**P540**

## NEXT GENERATION SEQUENCING TECHNIQUES REVEAL MOLECULAR MECHANISMS OF MYB REGULATION AND FUNCTION IN MLL-AF9 LEUKEMIA

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**Background:** Mutations involving the *MLL* gene at 11q23 are found in 10% of adult and 18% of childhood acute myeloid leukaemia (AML) cases. The most frequently occurring *MLL* mutations are chromosome translocations that fuse the *MLL* gene in-frame with a second partner gene, creating novel fusion proteins (MLL-FPs). MLL-AF9 is the most common MLL-FP in AML. Despite much progress in the overall management of AML, patients carrying *MLL*-rearrangements still have a poor survival prognosis and limited response to existing therapy. This is in part due to the low therapeutic indices and narrow therapeutic windows of current chemotherapeutic agents, therefore underscoring the need to develop improved, targeted therapies. *MYB* is a direct downstream target of MLL-AF9. Recent studies indicate that MLL-AF9 leukemia cells are more affected by *MYB* knockdown compared to normal hematopoietic stem progenitor cells. This is despite the fact that *MYB* is known to be essential for the establishment of definitive hematopoiesis. This suggests that a therapeutic window may be achieved through targeting *MYB.* Therefore, by understanding more about the role of *MYB* in MLL-AF9 leukemia and the network it regulates, we maybe able to exploit this knowledge to target *MYB* directly by interfering with its function or indirectly via its downstream targets.
**Aims:** To understand the molecular function of *MYB* in MLL-AF9 leukaemia.
**Methods:** We performed genome-wide MYB, MLL-AF9, H3K27ac, H3K4me3 and H3K4me1 chromatin immunoprecipitation sequencing (ChIP-seq) and Assay for Transposase-Accessible Chromatin with high throughput sequencing (ATAC-seq) in two MLL-AF9 leukemia models to identify putative regulatory regions of *MYB* and those of a direct MYB gene target, *BCL2*. The chromatin conformation capture technique, Capture-C (one *vs* all) was used to further characterize interactions from the MYB promoter. We then performed siRNA knockdown of *MYB* and assessed the effect of MYB loss on its downstream druggable target *BCL2*, using RT qPCR, Western blotting and ChIP qPCR.
**Results:** We identified MLL-AF9 binding to novel putative enhancers of *MYB* as defined by regions co-bound by H3K27ac, H3K4me1 and marked by open chromatin on ATAC-seq. Furthermore, Capture-C from the MYB promoter identified novel putative enhancer-promoter interacting domains 100-200kb apart that are co-bound by MYB but not MLL-AF9. This suggests long-range autoregulation of MYB. Next, siRNA knockdown of MYB results in loss of MYB binding at the BCL2 promoter and its downstream enhancer by ChIP qPCR. There is a corresponding loss of *BCL2* mRNA and protein expression in MYB knocked-down cells compared with control, confirming that BCL2 is directly regulated by MYB.
**Summary/Conclusions:** We have identified for the first time, regulation of *MYB* by MLL-AF9 via putative enhancers, and also an autoregulatory role of MYB involving long-range cis-interactions. Furthermore, we confirm that *BCL2* is directly regulated by MYB in MLL-AF9 leukemia, suggesting a molecular rational for using BCL2 inhibitors in MLL-AF9 leukemia therapy.

**P541**

## CD123-SPECIFIC CHIMERIC ANTIGEN RECEPTOR T-CELL THERAPY IN ACUTE MYELOID LEUKAEMIA

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**Background:** Acute myeloid leukaemia (AML) is a heterogeneous disease characterized by clonal evolution of myeloid precursors in bone marrow and peripheral blood resulting in accumulation of leukemic blasts and severe impairment of normal haematopoiesis. Despite advances in our understanding of AML biology, development of novel therapies has been limited with 43% relapse rate and 18% of patients never attaining clinical remission (CR) with frontline induction treatment. Chimeric antigen receptor (CARs) T cells specific for tumour-associated antigens are emerging to be an effective form of immunotherapy for AML. A small number of *in vitro* and *in vivo* studies have evaluated the efficacy and specificity of CAR T cell immunotherapy in AML by targeting interleukin three receptor alpha (IL3RA; CD123), a molecule over expressed on AML blasts and leukaemia stem cells (LSC) compared to normal haematopoietic stem cells (HSCs).
**Aims:** In this study, we investigated the efficacy of a second generation CAR expressing six single-chain variable fragments (scFv) with different affinities for CD123 and evaluated the cytotoxic effect of different co-stimulatory domains (CD28 *versus* 41BB) using a co-culture assay. Furthermore, we also evaluated the cytotoxic effects of a dual targeting CAR (against CD123 and CD33) using the same assay conditions.
**Methods:** Six lentiviral constructs (two high, two moderate & two low affinity) were transduced (MOI 1:5) into peripheral blood mononuclear cells (PBMCs) from healthy donors and their cytotoxicty was examined by flowcytometry on leukaemic cell lines; KG1 (CD123$^+$, CD34$^+$, CD33$^+$) [Fig:1a], Kasumi-1 (CD123$^+$, CD34$^+$, CD33$^+$), U937 (CD123$^+$, CD34$^-$, CD33$^+$), K562 (CD123$^-$, CD34$^-$, CD33$^+$) and AML mononucelar cells (MNCs).
**Results:** Flowcytometric analysis confirmed the expansion of T cells from PBMCs and the cytotoxicity of the six CARCD123 constructs against CD123$^{+ve}$ cells. The high affinity CARCD123 (4nM kD & 4nM kD K136Q) T cells demonstrated enhanced cytotoxicity compared to moderate (56nM kD, 56nM kD A105G) and low affinity (101nM kD, 101nM kD V24G) CARCD123 in both leukaemic cell lines and also in allogenic AML MNCs. Both the highest affinity CARCD123 constructs were also tested in cell lines using increasing effector: target ratios (1:2, 1:4 & 1:10) displaying consistent cytotoxicity and were also effective against autologous AML MNCs (target cells) and PBMCs (effector cells) from two patients. T cell activation was confirmed by ELISA and showed increased IFN-γ (500-2000 fold) and TNF-α (150-200 fold) levels. Previous studies have confirmed the distinction in CAR efficiency using CD28 *versus* 41BB co-stimulatory domains; CD28 co-stimulation augmented, whereas 4-1BB co-stimulation reduced T cell exhaustion induced by continuous CAR signaling. To confirm persistence of the CAR cytoxicity, we constructed a high affinity CAR substituting CD28 with a 4-1BB co-stimulatory domain and obtained similar cytotoxicity results on K562 and U937 cell lines. Furthermore, a novel a dual targeting CAR in which the activation domain (CD3ζ) is directed against CD33 and the costimulatory domain (CD28) directed against CD123 enhanced the specificity of the CAR towards leukaemic cells; reducing "on-target but off-organ effects". Results obtained in co-culture assay against KG1 [Fig:1b] and K562 cell lines [Fig:1c] with varying effector: target ratios were demonstrated results similar to the high affinity single targeting CAR.

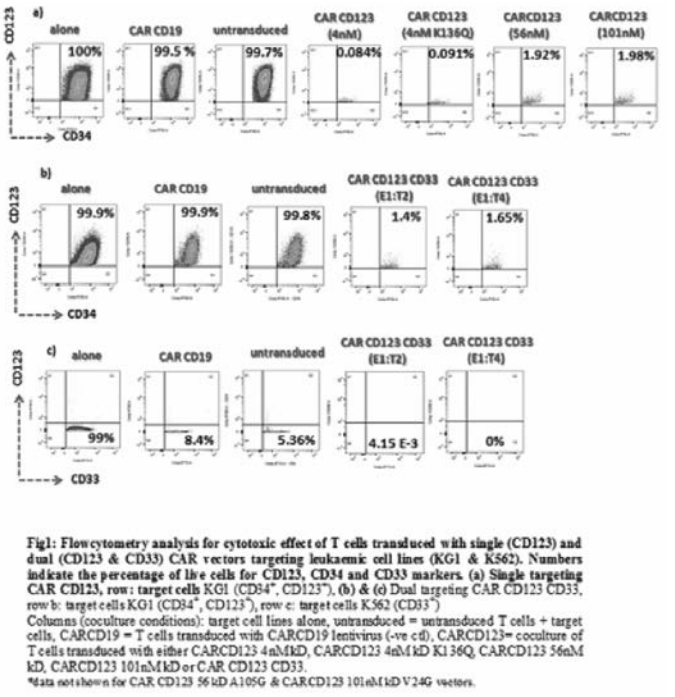


**Fig1: Flowcytometry analysis for cytotoxic effect of T cells transduced with single (CD123) and dual (CD123 & CD33) CAR vectors targeting leukaemic cell lines (KG1 & K562). Numbers indicate the percentage of live cells for CD123, CD34 and CD33 markers. (a) Single targeting CAR CD123, row: target cells** KG1 (CD34$^+$, CD123$^+$), **(b) & (c)** Dual targeting CAR CD123 CD33, row b: target cells KG1 (CD34$^+$, CD123$^+$), row c: target cells K562 (CD33$^+$)
Columns (coculture conditions): target cell lines alone, untransduced = untransduced T cells + target cells, CARCD19 = T cells transduced with CARCD19 lentivirus (-ve ctl), CARCD123= coculture of T cells transduced with either CARCD123 4nMkD, CARCD123 4nM kD K136Q, CARCD123 56nM kD, CARCD123 101nM kD or CAR CD123 CD33.
*data not shown for CAR CD123 56 kD A105G & CARCD123 101nM kD V24G vectors.

**Figure 1.**

**Summary/Conclusions:** In conclusion, we demonstrate the importance of the scFv on CAR T cell cytoxicity and have constructed and validated the efficacy of a dual targeting CAR vector in the context of AML.

**P542**

## TARGETED COMBINATION THERAPY WITH CDK4/6 INHIBITOR PALBOCICLIB IN AML

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**Background:** Acute myeloid leukemia (AML) is a clonal hematologic disorder marked by clinical and biological heterogeneity. AML remains incurable for a significant proportion of adult patients while no therapeutic option exists for patients with relapsed and refractory AML. Mutations of the fms-like tyrosine kinase 3 (*FLT3*) gene are among the most frequent events in AML and usually involves internal tandem duplication (ITD) of the juxtamembrane domain coding region or point mutations of the tyrosine kinase domain. There have been considerable efforts to develop FLT3 tyrosine kinase inhibitors (TKI). The clinical impact of FLT3-TKI has been limited as resistant clones have emerged rapidly.