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## Selected abstract
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# Abstract 555

## The Enhanced Functionality of Low-Affinity CD19 CAR T Cells Is Associated with Activation Priming and Polyfunctional Cytokine Phenotype

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We have recently described a low-affinity second-generation anti-CD19 Chimeric Antigen Receptor (CAR) (CAT), characterized by faster antigen dissociation rate which showed enhanced expansion, cytotoxicity and anti-tumour efficacy compared with the high affinity (FMC63 based) CAR used in Tisagenlecleucel in pre-clinical models. Furthermore, CAT CAR T cells showed an excellent toxicity profile, enhanced *in vivo* expansion and long-term persistence in a Phase I clinical study (Ghorashian *et al* Nature Med 2019). However the molecular mechanisms behind the improved properties of CAT CAR T cells remain unknown. Herein, we performed a systematic *in vitro* characterization of the transcriptomic (bulk RNA-seq) and protein (CyTOF) changes occurring in CAR T cells expressing a low-affinity (CAT) *vs* high affinity (FMC63) anti-CD19 CARs following stimulation with CD19 expressing targets.

Untransduced (UT) controls and T cells lentivirally transduced to express CAT or FMC63 CD19 CARs were compared both at baseline and following stimulation with $CD19^+$ Acute Lymphoblastic Leukaemia cell line NALM6. In Principal Component Analysis for both RNA-seq and protein results, we found that the major variance across conditions was explained by CD19-mediated CAR T activation. Strikingly, unstimulated CAT CAR T cells showed an intermediate degree of activation between UT T cells and antigen stimulated CAR T cells. Indeed, when comparing RNA-seq results of unstimulated CAT *vs* FMC63, we found enhanced expression (FDR <0.1) of genes involved in cytotoxicity (*GNLY, GZMK*) and T cell activation (*HLA-DRA* and *HLA-DPA1*) (Figure 1a), confirmed at protein level by CyTOF. This "activation priming" observed in CAT CAR T cells was associated with and may be driven by residual CD19-expressing B-cells present in the manufacture product, preferentially inducing a T Central Memory ($T_{CM}$) phenotype in CAT vs FMC63, in both CD4 and CD8 T cells. Such priming is likely to be instrumental to CAT CAR T cells more potent cytotoxic response upon NALM6 stimulation, when they displayed further increase in the expression of immune stimulatory cytokines (*IFNG, CSF2*), chemokines (*CCL3L1, CCL4, CXCL8*) and *IFN*g responsive genes (*CIITA*) by RNA-seq, as well as augmented T cell activation (CD25, NFAT1) and proliferation (pRB) markers by CyTOF.

To identify the mechanisms underlying the stronger basal activation of CAT CAR T cells, we analysed cytokine expression at the single cell level by mass cytometry. Interestingly, rather than an increment in the expression of individual cytokines, we found that the distinctive feature of CAT CAR T cells was a shift toward a cytokine polyfunctional phenotype, with a marked increase in the proportion of cells co-expressing 3 or more cytokines (17.50% CAT *vs* 7.33% FMC63) (Figure 1b). Of note, cytokine polyfunctionality (expression of more than 1 cytokine/cell) in pre-infusion CAR T cell products has been associated to improved clinical efficacy. The functional phenotype observed in CAT CAR T cells was linked to the preferential activation of the p38 MAPK phospo-signalling, which is activated downstream of TCR CD3ζ chain (present in the CARs) but is also central to cytokine-dependent T cell activation in memory T cells.

Interestingly, cytokine polyfunctional CAT CAR T cells were enriched in the $CD3^+CD19^+$ trogocytic (trog+) population, found at higher proportion in CAT *vs* FMC63 at 24h post antigen stimulation. Although trogocytosis has been associated to CAR T cell fratricide killing, trog+ CAT CAR T cells displayed higher levels of proliferation (pRB), activation (CD25, NFAT1) and cytotoxic (Granzyme B, Perforin B) markers, pointing at a stimulatory role of trogocytosis over fratricide killing, potentially due to the low-affinity CAR T cells distinctive property of better discriminating between low (trog+ CAR T cells) and high (tumour cells) target expression levels.

In conclusion, we described the molecular mechanisms underlying the low affinity CAT CAR T cells functional phenotype. Our results show that the potent and long-term anti-tumour responses observed with CAT may be sustained by the establishment of CAR T cells self-reinforcing circuits activated through polyfunctional cytokine crosstalk. This work may inform the future design of versatile CAR T cells, capable of balancing safety, efficacy and long-term persistence.

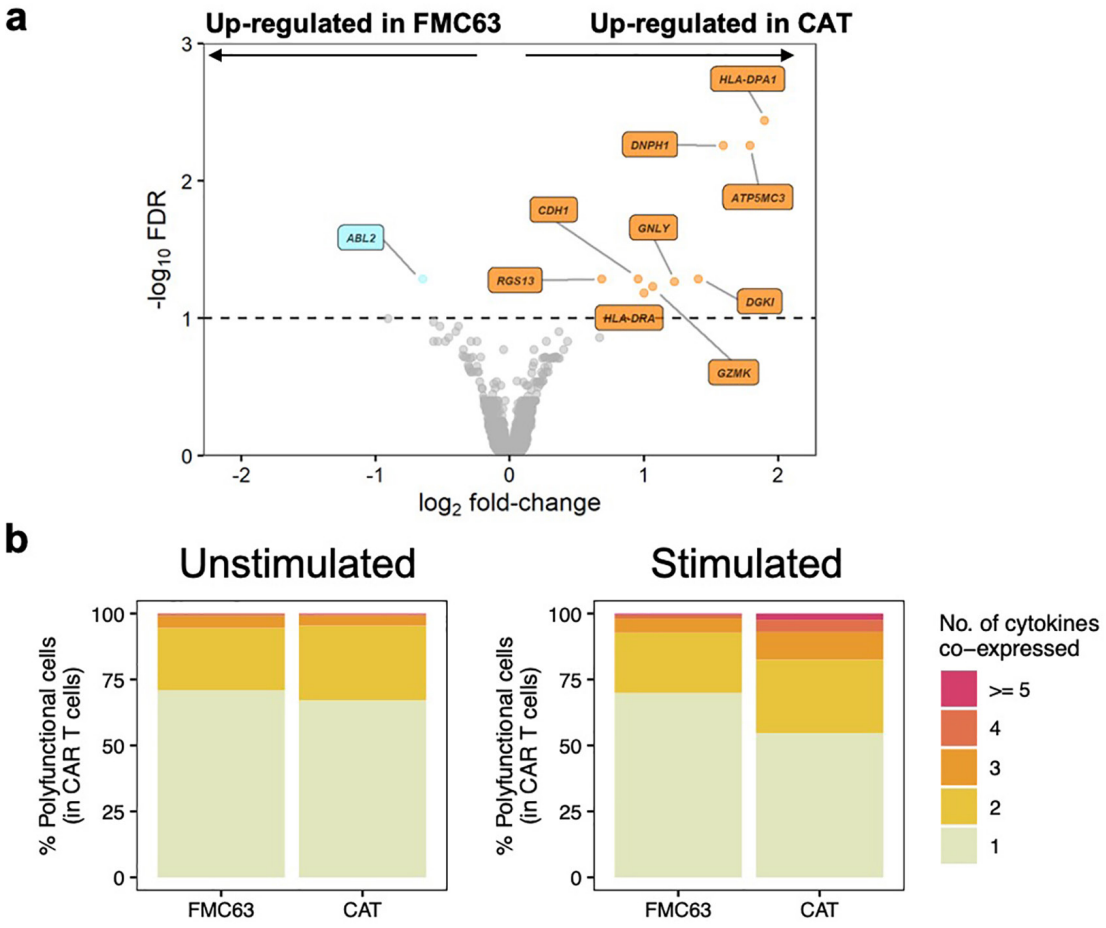


**Figure 1**
**a)** RNA-seq. Volcano plot showing differentially expressed genes (FDR<0.1) between unstimulated CAT and FMC63 CAR T cells (n=6, 2 experiments). **b)** CyTOF. Stacked bar plots showing the number of co-expressed cytokines in both unstimulated (left) and antigen stimulated (right) FMC63 and CAT CAR T cells (n=4, 1 experiment).

***Disclosures:***
**Ghorashian:** *Amgen:* Honoraria; *UCLB:* Patents & Royalties; *Novartis:* Honoraria. *Pule:* Autolus: Current Employment, Other: owns stock in and receives royalties, Patents & Royalties; *UCLB:* Patents & Royalties; *Mana Therapeutics:* Other: entitled to share of revenue from patents filed by UCL.

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