cell function in the presence of supernatant collected from the culture of primary bone marrow-derived AML cells from one patient (AML047) either alone or co-cultured with HS5 cells for 48 hours under hypoxic conditions. AML047 was selected because results presented in Chapter 3 section 3.2.5 showed that co-culture of this sample with HS5 cells consistently induced comparatively high suppression of T cells from healthy individuals. In addition to assessment of suppression of the T-cell response to CMV, each sample from allogeneic HSCT patients was also tested for suppression of the T-cell response to stimulation with αCD3/CD28 beads. Statistical analysis was not performed on data from these experiments because only four samples were available for study.

**Table 4-1 Clinical characteristics of the allogeneic HSCT patients**

| Patient Code | Disease | HSCT regimen | Relapse after HSCT | CMV Patient / donor status Reactivation | GVHD ≥Grade 2 acute or chronic GVHD Treatment | CD3 chimerism at sample relevant time points Days post HSCT (% CD3 donor) |
|---|---|---|---|---|---|---|
| 1 | AML | BCyC | No | +ve/+ve Yes (d20-63 & 377-384) | No | 280 (37%) |
| 2 | AML | FBC | No | +ve/-ve Yes (d40-130 & 175-229, & 438-473) | Acute Grade 3 (d55-75) Methylprednisolone, budesonide | 426 (96%), 495 (100%) |
| 3 | AML | FB4C | No | +ve/-ve Yes (d28-58) | No | 484 (96%) |

F indicates fludarabine, B busulphan, C campath, Cy cyclophosphamide
Cyclosporin weaning from day 56 and completed by day 100

A representative example to illustrate the gating strategy and expression of the activation marker CD137 by $CD8_+$ T cells after stimulation with the pp65 and IE-1 CMV peptide libraries or αCD3/CD28 beads is shown in ***Figure*** 4-1. In this case, the background frequency of $CD137_+$ $CD8_+$ T cells for patient 3 at day 484 after allogeneic HSCT was 0.75%, a very high proportion responded to stimulation with the CMV peptide libraries, with 55.6% of $CD8_+$ T cells activated and almost all $CD8_+$ T cells were activated by the non-specific stimulus of αCD3/CD28 beads (95.4%). Similarly, the background frequency of $CD137_+$ $CD8_+$ T cells in the absence of stimulation was low for the other patient samples (<1.5%) and the majority were activated by αCD3/CD28 beads (>50%). The frequencies activated by stimulation with the CMV peptide libraries were 13.1% for patient 2 at day 473, 7.04% for patient 2 at day 390 and 11% for patient 1 at day 280. These

frequencies of CMV-specific CD8+ T cells were comparable to previous results obtained with aliquots from these samples (Monera Al Rukhayes, personal communication).

Several of the patient samples contained populations of CD3+ T cells that were double negative or double positive for CD4 and CD8, as illustrated by the represented example shown ***Figure*** 4-1. Although it has been reported that CD4+ CD8+ T cells are enriched for virus-specific memory and effector T cells [320], few responded to stimulation with the CMV peptide libraries (1.8% in the example shown and <1% for the other samples). Consequently, these populations were not included in the analysis.

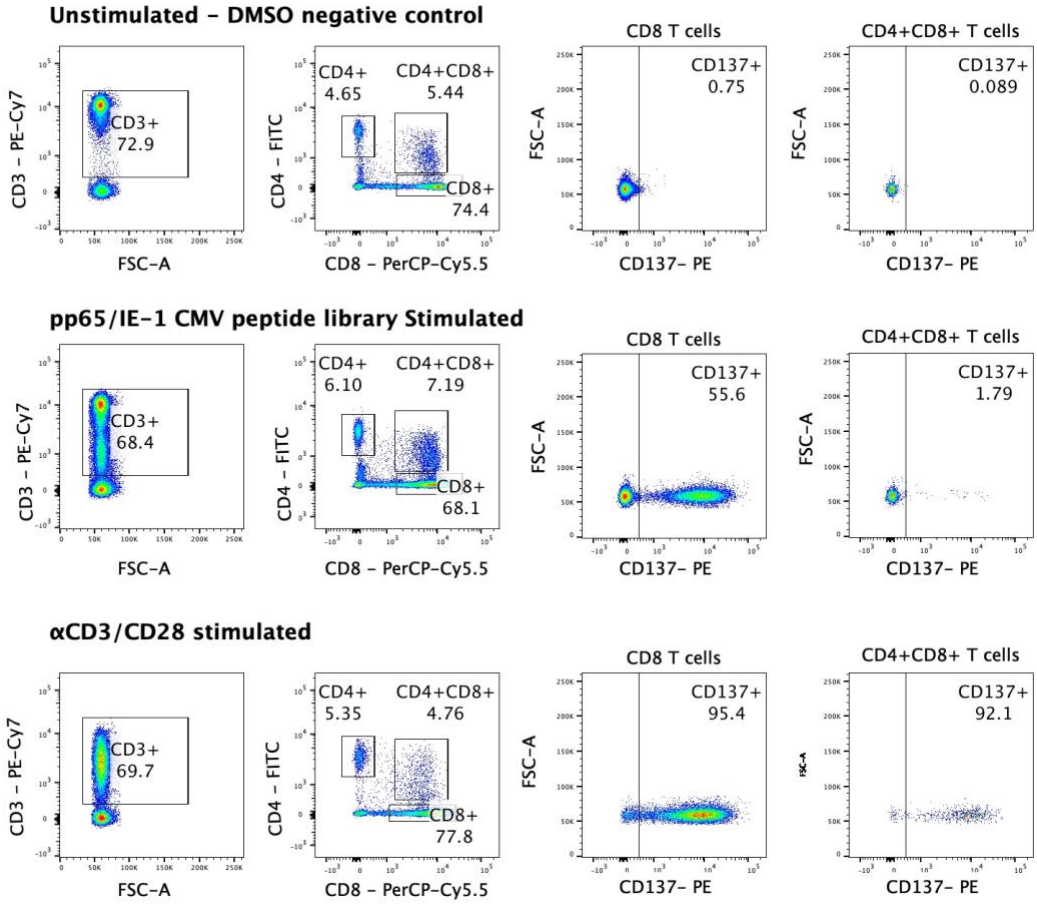



**Figure 4-1 Representative example of scatter plots of samples from one patient to illustrate the gating strategy for assessing CD137 expression on CD8+ T cells stimulated with pp65 and IE-1 CMV peptide libraries or αCD3/CD28 beads**

Debris, cell doublets and dead cells were excluded. Viable CD3+ cells were gated and segregated based on expression of CD8+ or CD4+. Position of the gate to distinguish CD137+ CD8+ T cells was based on the unstimulated negative control (DMSO only) sample. Top panel shows

unstimulated T cells, middle panel is stimulation with the CMV peptide libraries and bottom panel is stimulation with αCD3/CD28 beads.

#### 4.2.1.1 Activation and function of αCD3/CD28 stimulated T cells from allogeneic HSCT patients can be suppressed in the AML microenvironment

Results presented in Figure 4-2 show that the response of T cells from allogeneic HSCT patients to non-specific stimulation with αCD3/CD28 beads was suppressed in the presence of supernatant from the AML microenvironment. This finding was similar to observations made using T cells from healthy individuals described in the previous chapter (3.2.5). Supernatant from AML047 cells cultured alone had no impact on the frequency and expression levels of the CD137 activation marker by $CD8_+$ T cells for the sample from patient 3 at day 484 and enhanced activation in the other three samples (Figure 4-2A). In contrast, supernatant from co-culture of the same AML cells with HS5 cells suppressed activation of αCD3/CD28 bead stimulated $CD8_+$ T cells in all four samples (Figure 4-2A), relative to supernatant from the AML cells cultured alone. Evidence was also obtained indicating suppression of cytokine production in the AML microenvironment, although results were not uniform (Figure 4-2B). There was suppressed secretion of both IFN-γ and TNF-α for the sample from patient 3 at day 484 in the presence of supernatant from AML047 cells alone and suppression was greater with supernatant from AML047 cells co-cultured with HS5 cells. The same IFN-γ secretion profile was also observed for samples from patients 1 at day 280 and 2 at day 390; namely reduced production with supernatant from AML047 cells alone and even greater suppression with supernatant from AML047 cells co-cultured with HS5 cells. However, AML047 cell culture supernatant increased IFN-γ secretion for the sample from patient 2 at day 473 and had no effect on secretion of TNF-α for three of the four samples tested. Results presented previously in section 3.2.5 Figure 3-9 showed that IFN-γ and TNF-α were not present in supernants from AML cells or AML cells co-cultured with HS5 cells. AML047 was included in this analysis, therefore the cytokines detected in the experiments presented in Figure 4-2B and Figure 4-3B can be ascribed to production by T cells.

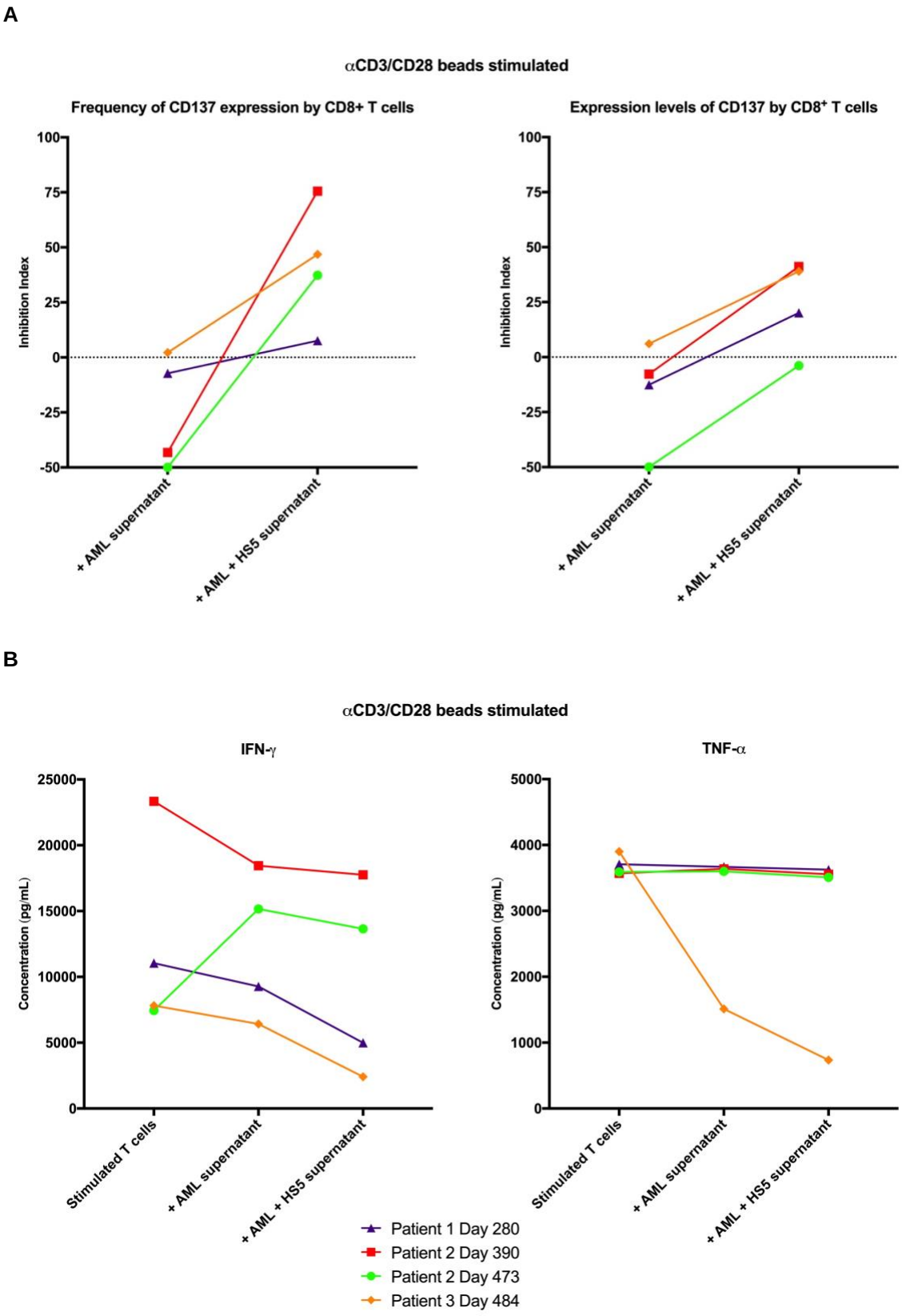



**Figure 4-2 Supernatant from AML and HS5 co-cultured cells suppresses the activation and function of αCD3/CD28 bead stimulated T cells from allogeneic HSCT patients**

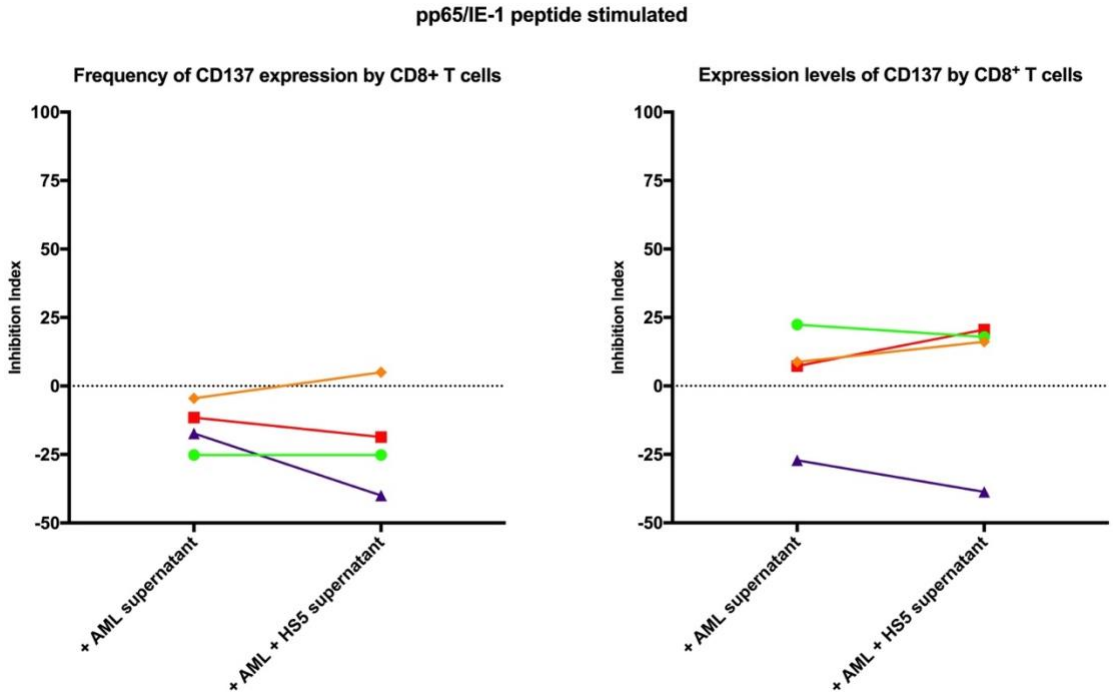
T cells from three allogeneic HSCT patients were stimulated with αCD3/CD28 beads either in culture media, supernatant from AML047 cells cultured alone, or supernatant from AML047 cells

co-cultured with HS5 cells under normoxic conditions overnight. (A) T cell activation was assessed by measuring the frequency and expression levels of CD137 by $CD8_+$ T cells using flow cytometry, with results presented as an inhibition index relative to the culture media positive control. (B) T cell function was assessed by measuring the concentration of IFN-$\gamma$ and TNF-$\alpha$ produced. Cell culture supernatants were diluted 1:25 and 1:5 for IFN-$\gamma$ and TNF-$\alpha$ respectively and cytokines quantified by ELISA.

#### 4.2.1.2 Function of CMV-specific T cells from allogeneic HSCT patients can be suppressed in the AML microenvironment

Analysis of the CMV-specific T-cell responses for the four samples from allogeneic HSCT patients was performed at the same time as the $\alpha$CD3/CD28 bead stimulation and showed that their function, but not expression of the CD137 activation marker, could be suppressed in the presence of supernatant from the AML microenvironment. Mirroring the results obtained with $\alpha$CD3/CD28 beads, the presence of supernatant from AML047 cells cultured alone increased the frequencies of $CD137_+$ CMV-specific $CD8_+$ T cells in three samples and had no impact for the sample from patient 3 at day 484 (Figure 4-3A). However, in contrast to $\alpha$CD3/CD28 bead stimulation, expression of $CD137_+$ CMV-specific $CD8_+$ T cells in the presence supernatant from AML cells cultured alone or supernatant from AML047 cells co-cultured with HS5 cells was similar (Figure 4-3A). Although supernant from the co-culture of AML cells with HS5 cells did not suppress activation marker expression of CMV-specific $CD8_+$ T cells from the four samples tested, the secretion of cytokines was reduced (Figure 4-3B). Relative to supernatant from AML047 cells alone, both IFN-$\gamma$ and TNF-$\alpha$ levels were lower for all four samples in the presence of supernatant from AML047 cells co-cultured with HS5 cells. Of note, in every case except for the IFN-$\gamma$ levels for patient 3 at day 484, supernatant from AML047 cells alone slightly increased cytokine production by CMV-specific T cells compared to fresh media.

**A**

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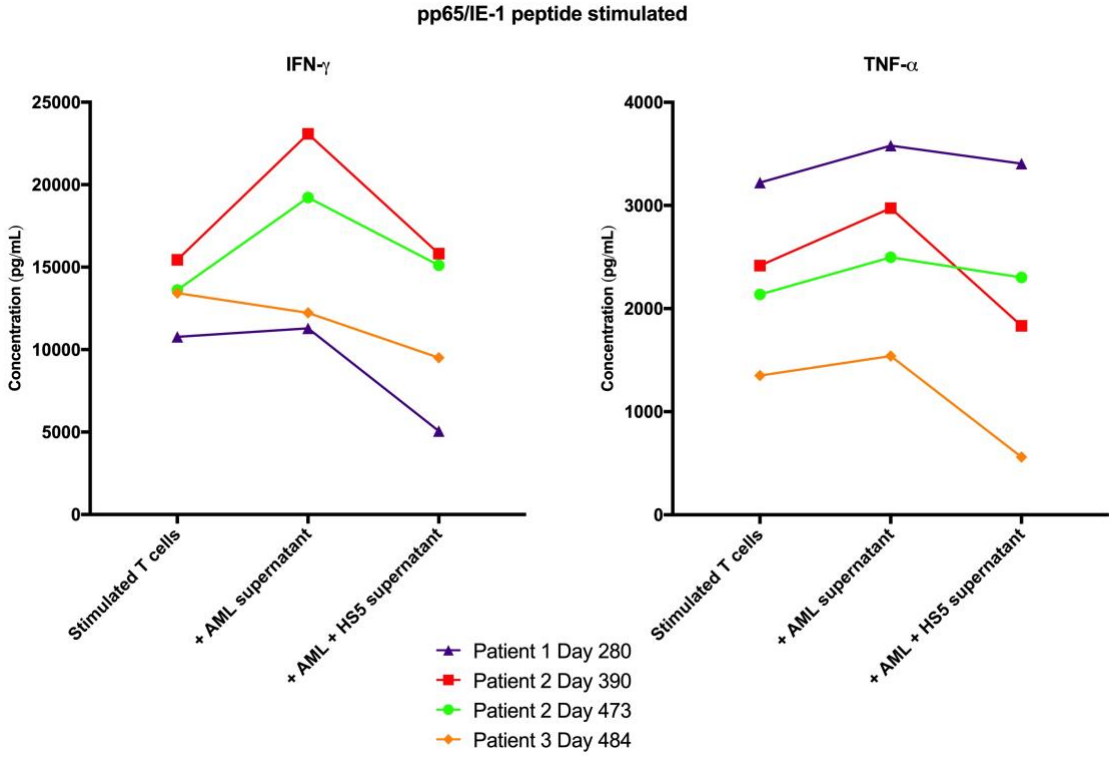



**Figure 4-3 Supernatant from AML and HS5 co-cultured cells suppresses cytokine production by CMV-specific T cells from allogeneic HSCT patients**

T cells from three allogeneic HSCT patients were stimulated with pp65 and IE-1 CMV peptide libraries either in culture media, supernatant from AML047 cells cultured alone, or supernatant

from AML047 cells co-cultured with HS5 cells under normoxic conditions overnight. (A) T cell activation was assessed by measuring the frequency and expression levels of CD137 by CD8+ T cells using flow cytometry, with results presented as an inhibition index relative to the culture media positive control. (B) T cell function was assessed by measuring the concentration of IFN-$\gamma$ and TNF-$\alpha$ produced. Cell culture supernatants were diluted 1:25 and 1:5 for IFN-$\gamma$ and TNF-$\alpha$ respectively and cytokines quantified by ELISA.

### 4.2.2 Assessment of the suppression of Vγ9Vδ2 T cells in the AML microenvironment

Although the results described above using patient T cells are not conclusive due to the low number of samples available for study, they provide additional indication that the AML microenvironment suppresses T-cell activity. To further investigate the impact on T cell responses, the recognition of AML cells treated with the aminobisphosphonate zoledronic acid (ZA) by Vγ9Vδ2 T cells in the absence or presence of HS5 cells was studied. The response of Vγ9Vδ2 T cells to AML cells was assessed using the AML cell line U937. This AML cell line was selected because it has previously been observed that ZA treated U937 cells induce Vγ9Vδ2 T cell proliferation[321] and the functional responses are robust compared with other AML cell lines (Dr John Maher and Dr Ana Parente-Pereira, personal communication). Furthermore, results presented in the previous chapter (section 3.2.3) showed that the microenvironment created by co-culture of U937 and HS5 cells significantly suppressed the expression of activation markers and secretion of cytokines by αβ T cells stimulated with αCD3/CD28 beads.

#### 4.2.2.1 Activation of αCD3/CD28 stimulated Vδ2 T cells is suppressed in the presence of U937 with HS5 cells

To assess whether the combination of U937 and HS5 cells promoted suppression of γδ T cells stimulated with αCD3/CD28 beads, γδ T cells were isolated from PBMC of three healthy individuals and tested using the *in vitro* three-cell co-culture model. A representative example of γδ T-cell purity and the proportion of Vδ2$^+$ cells presented in Figure 4-4 shows 94.3% purity for γδ T cells, of which 78.5% were Vδ2$^+$. Given the majority of Vδ2$^+$ are Vγ9Vδ2, use of Vδ2$^+$ only to identify the subset is routinely reported[201, 322, 323]. Although cell purity was consistently high, the absolute number of cells obtained was low; mean of $1.5x10^4$ Vδ2 T cells per $1x10^6$ PBMC (1.5%), consistent with reported frequencies of between 0.5-5% within the CD3$^+$ T cell population[10]. Due to the low Vδ2$^+$ T cell numbers available, co-cultures were scaled down to a 96-well plate format with cell and αCD3/CD28 bead ratios maintained. After culture for 48 hours under normoxic conditions, cells were analysed by flow cytometry to assess expression of CD137. This activation marker was selected for assessment to be consistent with the study of αβ T-cell activation, and it has been previously reported to be rapidly induced on Vδ2$^+$ T cells upon antigenic stimulation[324].

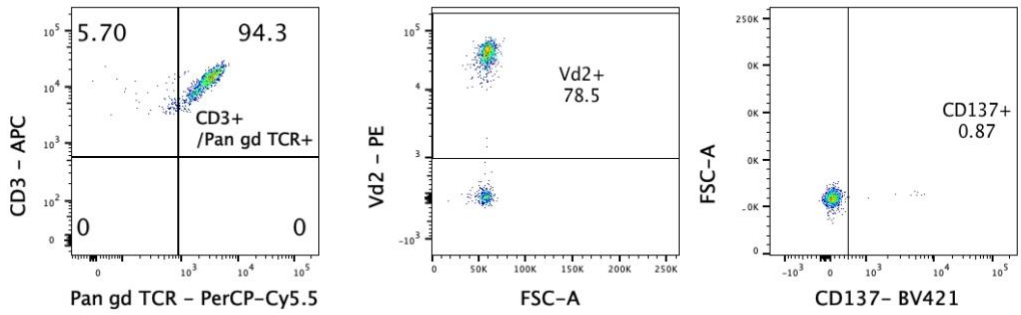


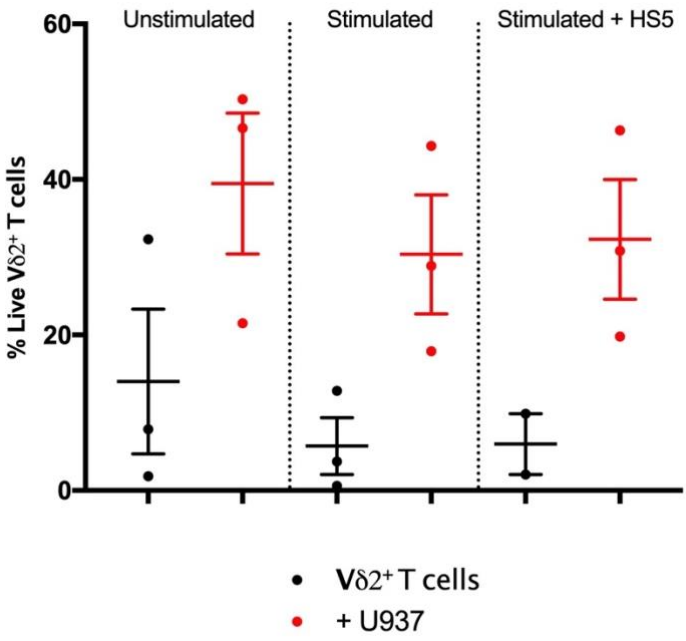

**Figure 4-4 Representative example of the purity of γδ T cells after isolation from PBMC**

Debris, cell doublets and dead cells were excluded. Viable CD3+ cells were gated and further distinguished as TCR γδ+ and Vδ2+ T cells. After purification, 94.3% of cells were TCR γδ+, and 78.5% of TCR γδ+ T cells were Vδ2+. Position of the gate to distinguish CD137+ γδ+ T cells was based on the unstimulated population analysed immediately after purification because unstimulated cells died during in vitro culture.

Poor viability of both Vδ2- and Vδ2+ γδ T-cell subsets after *in vitro* culture was a consistent problem in samples from all three healthy individuals (Figure 4-5A). Cell viability was low when Vδ2+ T cells were cultured alone but improved by presence of U937 cells. Mean viability increased from 14.0% to 39.5% for unstimulated Vδ2+ T cells, 5.7% to 28.9% for αCD3/CD28 bead stimulated Vδ2+ T cells and 6% to 30.8% for αCD3/CD28 bead stimulated Vδ2+ T cells cultured with HS5 cells. There was some activation of Vδ2+ T cells by U937 cells, indicated by a small increase in CD137 expression by Vδ2+ T cells in the presence of U937 cells without αCD3/CD28 bead stimulation (Figure 4-6) compared to CD137 expression *ex vivo* (Figure 4-4), that perhaps promoted Vδ2+ T-cell survival. In contrast, presence of U937 cells reduced the viability of Vδ2- T cells (Figure 4-5B), from 6.65% to 2.25% for unstimulated Vδ2- T cells, 9.17% to 2.59% for αCD3/CD28 bead stimulated Vδ2- T cells and 7.5% to 1.31% for αCD3/CD28 bead stimulated Vδ2- T cells cultured with HS5 cells. Due to very poor viability of the Vδ2- T cells, activation marker expression on these cells was not assessed. The analysis of CD137 expression by Vδ2+ T cells presented in Figure 4-6 shows that the presence of U937 cells together with HS5 cells suppressed activation marker expression by Vδ2+ T cells stimulated with αCD3/CD28 beads. The frequency and expression level of CD137 by Vδ2+ T cells was reduced from a mean of 77.8% with 5826.7 MFI in the presence of U937 cells to 46.8% with 1658.3 MFI when HS5 cells were also present.

Data could not be presented as an inhibition index because of the very poor viability of the positive control αCD3/CD28 bead stimulated Vδ2+ T cell cultures. Cytokine secretion was not evaluated.

**A**

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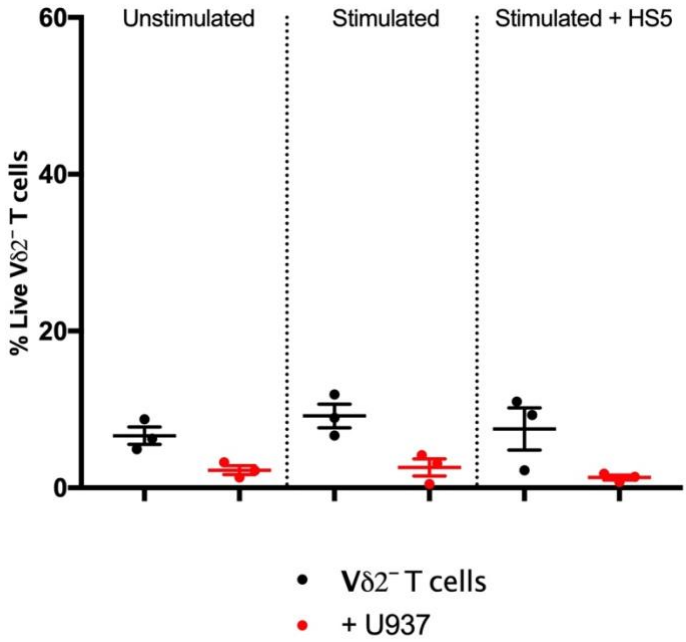


**Figure 4-5 Presence of U937 cells sustains Vδ2+ T cells but not Vδ2- T cells during in vitro culture**

γδ T cells were co-cultured alone (black) or with U937 cells (red) at a 1:1 ratio for 48 hours under normoxic conditions. Cultures were either unstimulated, stimulated with αCD3/CD28 beads or stimulated with αCD3/CD28 beads in the presence of HS5 cells. Viability of (**A**) Vδ2+ and (**B**) Vδ2-

T cells after culture was assessed by flow cytometry. Debris and cell doublets were excluded, Vδ2+ and Vδ2- cells were gated and % live cells in each population determined. Data shown represents the mean ± SEM.

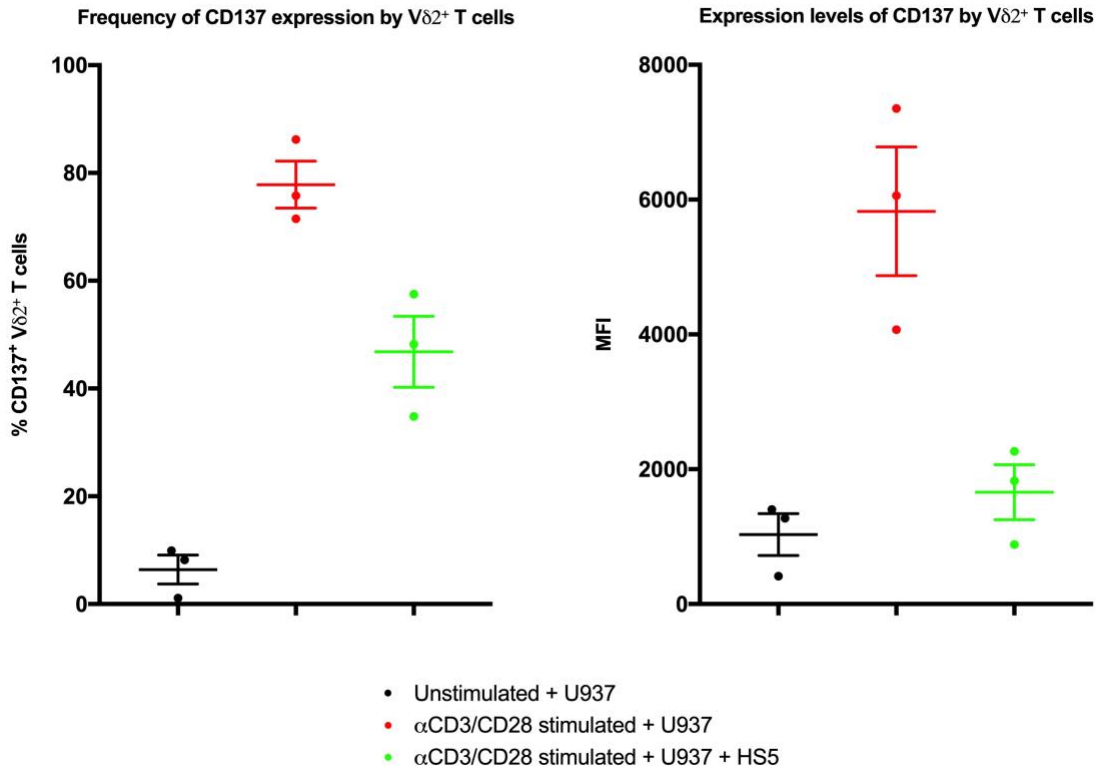


**Figure 4-6 Expression of the activation marker CD137 by Vδ2+ T cells stimulated with αCD3/CD28 beads is suppressed in the presence of U937 cells co-cultured with HS5 cells**

Purified γδ T cells were cultured in the presence of U937 either without stimulation (black), stimulated with αCD3/CD28 beads (red) or stimulated with αCD3/CD28 beads in the presence of HS5 cells (green) under normoxic conditions for 48 hours. Frequency and expression level of CD137 by Vδ2+ T cells was assessed by flow cytometry. Vδ2- T cells and unstimulated Vδ2+ T cells alone were not assessed due to poor cell viability. Unstimulated Vδ2+ T cells co-cultured in the presence of U937 alone is the negative control. Data shown represents the mean ± SEM.

#### 4.2.2.2 Activation of Vδ2+ T cells stimulated with ZA-treated U937 cells, but not IFN-γ production, is suppressed in the presence of HS5 cells

Analysis of the response of purified Vδ2+ T cells from three healthy individuals to ZA-treated U937 cells was performed at the same time as the αCD3/CD28 bead stimulation. The U937 cells were pre-treated with 3μg/mL ZA overnight and washed before use to stimulate γδ T cells for 48 hours under normoxic conditions, either in the absence or presence of HS5 cells. The ZA concentration used was based on previous titration studies that determined the maximum sensitization of AML cell lines for recognition by Vγ9Vδ2 was achieved at 3μg/mL (Dr Ana Parente-Pereira, personal communication). The expression of CD137 by Vδ2+ T cells and secretion of IFN-γ were then analysed.

Results presented in Figure 4-7A show that a high frequency of Vδ2+ T cells were activated by stimulation with ZA-treated U937 cells (mean 78.8% Vδ2+ T cells were CD137+). The frequency was also high in co-cultures that contained HS5 cells (mean 83.7% Vδ2+ T cells were CD137+) but the level of activation marker expression was consistently lower. The mean CD137 expression level was 13882.3 MFI in the absence and 6770 MFI in the presence of HS5 cells. The analysis of IFN-γ production in response to stimulation with ZA-treated U937 cells presented in Figure 4-7B shows a robust functional response that was consistently higher in the presence of HS5 cells, with a mean of 925.1 pg/mL in the absence and 1358.4 pg/mL in the presence of HS5 cells. Therefore, the activation of Vδ2+ T cells but not secretion of IFN-γ in response to stimulation with ZA-sensitized AML cells was suppressed when the HS5 cells were present. These assays were very challenging to set up, and the analysis was limited because of the low numbers of Vδ2+ T cells obtained and their poor viability when cultured. The experiment design was therefore modified to expand Vδ2+ T-cell numbers by *in vitro* stimulation before assessment of the impact of the AML microenvironment on their function.

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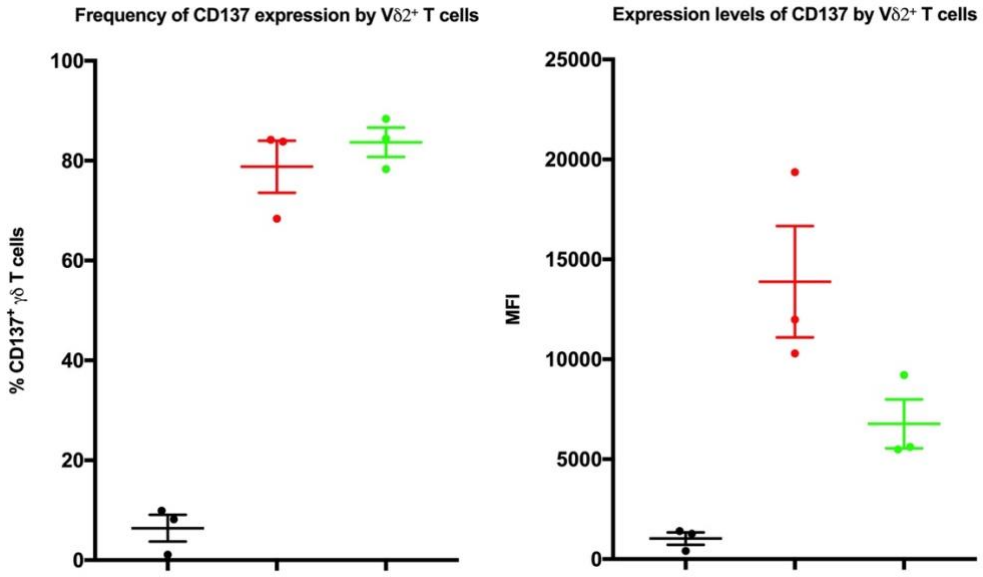


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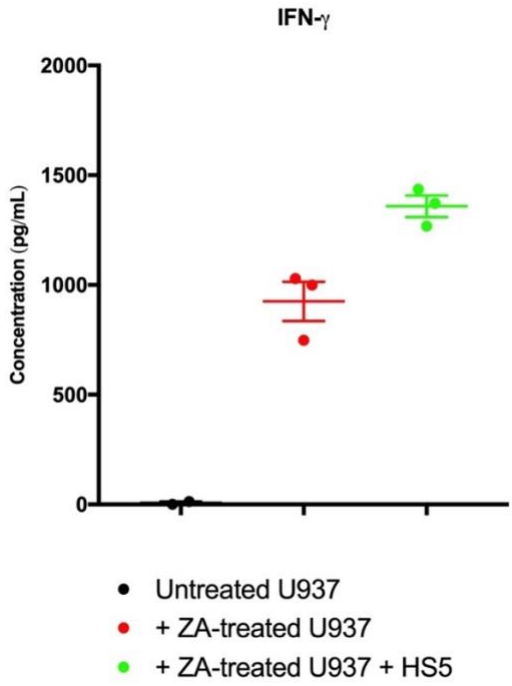



**Figure 4-7 Presence of HS5 cells suppresses the expression level of the activation marker CD137 by Vδ2 T cells stimulated with ZA-treated U937 cells but not IFN-γ production**

Purified γδ T cells were cultured in the presence of untreated U937 (black), ZA-treated U937 alone (red) or ZA-treated U937 cells with HS5 cells (green) under normoxic conditions for 48 hours. (A) Frequency and level of expression of CD137 by Vδ2 T cells was assessed by flow cytometry. (B) Concentration of IFN-γ secreted was assessed by ELISA. Data shown represents the mean ± SEM.

### 4.2.3 Cytolytic activity of *in vitro* expanded Vδ2+ T cells in response to recognition of ZA-treated U937 cells is suppressed in the presence of HS5 cells

In order to study the effect of the AML microenvironment on the function of Vδ2+ T cells, it was necessary to expand the population by *in vitro* stimulation to obtain sufficient numbers. Previous studies have reported expansion of Vδ2+ T cells by *in vitro* stimulation with ZA in the presence of IL-2 or endotoxins [53, 197, 325]. The *in vitro* expansion conditions previously described by colleagues Dr John Maher and Dr Ana Parente-Pereira at King's College London were used.[197] PBMC were isolated from peripheral blood collected in citrate vacutainers from one of the three healthy individuals studied in 4.2.2.1, selected because the highest number of γδ T cells were consistently obtained from this individual. Freshly isolated PBMC were cultured in RPMI supplemented with 10% AB serum, 100 U/mL IL-2, 10 ng/mL IL-15 and 1 μg/mL ZA for 14 days, as described in Chapter 2 section 2.3.6.1. In the setting of ZA-treated PBMC, expansion of Vδ2+ T cells has been shown to be mediated by accumulation of the phospho-antigen isopentenyl diphosphate in the peripheral blood monocytes[326].

Cell composition and kinetics of the Vδ2+ T-cell expansion analysed at days 7 and 14 are shown in Figure 4-8. After 7 days, Vδ2+ T-cell numbers were approximately 5-fold higher in the culture containing ZA, with a mean of 4875 cells/mL compared to 914 cells/mL in the absence of ZA, and percentage composition enriched to a mean of 9.6% compared to 1.4% (Figure 4-8A**)**. By day 14, ZA had promoted expansion of Vδ2+ T-cell numbers to a mean of 15704 cells/mL and comprised 26.1% of total live cells, compared to 624 cells/mL and 1.2% in the absence of ZA (Figure 4-8B). The presence of ZA promoted only a marginal increase in the proportion of Vδ2- T cells from 4.2% to 5.0% present at day 14 (Figure 4-8C), indicating selective expansion of the Vδ2+ subset. The enrichment of Vδ2+ observed was consistent with the 30% range previously reported using this protocol[197, 322]. The other cells present at day 14 were predominantly CD3 negative (Figure 4-8D), with a mean of 86.7% in the absence and 60.0% in the presence of ZA. These may be NK cells that are known to be sustained by the concentrations of IL-2 and IL-15 used in the protocol[327, 328].

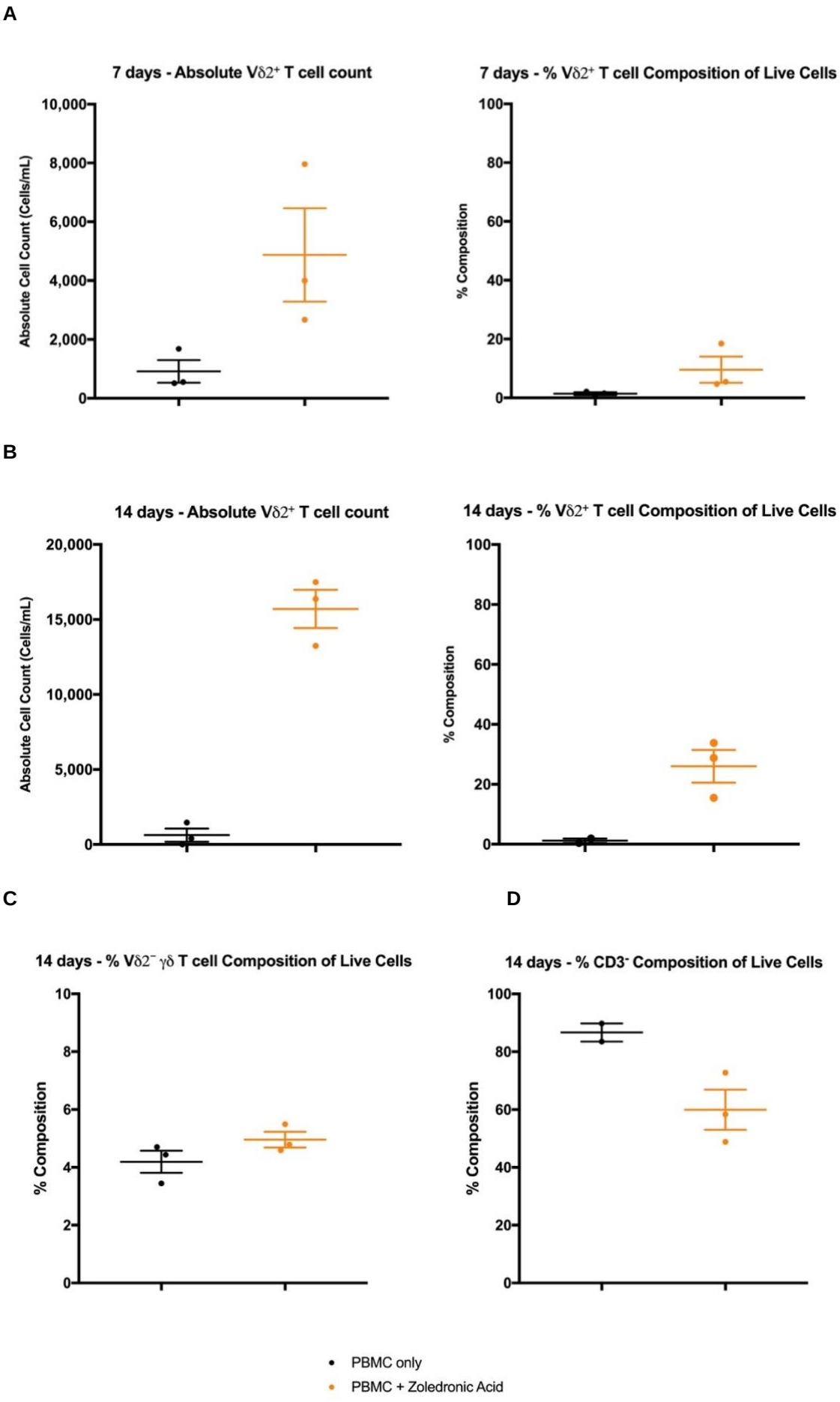



**Figure 4-8 Kinetics of in vitro expansion of Vδ2+ T cells from PBMC of a healthy individual**

In vitro expansion of γδ T cells in PBMC from a healthy individual by stimulation with ZA in media containing IL-2 and IL-15 was performed on three separate occasions and cultures analysed by flow cytometry with counting beads. Viable Vδ2+ T-cell numbers and percentage composition were assessed at day 7 (A) and day 14 (B). Percentage of viable Vδ2- γδ T cells (C) and CD3- cells (D) were assessed at day 14. Data shown represents the mean ± SEM.

Cytolytic activity is key to the anti-tumour response mediated by γδ T cells. The availability of a larger number of γδ T cells after *in vitro* expansion enabled the performance of assays to measure killing of ZA-treated U937 cells, and the impact of presence of HS5 cells to be studied. γδ T cells were purified from the cultures at day 14 and incubated with ZA-treated U937 target cells for 6 hours under normoxic condition. A target cell killing assay was developed using a modified version of a flow cytometry-based protocol previously reported by Jedema et al[329]. Live U937 cells were identified by expression of CD33 and enumerated using counting beads. Percent specific killing of ZA-treated U937 cells by γδ T cells was calculated by comparison to the appropriate negative control; namely the number of viable untreated U937 cells after incubation with γδ T cells in either the absence or presence of HS5 cells.

Results presented in Figure 4-9A show the *ex vivo* expanded γδ T-cell exhibited cytolytic activity specific to ZA sensitized AML cells. Numbers of U937 cells were similar in the cultures of U937 alone (mean 661,605 cells/mL), untreated U937 cells after incubation with γδ T cells (mean 607,458 cells/mL) and untreated U937 cells after incubation with γδ T cells with HS5 (mean 611,113 cells/mL). The numbers were substantially lower in the culture of ZA-treated U937 cells after incubation with γδ T cells (mean 412,706 cells/mL) representing 32% killing. In the presence of HS5 cells, the killing of ZA-treated U937 cells was lower, at 19.5% killing (mean 491,708 cells/mL), indicating that γδ T-cell cytolytic activity against AML cells was suppressed in the presence of stromal cells.

Cytokine secretion was also assessed and a specific response to stimulation with ZA sensitized AML cells was observed, although levels were low due to the short 6-hour stimulation (Figure 4-9B). No IFN-γ or TNF-α was detected in cultures containing untreated U937 cells whereas means levels were 126.6 pg/mL and 143.5 pg/mL respectively in the cultures with ZA-treated

U937 cells. Akin to results obtained with γδ T cells *ex vivo*, the presence of HS5 cells did not suppress cytokine production and was actually higher with mean levels of 142.2 pg/mL for IFN-γ and 221.9 pg/mL for TNF-α. The IFN-γ levels were comparable in the absence and presence of HS5 cells for two of the three replicate experiments and increased in one experiment. TNF-α production was substantially higher in the presence of HS5 cells in all three experiments. A limitation of measuring cytokines in the supernatant from a co-culture of multiple cell types was that the source of cytokines was not determined. Expression of the activation marker CD137 was not assessed because it was not upregulated during the short assay duration.

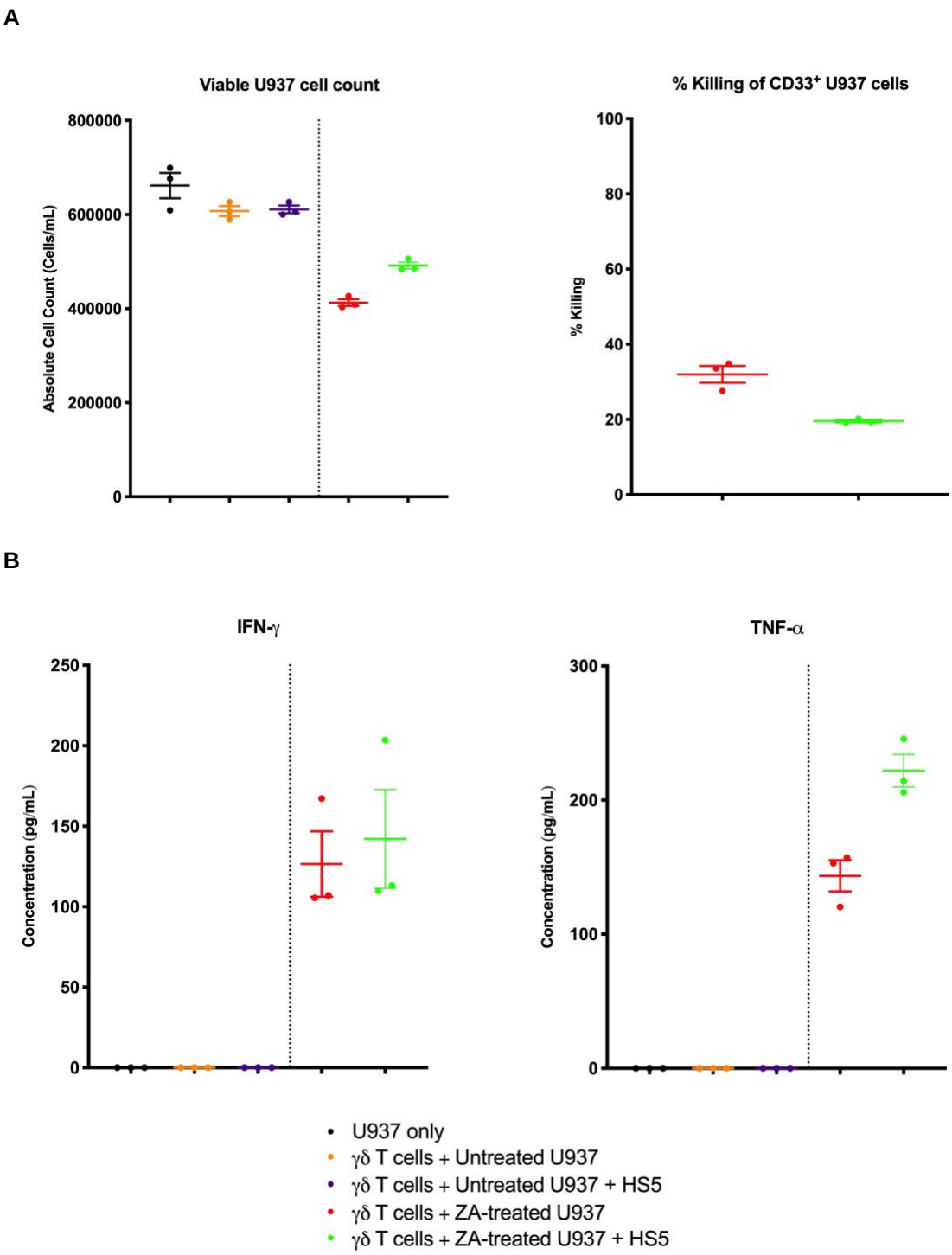



**Figure 4-9 Presence of HS5 cells suppresses the cytolytic activity of in vitro expanded γδ T cells to ZA-treated U937 cells, but not cytokine production**

Function of γδ T cells in the three in vitro expansions from PBMC of a healthy individual was assessed at day 14. Purified γδ T cells were stimulated under normoxic conditions for 6 hours with untreated U937 cells (orange), untreated U937 cells in the presence of HS5 cells (purple), ZA treated U937 cells (red) or ZA treated U937 cells in the presence of HS5 cells (green). (A) Cytolytic activity was assessed by counting the number of viable CD33+ U937 cells using flow

cytometry with counting beads and presented as % killing relative to the appropriate negative control; namely untreated U937 cells after incubation with γδ T cells in either the absence or presence of HS5 cells. (B) Concentration of IFN-γ and TNF-α secreted was assessed by ELISA without dilution of supernatants. Data shown represents the mean ± SEM.

### 4.2.4 Assessment of the suppression of αCD123 CAR T cells in the AML microenvironment

The use of CAR T cells as a novel immunotherapy for AML is being explored. To assess the potential impact of the immunosuppressive microenvironment on CAR T cell function, the recognition of AML cells by αCD123 CAR T cells in the absence or presence of HS5 cells was studied.

#### 4.2.4.1 Production of CAR T cells

The αCD123 CAR lentivirus vector construct was a kind gift provided by Georg Fey (Friedrich-Alexander-Universität Erlangen-Nürnberg) and the αPSMA CAR retrovirus vector construct was a kind gift provided by John Maher (King's College London). Both CAR constructs are 2nd generation CAR containing αCD123 or αPSMA single chain variable fragment (scFv) and a CD28ζ co-stimulatory domain. CAR T cells were produced as described in (Chapter 2 section 2.3.7.5). Purified T cells from a healthy individual were transduced with the αCD123 CAR vector or αPSMA CAR vector (negative control CAR) and expanded for 14 days using αCD3/CD28 beads in the presence of IL-2.

After 14 days of expansion, the percentage of transduced T cells was determined by measuring transgene expression using flow cytometry. The αCD123 CAR construct contains a sequence encoding a c-myc tag peptide for tracking transduction efficiency. The αPSMA CAR construct does not contain a tag, therefore transduction efficiency was determined by measuring the frequency of T cells expressing the mouse scFv. The flow cytometry analysis presented in Figure 4-10 shows that 61.3% of $CD8^+$ T cells and 89.6% of $CD4^+$ T cells expressed the c-myc tag following transduction with the αCD123 CAR construct and 30.8% of $CD3^+$ T cells expressed the mouse scFv following transduction with the αPSMA CAR construct; $CD8^+$ and $CD4^+$ T cells were not assessed for αPSMA CAR transduction efficiency as they were not stained for CD4 and CD8. The relatively low transduction efficiency for the αPSMA CAR was consistent with previous

observations from the laboratory of John Maher (personal communication). The T-cell populations were cryopreserved in aliquots to enable all experiments to be performed using the same batches of transduced cells. Viability after thawing was consistently around 65%. The αPSMA CAR T cells, which recognise the prostate-specific membrane antigen and therefore not targeted to AML cells, were used as a negative control to assess the non-specific activity of CAR T cells.

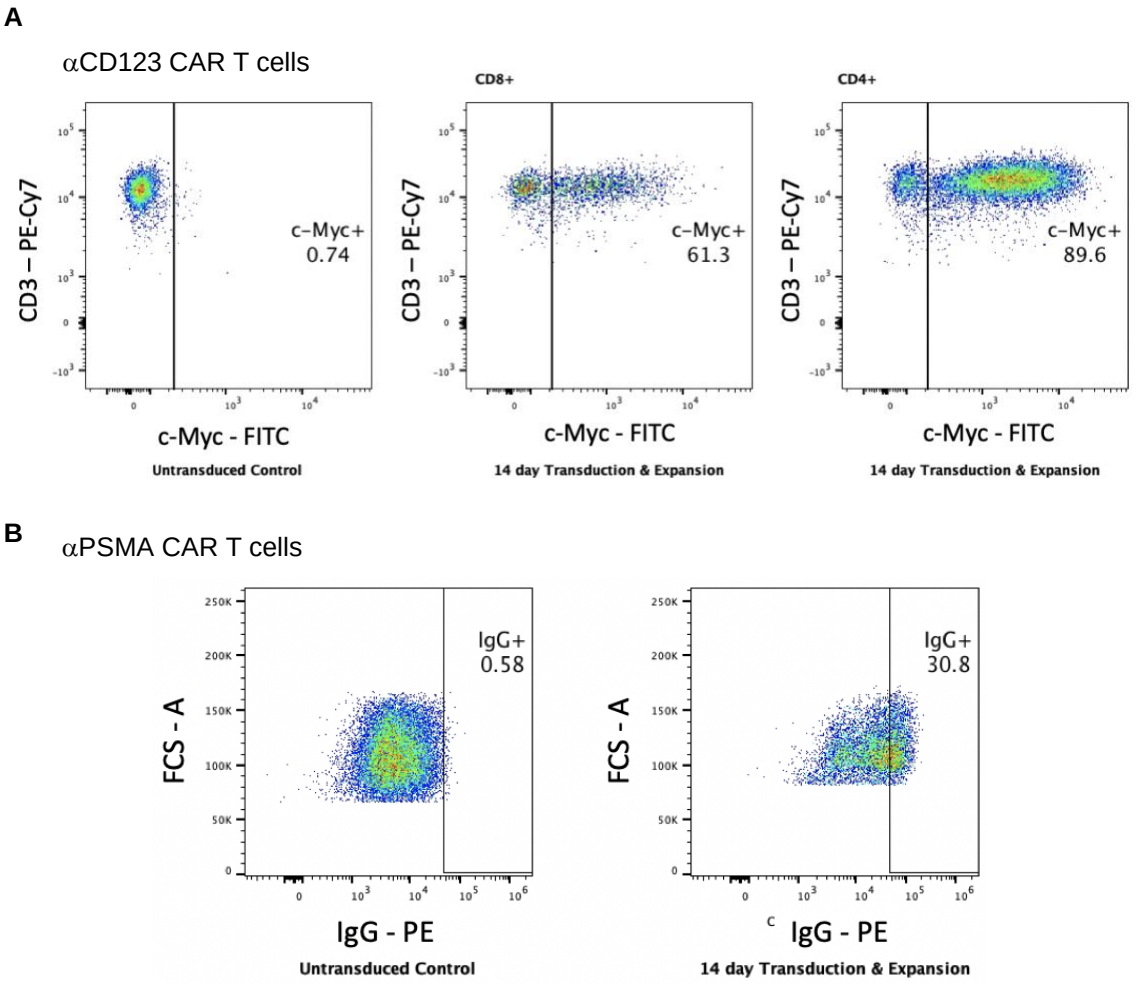



**Figure 4-10 T-cell transduction with αCD123 CAR or αPSMA-P28z CAR constructs**

Flow cytometry was performed after in vitro expansion for 14 days. Debris, cell doublets and dead cells were excluded. (A) Viable $CD3_+$ $CD8_+$ and $CD4_+$ cells were gated and frequencies of T cells expressing the c-myc tag (αCD123 CAR) or (B) mouse scFv expressing $CD3_+$ cells (αPSMA CAR) were determined; $CD8_+$ and $CD4_+$ T cells were not assessed for αPSMA CAR transduction efficiency. Position of the gate to distinguish transduced T cells was based on the untransduced control population.

#### 4.2.4.2 AML cell line phenotyping for CD123 expression

The expression of CD123 by primary AML cell varies between patients[151, 179, 192, 315] and the bone marrow samples from patients have heterogeneous cell composition. AML cell lines were therefore used as targets. Six different AML cell lines were screened for CD123 expression by flow cytometry because CD123 is not expressed by all AML subtypes. Results presented in Figure 4-11) show that U937 cells do not express CD123 and the other cell lines grouped into two categories, expressing either low or high levels of CD123. The three AML cell lines THP-1, MOLM14, and KG1 that express high levels of CD123 were used as targets to assess the function of αCD123 CAR T cells and U937 cells were used as a negative control.

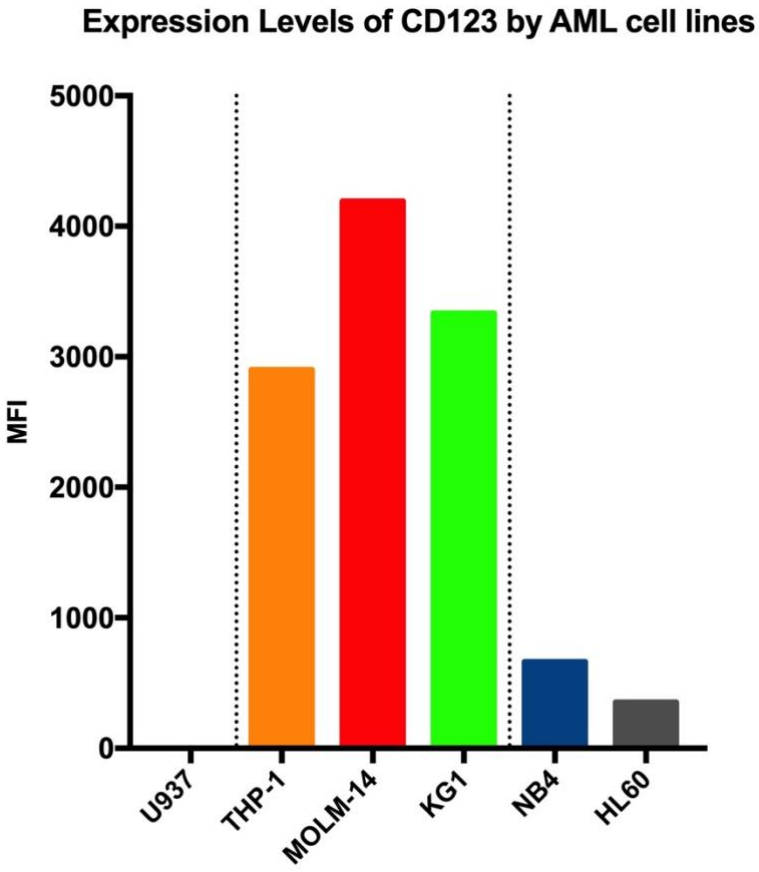



**Figure 4-11 CD123 expression by AML cell lines**

Debris, cell doublets and dead cells were excluded. Viable $CD33^+$ cells were gated and expression of CD123 evaluated based on comparison to an FMO negative control. Expression levels are presented as geometric median fluorescence intensity (MFI) of cells that are positive for CD123.

#### 4.2.4.3 Cytolytic activity and cytokine production by αCD123 CAR T cells are significantly suppressed in the presence of HS5 cells

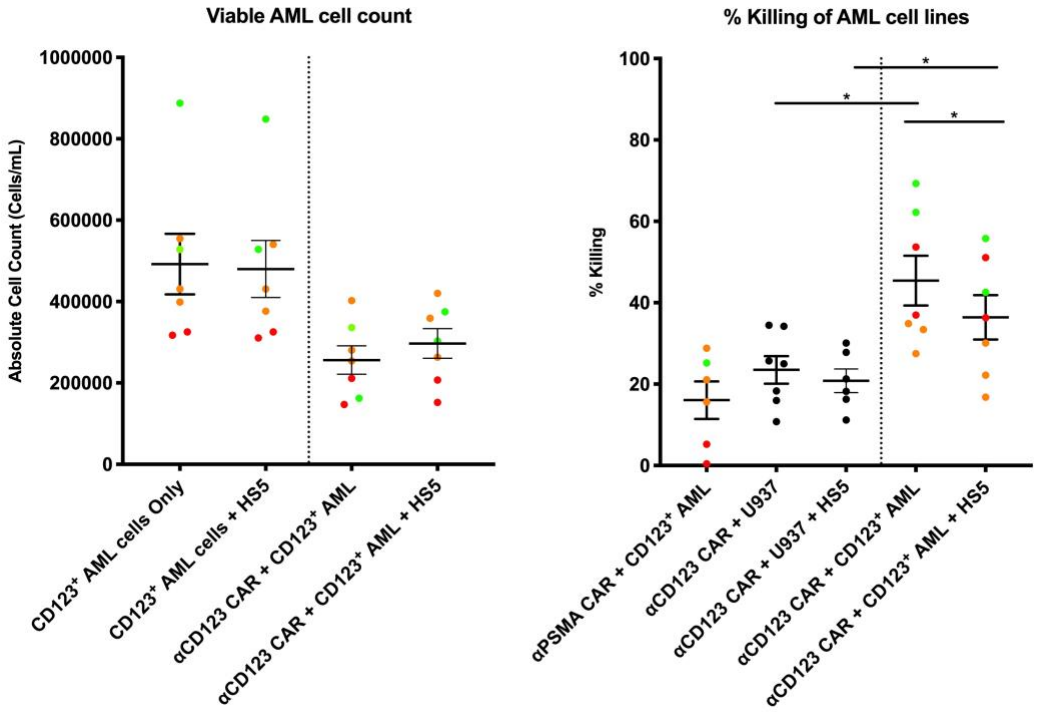
The cytolytic capability of αCD123 CAR T cells is an important measure of anti-leukaemia activity. The impact of presence of HS5 cells on killing of AML cells was assessed using a flow cytometry-based assay. The CD123+ AML cell lines THP-1, MOLM14, and KG1, and the CD123 negative control cell line U937 were labelled with CellTrace Violet immediately prior to incubation with CAR T cells in the absence or presence of HS5 cells for 4 hours. Cells were then harvested and analysed by flow cytometry with counting beads to enumerate the live CellTrace Violet positive AML cells. Percent specific killing of AML cells by CAR T cells was calculated by comparison to the appropriate negative control; namely the number of viable cells for each AML cell line after incubation without CAR T cells in either the absence or presence of HS5 cells.

Results presented in Figure 4-12A show that the αCD123 CAR T cells exhibited cytolytic activity specific for CD123+ AML cells and that presence of HS5 cells significantly inhibited cytolytic activity. Background levels of non-specific recognition of AML cell lines by the irrelevant αPSMA CAR T cells were mean 16% killing or the CD123 negative AML cell line U937 alone or with HS5 by αCD123 CAR T cells were mean 23.5% killing and 22.7% killing respectively. Numbers of CD123+ AML cells were similar in the cultures of CD123+ AML cells alone (mean 491,957 cells/mL), and with HS5 (mean 480,065 cells/mL). The αCD123 CAR T cells killed CD123+ AML cells indicated by their significant increase in killing to a mean of 45.8% (256,236 cells/mL) ($p$=0.02), but % killing was significantly lower at 36.4% in the presence of HS5 cells (297,096 cells/mL) ($p$=0.02), indicating that cytolytic activity was suppressed in the AML microenvironment.

Cytokine secretion was also assessed. A specific response by αCD123 CAR T cells to CD123+ AML cells was observed and significantly inhibited by presence of HS5 cells, although levels were low due to the short 4-hour assay (Figure 4-12B***)***. No IFN-γ or TNF-α was detected in the culture supernatants from αPSMA CAR T cells incubated with AML cell lines or αCD123 CAR T cells incubated with the CD123 negative AML cell line U937. Mean levels produced by αCD123 CAR T cells in response to recognition of CD123+ AML cells were 136.9 pg/mL for IFN-γ and 1051.0 pg/mL for TNF-α. In the presence of HS5 cells, levels were significantly reduced to 56.8 pg/mL ($p$=0.02) and 489.9 pg/mL ($p$=0.02) respectively.

Although the number of replicate assays for each of the CD123+ AML cell lines was low (THP-1 n=3, KG1 n=2, MOLM14 n=2), there was an indication of consistent differences in the functional activity of the αCD123 CAR T cells in response to each cell line. The specific killing activity was highest with KG1 cells and lowest with THP-1 cells. In contrast, production of IFN-$\gamma$ was highest with THP-1 cells and lowest with MOLM-14 cells whereas production of TNF-$\alpha$ was highest with MOLM-14 cells and lowest with KG1 cells. Response profiles did not correlate with levels of CD123 expression which was highest for MOLM-14 and lowest for THP-1 (Figure 4-11). Functional responses to all AML cell lines were diminished in the presence of HS5 cells.

**A**




**B**

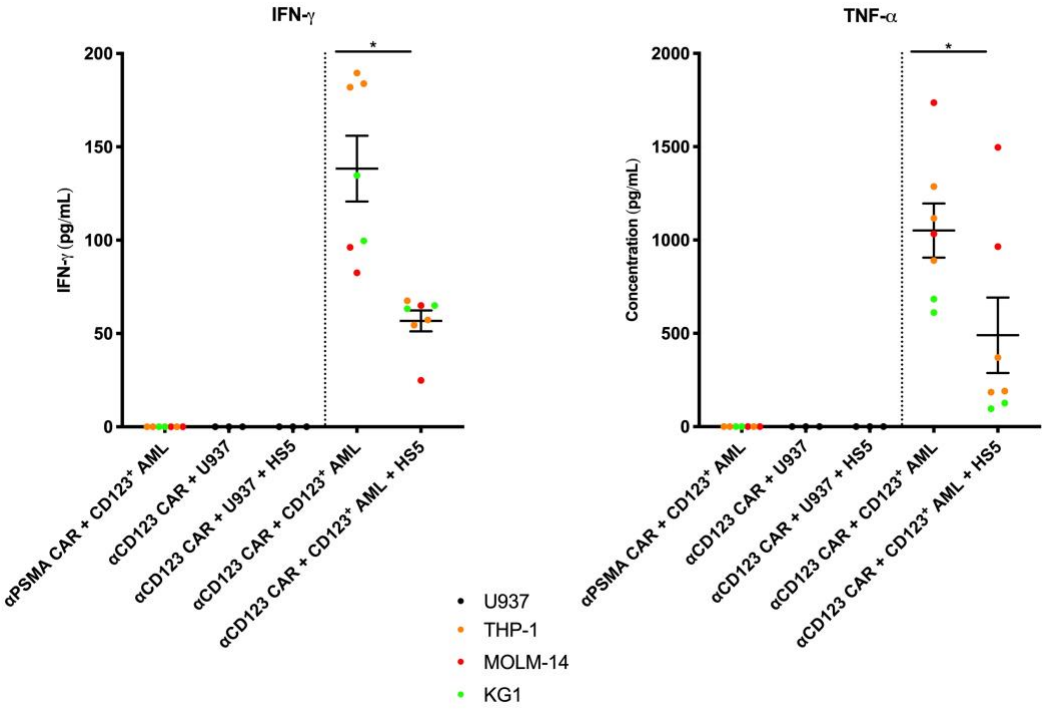



**Figure 4-12 Presence of HS5 cells significantly suppresses the cytolytic activity and cytokine production by AML-targeted CD123 CAR T cells**

CAR T cells were cultured with AML cell lines in the absence or presence of HS5 cells for 4 hours. A. Cytolytic activity was assessed by counting the number of viable CellTrace Violet labelled AML cells using flow cytometry with counting beads and presented as % killing relative to the

appropriate negative control; namely the number of viable cells for each AML cell line after incubation without CAR T cells in either the absence or presence of HS5 cells. (B) Concentration of IFN-$\gamma$ and TNF-$\alpha$ secreted was assessed by ELISA without dilution of supernatants. Data shown represents the mean $\pm$ SEM. $^{*}p<0.05$, determined by non-parametric Wilcoxon matched paired t test.

## 4.3 Discussion

The important curative role of GvL in AML patients after treatment by allo-HSCT is well established, but approximately 40% of patients suffer relapse of the disease[85]. Outcomes of clinical testing of some novel immunotherapies for AML have been disappointing. One important reason for the variable efficacy of immunotherapies is likely to be the immunosuppressive microenvironment created by AML. The work presented in this chapter used the previously described *in vitro* co-culture model to assess the impact of the AML microenvironment on several different types of antigen-specific T-cell response; CMV-specific T cells from AML patient post allo-HSCT and the novel T-cell based immunotherapeutics, $\gamma\delta$ T cells and CAR T cells.

CMV-specific $CD8_+$ T cells from AML patients after allo-HSCT were used as a surrogate model of GvL because the number of known AML epitopes and frequencies of responding T cells are low[154, 261, 306]. Only four patient samples were available for study and therefore results obtained were insufficent for definitive conclusions to be made. Some evidence was obtained to indicate that T cells from patients after allo-HSCT are susceptible to suppression in the AML microenvironment. In the context of non-specific stimulation with $\alpha$CD3/CD28 beads, the impact of the AML microenvironment on responses with cells from allo-HSCT patients was similar to that observed with T cells from healthy individuals (shown previously Figure 3-7 and Figure 3-8). The frequency and expression level of the CD137 activation marker by $CD8_+$ T cells in all four samples were suppressed and IFN-$\gamma$ production was suppressed for three samples. In contrast, impact of the AML microenvironment on responses for CMV antigen-specific T cells from allo-HSCT patients was less consistent. Unlike responses to non-specific stimulation of T cells from healthy individuals and patients, the frequency and expression levels of the CD137 activation marker by CMV-specific $CD8_+$ T cells from patients were not suppressed in the AML microenvironment. Some suppression of cytokine production in response to stimulation with CMV antigens was observed in the AML microenvironment, but only for IFN-$\gamma$ in samples from patients 1 and 3 and TNF-$\alpha$ in samples from patients 2 (day 390) and 3.

A larger number of samples would be need to be studied to explore the variable susceptibility of CMV-specific T cells to suppression of cytokine production in the AML microenvironment and to substantiate whether expression of CD137 by CMV-specific $CD8_+$ T cells is consistently resistant

to suppression. If the latter is confirmed, it might be a consequence of the effector phenotype of CMV-specific T cells, contrasting with non-specific stimulation with αCD3/CD28 beads that activates naïve, memory and effector T cells. A synergistic link between CD28 stimulation and expression of CD137 is indicated by a report that CD28 blocking antibodies suppress expression of CD137[330]. The co-stimulatory molecule CD28 is expressed by naïve and memory T cells but downregulated on effector T cells[331, 332]. Previous studies have reported that the majority of CMV-specific $CD8_+$ T cells from CMV seropositive individuals do not express CD28[333, 334]. This phenomenon has also been observed in the allo-HSCT patients that were used in this study (Monera Al Rukhayes; personal communication). Consequently, the CD28 signalling pathway has limited involvement in responses of effector CMV-specific $CD8_+$ T cells to antigen. The CD28 pathway is known to have an important role in immune tolerance mediated by CTLA-4[335, 336] and PD-1[337] and is exploited by cancer cells for immune evasion[229]. This indicates that $CD28_+$ naïve and memory T cells might be more susceptible to suppression in the AML microenvironment than effector T cells. If confirmed, it suggests that priming of naïve leukaemia-reactive T cells and activation of memory T cells will be prone to being suppressed in the AML microenvironment.

The requirement for processing and presentation of CMV peptides by APCs necessitated the use of supernatants from AML cell cultures to assess suppression in these experiments, rather than the multi-cell co-culture system used in the studies described in chapter Chapter 3. By definition, the suppression observed with supernatant from primary AML cells co-cultured with HS5 cells must be induced by soluble factors. Suppression of T-cell production of IFN-γ production by a soluble factor produced by primary AML cells was first reported by Buggins et al[212, 276]. The phenomenon was not mediated by any of the endogenous immunosuppressors known at the time; namely gangliosides, nitric oxide, TGF-β, IL-10, VEGF or prostaglandin[212, 276]. Our knowledge of the immunosuppressive mechanisms exploited by AML cells has increased substantially since these studies were performed (reviewed in section 1.4.2). Potential candidates for soluble factors produced by AML cells that might mediate the suppression observed are IDO,arginase II, CD39, or CD73[220, 221, 225, 226].

In the current study, there were several instances where supernatant from primary AML cells cultured alone promoted increased T-cell activity, which contrasts with findings previously

reported by Buggins et al[212]. The inhibition index values for frequency of CD137+ T cells after stimulation with αCD3/CD28 beads or CMV peptides in the presence of supernatant from AML047 cells cultured alone were negative for three of the four samples tested, reflecting increased activation. The production of IFN-γ was increased for three samples and TNF-α slightly increased for all four sample after stimulation with CMV peptides in the presence of supernatant from AML cells cultured alone. Significantly increased production of IFN-γ in the co-cultures of T cells from healthy individuals with primary AML cells (without HS5 cells) had been observed in the experiments described in section 3.2.5 and expression of activation markers was also increased for some samples. It was speculated that this might be due to presence of patient T cells in the AML samples contributing to the response to αCD3/CD28 beads. This, however, is not a plausible explanation for observations using the CMV T-cell model because only AML supernatants were used in this setting. It has been reported than some AML cells secrete IL-1[98, 338, 339] which could stimulate T-cell activation and function. Another potential stimulus could be IL-12, which can be produced by leukaemia-derived dendritic cells[340].

The basis for the descrepancy between results reported by Buggins et al and observations described here may be due to differences in the experiment design. In the previous study, primary AML cells were separated from T-cells using a cell culture insert to prevent cell contact, co-cultured for 16 hours in the presence of a protein transport inhibitor and intracellular accumulation of cytokines was measured[212]. In the current study, supernatants collected from primary AML cells cultured alone or together with HS5 cells for 48 hours were used and cytokine secretion was measured. Only one AML patient sample was tested in the current study. More samples would need to be evaluated to determine if differing results are consistently observed.

The studies described so far in this thesis have assessed the impact of the AML microenvironment on activity of T cells that express the αβ TCR. Given the evidence that Vδ2+ γδ T cells exhibit anti-leukemic activity, studies were extended to investigate the activation and function of these T cells in the AML microenvironment. Due to the low frequency of γδ T cells in peripheral blood, the *in vitro* co-culture model was adapted from a 24-well to a 96-well format. The scaled down model was successfully used to demonstrate that frequency and expression level of the CD137 activation marker by Vδ2+ T cells stimulated non-specifically with αCD3/CD28 beads are

suppressed in the AML microenvironment, consistent with the findings using αβ T cells. However, these experiments were hampered by the poor viability of purified γδ T cells. Interestingly, survival of the Vδ2+ T cells was improved in the presence of U937 cells. This might be due to provision of a survival stimulus to Vδ2+ T cells by stress-induced surface proteins expressed on tumour cells, such as F1-ATPase or heat shock proteins, that are recognised by Vδ2+ T cells[41, 341, 342].

The ability to sensitize AML cells for recognition by Vδ2+ T cells by treatment with ZA to promote phospho-antigen expression provided an opportunity to study the impact of the AML microenvironment on an anti-leukaemia T-cell response. The lower levels of CD137 expression seen in the presence of HS5 cells provided some evidence to indicate that the recognition of leukaemic cells by Vδ2+ T cells is susceptible to suppression in the AML microenvironment. However, the frequency of CD137+ Vδ2+ T cells and production of IFN-γ was actually higher in the presence of HS5 cells compared to stimulation with ZA-sensitized AML cells alone. To overcome the difficulty of performing these experiments due to low numbers of γδ T cells obtained from PBMC, *ex vivo* expansion was performed. This enabled the assessment of cytolytic activity. Evidence was obtained that the killing of ZA-sensitized AML cells by Vδ2+ T cells is suppressed in the leukaemia microenvironment. The cytolytic activity observed in these assays was low (mean 37.3% in the absence of HS5 cells). However, the results are similar to the levels of AML target cell killing by *ex vivo* expanded γδ T cells previously reported by Gertner-Dardenne *et al.* and Idrees *et al.*, where the effector to target cell ratios of 5:1 and 40:1 respectively were actually higher than the 1:1 used here[54, 343].

Despite suppression of tumour cell-killing by the *ex vivo* expanded γδ T cells, overall impact of the AML microenvironment was ambiguous because cytokine production by γδ T cells in response to stimulation with ZA-sensitized AML cells was consistently higher in the presence of HS5 cells, albeit only six samples were tested. In contrast, IFN-γ and TNF-α production in assays using αβ T cells was consistently reduced in the co-cultures of AML cell lines (Figure 3-6) or primary AML cells (Figure 3-9) with HS5 cells compared to AML cells alone, and seen in the context of stimulation with αCD3/CD28 beads or CMV peptides (Figure 4-2 and Figure 4-3). Cytokine production was assessed by measuring secretion. This approach was chosen to avoid treatment with protein transport inhibitors that is required for intracellular detection because these agents

would disrupt production of soluble factors from the AML and HS5 cells that likely contribute to the immunosuppressive microenvironment. A consequence of measuring secreted cytokines, however, is that the cell type producing IFN-γ and TNF-α is not identified. A potential explanation for the increased levels is that γδ T cells are stimulated to produce more IFN-γ and TNF-α in the additional presence of HS5 cells. A murine equivalent of the HS5 stromal cell line is known to produce IL-18, and this cytokine has been shown to increase IFN-γ and TNF-α production by human γδ T cells, which sets a precedent for this possibility[344, 345]. Alternatively, activated γδ T cells may induce secretion of IFN-γ and TNF-α by the AML and/or HS5 cells. Unlike αβ T cells, γδ T cells have both innate and adaptive functional attributes[10], and also characteristics of antigen-presenting cells, which may influence responses in the three-cell co-culture model[346]. Further investigation of the intriguing possibility that some aspects of γδ T-cells function may be less susceptible than αβ T cells to suppression in the AML microenvironment is required. The study conducted used γδ T cells from one healthy donor and assessed recognition of the AML cell line U937. Next steps could be to test γδ T cells from multiple individuals and their responses to other AML cell lines and primary AML cells. To determine the cell types producing cytokines in a multi-cell co-culture without using protein transport inhibitors, an option would be to purity the cell types after co-culture using antibody-coated magnetic beads or FACS and measure cytokine gene expression levels.

The impact of the AML micorenvironment on an anti-leukaemia T-cell response was also investigated in the context of T cells modified to express an αCD123 CAR. This novel immunotherapic strategy has been particularly successful in the form of αCD19 CAR T cells for treatment of B-ALL[187, 316, 347]. However, not all patients respond to CAR T-cell therapy and many others relapse after an initial response, as only 50% of patients survived one year after receiving treatment[316, 347]. One reason for the variable efficacy might be immunosuppression in the tumour microenvironment. Evaluation of αCD123 CAR T-cell recognition of AML cells in the presence of HS5 cells using the *in vitro* co-culture model showed that both cytolytic activity and cytokine production were significantly suppressed in the AML microenvironment. Inhibition was consistently observed with three different $CD123^+$ AML cell lines.

The mean AML target cell killing of 45.8% observed in the absence of HS5 cells is comparable to levels previously reported for αCD123 CAR T cells[192, 194, 315]. The construct used shares similarity with the constructs generated by Mardiros et al and Tettamanti et al as the endodomain all contain CD28ζ, but the scFv are different patented antibody sequences.[192, 194, 348] The negative control αPSMA CAR T cells included in experiments were not ideal because transduction efficiency was substantially lower compared to the αCD123 CAR T cells (30.3% of $CD3^+$ T cells compared to 61.3% of $CD8^+$ T cells and 89.6% of $CD4^+$ T cells). However, this was not a major concern because some published studies have used mock-transduced or non-transduced T cells to assess non-specific killing[192, 315]. Furthermore, the CD123 negative AML cell line U937 was included in experiments representing the important specificity control for αCD123 CAR T-cell activity.

The previous *in vitro* studies of αCD123 CAR T-cell activity have only assessed responses in the context of T cells and target cells. The findings in this study using the three-cell *in vitro* co-culture model including HS5 cells represent the first evidence that CAR T cells might be susceptible to suppression in the AML microenvironment. Although *in vivo* elimination of AML cells has been studied using xenogeneic humanised mouse models, these experiments do not recapitulate the immunosuppressive AML microenvironment[194, 315]. This is because the tumour cells injected via tail did not localised to the bone marrow and cross species differences likely preclude normal inter-cellular interactions[315, 349].

Cytolytic killing and production of IFN-γ and TNF-α by αCD123 CAR T cells were all found to be suppressed in the AML microenvironment. These are important effector functions involved in leukaemia control. The *in vitro* three-cell co-culture model is a potentially useful tool for evaluating strategies to circumvent suppression of αCD123 CAR T-cell function. This is likely to be particularly pertinent if αCD123 CAR T cells are used for the treatment of AML relapse after allogeneic HSCT. This is currently being evaluated in phase I clinical trials (NCT02623582, NCT03796390, and NCT02937103), but it is a setting where the T-cell mediated GvL effect has previously failed to eliminate disease and recent evidence highlights that immune escape might play a key role in treatment failure[210, 211].

In summary, this chapter describes an investigation into the activity of antigen-specific T cells in the AML microenvironment using the *in vitro* co-culture model. Consistent with results reported in the previous chapter where investigation of responses to non-specific T cell activation were described, evidence was found that the presence of AML cells with stromal cells inhibits the function of antigen-specific T cells, including leukaemia-specific T-cell responses. Improving the efficacy of T-cell based immunotherapies for the treatment of AML will require implementation of strategies to circumvent suppression in the AML microenvironment. The *in vitro* co-culture model was employed to explore the mechanisms of T-cell inhibition and attempt to prevent suppression with immunomodulatory drugs. Findings are described in the next chapter.

# Chapter 5 Investigation of the mechanistic basis for suppression of T cells in the AML microenvironment

## 5.1 Introduction

AML cells employ multiple immunosuppressive mechanisms (reviewed Chapter 1 section 1.4.2). These include inducing a metabolically unfavourable microenvironment, expression of ligands for inhibitory receptors and defective immune synapse formation. Attempts to circumvent suppression and thereby boost the efficacy of anti-leukaemia immune responses in AML patients have so far focused on immune checkpoint blockade and use of the immunomodulatory agent lenalidomide.

Interest in anti PD-1 and PD-L1 blocking antibodies has been encouraged by studies that showed T cells from AML patients express the PD-1 receptor[231, 233, 350], the PD-L1 ligand is often expressed on AML cells[231, 233], and PD-1 engagement with PD-L1 is known to impair T cell proliferation, cytokine production and survival through inactivation of the PI3K/AKT pathway[351]. However, clinical studies undertaken to date report that treatment with single agent PD-1/PD-L1 inhibitors has only modest anti-leukaemia effect[253, 352, 353].

Lenalidomide is a thalidomide derivative that has anti-leukaemia activity which is mediated by a combination of direct inhibitory effects on malignant cells and modulation of immune responses[354]. Efficacy has been most widely investigated for chronic lymphocytic leukaemia (CLL)[355]. Among the multiple modes of action are ability to stimulate T cell proliferation and production of IL-2 and IFN-γ[356, 357, 358], and repair of defective T cell synapse formation[359]. Use of lenalidomide for treatment for AML is being evaluated[255], but single agent response rates have been relatively low[257, 258, 259, 360].

Evidence-based selection of combinations of immunotherapies with strategies to mitigate immunosuppression by AML cells is required. The aim of the work described in this chapter was to use the *in vitro* co-culture model of the AML microenvironment to investigate the mechanisms responsible for T cell suppression and explore use of the platform for testing strategies for circumventing suppression.

## 5.2 Results

### 5.2.1 Investigation of cell contact requirements for suppression of T cell responses in the AML microenvironment

A role for soluble factors produced by AML cells in the suppression of T cell function was reported by Buggins et al[212]. More recent studies have also revealed a role for receptor - ligand interactions, such as the immune checkpoint molecules that modulate T cell responses in the AML microenvironment[231, 233, 235, 247, 249, 361, 362]. By definition, these interactions require contact between cells.

Results presented in Chapter 4 section 4.2.1.1 of this study showed that supernatants from co-cultures of primary AML cells with HS5 cells suppressed the activation and production of cytokines by T cells, which indicated a role for soluble factors. Experiments were performed to investigate whether contact between cells also contributed to suppression of T cell responses in the *in vitro* co-culture model of the AML microenvironment. Permeable polycarbonate membrane inserts, known as transwell chambers, were used to assess activity of T cells from the PBMC of four healthy individuals stimulated with anti-CD3/CD28 beads under hypoxic conditions. A total of three AML patient samples were used (AML047, AML048, and AML075). Table 3-1 lists the different co-culture conditions tested, with the contact / no contact between T cells and AML cells indicated. Due to their adherent nature, the stromal HS5 cells had to be cultured at the bottom compartment of the transwell chamber. Activation status of T cells after stimulation for 48 hours was determined by flow cytometric assessment of the activation markers CD137 for CD8 T cells and CD154 for CD4 T cells. Cytokine secretion was measured by ELISA.

**Table 5-1 Co-culture conditions for assessing cell contact requirements for suppression of T cell responses**

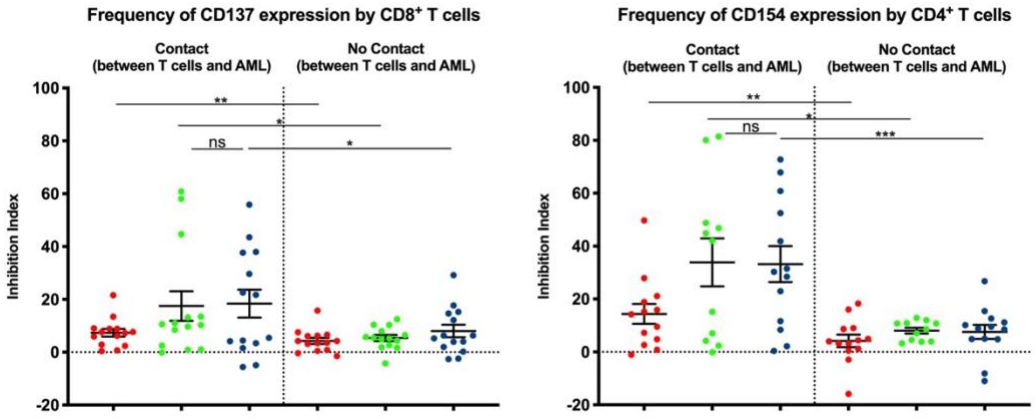
| Cell culture composition | Contact between T cells and AML cells |
|---|---|
| Stimulated T cells + AML | Contact |
| Stimulated T cells + (Transwell AML) | No Contact |
| Stimulated T cells + HS5 + AML | Contact |
| Stimulated T cells + HS5 + (Transwell AML) | No Contact |
| HS5 + (Transwell Stimulated T cells + AML) | Contact |
| AML + HS5 + (Transwell Stimulated T cells) | No Contact |

The results presented in Figure 5-1A show that suppression of the frequency of T cells induced to express activation markers after stimulation with anti CD3/CD28 beads was significantly higher when there was contact between T cells and primary AML cells. There was some suppression of the frequency of T cell activation marker expression when co-cultured with AML cells (red conditions) and this was significantly higher when the two cell types were in contact. Inhibition index for the frequency of $CD137_+$ CD8 T cells increased from mean 4.3% to 7.3% ($p$=0.003) when there was contact with AML cells and increased from 4.0% to 14.4% ($p$=0.005) for frequency of $CD154_+$ CD4 T cells. Consistent with results presented previously, suppression was enhanced in the presence of HS5 cells (green and blue conditions), and the experiments using transwell chambers showed that suppression in the three-cell co-culture model was substantially higher when there was contact between T cells and primary AML cells. Inhibition index for the frequency of activation marker expressing T cells was only 5.4% for $CD137_+$ and 8.1% for $CD154_+$ when the T cells were separated from AML cells, but suppression was significantly higher at 17.5% ($p$=0.04) and 33.9% ($p$=0.01) respectively when all three cell types were together (green conditions). The conditions shown in blue assessed the requirement for contact with HS5 cells. Results showed that the enhanced suppression of T cell activation seen in the presence of the HS5 stromal cells did not require contact with the T cells and AML cells. Inhibition index values for the frequency of activation marker expressing T cells when HS5 cells were separated from the T cells and AML

cells were very similar to suppression seen when all three cell types were together (18.4% compared to 17.5% for CD137+ and 33.2% compared to 33.9% for CD154+).

Analysis of the expression levels of the activation markers presented in Figure 5-1B similarly showed that suppression was significantly higher when AML cells were in contact with T cells, but contact with HS5 cells was not required. Inhibition index for the activation marker MFI was mean 20.0% for CD137+ and 19.2% for CD154+ when the T cells were separated from AML cells (red conditions), but the suppression was significantly higher at 45.2% ($p$<0.001) and 45.6% ($p$=0.01) respectively when all three cell types were together (green conditions). Inhibition index values for the MFI of activation marker expressing T cells when HS5 cells were separated from the T cells and AML cells (blue conditions) were slightly lower but not significantly different compared to suppression seen when all three cell types were together (40.4% compared to 45.2% for CD137+ $p$=0.53 and 39.9% compared to 45.6% for CD154+ $p$=0.85).

**A**




**B**

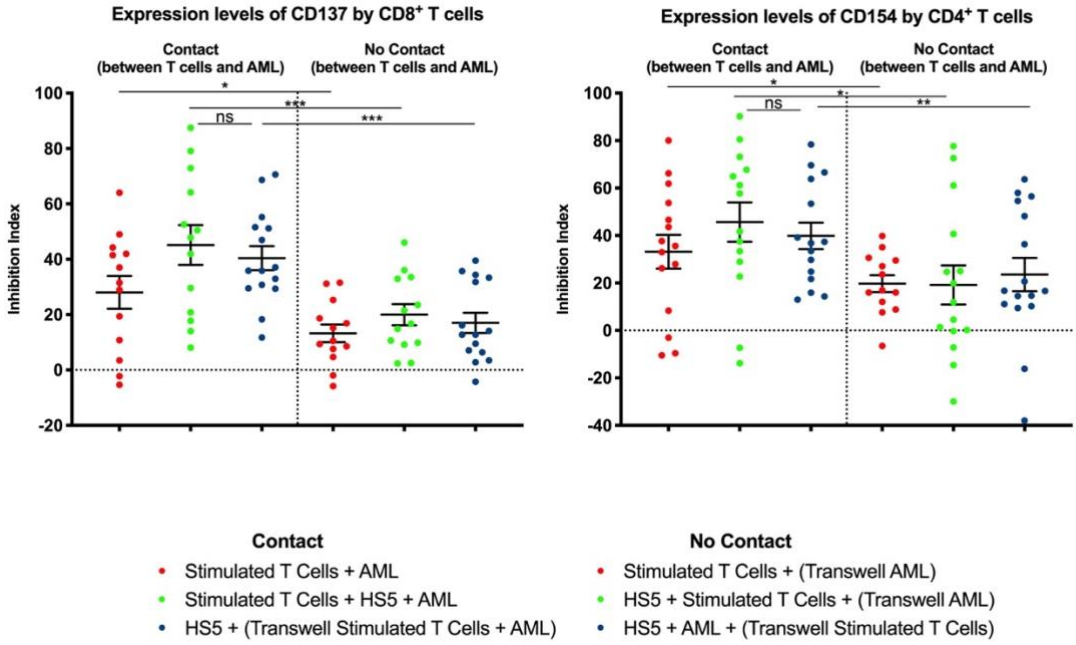



**Figure 5-1 Activation of αCD3/CD28 bead stimulated T cells in the AML is inhibited to greater extent when there is contact between T cells and primary AML cells**

T cells from four healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells in the absence or presence of HS5 cells for 48 hours under hypoxic conditions. The requirements for cell contact in the co-cultures were assessed using transwell chambers. T cell activation was assessed by measuring the frequency (A) and expression levels (B) of CD137 by $CD8_+$ T cells or CD154 by CD4 T cells using flow cytometry. Results are presented as an inhibition index, whereby T cells + AML cells were normalised to T cells alone and T cells + AML cells + HS5 cells were normalised to T cells + HS5 cells. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, determined by paired t test.

Analysis of the concentrations of IFNγ, TNF-α, and IL-4 in supernatants from co-cultures using transwell chambers shown Figure 5-2 substantiated that suppression was greatest when AML cells were in contact with T cells. When all three cell types were cultured together (green conditions), production of IFN-γ was mean 4075.4 pg/mL compared to 4738.4 pg/mL when T cells were separated from the AML cells ($p$=0.04), TNF-α was 532.6 pg/mL compared to 1058.3 pg/mL ($p$=0.02), and IL-4 was 143.8 pg/mL compared to 288.5 pg/mL ($p$=0.009).

Cytokine production was not significantly different in cultures where all three cell types were together (green conditions) compared to when AML with T cells were separated from the HS5 cells (blue conditions). Production of IFN-γ was mean 4075.4 pg/mL when all three cell types were together compared to 3603.6 pg/mL when HS5 cells were separated from the AML with T cells ($p$=0.07), TNF-α was 532.6 pg/mL compared to 397.4 pg/mL ($p$=0.36), and IL-4 was 143.8 pg/mL compared to 146.0 pg/mL ($p$=0.94). These results substantiate that suppression does not require contact with HS5 cells.

In the two-cell co-cultures of AML and T cells (red conditions), production of TNF-α was similar to the control condition of stimulated T cells alone in both the contact and no contact conditions. The lack of suppression may be due to poor viability of AML cells in the absence of HS5 cells. Production of IFN-γ was significantly increased when T cells and AML cells were in contact compared to the control condition of stimulated T cells alone (mean 7125.8 pg/mL compared to 3992.6, $p$<0.001). This phenomenon was also seen in the results presented section 3.2.5 Figure 3-9. Consistent with speculation that T cells within the AML sample may contribute to the production of IFNγ, levels were significantly lower (4816.8 pg/mL, $p$=0. 0.004) when there was no contact between T cells and AML cells. In this setting, the AML cells (plus any patient T cells) in the lower chamber of the transwell were not exposed to the αCD3/CD28 beads (diameter 4.5µm) retained in the upper chamber by the 0.4µm membrane. Production of IL-4 when T cells were in contact with AML cells (mean 193.2 pg/mL) was similar to the control condition of stimulated T cells alone (223.4 pg/mL) but there was a surprising significant increase when cells were separated (287.3 pg/mL, $p$=0.03), mainly attributed to high IL-4 levels produced in three of the eleven cultures.

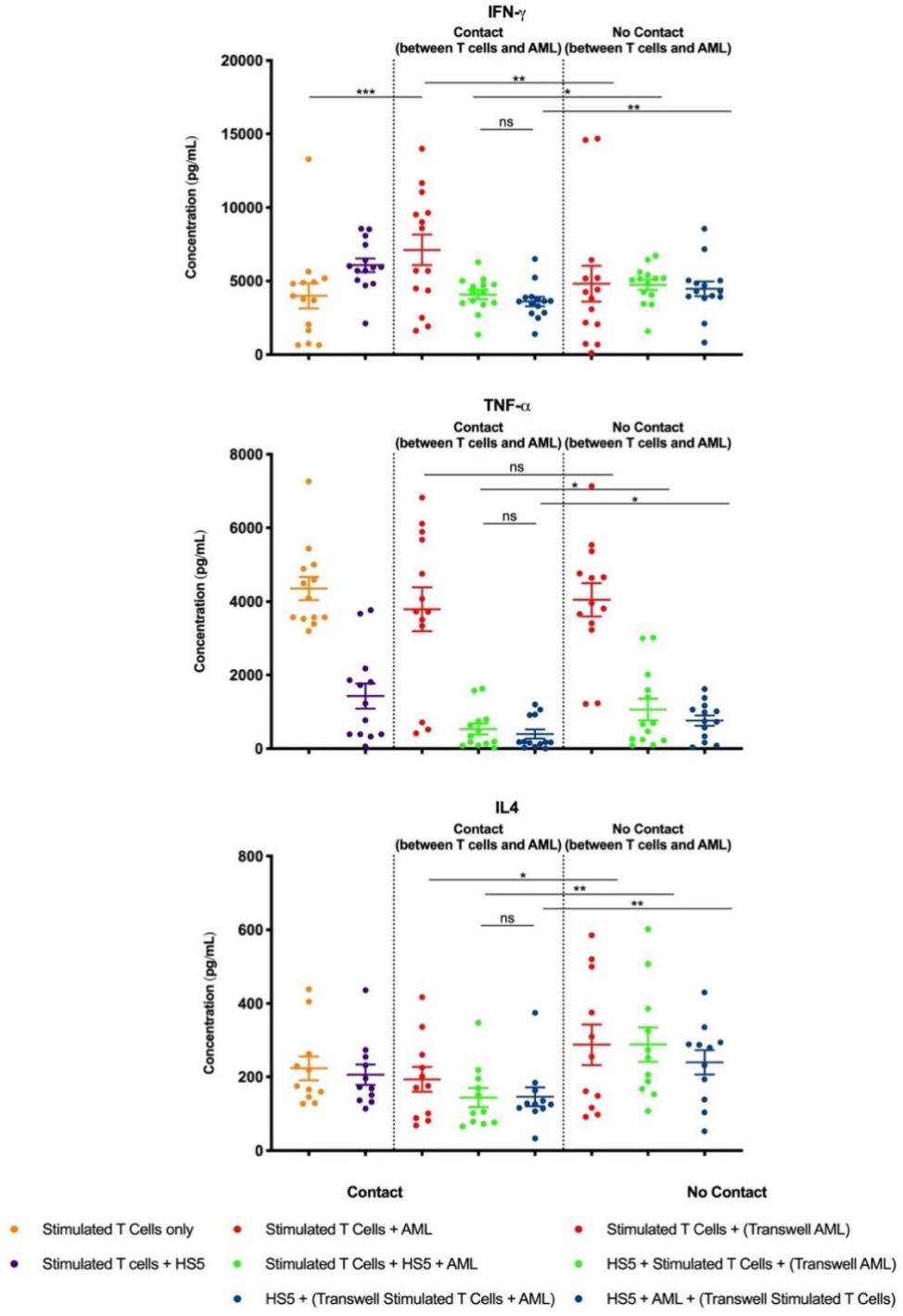



**Figure 5-2 Cytokine production in cultures of αCD3/CD28 bead stimulated T cells is inhibited to greater extent when there is contact between T cells and primary AML cells**

T cells from healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells in the absence or presence of HS5 cells for 48 hours under hypoxic conditions. The requirements for cell contact in the co-cultures were assessed using transwell chambers. Cytokine production was measured by ELISA using cell culture supernatants diluted 1:25 for IFNγ, 1:5 for TNF-α or 1:5 for IL-4. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, determined by paired t test

### 5.2.2 Assessment of the contribution of PD-1 to suppression of T cell responses in the AML microenvironment

The experiments with transwell chambers demonstrated the importance of contact-mediated inhibition of T cell function by AML cells. Given the evidence that immune checkpoint molecules can play a key role in immune evasion by tumour cells, the contribution of the interaction between PD-1 and PD-L1 to contact-mediated suppression of T cells in the *in vitro* co-culture model was explored.

#### 5.2.2.1 PD-L1 expression by primary AML cells is upregulated in the presence of αCD3/CD28 stimulated T cells

The expression of PD-L1 on primary AML cells was assessed after culture for 48 hours under hypoxic conditions by flow cytometry. A representative example to illustrate the gating strategy is shown in Figure 5-3. AML samples from eight different patients were assessed. The frequency of PD-L1+ AML cells and expression levels are presented in Figure 5-4. Results for the AML cells cultured alone showed that samples from two patients did not express PD-L1 and only a small percentage of cells in the other samples expressed PD-L1 (range 0.28-49.1%) at low expression levels (range 503 - 1920 MFI). Co-culture with HS5 cells promoted an increase in the frequency of PD-L1+ AML cells from mean 17.6% to 22.9% ($p$=0.02), although one sample remained negative, and expression levels rose slightly from mean 1196.4 MFI to 1725.9 MFI ($p$=0.06). Co-culture with αCD3/CD28 stimulated T cells led to a substantial increase in both the frequency and expression levels of PD-L1 for all AML samples. The mean frequency of PD-L1+ AML cells increased to 80.6% ($p$<0.001) and expression levels rose more than 8-fold to 9689.6 MFI ($p$=0.002) in the AML and T cell co-cultures compared to AML cells alone. Similar upregulation was observed in the three-cell co-cultures in the presence of HS5 cells, with the mean frequency of PD-L1+ AML cells increased to 81% ($p$<0.001) and expression levels increased to 8125.3 MFI ($p$=0.002) compared to AML cells cultured with HS5 cells. Of note, although the expression level rose, the magnitude of the increase was significantly lower compared to AML cells co-cultured with T cells in the absence of HS5 cells ($p$=0.047).

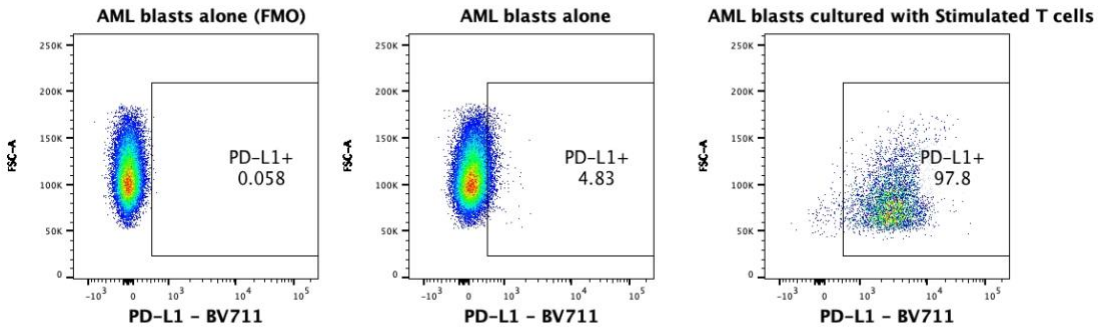



**Figure 5-3 Representative scatter plots for PD-L1 expression on AML cells**

AML cells were gated on viable CD33+ or CD34+ cells. A fluorescence minus one (FMO) control was used to determine the location of the PD-L1 negative population and positioning of the PD-L1+ gate. PD-L1 expression levels were measured using geometric median fluorescence intensity of the cells that are positive for the marker.

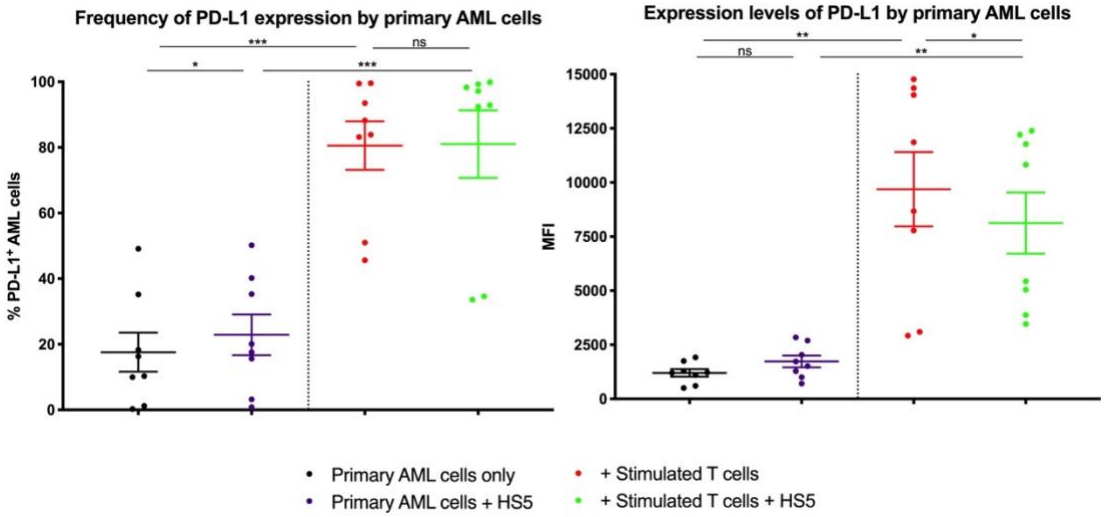



**Figure 5-4 PD-L1 expression by primary AML cells is significantly increased in the presence of T cells stimulated with αCD3/CD28 beads**

Primary AML cells from eight patients were cultured alone or together with HS5 cells and/or T cells from healthy individuals stimulated with αCD3/CD28 beads for 48 hours under hypoxic conditions. Upregulation of PD-L1 on AML cells was assessed by measuring the frequency and expression levels using flow cytometry. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, determined by paired t test.

#### 5.2.2.2 PD-1 expression is upregulated on αCD3/CD28 stimulated T cells

The expression of PD-1 on T cells was assessed after stimulation with αCD3/CD28 beads for 48 hours under hypoxic conditions by flow cytometry. A representative example to illustrate the gating strategy is shown in Figure 5-5. The results presented in Figure 5-6 show that αCD3/CD28 stimulation promoted a substantial increase in the frequency and expression level of PD-1 by T cells. The frequency of PD-1+ CD8+ T cells rose from mean 43.15% without stimulation to 97.1% ($p$<0.001) and the level of PD-1 expression rose from a mean 756.1 MFI to 4145.5 MFI ($p$<0.001) after stimulation (Figure 5-6A). The frequency of PD-1+ CD4+ T cells rose from mean 31.8% to 97.5% ($p$<0.001) and the level of PD-1 expression rose from a mean 729.6 MFI to 6260.3 MFI ($p$<0.001) after stimulation Figure 5-6B.

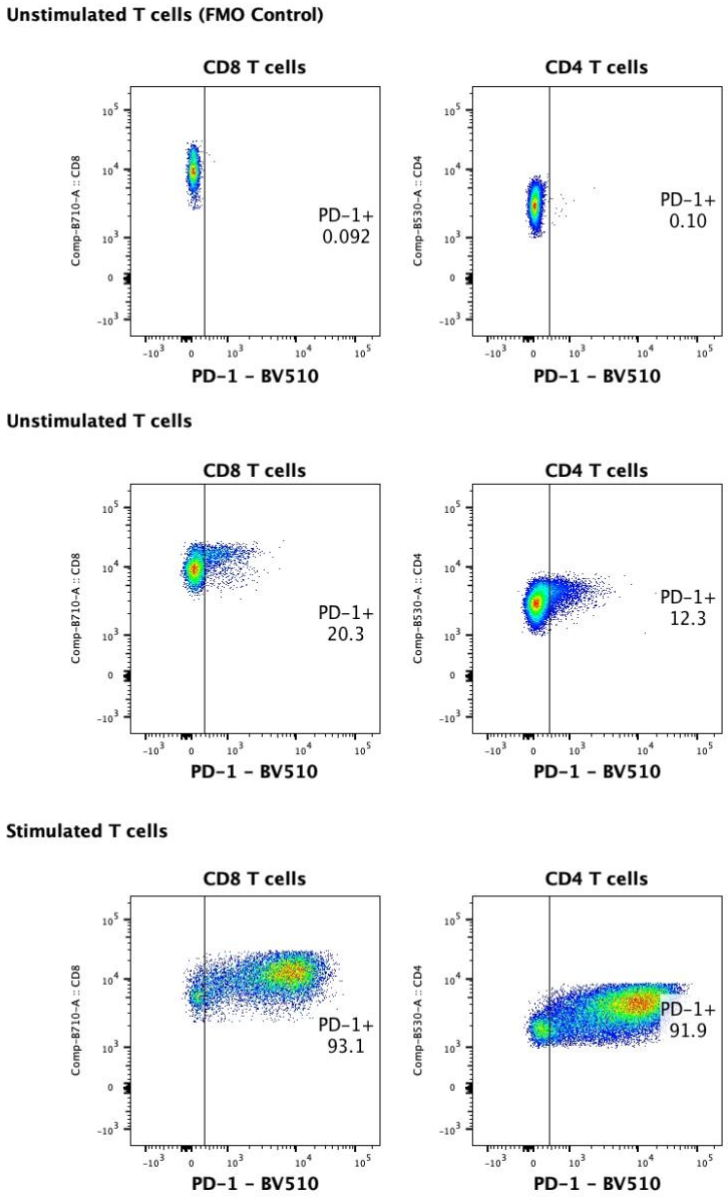
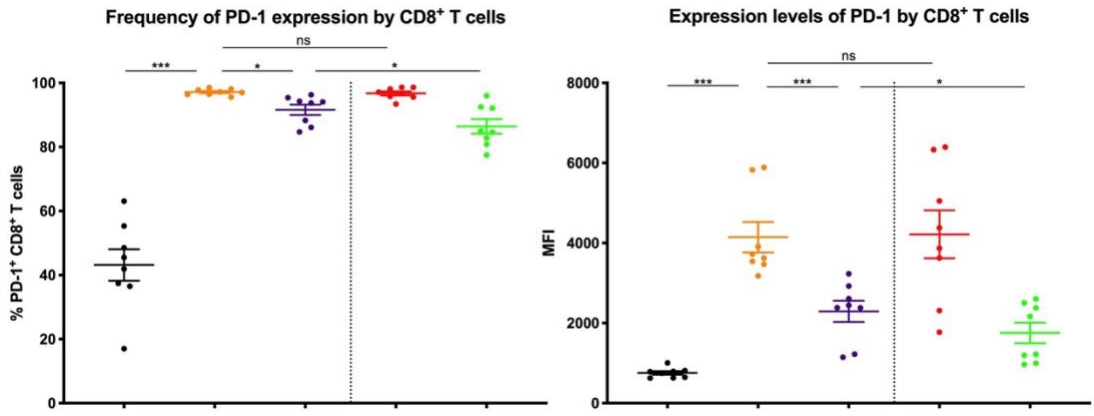



**Figure 5-5 Representative scatter plots for PD-1 expression on CD8+ and CD4+ T cells**

AML cells (CD33+ or CD34+) and HS5 cells (CD29+) were excluded. T cells were gated on CD3+ viable cells. CD3+ T cells were then further distinguished as CD8+ and CD4+ T cells. A fluorescence minus one (FMO) control with unstimulated T cells was used to determine the location of the PD-1 negative population and positioning of the PD-1+ gate (top panel). PD-1 expression levels were measured using geometric median fluorescence intensity of the cells that were positive for the marker.

**A**




**B**

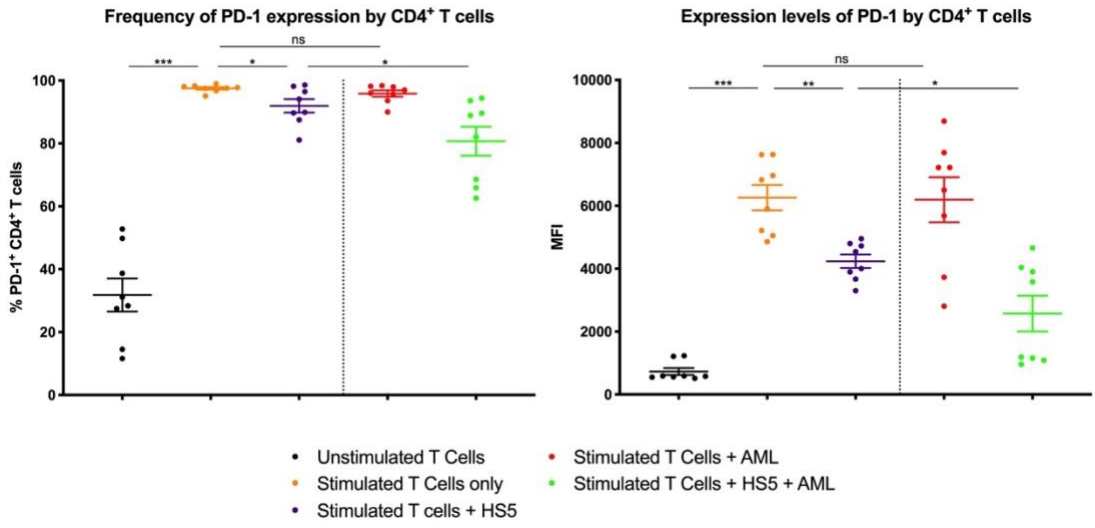



**Figure 5-6 PD-1 expression is upregulated on T cells stimulated with αCD3/CD28 beads but the magnitude is significantly diminished in the presence of AML cells with HS5 cells**

T cells from healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells in the absence or presence of HS5 cells for 48 hours under hypoxic conditions. Upregulation of PD-1 on CD8 T cells (A) and CD4 T cells (B) was assessed by measuring the

frequency and expression levels using flow cytometry. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, determined by paired t test.

The presence of primary AML cells did not have an impact on the upregulation of PD-1 expression on αCD3/CD28 stimulated T cells, but there was significantly diminished expression in the presence of HS5 cells and there was a further significant reduction in the three-cell co-culture. The upregulation of PD-1 expression by CD8 T cells was reduced to mean 91.6% ($p$=0.01) 2293.9 MFI ($p$<0.001) in the presence of HS5 cells and 86.4% ($p$=0.04) 1754.3 MFI ($p$=0.01) in the presence of AML cells with HS5 cells (Figure 5-6A). The upregulation of PD-1 expression by CD4 T cells was reduced to mean 92.0% ($p$=0.04), 4237.0 MFI ($p$=0.002) in the presence of HS5 cells and 81.7% ($p$=0.03) 2573.1 MFI ($p$=0.02) in the presence of AML cells with HS5 cells (Figure 5-6B). Although the increase in frequency of PD-1+ T cells after stimulation was significantly lower in the AML microenvironment, the majority (>80%) were positive but the expression levels were substantially lower at approximately half the surface density of stimulated T cells cultured alone.

#### 5.2.2.3 Anti-PD-1 and anti-PD-L1 blocking antibodies do not counteract T cell suppression in the AML microenvironment

The investigation of expression of PD-L1 by AML cells and PD-1 by T cells in the *in vitro* co-culture model showing substantial upregulation of both molecules provided justification for assessing the role of this interaction in suppression of T cell activation and cytokine production in the AML microenvironment. A primary AML cell sample (AML048) was selected for study, based on knowledge that high levels of PD-L1 were expressed on the majority of cells when cultured with αCD3/CD28 stimulated T cells in the presence of HS5 cells. Co-cultures were set up with αCD3/CD28 stimulated T cells from three different healthy individuals and anti-PD-1 or anti-PD-L1 blocking antibodies (10μg/mL) were tested for ability to prevent suppression of T cell activation and cytokine production in the AML microenvironment. The concentrations of anti-PD-1 or anti-PD-L1 blocking antibodies used were based on supplier instructions and studies reported by Sakuishi et al and Wang et al that showed restoration of anti-tumour T cell functions when cells were treated with anti-PD-1 or anti-PD-L1 blocking antibodies at 10μg/mL[363, 364].

Results presented in Figure 5-7 show that anti-PD-1, anti-PD-L1 or a combination of both blocking antibodies did not counteract inhibition of T cell activation marker expression in the presence of AML cells with HS5 cells. The inhibition index values of CD137 on CD8 T cells and CD154 on CD4 T cells for frequencies (Figure 5-7A) and expression levels (Figure 5-7B) for T cells from two of the three different healthy individuals (distinguished based on symbol shape) were similar in the absence or presence of blocking antibodies. Treatment with anti-PD-1, anti-PD-L1 or a combination of both blocking antibodies also did not counteract suppression of cytokine production (Figure 5-8). These results indicate that the interaction of PD-1 with PD-L1 is not the main mechanism for contact-dependent inhibition of T cell function in the AML microenvironment *in vitro* co-culture model.

**A**

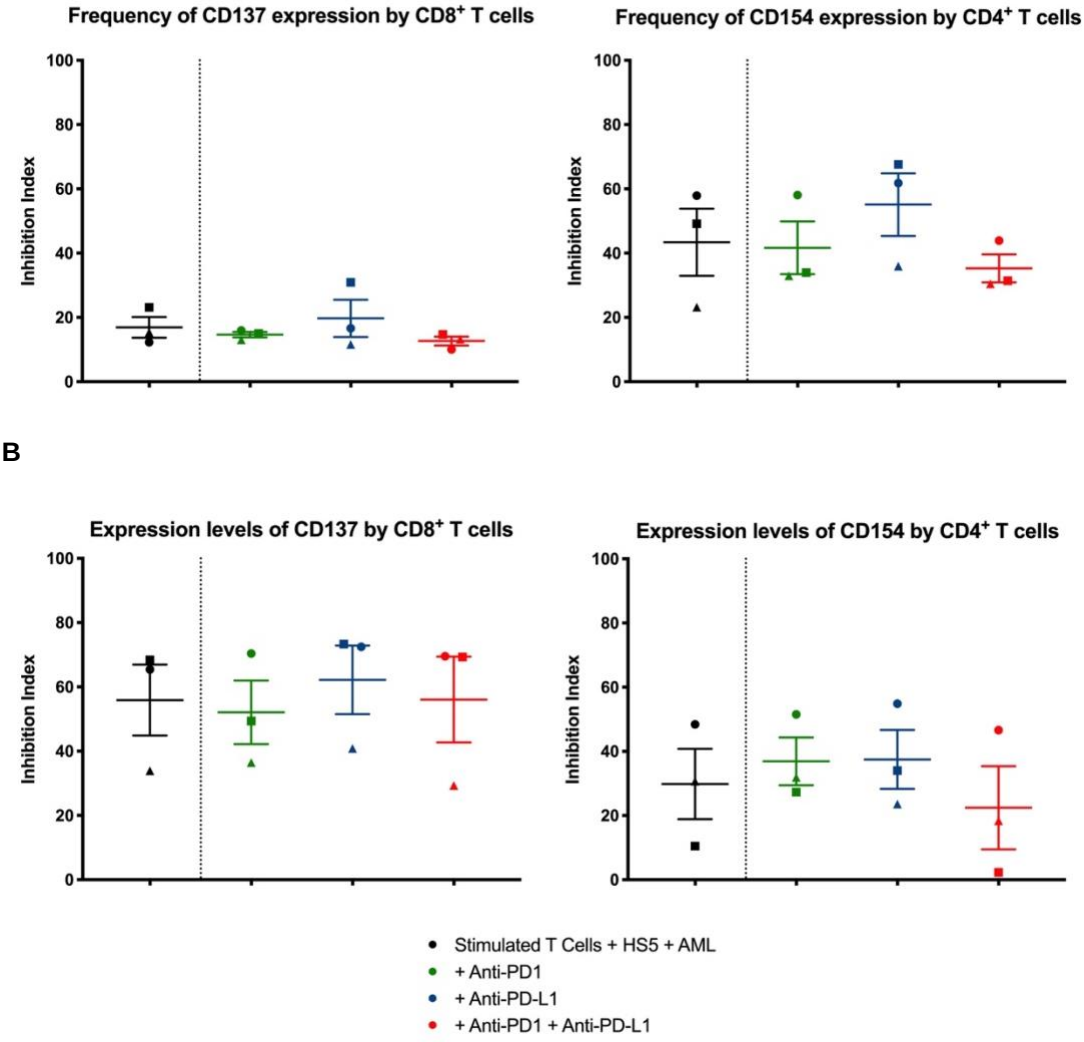



**Figure 5-7 Anti-PD-1 or anti-PD-L1 blocking antibodies do not counteract the suppression of activation of αCD3/CD28 bead stimulated T cells in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells (AML048) in presence of HS5 cells for 48 hours under hypoxic conditions. Cultures were treated with anti-PD-1 (10μg/mL), anti-PD-L1 (10μg/mL) or a combination of both. T cell activation was assessed by measuring the frequency (A) and expression levels (B) of CD137 by $CD8_+$ T cells or CD154 by CD4 T cells using flow cytometry. Results are presented as an inhibition index, whereby T cells + HS5 cells were normalised to T cells alone, T cells + AML cells were normalised to T cells alone and T cells + AML cells + HS5 cells were normalised to T cells + HS5 cells. The data points are shown as circle, square or triangle symbol shapes to illustrate T cells from each of the three different healthy individuals and thereby facilitate tracking across culture conditions. Data shown represents the mean ± SEM.

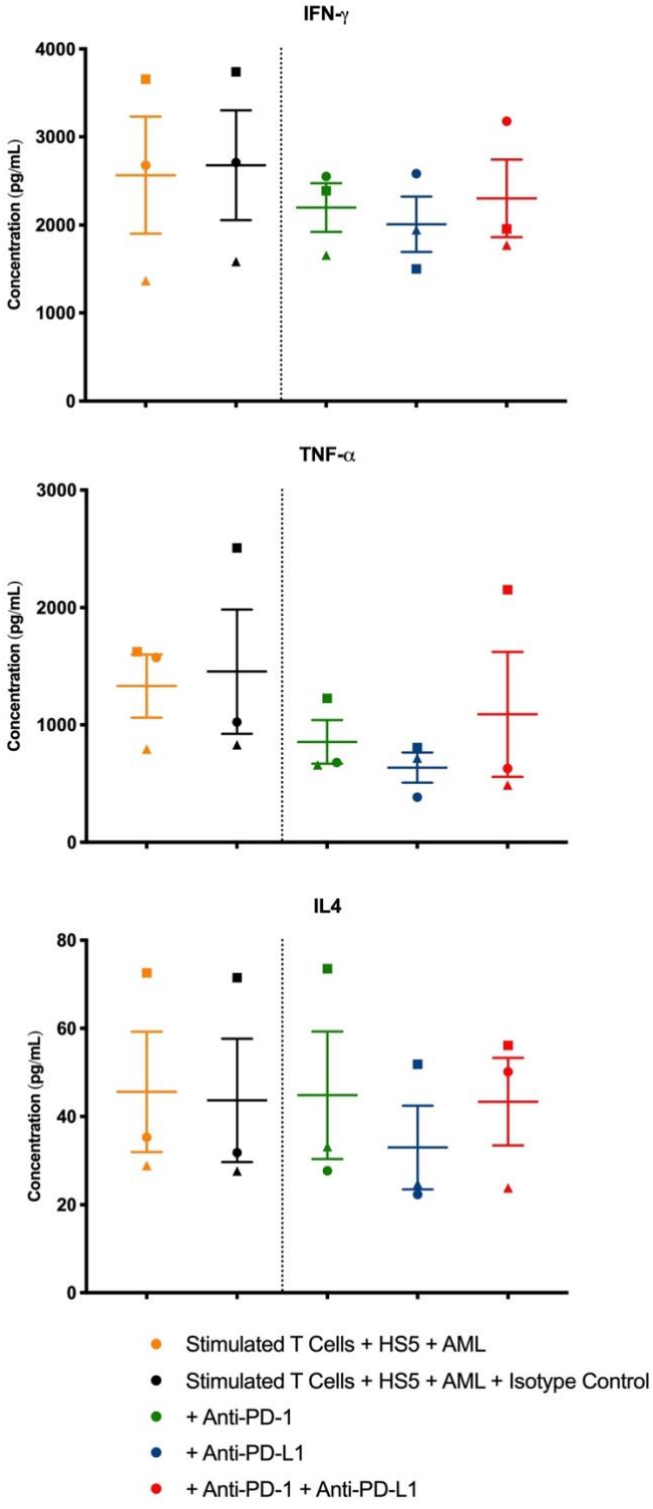



**Figure 5-8 Anti-PD-1 or anti-PD-L1 blocking antibodies do not counteract suppression of cytokine production by αCD3/CD28 bead stimulated T cells in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells (AML048) and HS5 cells for 48 hours under hypoxic conditions. Cultures

were treated with anti-PD-1 (10μg/mL), anti-PD-L1 (10μg/mL) or a combination of both. Cytokine production was measured by ELISA using cell culture supernatants diluted 1:25 for IFNγ, 1:5 for TNF-α or 1:5 for IL-4. The data points are shown as circle, square or triangle symbol shapes to illustrate T cells from each of the three different healthy individuals and thereby facilitate tracking across culture conditions. Data shown represents the mean ± SEM.

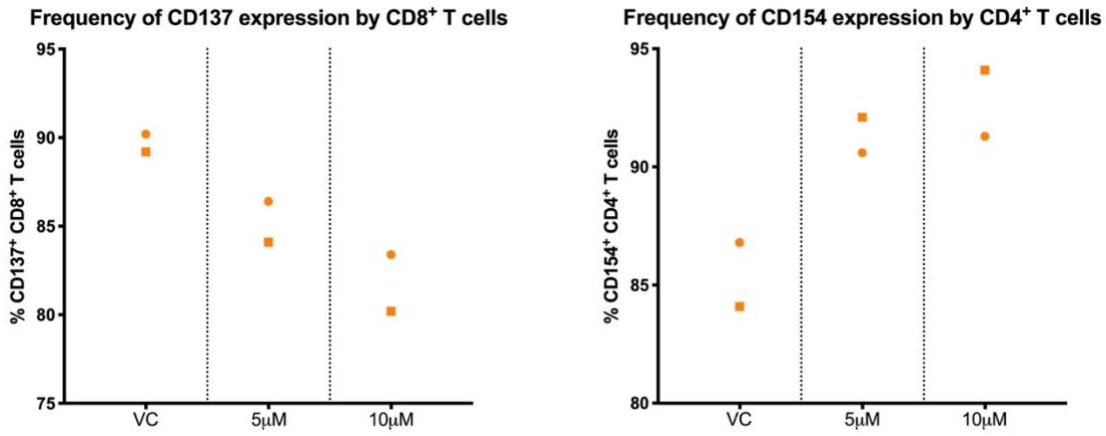
### 5.2.3 Evaluation of the ability of lenalidomide to modulate T cell suppression in the AML microenvironment

It is known that T cells from AML patients fail to form effective synapses with autologous AML cells[244]. This defect represents another potential mechanism for contact-dependent suppression of T-cell function in the AML microenvironment *in vitro* co-culture model. The immunomodulatory agent lenalidomide has previously been shown to overcome inhibition of *in vitro* T cell function seen in the presence of malignant cells of the lymphoid lineage by reversing defective immune synapse formation.[359] The ability of lenalidomide to modulate suppression of T cell responses in the presence of AML cells with HS5 cells was therefore evaluated.

Lenalidomide directly enhances the activity of T cells *in vitro*.[357, 365] This phenomenon was exploited to determine an effective drug concentration for use in the AML microenvironment co-culture model. T cells from two healthy individuals were stimulated with αCD3/CD28 beads for 48 hours under normoxic conditions. Previous studies have used lenalidomide at concentrations of 1-10μM for *in vitro* enhancement of T cell activity[365, 366, 367]. Lenalidomide was therefore tested at 5 μM and 10 μM, and compared to a vehicle control comprising DMSO. Lenalidomide promoted a small increase in the frequency of CD154+ CD4 T cells (Figure 5-9A) and CD154 expression levels (Figure 5-9B). In contrast, there was a small decrease in the frequency of CD137+ CD8 T cells (Figure 5-9A) and a decrease in expression levels in cultures treated with lenalidomide (Figure 5-9B). The changes in activation marker expression produced by treatment with lenalidomide were similar at both concentrations tested. The analysis of cytokine production presented in Figure 5-10 shows that lenalidomide promoted increased production of IFNγ. It has been previously reported that lenalidomide induces IFN-γ production through the downregulation of suppressor of cytokine signalling (SOCS1)[365]. Treatment of T cells in the *in vitro* model with

lenalidomide reproduced the finding for IFN-γ. The increase in IFN-γ production was highest when treated with 5 μM lenalidomide, which was therefore used for subsequent experiments.

**A**

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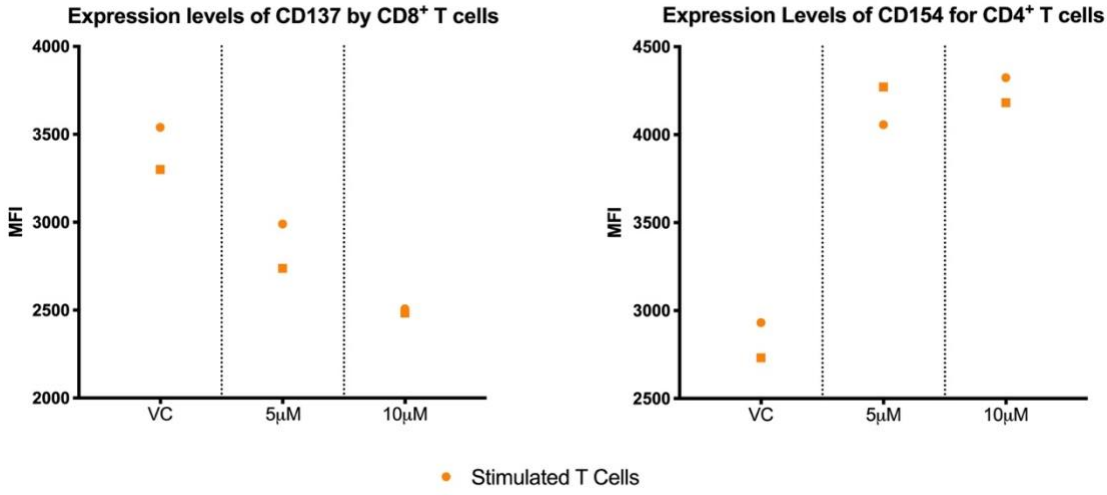



**Figure 5-9 Lenalidomide has limited impact on activation marker expression by αCD3/CD28 stimulated T cells**

T cells from two healthy individuals were stimulated with αCD3/CD28 beads for 48 hours under normoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide at 5 μM or 10 μM. T cell activation was assessed by measuring the frequency (A) and expression levels (B) of CD137 by CD8 T cells or CD154 by CD4 T cells using flow cytometry. The data points are shown as circle or square symbol shapes to illustrate T cells from two different healthy individuals and thereby facilitate tracking across treatments.

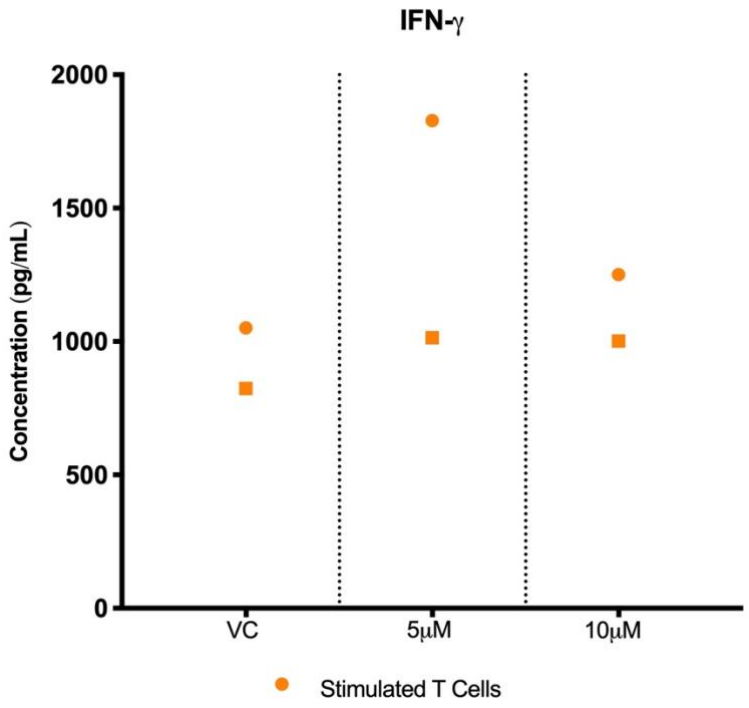



**Figure 5-10 Lenalidomide improves production of IFN-γ by αCD3/CD28 stimulated T cells**

T cells from two healthy individuals were stimulated with αCD3/CD28 beads for 48 hours under normoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide at 5 μM or 10 μM. Cytokine production was measured by ELISA using cell culture supernatants diluted 1:25 for IFNγ. The data points are shown as circle or square symbol shapes to illustrate T cells from two different healthy individuals and thereby facilitate tracking across treatments.

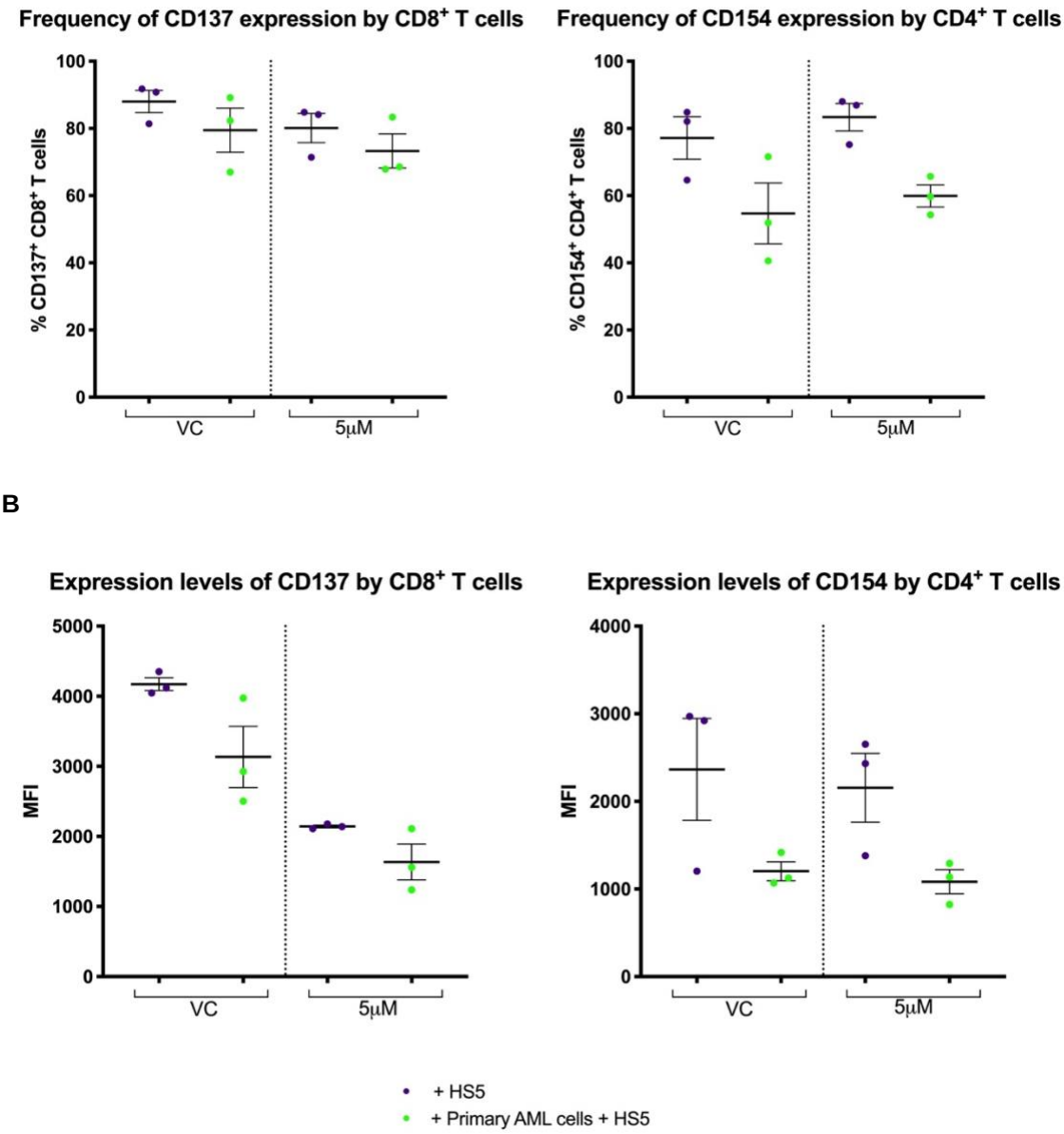
#### 5.2.3.1 Lenalidomide has limited impact on suppression of αCD3/CD28 stimulated T cells in the AML microenvironment

T cells from three different healthy individuals were stimulated with αCD3/CD28 beads in the presence of AML cells (AML048) with HS5 cells for 48 hours under hypoxic conditions, and lenalidomide at 5 μM was tested for the ability to overcome suppression of T cell activation and cytokine production. As expected, the frequency and expression levels of activation markers by αCD3/CD28 stimulated T cells (Figure 5-11) and cytokine production (Figure 5-12) were lower in the presence of AML cells with HS5 cells compared to the positive control with HS5 cells. However, there was little change when treated with lenalidomide. The mean frequency of CD137+ CD8 T cells in the presence of AML cells with HS5 cells decreased from 79.5% to 73.3% and CD154+ CD4 T cells increased from 54.7% to 59.9% when treated with lenalidomide (Figure 5-11A). Mean expression levels of CD154 decreased slightly from 1203.3 MFI to 1083.7 MFI, and

a more substantial decrease was observed for CD137 from 3134.3 MFI to 1635.7 MFI (Figure 5-11B). Analysis of cytokine production also showed limited and variable effects of treatment with lenalidomide (Figure 5-12). There was a small increase in the level of IFN-$\gamma$ from a mean concentration of 3649.0 pg/mL to 3958.9 pg/mL and a more substantial increase for TNF-$\alpha$ from 1817.5 pg/mL to 2723.9 pg/mL. In contrast, levels of IL-4 were decreased from a mean of 35.3 pg/mL to 14.0 pg/mL. The observation of reduced TNF-$\alpha$ production in some of the cultures and reduced IL-4 production is consistent with what has been previously published, as it has been shown that lenalidomide reduces levels of TNF-$\alpha$ and IL-4 produced by T cells[356, 368, 369].

One of the mechanisms by which lenalidomide promotes T cell function is via tyrosine phosphorylation of CD28 and subsequent activation of NF$\kappa$B[357]. The limited impact of lenalidomide observed may be a consequence of using $\alpha$CD3/CD28 beads for stimulation, which potentially supersedes any substantial benefit of treatment with the immunomodulatory agent. The experiment was therefore repeated using $\alpha$CD3 coated beads for T cell stimulation.

**A**




**B**




**Figure 5-11 Lenalidomide has limited impact on suppression of activation marker expression by αCD3/CD28 stimulated T cells in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells (AML048) and HS5 cells for 48 hours under hypoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide at 5 μM. T cell activation was assessed by measuring the frequency (A) and expression levels (B) of CD137 by $CD8_+$ T cells or CD154 by CD4 T cells using flow cytometry. Data shown represents the mean ± SEM.

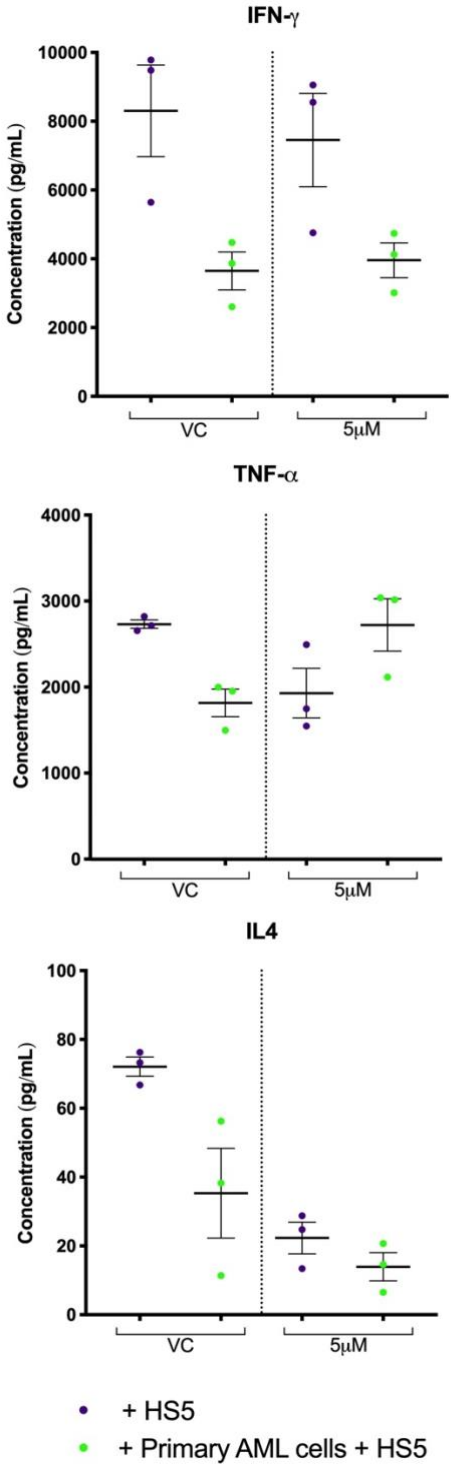



**Figure 5-12 Lenalidomide slightly modulates suppression of TNF-α production by αCD3/CD28 stimulated T cells in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3/CD28 beads and co-cultured with primary AML cells (AML048) and HS5 cells for 48 hours under hypoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide (5 μM). Cell culture supernatants were diluted as 1:25, 1:5, and 1:5 for IFNγ, TNF-α, and IL-4 respectively. Cytokine production was

measured by ELISA using cell culture supernatants diluted 1:25 for IFNγ, 1:5 for TNF-α or 1:5 for IL-4. Data shown represents the mean ± SEM.

#### 5.2.3.2 Activity of αCD3 stimulated T cells is not suppressed in the AML microenvironment

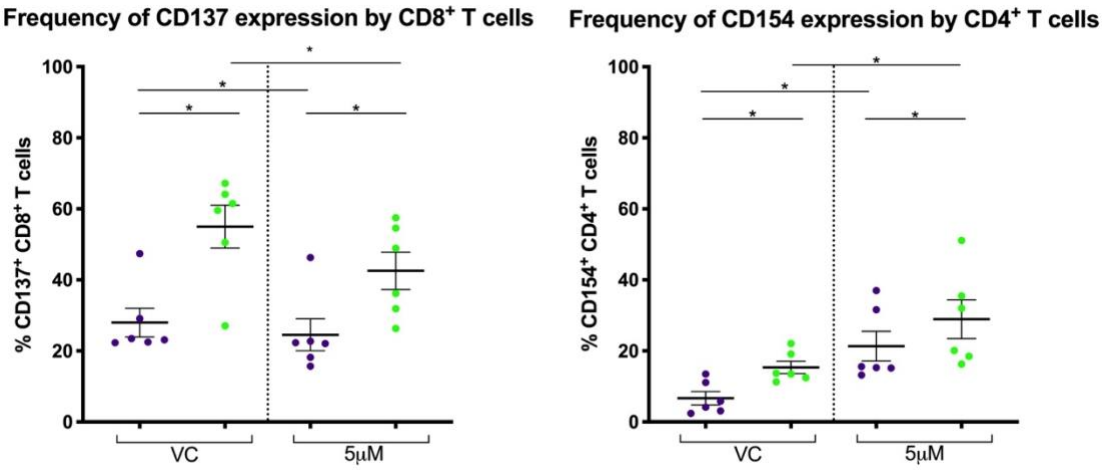
Stimulation of T cells with αCD3 beads revealed the interesting finding that there was no inhibition of activity in the presence of AML cells with HS5 cells, compared to with HS5 cells alone. This indicates that the mechanisms of immune suppression by AML cells target the CD28 pathway. Results presented previously using αCD3/CD28 beads to stimulate T cells have consistently shown lower activation marker expression and cytokine production in the three-cell co-cultures of AML cells with HS5 cells compared to the two-cell co-cultures with HS5 cells. In contrast, the frequency of activation marker expressing T cells and production of IFN-γ and TNF-α were all significantly higher in three-cell co-cultures stimulated with αCD3 beads compared to the two-cell co-cultures with HS5 cells.

In the vehicle control treated cultures, the mean frequency of CD137+ CD8 T cells increased from 28.0% with HS5 cells to 55.0% in the presence of AML cells with HS5 cells ($p$=0.03) and the mean frequency of CD154+ CD4 T cells rose from 6.7% to 15.4% ($p$=0.03) (Figure 5-13A) . Expression levels of the activation markers were similar in both the co-cultures with HS5 cells and the three-cell co-cultures including AML cells (Figure 5-13B). Production of IFN-γ and TNF-α was significantly increased from mean 398.2 pg/mL to 673.5 pg/mL ($p$=0.03) and 37.4 pg/mL to 82.5 pg/mL ($p$=0.03) respectively, and IL-4 production also increased from 12.3 pg/mL to 14.6 pg/mL ($p$=0.06) (Figure 5-14). As expected, the overall level of T cell activation and cytokine production was substantially lower after stimulation with αCD3 beads compared to previous results with αCD3/CD28 beads due to the absence of co-stimulation. The increased responses observed in the presence of AML cells with HS5 compared to with HS5 cells alone might be a consequence of the allogeneic composition of the co-cultures comprising patient AML cells and T cells from healthy individuals. Increased cytokine production might also be a consequence of contributions from patient T cells within the AML sample.

Given that the activity of T cells stimulated with αCD3 beads was not suppressed in the presence of AML cells with HS5 cells, it was not possible to evaluate the ability of lenalidomide to modulate

suppression. Changes in the profiles of activation marker expression by T cells and cytokine production were seen in the presence of lenalidomide, and the varying direct of changes mirrored those observed in the context of stimulation with αCD3/CD28 beads. The mean frequency of CD137+ CD8 T cells in the presence of AML cells with HS5 cells decreased from 55.0% to 42.6% (*p*=0.03) and CD154+ CD4 T cells increased from 15.3% to 28.9% (*p*=0.03) when treated with lenalidomide (Figure 5-13A). Mean expression levels of CD154 were similar (678.5 MFI and 681.2 MFI) and CD137 levels were substantially decreased from 1903.2 MFI to 1157.0 MFI (*p*=0.03) (Figure 5-13B). The increased production of IFN-γ and TNF-α observed in the co-cultures of αCD3 stimulated T cells in the presence of AML cells with HS5 cells was potentiated by treatment with lenalidomide. IFN-γ production increased from a mean of 673.5 pg/mL to 1179.3 pg/mL (*p*=0.03) and TNF-α rose from a mean of 82.5 pg/mL to 157.3 pg/mL (*p*=0.03) (Figure 5-14). In contrast, and consistent with the profile observed previously, production of IL-4 was decreased from 14.6 pg/mL to 10.7 pg/mL (*p*=0.03) (Figure 5-14).

**A**

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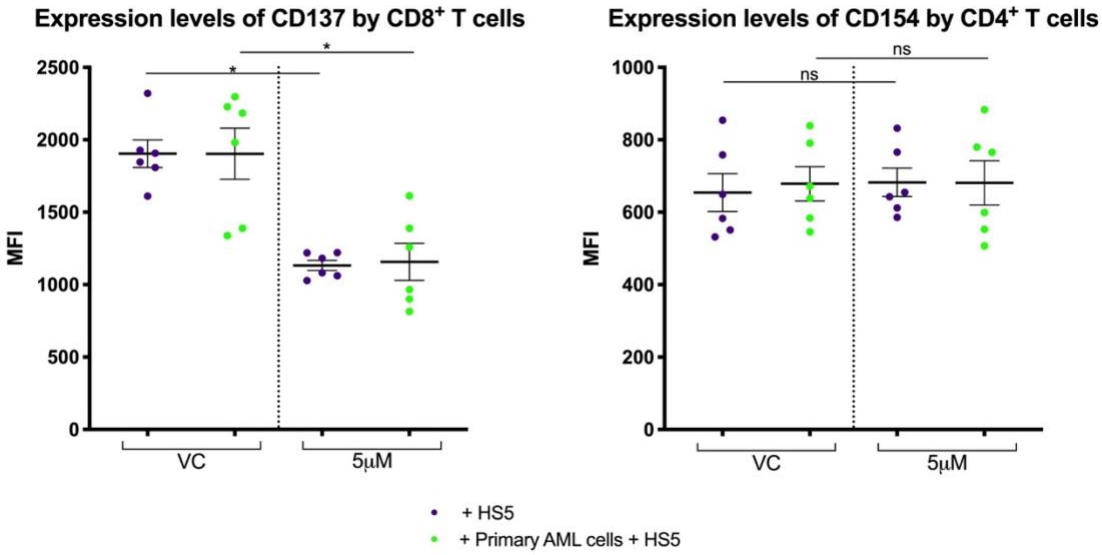


**Figure 5-13 Activation of αCD3 stimulated T cells is not suppressed in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3 beads and co-cultured with primary AML cells (AML047 or AML048) and HS5 cells for 48 hours under hypoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide (5 μM). T cell activation was assessed by measuring the frequency (A) and expression levels (B) of CD137 by CD8+ T cells or CD154 by CD4 T cells using flow cytometry. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, determined by non-parametric Wilcoxon matched paired t test.

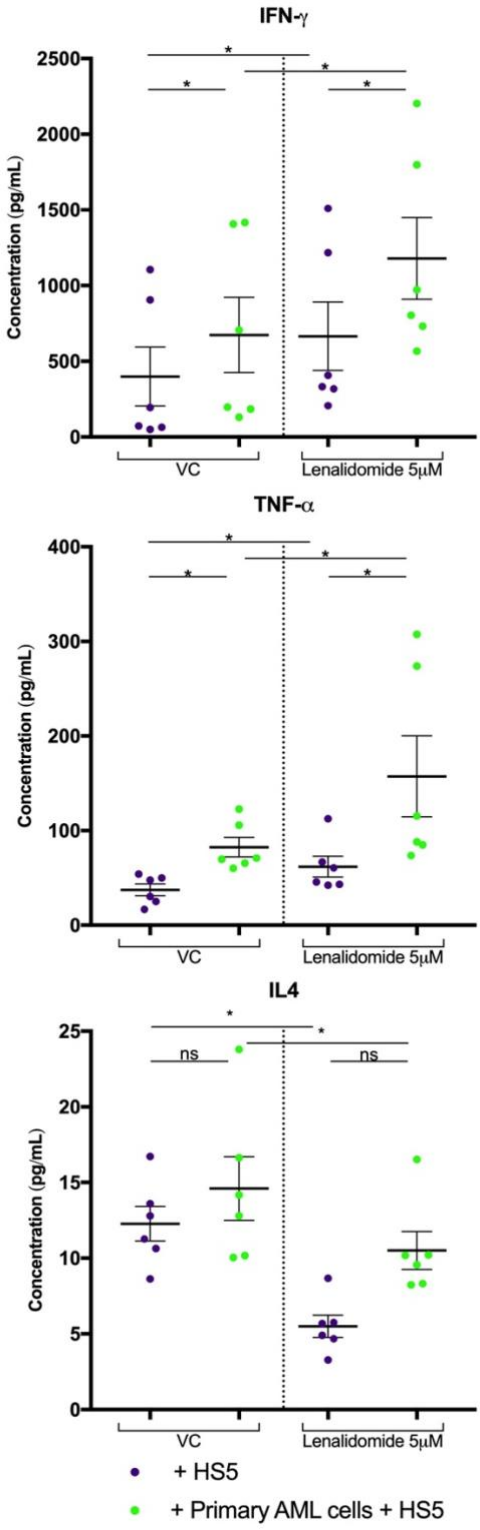


**Figure 5-14 Cytokine production by αCD3 stimulated T cells is not suppressed in the presence of AML cells with HS5 cells**

T cells from three healthy individuals were stimulated with αCD3 beads and co-cultured with primary AML cells (AML047 or AML048) and HS5 cells for 48 hours under hypoxic conditions. Cultures were treated with vehicle control (VC) or lenalidomide at 5 μM. Cytokine production was measured by ELISA using cell culture supernatants diluted 1:25 for IFNγ, 1:5 for TNF-α or 1:5 for

IL-4. Data shown represents the mean ± SEM. $^{ns}p>0.05$, $^{*}p<0.05$, determined by non-parametric Wilcoxon matched paired t test.

### 5.2.4 Subtle changes in the gene expression profile of CD8+ T cells cultured in the presence of primary AML cells with HS5 cells

The gene expression profile of T cells stimulated with αCD3/CD28 beads in the presence of AML cells with HS5 cells was examined to obtain a global picture of the impact of suppression in the AML microenvironment. To standardise the responding population, aliquots of cryopreserved T cells from one healthy individual were used and co-cultured with primary AML cells from five patients (AML017, AML047, AML048, AML067 and AML075). These AML samples were selected because they consistently suppressed T cell activation and cytokine production to greatest extent. T cells were stimulated with αCD3/CD28 beads for 48 hours under hypoxic conditions in the presence of AML cells with HS5 cells. The controls comprised stimulated T cells cultured with HS5 cells and unstimulated T cells cultured for 48 hours under hypoxic conditions. The controls were performed in triplicate. After culture, live CD3+ CD8+ T cells were purified by FACS. A representative example of the gating for cell sorting is shown in Figure 5-15. Limited finances meant that only one T cell subset could be analysed. CD8 T cells were chosen because this population exhibited the most suppression in the *in vitro* co-culture model. Mean purity of CD8 T cells recovered was 99.3% (range 98.9%-99.6%) and mean cell number was 250,000 (range 107000 - 404000). Cells were pelleted, resuspended in Tri-Reagent immediately after sorting and stored at -80°C prior to RNA extraction. The RNA Integrity Numbers (RIN) for the samples are shown in Table 5-2. The range of 7.7 to 9 all passed the quality check, based on the report by Schroeder et al. that RIN values of 7-10 indicate little / no degradation of RNA[370]. RNA sequencing was performed by Novogene (Bioinformatics Institute, Beijing, China) on a HiSeq 4000 platform with paired-end 150bp reads and a sequencing depth of 30M reads. Pair-ended 150bp reads were selected to enable more coverage of transcripts, and identification of alternative splicing[371]. The ENCODE Project Consortium has shown that 80% of transcripts that are expressed at more than 10 Fragments per kilobase of exon per million reads mapped (FPKM) can be accurately quantified with ~36M reads[372]. Data analysis was performed using the Novogene platform.

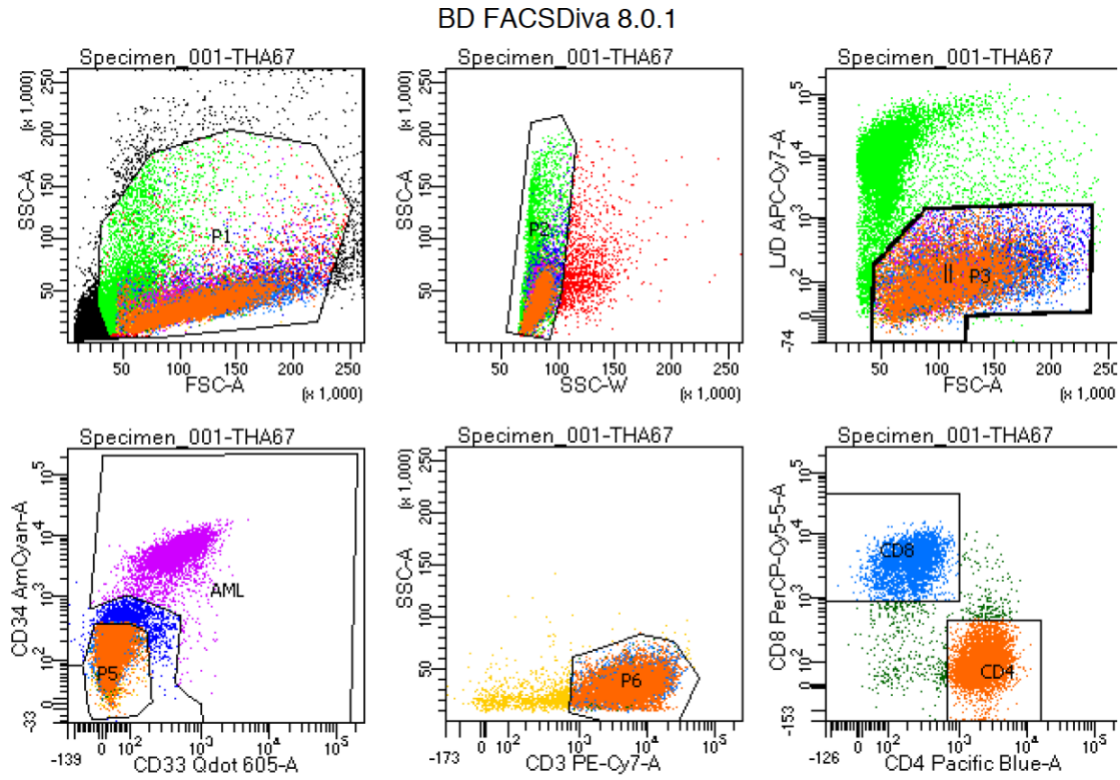


**Figure 5-15 Representative gating for cell sorting**

T cells were gated on CD3+ viable cells after exclusion of AML cells (CD33+ or CD34+) and HS5 cells (size). CD3+ T cells were then further distinguished as CD8+ and CD4+ T cells. Viable CD3+ CD8+ Cells were sorted for RNA extraction.

**Table 5-2 RNA Integrity Number for the CD8 T cell samples**

| Sample Name | RIN |
|---|---|
| Unstim 1 | 7.7 |
| Unstim 2 | 7.8 |
| Unstim 3 | 8.4 |
| TH1 | 8.7 |
| TH2 | 8 |
| TH3 | 8.7 |
| THA17 | 8.7 |
| THA47 | 8.7 |
| THA48 | 8.8 |
| THA67 | 9 |
| THA75 | 9 |

To assess the correlation and variation between samples subjected to the same culture conditions, the coefficient of determination ($R_2$) based on Pearson correlation coefficient of sample pairs were calculated. Table 5-3 shows the $R_2$ value ranged of 0.98 to 0.992 for unstimulated T cells, 0.988 to 0.992 for T cells stimulated with αCD3/CD28 beads in the presence of HS5 cells and 0.969 to 0.991 for T cells stimulated with αCD3/CD28 beads in the presence of AML cells with HS5 cells. Values were all above 0.92, which indicates a high correlation between samples in each culture condition. Consequently, the RNA sequencing results for samples within each condition were grouped together for analysis.

**Table 5-3 Coefficient of determination ($R_2$) values for sample pairs subjected to the same culture conditions**

| | Unstimulated T cells Replicate 1 | Unstimulated T cells Replicate 2 | Unstimulated T cells Replicate 3 |
|---|---|---|---|
| Unstimulated T cells Replicate 1 | 1 | | |
| Unstimulated T cells Replicate 2 | 0.992 | 1 | |
| Unstimulated T cells Replicate 3 | 0.981 | 0.98 | 1 |

| | Stimulated T cells + HS5 cells Replicate 1 | Stimulated T cells + HS5 cells Replicate 2 | Stimulated T cells + HS5 cells Replicate 3 |
|---|---|---|---|
| Stimulated T cells + HS5 cells Replicate 1 | 1 | | |
| Stimulated T cells + HS5 cells Replicate 2 | 0.988 | 1 | |
| Stimulated T cells + HS5 cells Replicate 3 | 0.992 | 0.989 | 1 |

| | Stimulated T cells + HS5 cells + AML017 | Stimulated T cells + HS5 cells + AML047 | Stimulated T cells + HS5 cells + AML048 | Stimulated T cells + HS5 cells + AML067 | Stimulated T cells + HS5 cells + AML075 |
|---|---|---|---|---|---|
| Stimulated T cells + HS5 cells + AML017 | 1 | | | | |
| Stimulated T cells + HS5 cells + AML047 | 0.981 | 1 | | | |
| Stimulated T cells + HS5 cells + AML048 | 0.981 | 0.986 | 1 | | |
| Stimulated T cells + HS5 cells + AML067 | 0.969 | 0.989 | 0.987 | 1 | |
| Stimulated T cells + HS5 cells + AML075 | 0.976 | 0.991 | 0.985 | 0.99 | 1 |

Unsupervised clustering of all samples based on FPKM and hierarchical clustering is illustrated schematically in the heatmap shown Figure 5-16. There was a clear separation between unstimulated $CD8_+$ T cells (unstim) and $\alpha$CD3/CD28 stimulated $CD8_+$ T cells in the presence of HS5 cells (TH). Using a false discovery rate (FDR) adjustment calculated with the Benjamin-Hochberg estimation method of *p* adjusted (adj) value <0.05, 11,264 differentially expressed genes (DEG) were identified and fold changes ranged from -8.9731 to 12.687 with 462 DEG greater than fold change of 5.

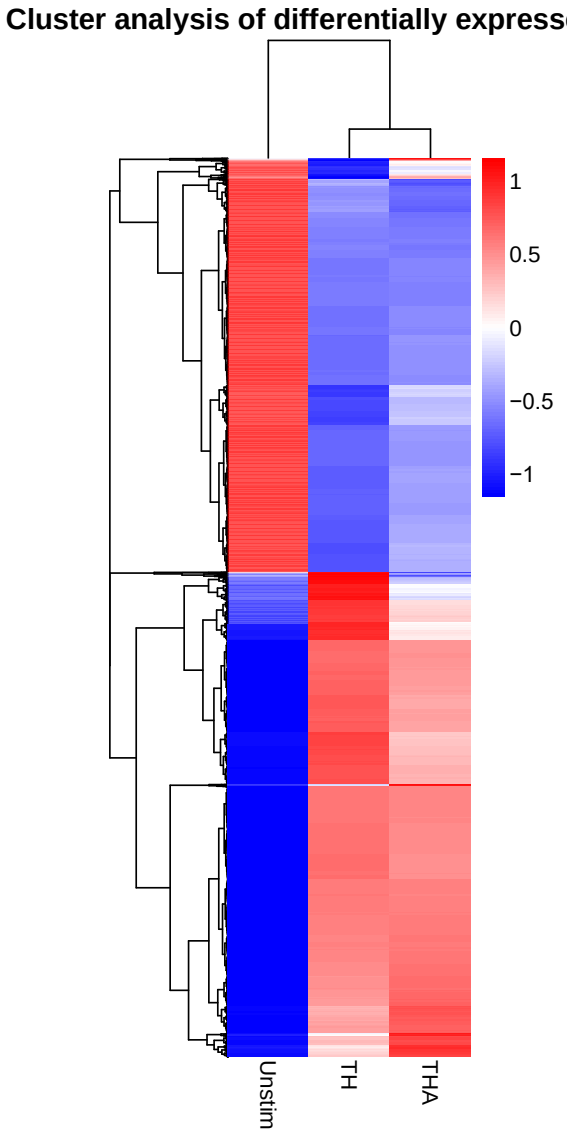


**Figure 5-16 Unsupervised cluster analysis of DEGs comparing unstimulated to αCD3/CD28 stimulated CD8+ T cells in the absence or presence of AML cells**

Gene expression levels (FPKMs) were obtained for each sample, and replicates of samples were grouped together as the coefficient of determination showed high correlation between replicate samples. Profiles for unstimulated CD8 T cells (unstim) were compared to CD8 T cells stimulated with αCD3/CD28 beads in cultures with HS5 cells in the absence (TH) or presence of AML cells (THA). Heatmap was created using unsupervised hierarchical clustering. Relative expression levels are depicted in colours from blue to red; downregulated genes are in blue and upregulated genes are in red.

Using the KEGG pathway database, the genes that were differentially expressed with a fold change of 5 or greater in the comparison of unstimulated CD8+ T cells to αCD3/CD28 stimulated CD8+ T cells in the presence of HS5 cells were grouped based on knowledge of their involvement

in specific cellular pathways. The top 10 most significantly enriched pathways are shown in the KEGG scatter plot in Figure 5-17. Of the 462 DEG, 25 are associated with the cytokine-cytokine receptor interaction pathway, 17 with the cell cycle pathway, 12 with the JAK/STAT signalling pathway, and 8 with the p53 signalling pathway. These are pathways where differences would be expected because proliferation, cytokine secretion, and death are features of $CD8_+$ T cells upon activation.

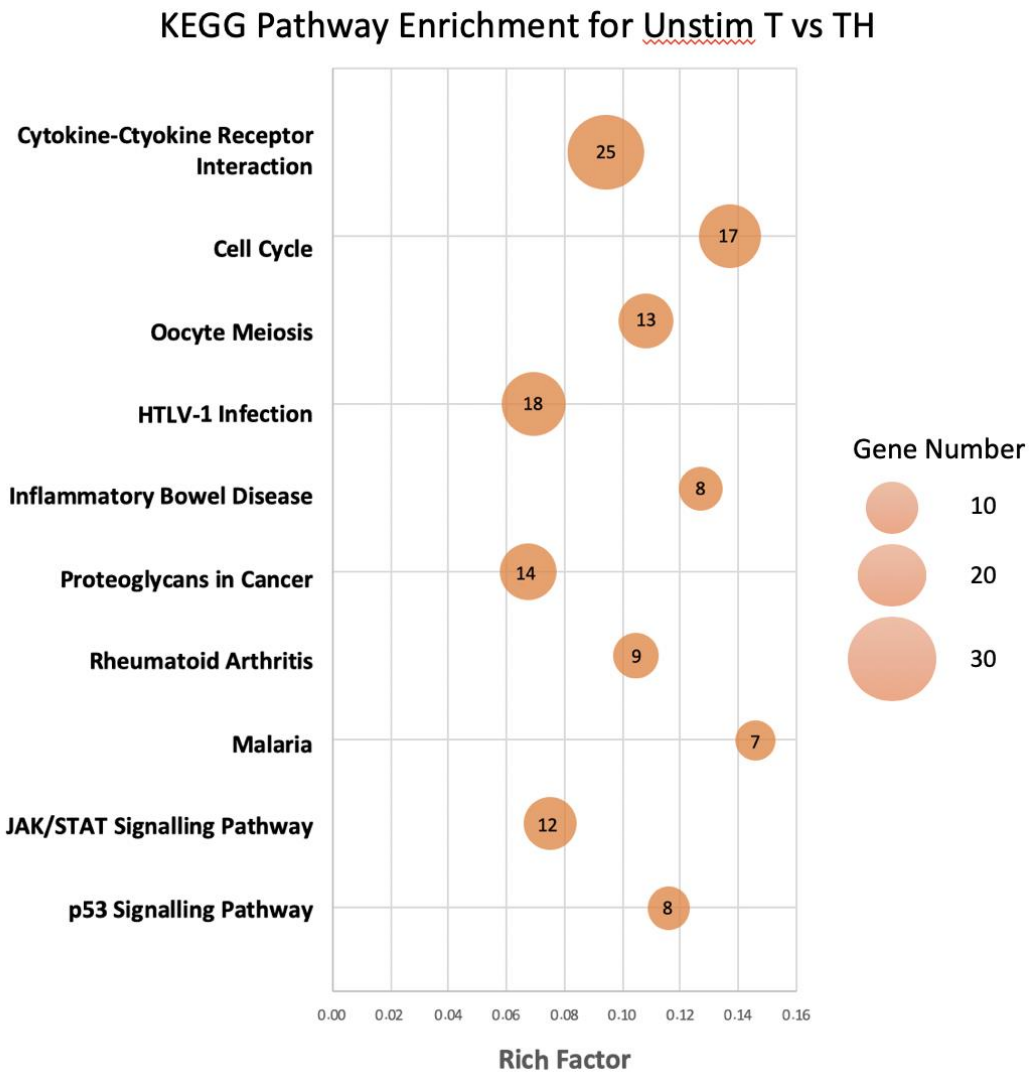


**Figure 5-17 Top 10 enriched pathways for genes with *p* adj <0.05 in the comparison of unstimulated $CD8_+$ T cells to αCD3/CD28 stimulated $CD8_+$ T cells with HS5 cells**

KEGG pathway enrichment analysis of DEG with a fold change of 5 or greater in unstimulated $CD8_+$ T cells compared to αCD3/CD28 stimulated $CD8_+$ T cells in the presence of HS5 cells. X-axis labels represent Rich factor, which is the ratio of differentially expressed gene numbers annotated in this pathway to all gene numbers annotated in this pathway. The greater the Rich

factor, the greater the degree of pathway enrichment. The number within the bubble represents the number of DEG involved in the particular pathway.

In contrast, to the 11,264 DEG with a *p* adj value <0.05 in the comparison of unstim and TH samples, only four DEG were found in the comparison of TH samples with the five αCD3/CD28 stimulated $CD8_+$ T cells in the presence of AML cells with HS5 cells (THA) samples. All had a fold change less than 2. They are listed in Table 5-4. This analysis showed that the immunosuppressive AML microenvironment does not dramatically alter the gene expression profile of αCD3/CD28 stimulated $CD8_+$ T cells.

**Table 5-4 Summary statistics of the four differentially expressed genes with p adj value <0.05 for CD8 T cells stimulated with αCD3/CD28 beads in the absence or presence of AML cells**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval | P*adj* (FDR) |
|---|---|---|---|---|---|
| BBC3 | 1735.42315 | 3555.27668 | -1.0347 | 3.11E-08 | 0.00072806 |
| ADGRD1 | 19.88707 | 68.0015409 | -1.7737 | 4.37E-08 | 0.00072806 |
| ZNF703 | 89.7265654 | 188.577147 | -1.0715 | 3.90E-06 | 0.043375 |
| AL391121.1 | 866.529132 | 1575.91787 | -0.86287 | 5.87E-06 | 0.048869 |

Of the four DEG identified, only BBC3 and ADGRD1 have documented roles in T cells. Expression of both was significantly down-regulated when $CD8_+$ T cells were stimulated in the presence of AML cells. BBC3, also known as p53 upregulated modulator of apoptosis, is a pro-apoptotic protein with a role in mediating p53-dependent and independent apoptotic pathways[373]. ADGRD1 is an orphan G-protein coupled receptor with a role in the adenyl cyclase pathway; furthermore, increased expression of ADGRD1 has been shown to increase intracellular cAMP levels which modulates T cell function[374, 375]. Little is known about the other two DEG, both of which were also downregulated. ZNF703 encodes a zinc finger protein, and its upregulation has been associated with promotion of tumour cell proliferation[376]. AL391121.1 is a long non-coding

RNA (lncRNA), and reduced levels have been associated with lower mRNA levels of adjoining genes[377].

Although the expression levels of only four genes remained statistically different after the false discovery correction of the comparison of αCD3/CD28 stimulated $CD8_+$ T cells with HS5 cells in the absence or presence of AML cells, there were 819 genes with a *p* value <0.05. The substantial loss of statistical significance after the false discovery adjustment was due to the relatively small differences in expression levels, with fold changes ranging from -4.4056 to 7.5951, combined with the large number of multiple comparisons performed for the 33,088 different genes. To illustrate the impact, in the comparison of unstimulated $CD8_+$ T cells to αCD3/CD28 stimulated $CD8_+$ T cells with HS5 cells, where fold changes were larger (ranged from -8.9731 to 12.687) for 32,489 different genes, the 11,264 genes with *p* adj <0.05 was not substantially lower than the 12,715 genes with *p* value <0.05.

The false discovery adjustment method used in this study (the Benjamini-Hochberg procedure) is less conservative than familywise error rate procedures such as the Bonferroni correction. However, there is still a risk of dismissing true differences as non-significant when *p* values are not very low and sample size is small, as in the case of the comparison of the TH samples with the THA samples in this study. Although to be viewed with caution, provided a reader is aware of the number of multiple comparisons performed (in this case the number of different genes), data analysis based on non-adjusted *p* values can be undertaken[378]. Notably, of the 819 genes with a *p* value <0.05, 547 genes (67%) were expressed at lower levels in THA samples compared to TH samples. This perhaps suggests lower overall transcription activity of $CD8_+$ T cells in the presence AML cells. Pathway analysis was performed on these 819 genes with a *p* value <0.05, and the top 10 most significantly enriched pathways are shown in the KEGG scatter plot in Figure 5-18. Seven pathways are of particular potential interest. Expression levels of the five groups of genes associated with the metabolic pathways and the signalling pathways PI3K-Akt, MAPK, Rap1, and Ras provide an indication of general cellular activity. Two other groups of genes associated with actin cytoskeleton and cytokine-cytokine receptor interactions provide an indication of activity associated with specific T cell functions. Genes with a *p* value <0.05 from these seven pathways are listed, with information on read counts and fold change, in Appendix A.

For the 39 genes associated with metabolic pathways, 26 were expressed at lower levels in the THA samples. This suggests less metabolic activity in $CD8_+$ T cells stimulated in the presence of AML cells. Two of these genes were Cox-1 and Cox-2, whose expression is known to be regulated by the transcription factor NFκB[379, 380]. Their expression at lower levels is consistent with the expectation of reduced NFκB activity in T cells that exhibit less activation in the AML microenvironment. Most of the genes from the signalling pathways were also expressed at lower levels by $CD8_+$ T cells stimulated in the presence of AML cells. For the PI3K-Akt pathway, 15 of the 20 genes were expressed at lower levels; for the MAPK pathway, 15 of 17; for the Rap1 pathway, 11 of 14 and for the Ras pathway, 10 of 12. Noteworthy genes within these signalling pathway groups were JunD, NFATc and c-Rel because their expression is known to be regulated by the transcription factors AP-1, NFAT, and NFκB that play roles in activation of T cells[381, 382, 383]. Another gene of interest that was expressed at lower levels in the presence of AML cells is PKCα, which is known to be required for the activation of the IKK complex leading to activation of NFκB[384].

Like the genes associated with general cellular activities, the majority of genes from pathways with specific relevance to T cell function were also expressed at lower levels in the THA samples. For the genes in the cytokine-cytokine receptor interaction pathway, 10 of 14 were expressed at lower levels by $CD8_+$ T cells stimulated in the presence compared to absence of AML cells. Of these, previous studies have reported that TNFRSF18, CD70, and OX40 are upregulated upon T cell activation[385, 386, 387]. For the 15 genes in the regulation of the actin cytoskeleton pathway, 12 were expressed at lower levels in the THA samples. Differential expression of genes in this pathway is perhaps consistent with defective T cell synapse formation observed in the AML microenvironment[244]. Of these genes, WAVE1 is known to be necessary for actin polymerisation at the immunological synapse[388]. Also IRSp53, in association with WAVE2, promotes F-actin remodelling[389], and suppression of the WAVE2 complex has been shown to result in impaired immunological synapse formation due to the lack of F-actin[390].

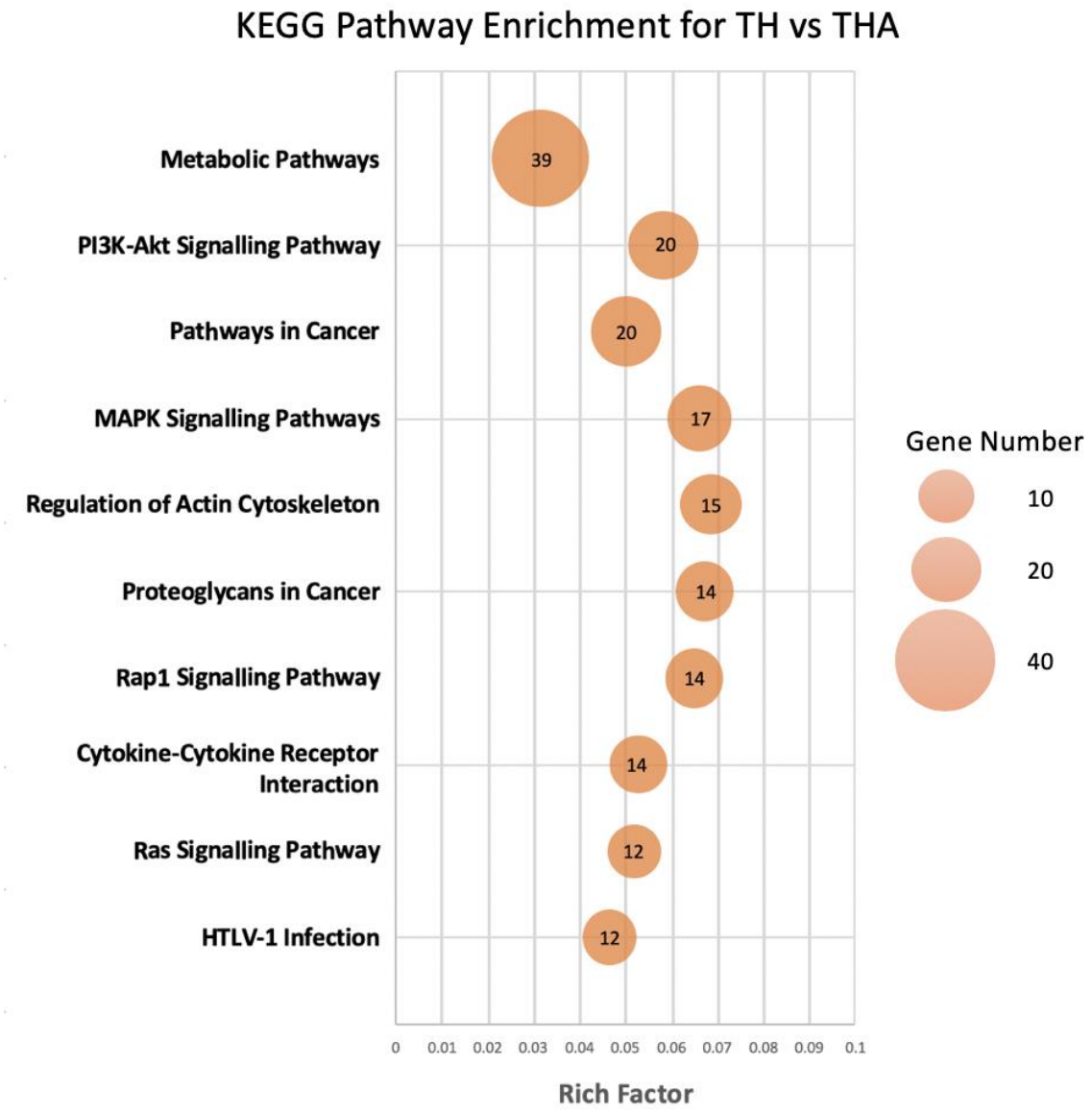


**Figure 5-18 Top 10 enriched pathways for genes with p<0.05 in the comparison of αCD3/CD28 stimulated CD8+ T cells in the absence or presence of AML cells**

KEGG pathway enrichment analysis of the 819 genes with $p$<0.05 in the comparison of αCD3/CD28 stimulated CD8+ T cells with HS5 cells in the absence (TH) or presence of AML cells (THA). X-axis labels represent Rich factor, which is the ratio of differentially expressed gene numbers annotated in this pathway to all gene numbers annotated in this pathway. The greater the Rich factor, the greater the degree of pathway enrichment. The number within the bubble represents the number of DEG involved in the particular pathway.

## 5.3 Discussion

The work presented in this chapter was designed to investigate the mechanistic basis for suppression of T cell activity when co-cultured with AML cells and HS5 cells in the *in vitro* model. An understanding the relative importance of the various mechanisms of inhibition exploited by AML cells will be crucial to the successful implementation of T cell-based immunotherapies.

Several reports have previously explored the role of soluble factors in the immunosuppressive microenvironment created by primary AML cells using transwell inserts, but the role of contact between AML cells and T cells was not assessed simultaneously[212, 276]. Studies were therefore performed to assess the relative contribution of soluble factors and contact-dependent mechanisms to the inhibition of T cell activity observed. A role for soluble factors in the immunosuppression of T cells has been well documented[212, 219, 221]. Findings from the transwell co-cultures performed in this study were consistent with the notion that soluble factor mediated immunosuppression plays a role. However, the work presented in this chapter also demonstrated the importance of contact between T cells and AML cells to inhibition, which highlights the multifactorial nature and complexity of the immunosuppressive AML microenvironment. The *in vitro* three-cell co-culture model represents a good platform for assessing immune suppression in the AML microenvironment because both soluble factors and contact-dependent mechanisms can exert their effect.

The finding that cell contact plays an important role has implications for the study of suppression of CMV-specific T cells in the AML microenvironment presented in Chapter 4 Section 4.2.1.2. Due to the need for CMV antigen processing and presentation, these experiments were performed using supernatant from AML cell and HS5 cell co-cultures to ensure the required contact between T cells and dendritic cells was not compromised. Although inhibition of T-cell activation was observed, the extent of suppression in the AML microenvironment may have been under-estimated because mechanisms involving contact between T cells and AML cells were not assessed.

The possibility that the immune checkpoint receptor PD-1 and its ligand PD-L1 contributed to the contact dependent suppression of T cells in the presence of AML cells was evaluated. Immunophenotyping showed that expression of the PD-1 and PD-L1 was low on *ex vivo* T cells and AML cells respectively, but substantially increased during *in vitro* co-culture. Upregulation of PD-L1 expression by primary AML cells in the presence of stimulated T cells was unsurprising. The IFN-γ produced by stimulated T cells is known to promote PD-L1 expression[391]. Studies specifically in the context of primary AML cells have shown that PD-L1 is not always constitutively expressed, but can be induced by IFN-γ[230, 231, 392]. Despite expression of the receptor and ligand by cells in the *in vitro* co-culture model, treatment with anti-PD-1 and anti-PD-L1 antibodies did not reverse T cell suppression in the AML microenvironment. This finding was observed for T cells from three different healthy individuals using one source of primary AML cells that were known to express high levels of PD-L1 and promote potent T cell suppression. Substantiation of this finding would require further experiments using primary AML cells from additional patients.

A potential reason why blocking the interaction of PD-1 and PD-L1 did not counteract immunosuppression in the *in vitro* model might be because these molecules were not expressed by cells at the start of the co-culture. Instead, their expression was induced as a consequence of the co-culture. In this setting, PD-1 behaved as a marker of activation; being induced in response to T-cell stimulation. Consistent with this interpretation, the induced expression of PD-1 was lower on T cells in the AML microenvironment, reflecting their diminished activation status. Other studies that show blocking PD-1 and PD-L1 antibodies reverse inhibition of T cell function have used cells that already expressed high levels of PD-1 and PD-L1[231, 247]. Attempts were made to induce PD-1 expression on T cells by pre-treatment with anti-CD3/CD28 beads and then co-culture with AML cells. However, once stimulated, subsequent exposure to the AML microenvironment did not curtail activation or cytokine production by T cells (data not shown). Another approach that could be considered would be to isolate PD-1 expressing T cells from *ex vivo* samples for testing in the *in vitro* co-culture model. The potential role of other immune checkpoints, such as CTLA-4, also needs to be explored.

Another potential mechanistic basis for the contact dependent suppression of T cells in the presence of AML cells was explored by treatment with the immunomodulatory drug lenalidomide, which has previously been shown to reverse defective immune synapse formation between T cells and malignant cells of the lymphoid lineage[359]. However, lenalidomide did not substantially improve the function of αCD3/CD28 stimulated T cells in the AML microenvironment. A potential limitation of this experiment design was that stimulation of the CD28 pathway may thwart the possibility of observing further enhancement of T-cell function because lenalidomide promotes T cell function is via tyrosine phosphorylation of CD28 and subsequent activation of NFκB[357]. An attempt was therefore made to use αCD3 stimulation alone. This led to the surprising, and potentially important, observation that activation and cytokine production by αCD3 stimulated T cells was not suppressed in the AML microenvironment. Targeting of the CD28 pathway by the immunosuppressive mechanisms utilised by AML cells was also suggested by findings from experiments with CMV-specific T cells presented in Chapter 4 Section 4.2.1.2. These antigen-specific T cells with memory or effector phenotype do not express CD28[331, 334] and were found to be less susceptible to suppression in the AML microenvironment than T cells from the same individual stimulated with αCD3/CD28 beads. If suppression preferentially targets the CD28 pathway, it suggests that priming of naive leukaemia reactive T cells and activation of quiescent memory T cells, that by definition express CD28, will be particularly susceptible to suppression in the AML microenvironment.

The lacklustre impact of PD-1 and PD-L1 blocking antibodies and lenalidomide on reversal of T cell suppression in the *in vitro* model is consistent with modest clinical results of monotherapy with these agents[253, 254, 257]. Gene expression profiling of T cells was performed by RNA-seq to search for other mechanisms underlying diminished activity in the AML microenvironment. The global gene expression profiling comparing $CD8^+$ T cells co-cultured in the presence of HS5 cells or HS5 cells with AML cells revealed that only four genes showed statistically significant differences in expression. The pro-apoptotic gene BBC3 was expressed at lower levels by T cells stimulated in the presence of primary AML cells with HS5 cells. This finding is consistent with two previous reports of reduced apoptosis of stimulated T cells in the AML microenvironment[212, 282].

ADGRD1 was also expressed at lower levels by αCD3/CD28 stimulated T cells cultured in the presence of AML cells with HS5 cells. Previous studies have demonstrated that activated T cells have elevated levels of cAMP[393, 394]. A potential explanation for the lower levels of ADGRD1 transcription may be that expression is correlated to the activation status of T cells, as ADGRD1 signals through the adenyl-cyclase pathway resulting in accumulation of cAMP[374].

Although only four differentially expressed genes were identified, 819 genes had a *p* value <0.05 but the data was insufficiently robust to remain statistically significant after adjustment to correct for multiple comparison testing. A KEGG pathway analysis of these genes was performed. Of potential interest were those that grouped into pathways associated with metabolic activity and cell signalling, together with the observation that the majority were expressed at lower levels in the presence of AML cells. Several of the genes expressed at lower levels in these pathways; namely Cox-1, Cox-2, NFATc, c-rel, PKCα, and JunD, are linked with activity of the transcription factors NF-κB and NFATc which have previously been shown to be less active in T cells exposed to the AML microenvironment[212].

The subtle nature of differences between the gene expression profiles of $CD8^+$ T cells stimulated in the presence of HS5 alone or AML cells with HS5 cells indicates that there is no dramatic change in the properties of T cells in the AML microenvironment. No evidence of a role for inhibitory T cell signalling pathways was obtained. Instead, findings point to lower levels of overall activity when T cells are stimulated in the AML microenvironment.

There were limitations to the gene expression profiling performed. The small sample numbers compromised ability to detect differentially expressed genes in the context of the subtle differences between T cells stimulated in the absence or presence of AML cells. Although pathway analysis of the 819 genes with $p<0.05$ revealed some seemingly rationale differences, the data was not adequate for robust statistical analyses. The power to discover differences in expression of novel transcripts and genes expressed at low levels is dependent on the read depth; deeper level of sequencing would be more precise and more transcripts would be detected[395].

Increasing the read depth from 30M to 100M may allow identification of novel transcripts and differences between genes expressed at low levels. Although the RNA-seq analysis performed assessed global differential gene expression, there may be advantages to a more focused approach, such as targeted RNA-seq or qRT-PCR of selected genes. Reducing the number of multiple comparisons performed increases the likelihood of detecting statistically significant differences. Specifically, analysis of genes associated with the CD28 pathway could be considered, given some indications that suppression might target this pathway. Any form of RNA-based analysis may not accurately portray levels of protein expression, therefore techniques such as western blot could be considered[396].

In summary, this chapter describes exploration of the contribution of soluble factors and cell contact dependent mechanisms to the inhibition of T cell activity in the AML microenvironment and shows a multi-factorial basis. Anti-PD-1 and anti-PD-L1 blocking antibodies or lenalidomide did not reverse suppression of T cell activity. Gene expression profiling of T cells indicated overall lower levels of T cell activity in the AML microenvironment. Some findings suggest that inhibition of T cell function in the AML microenvironment may target the CD28 pathway.

# Chapter 6 Conclusions and future studies

## 6.1 Establishment of an *in vitro* co-culture model for evaluating T-cell activity in the AML microenvironment

One of the major factors contributing to the poor prognosis[85] and the relatively high incidence of disease relapse after treatment[85] is the immunosuppressive microenvironment created by AML. The first studies to investigate the mechanisms exploited by AML cells for protection from the anti-leukaemia immune response reported a role for soluble factors[212, 213, 276]. Subsequent studies have shown that multiple mechanisms of immune escape can be utilised by AML[220, 221, 227, 230, 397]. The importance of immune escape in AML has been recently highlighted by the finding that disease relapse after allogeneic HSCT is associated with dysregulation of pathways that influence immune function[210, 211].

Studying the impact of the AML microenvironment on immune responses and evaluation of strategies to circumvent immunosuppression has been impeded by the poor viability of primary AML cells during *in vitro* culture. AML is a disease of the BM, where the leukaemic cells are sustained by complex interactions involving direct contact and cytokine and chemokine signalling with components of the BM environment including stromal cells, adipocytes and endothelial cells[103, 283, 398, 399]. A key aspect of the work reported in this thesis was the development of an *in vitro* co-culture model to recapitulate the physiological AML microenvironment in which the impact of immunosuppression by AML cells on T-cell functions could be assessed. The co-culture model established builds on the *in vitro* model previously described by Garrido et al in which primary AML cells are protected from apoptotic cell death by culture with the human stromal cell line HS5.[283] The sustainment of primary AML cells was reproduced, and the model then developed by addition of allogeneic T cells. Inhibition of T-cell activity in the presence of AML cells with HS5 cells was demonstrated and the model used to investigate the mechanistic basis and to test strategies for preventing inhibition.

The three-cell co-culture model was established in a 24-well format and subsequently successfully scaled down to a 96-well plate format, which illustrates its potential for use as a high-

throughput screening platform. The model also has scope for further development. The role of cells types other than stromal cells to the immunosuppressive AML microenvironment, such as adipocytes, endothelial cells, and fibroblasts[90], could be investigated. Use of e*x-vivo* patient mesenchymal stromal cells may improve recapitulation of the BM niche in the disease state because studies have shown AML cells reprogram stromal cells to favour their survival[228, 400]. If combined with use of e*x-vivo* patient T cells, the model would represent a completely autologous patient-specific screening platform.

## 6.2 Use of the *in vitro* co-culture model to show suppression of T-cell responses in the AML microenvironment

The presence of AML cells obtained from eleven different patients who experienced disease relapse after allogeneic HSCT was found to suppress the activation of both $CD4^+$ and $CD8^+$ T cells and secretion of the cytokines IFN-$\gamma$, TNF-$\alpha$ and IL-4. A panel of five immortalised AML cell lines representing a range of different disease subtypes were also tested. Although HS5 cells were not required to promote survival of the AML cells in this setting, the finding that there was still a trend for greater suppression of T-cell function in the presence of AML cells with HS5 cells compared to AML cells alone shows that these stromal cells contribute more to the microenvironment than just sustaining AML cells.

Co-culture with HS5 cells alone did not suppress T cell-activity. A speculated basis for the synergistic interaction between AML cells and HS5 cells that enhances inhibition of T-cell responses is metabolic reprogramming of AML cells, because a shift towards aerobic glycolysis in the presence of HS5 cells has been previously reported[401]. A consequence of upregulated aerobic glycolysis in AML cells is acidosis of the local environment. High lactic acid concentration within the tumour microenvironment is known to prevent lactic acid export from T cells, which in turn inhibits T-cell proliferation, cytokine secretion, and cytotoxicity activity[402].

## 6.3 Use of the *in vitro* co-culture model to investigate mechanisms of T-cell suppression in the AML microenvironment

Although it is known that AML cells employ multiple mechanisms to suppress T-cell activity, *in vitro* investigations reported to date have studied T-cell responses only in the presence of AML cells. Having shown that presence of HS5 stromal cells enhances the immunosuppression of T-cell activity by AML cells, the mechanisms of inhibition operating in the three-cell *in vitro* co-culture model were explored. The enhanced suppression of T-cell activity by AML cells promoted by presence of HS5 cells did not require contact between AML cells and the HS5 cells. In contrast, the enhancement of suppression by HS5 cells was greatest when there was contact between AML cells and T cells.

Soluble factors known to be produced by HS5 cells that could potentially influence the immunosuppressive properties of AML cells are IL-6, GM-CSF and SCF, as they have been shown to promote AML survival and tumourigenesis[110, 111, 113]. A candidate for the contact-dependent suppression of T-cell activity by AML cells is the binding of PD-1 by PD-L1. T-cell mediated inhibition by the PD-1/PD-L1 interaction has been demonstrated in studies of both AML patients and murine AML models.[231, 251, 403] However, although the expression of PD-1 and PD-L1 by cells in the co-culture model was observed, the use of αPD-1 and αPD-L1 blocking antibody did not counteract contact mediated suppression. A speculation can be that the inhibition observed was not mediated by PD-1 and PD-L1 interaction, as a recent study by Minuzo et al has demonstrated a significant attenuation in PD-1 mediated inhibition in the presence of strong CD28 co-stimulation[404]. Therefore, taken together with the αCD3/CD28 beads stimulation method used in our experiments, the contact-mediated inhibition observed may not be mediated by PD-1 and PD-L1 interaction, thus the use of αPD-1 and αPD-L1 blocking antibody did not counteract inhibition observed. A limitation to the αPD-1 and αPD-L1 blocking antibody study is the low sample numbers. To further substantiate the observation, additional primary AML samples and healthy donors will be required, as only one source of primary AML cells with high levels of PD-L1 expression were used.

Another candidate mechanism for contact-dependent suppression of T-cell function may relate to the reported observation that patient T cells fail to form effective synapses with autologous AML cells.[244] The immunomodulatory agent lenalidomide has previously been shown to overcome inhibition of T-cell function by reversing defective immune synapse formation[359, 405], however, addition of lenalidomide to the *in vitro* co-culture model did not overcome suppression of T-cell activity in the AML microenvironment.

Further study is required to determine the mechanisms of T-cell suppression operating in the *in vitro* co-culture model. There are several other known mechanisms of suppression that have not been explored. An example of a cell contact dependent mechanism is membrane proteinase 3 (mP3) that is known to be expressed on AML cells and has been shown to inhibit T-cell proliferation[406]. Enzyme mediated suppression involving IDO, arginase II or CD39 could be investigated using blocking antibodies or small molecule enzymatic inhibitors[221, 227, 406, 407]. Another potential strategy is the use of imatinib, a tyrosine kinase inhibitor, as it has been shown to both reduce IDO expression in the context of a solid tumour and inhibit c-kit tyrosine phosphorylation for c-kit positive AML cells, as c-kit is known to be produced by HS5 cells[408, 409]. Therefore, imatinib may also be a potential candidate as it targets both a potential suppressive mechanism and proliferation of AML cells.

An approach taken to assess the global impact of exposure to the immunosuppressive AML microenvironment on T cells was to perform gene expression profiling. The differential gene expression analysis of results obtained by RNA sequencing revealed only subtle differences between $CD8^+$ T cells co-cultured with HS5 cells in the absence or presence of AML cells. No evidence was obtained to indicate that T-cell suppression in the AML microenvironment involved signalling pathways known to be involved in down-regulating T-cell activity. This perhaps points to the mechanisms of immunosuppression producing an overall slowing of T-cell activation and responsiveness in the AML microenvironment, rather than overt changes in behaviour. Limitations to the RNA-seq experiment were small sample number, which compromises robust statistical analyses, and the 30M read depth conducted that precludes detection of genes expressed at low level.

A previous study of the impact of presence of primary AML cells on pathways involved in T-cell activation and proliferation in response to stimulation for 16 hours with anti-CD3/CD28 beads was reported by Buggins et al[212]. Western blotting was used to assess protein expression levels and phosphorylation status, and confocal microscopy was used to monitor nuclear translocation of transcription factors. These analytical approaches showed that AML cells inhibited phosphorylation of the retinoblastoma family of proteins and induction of c-Myc, which would lead to suppressed cell cycle progression, and inhibited nuclear translocation of NF-κB and NFATc, which would lead to reduced T-cell function. Activity of these pathways in T cells was still induced, but delayed in the presence of AML cells. This previous observation is consistent with the results of the gene expression profiling reported here that suggests the AML microenvironment may slow the T-cell response to stimulation, but not radically alter behaviour. The gene expression profiling was performed on T cells after stimulation for 48 hours. It may be informative to undertake the analysis across a time course to determine if there is a global delay in expression of genes associated with T-cell activation in the AML microenvironment.

Two observations were made during this study that indicate a role for the CD28 signalling pathway in the suppression of T cells in the AML microenvironment. Firstly, in contrast to stimulation with αCD3/CD28 beads, T cells stimulated with αCD3 beads were not suppressed in the AML microenvironment. Secondly, CMV-specific T cells of memory / effector phenotype that by definition do not require co-stimulation via CD28 were also not suppressed in the AML microenvironment. Differences in expression of genes associated with the CD28 pathway were not found. However, the low number of samples analysed, single time point studied, and large number of multiple comparisons performed may have compromised detection. Alternative approaches that could be utilised to explore the role of the CD28 pathway are targeted RNA-seq or proteomics techniques, such as gel-based quantification, mass spectrometry, or protein array focusing only on genes / proteins known to be associated with CD28 signalling.

## 6.4 Use of the *In vitro* co-culture model to show that novel T-cell immunotherapies are suppressed in the AML microenvironment

A main objective of this study was to test the hypothesis that the efficacy of T-cell mediated immunotherapies for AML are diminished by the environment conditioned by the disease. There is considerable interest and effort directed to the development of novel immunotherapeutic approaches for treatment of AML, spurred by realisation of the important role played by donor-derived T cells in the GvL effect after allogeneic HSCT. [146, 273] *In vitro* studies demonstrating the anti-leukaemic activity of novel immunotherapies such as LAA-specific T cells, CD123 CAR T cells and exploitation of the anti-leukaemic activity of γδ T cells are encouraging[54, 194, 306, 315]. However, these studies have been performed using AML cells in isolation as targets for functional assays. Given the finding in this study that activity of αCD3/CD28 stimulated T cells is significantly curtailed when AML cells were present in the context of their microenvironment with stromal cells, it was important to re-evaluate the efficacy of T-cell mediated immunotherapies in conditions that more closely recapitulate the AML microenvironment.

Interestingly, it was found that the killing of AML cells by *in vitro* expanded Vδ2+ T cells and αCD123 CAR T cells was reduced in the presence of HS5 cells. In addition, levels of cytokine produced by αCD123 CAR T cells were significantly reduced, but this was not the case for Vδ2+ T cells. Limitations to the Vδ2+ T-cell study are the low number of replicates and number of donors used. The Vδ2+ T-cell experiments were performed in the final months in the lab, therefore, only 3 repeats were performed as the time used to optimise the *in vitro* expansion of γδ T cells and the three-cell co-culture system to a 96-well plate format were longer than anticipated. In addition, the ability to expand γδ T cells using the *in vitro* method varies between healthy donors (John Maher; personal communication). Consequently, the priority was given to the healthy donor with highest number of γδ T cells for *in vitro* expansion of γδ T cells. Therefore, to further substantiate our findings, further experiments using additional healthy donors will be required.

Although there has been much work in recent years in the advancement of Vδ2+ T-cell therapies and CAR T-cell therapies against AML[54, 192, 315]. this is believed to be the first time that these novel T-cell based immunotherapies have been evaluated in an *in vitro* model that recapitulates the immunosuppressive conditions. Findings obtained represent preliminary evidence that the potential efficacy of these novel T-cell immunotherapies being developed to treat AML may be compromised in the AML microenvironment.

## 6.5 Summary and implications

In summary, a three-cell *in vitro* co-culture model was successfully established and used to investigate T-cell suppression in the AML microenvironment. T cells stimulated with αCD3/CD28 beads in the presence of the stromal cell line HS5 with AML cells were significantly more suppressed compared to AML cells alone, indicating an enhanced immunosuppressive microenvironment. Contact between AML cells and T cells played a pivotal role in the suppression observed but was not mediated by the PD-1 / PD-L1 interaction. The gene expression profile of αCD3/CD28 stimulated T cells was not dramatically different in the AML microenvironment, which suggests reduced but not altered activity. Some evidence that suppression may target the CD28 pathway was obtained. A key finding reported for the first time was that efficacy of novel T-cell based immunotherapies being developed to treat AML are suppressed in the AML microenvironment.

The findings of the research reported here come at an important time. The prognosis for AML patients that relapse after allogeneic HSCT is very poor, so there is an unmet need for novel therapies to treat the disease. There are several promising novel immunotherapeutic options being developed, but the negative impact of the immunosuppressive AML microenvironment on their efficacy will need to be circumvented. One implication is to emphasise the importance of reducing disease burden as much as possible before treatment with immunotherapies so that the impact of the immunosuppressive AML microenvironment is limited. Another implication is realisation of the need to combine immunotherapy with treatments to counteract the immunosuppressive AML microenvironment. There is much enthusiasm for use of immune checkpoint inhibitors, but findings presented here indicate that blocking antibodies specific for PD-1 and PD-L1 may not be the hoped-for solution. To better combat the immunosuppressive AML microenvironment, a greater understanding of the interactions between AML cells and other cell types within the bone marrow niche is required. This study has highlighted that the communication between stromal cells and AML cells enhances the immunosuppressive microenvironment. Targeting this interaction to disrupt the microenvironment may represent a

strategy to prevent immunosuppression and achieve successful implementation of immunotherapies.

The *in vitro* co-culture model developed in this study represents a tool for testing the combination of immunotherapies with strategies to circumvent suppression in the AML microenvironment. An aspect not explored in this study is whether the efficacy of immunotherapies and impact of the immunosuppressive microenvironment varies for different AML types. It may be important to have an informed choice of immunotherapy and strategy to overcome immunosuppression that is tailored to the patient profile, based on AML type and immune competence. Further investigations of these areas are warranted.

# Appendix A.

Details of the genes with a *p* value <0.05 from the seven signalling pathways are listed below

**MAPK Signalling Pathway**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| DSUP8 | 608.196723 | 960.227584 | -0.65884 | 0.00070205 |
| NFATc1 | 4960.56677 | 7349.97998 | -0.56724 | 0.0069712 |
| PDGFA | 1471.68087 | 2606.30843 | -0.82454 | 0.0070196 |
| JUND | 25375.0433 | 35494.6155 | -0.48419 | 0.0089821 |
| PKCa | 2229.91539 | 3253.988 | -0.54522 | 0.0094309 |
| NR4A1 | 5061.87014 | 8376.42643 | -0.72666 | 0.015364 |
| FGFR3 | 108.13653 | 156.530045 | -0.53359 | 0.023197 |
| GADD45A | 1241.02477 | 2160.39021 | -0.79976 | 0.024972 |
| DUSP10 | 259.27083 | 350.562766 | -0.43521 | 0.033176 |
| PTPN7 | 7894.21505 | 10341.7136 | -0.38961 | 0.03381 |
| MAP2K3 | 3667.59754 | 4847.699 | -0.40246 | 0.035166 |
| PLA2G4A | 24.2674151 | 52.2128014 | -1.1054 | 0.038694 |
| ELK4 | 2355.46409 | 1714.70055 | 0.45805 | 0.038754 |
| FGF18 | 5.26605127 | 0.99023993 | 2.4109 | 0.039836 |
| FGFR1 | 492.760113 | 646.624624 | -0.39204 | 0.044063 |
| DUSP7 | 1985.68662 | 2565.99878 | -0.36988 | 0.049214 |

**PI3K-Akt Signalling Pathway**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| PGF | 38.3464829 | 89.7397492 | -1.2267 | 1.24E-05 |
| EFNA2 | 7.01398626 | 18.6352223 | -1.4097 | 0.0064619 |
| PDGFA | 1471.68087 | 2606.30843 | -0.82454 | 0.0070196 |

| | | | | |
|---|---|---|---|---|
| PKCa | 2229.91539 | 3253.988 | -0.54522 | 0.0094309 |
| NR4A1 | 5061.87014 | 8376.42643 | -0.72666 | 0.015364 |
| GNG3 | 6.05761415 | 14.9544157 | -1.3037 | 0.018658 |
| RBL2 | 2482.01075 | 1773.52289 | -0.44378 | 0.019463 |
| ITGB3 | 8.08405629 | 1.96550176 | 2.0402 | 0.019543 |
| IL7R | 4627.35783 | 3099.76193 | 0.57803 | 0.020137 |
| EPHA2 | 41.2628483 | 83.0597597 | -1.0093 | 0.020169 |
| FGFR3 | 108.13653 | 156.530045 | -0.53359 | 0.023197 |
| MDM2 | 1643.78879 | 2416.70791 | -0.55602 | 0.030649 |
| CDKN1A | 3780.45991 | 5211.4275 | -0.46312 | 0.032384 |
| FGF18 | 5.26605127 | 0.99023993 | 2.4109 | 0.039836 |
| SYK | 25.0902033 | 7.41747753 | 1.7581 | 0.04163 |
| LAMA5 | 82.5304512 | 117.493122 | -0.50958 | 0.041732 |
| PHLPP1 | 982.025895 | 1289.45949 | -0.39293 | 0.041789 |
| FGFR1 | 492.760113 | 646.624624 | -0.39204 | 0.044063 |
| GNG7 | 80.9381893 | 48.0772635 | 0.75147 | 0.049226 |
| CREB5 | 40.3181716 | 67.7052808 | -0.74784 | 0.049894 |

**Rap1 Signalling Pathway**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| PGF | 38.3464829 | 89.7397492 | -1.2267 | 1.24E-05 |
| ADORA2A | 87.5277401 | 158.929406 | -0.86057 | 0.00031194 |
| RAP1GAP | 125.363972 | 214.040714 | -0.77176 | 0.0007009 |
| EFNA2 | 7.01398626 | 18.6352223 | -1.4097 | 0.0064619 |
| PDGFA | 1471.68087 | 2606.30843 | -0.82454 | 0.0070196 |
| PKCa | 2229.91539 | 3253.988 | -0.54522 | 0.0094309 |
| BCAR1 | 162.223324 | 263.954824 | -0.70231 | 0.018368 |
| ITGB3 | 8.08405629 | 1.96550176 | 2.0402 | 0.019543 |

| | | | | |
|---|---|---|---|---|
| EPHA2 | 41.2628483 | 83.0597597 | -1.0093 | 0.020169 |
| FGFR3 | 108.13653 | 156.530045 | -0.53359 | 0.023197 |
| CDH1 | 363.458074 | 116.286325 | 1.6441 | 0.030439 |
| MAP2K3 | 3667.59754 | 4847.699 | -0.40246 | 0.035166 |
| FGF18 | 5.26605127 | 0.99023993 | 2.4109 | 0.039836 |
| FGFR1 | 492.760113 | 646.624624 | -0.39204 | 0.044063 |

**Ras Signalling Pathway**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| PGF | 38.3464829 | 89.7397492 | -1.2267 | 1.24E-05 |
| EFNA2 | 7.01398626 | 18.6352223 | -1.4097 | 0.0064619 |
| PDGFA | 1471.68087 | 2606.30843 | -0.82454 | 0.0070196 |
| PKCa | 2229.91539 | 3253.988 | -0.54522 | 0.0094309 |
| GNG3 | 6.05761415 | 14.9544157 | -1.3037 | 0.018658 |
| EPHA2 | 41.2628483 | 83.0597597 | -1.0093 | 0.020169 |
| FGFR3 | 108.13653 | 156.530045 | -0.53359 | 0.023197 |
| PLA2G4A | 24.2674151 | 52.2128014 | -1.1054 | 0.038694 |
| FGF18 | 5.26605127 | 0.99023993 | 2.4109 | 0.039836 |
| FGFR1 | 492.760113 | 646.624624 | -0.39204 | 0.044063 |
| c-Rel | 986.723222 | 1664.09092 | -0.75402 | 0.048816 |
| GNG7 | 80.9381893 | 48.0772635 | 0.75147 | 0.049226 |

**Regulation of Actin Cytoskeleton**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| IQGAP2 | 1066.66121 | 678.873228 | 0.65189 | 0.002624 |
| PDGFA | 1471.68087 | 2606.30843 | -0.82454 | 0.0070196 |

| | | | | |
|---|---|---|---|---|
| PIP5K1C | 2410.97898 | 3354.19549 | -0.47635 | 0.01092 |
| PIP5K1B | 19.5494949 | 50.961453 | -1.3823 | 0.016818 |
| NCKAP1 | 151.198687 | 246.887439 | -0.70741 | 0.018217 |
| BCAR1 | 162.223324 | 263.954824 | -0.70231 | 0.018368 |
| ITGB3 | 8.08405629 | 1.96550176 | 2.0402 | 0.019543 |
| FGFR3 | 108.13653 | 156.530045 | -0.53359 | 0.023197 |
| WASF1 | 291.841662 | 463.670572 | -0.66791 | 0.023347 |
| IQGAP3 | 595.620209 | 806.779837 | -0.43778 | 0.02586 |
| IRSp53 | 364.643762 | 490.66882 | -0.42826 | 0.032452 |
| FGF18 | 5.26605127 | 0.99023993 | 2.4109 | 0.039836 |
| MYH10 | 1115.76993 | 1557.63182 | -0.48131 | 0.04042 |
| FGFR1 | 492.760113 | 646.624624 | -0.39204 | 0.044063 |
| DOCK1 | 16.0338226 | 28.2278949 | -0.816 | 0.046255 |

**Metabolic Pathways**

| Gene | Read Count: THA | Read Count: TH | $Log_2$ Fold Change | Pval |
|---|---|---|---|---|
| DHRS3 | 1658.81847 | 2529.80933 | -0.60887 | 0.001173 |
| CEL | 96.026101 | 167.194365 | -0.80003 | 0.0015856 |
| PLCH1 | 76.9342021 | 41.6620849 | 0.88489 | 0.0023519 |
| A4GALT | 32.8270858 | 69.4505602 | -1.0811 | 0.0039562 |
| TPH1 | 48.6738361 | 24.5054555 | 0.99004 | 0.0040721 |
| CYP26A1 | 13.3293258 | 46.0763681 | -1.7894 | 0.0043066 |
| GALNT9 | 138.059681 | 221.2302 | -0.68026 | 0.0044915 |
| GCNT2 | 50.0725782 | 20.5539998 | 1.2846 | 0.0066752 |
| PIP3K1C | 2410.97898 | 3354.19549 | -0.47635 | 0.01092 |
| CYP2R1 | 445.381631 | 308.751659 | 0.5286 | 0.011479 |
| POLD4 | 138.493502 | 84.9970954 | 0.70433 | 0.011573 |

| | | | | |
|---|---|---|---|---|
| IDNK | 92.7277379 | 57.6300934 | 0.68618 | 0.012326 |
| RDH10 | 896.200184 | 1616.64253 | -0.85111 | 0.012549 |
| ACSBG2 | 7.88762506 | 1.63749536 | 2.2681 | 0.013152 |
| ABAT | 465.847254 | 709.861287 | -0.60768 | 0.015828 |
| PIP5K1B | 19.5494949 | 50.961453 | -1.3823 | 0.016818 |
| CYP2U1 | 142.626105 | 94.5396564 | 0.59325 | 0.01719 |
| MAOA | 50.6855857 | 129.767974 | -1.3563 | 0.019769 |
| MAOB | 6.36714181 | 17.9613123 | -1.4962 | 0.024142 |
| ADO | 2037.82156 | 2722.04741 | -0.41766 | 0.026248 |
| ALPL | 4.4416498 | 12.1155612 | -1.4477 | 0.028647 |
| IL1I1 | 1072.51901 | 1806.21193 | -0.75196 | 0.029698 |
| CYP2J2 | 101.006158 | 43.8818316 | 1.2027 | 0.030054 |
| PYGL | 69.6442168 | 102.638011 | -0.55949 | 0.0306 |
| RIMKLB | 336.152241 | 190.90869 | 0.81623 | 0.030817 |
| GK | 1030.29182 | 1367.81795 | -0.40882 | 0.034624 |
| PIP5KL1 | 49.3146723 | 25.6176743 | 0.94488 | 0.036831 |
| CRYL1 | 172.66724 | 122.782511 | 0.49189 | 0.037381 |
| AK8 | 6.08224585 | 13.818443 | -1.1839 | 0.037806 |
| PLA2G4A | 24.2674151 | 52.2128014 | -1.1054 | 0.038694 |
| AOC3 | 159.450124 | 242.380441 | -0.60417 | 0.039048 |
| PTGS2 | 90.5693004 | 157.254642 | -0.79601 | 0.039773 |
| COX2 | 90.5693004 | 157.254642 | -0.79601 | 0.039773 |
| LAMA5 | 82.5304512 | 117.493122 | -0.50958 | 0.041732 |
| FBP1 | 21.9126405 | 6.16498181 | 1.8296 | 0.042537 |
| PTGS1 | 15.6086523 | 29.9508464 | -0.94025 | 0.045615 |
| COX1 | 15.6086523 | 29.9508464 | -0.94025 | 0.045615 |
| CYP27B1 | 427.789412 | 579.843887 | -0.43876 | 0.045673 |
| ARG2 | 207.316239 | 287.719526 | -0.47283 | 0.046985 |

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