Development of a 2D *in vitro* CD8⁺ T Cell Killing Assay

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ABOUT CRUK-TDL

Cancer Research UK is the world's largest independent funder of cancer research, with contributions to the creation of 8/10 of the world's leading anti-cancer drugs. The primary goal of the charity is to conduct research into prevention, diagnosis, and new and current treatments, to increase patient survival from 50% to **75%** by 2034.

CRUK – Therapeutic Discovery Labs (TDL) focuses on target validation and disease positioning (TVDP), screening and compound profiling and medicinal chemistry. My role was within the immuno-oncology section of TVDP, based in Cambridge, therefore focused on the interaction of the immune system with cancer.

TECHNIQUES

At CRUK-TDL, I acquired multiple new techniques including: Mammalian cell culture, primary human cell isolation, Flow Cytometry, assay development, lentiviral production and tumour spheroid formation.

INTRODUCTION

CD8+ Cytotoxic T lymphocytes (CTLs) are potent anti-tumour lymphocytes that recognise specific antigens bound to MHC class I molecules through their T cell receptors (TCR). When tumour-associated antigen (TAA) recognition is coupled with cytokine and co-stimulation signals, an immune synapse forms, initiating pathways in the CD8+ CTL that result in tumour cell death (Alberts *et al*, 2002).

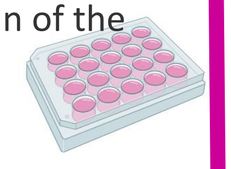
AIMS

- To transduce CD8+ CTLs with a lentivirus encoding the anti-NY-ESO-1 TCR, and optimise this process.
- To create an *in vitro* 2D CD8+ CTL killing assay by co-culturing tumour cells lines with the transduced CD8+ CTLs.
- To use the assay as an initial candidate compound screen, to determine their effect on CD8+ CTL-mediated tumour killing in a physiologically relevant environment.

METHODS

Generation of antigen-specific CD8+ CTLs

Primary Human CD8+ CTLs were isolated from peripheral blood mononuclear cells from healthy donors. These were transduced with lentiviral vectors encoding a puromycin resistance gene and either the anti-NY-ESO-1 TCR or an empty vector (EV) control. Following puromycin selection, the percentage of TRBV6-5+ (region on the β chain of the TCR) CTLs was determined by flow cytometry.



2D IncuCyte Killing assay

The EV+ and TCR+ CD8+ CTLs were co-cultured for five days at varying target: effector (T:E) ratios (1:2, 1:5, 1:10) with either A375 malignant melanoma or HCT-116 colon cancer cells, which are NY-ESO-1+ and NY-ESO-1- respectively.

Target cell growth was monitored by the IncuCyte Live-Cell Analysis System, using the tumour cell NucLight Red fluorescent label to give a red object count/mm^2. Additionally, Caspase-3/7-mediated apoptosis was measured in green objects/mm^2.

RESULTS

CD8+ CTL transduction efficiency measured by flow cytometry

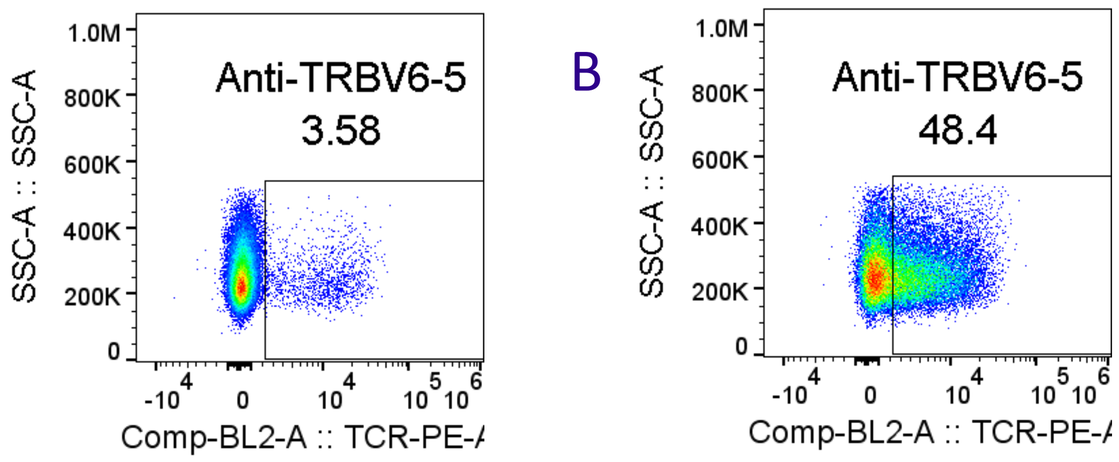


Figure 1: Flow cytometry analysis of the anti-TRBV6-5 antibody staining of CD8+ CTLs transduced using the RetroNectin-Bound Virus (RBV) method (Takara) with **(A)** EV and **(B)** TCR-encoding lentivirus. The TRBV6-5 region can be endogenously expressed by CTLs, therefore some detection of anti-TRBV6-5 expression in the EV-transduced CTLs was expected.

Anti-NY-ESO-1 TCR+ CD8+ CTLs specifically kill NY-ESO+ A375 cells

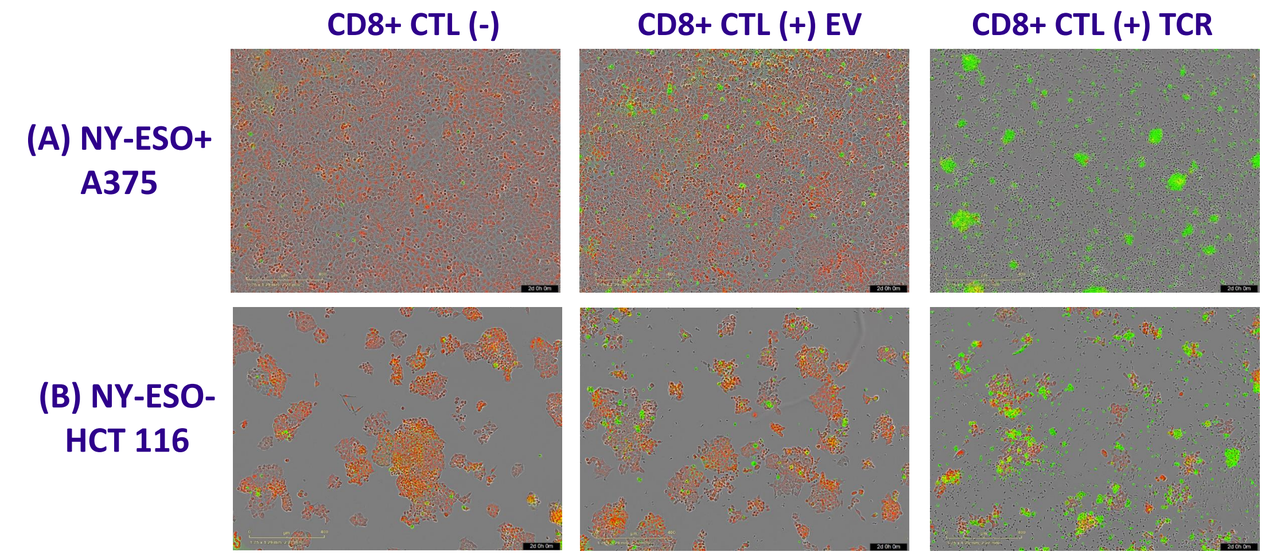


Figure 2. Representative IncuCyte® images indicated CD8+ CTL targeted killing of A375 cells. IncuCyte images (10x) acquired at the 48-hour timepoint of A375 and HCT-116 tumour cells with EV+ or TCR+ CD8+ CTLs at 1:5 T:E ratio. **(A)** The complete killing of A375 cells by TCR+ CD8+ CTLs, compared to minimal death by EV+ CD8+ CTLs indicated specificity of the TCR for NY-ESO-1. **(B)** TCR+ CD8+ CTLs show reduced killing of NY-ESO-1- HCT-116 cells compared to A375, further indicating TCR NY-ESO-1 specificity. The killing of HCT-116 cells by TCR+ CD8+ CTLs was likely an allogenic response, and the increased killing in the TCR+ well compared to the EV+ well was likely a result of the normalisation of TCR+ numbers to the transduction efficiency. The higher TCR+ CTL numbers would have increased waste products and competition for nutrients with the tumour cells. Additionally, CTL death was detected by the green Capase-3/7 dye, therefore may overestimate tumour cell death.

Anti-NY-ESO-1 TCR+ CTLs killed NY-ESO-1+ A375 cells in a dose-dependent manner

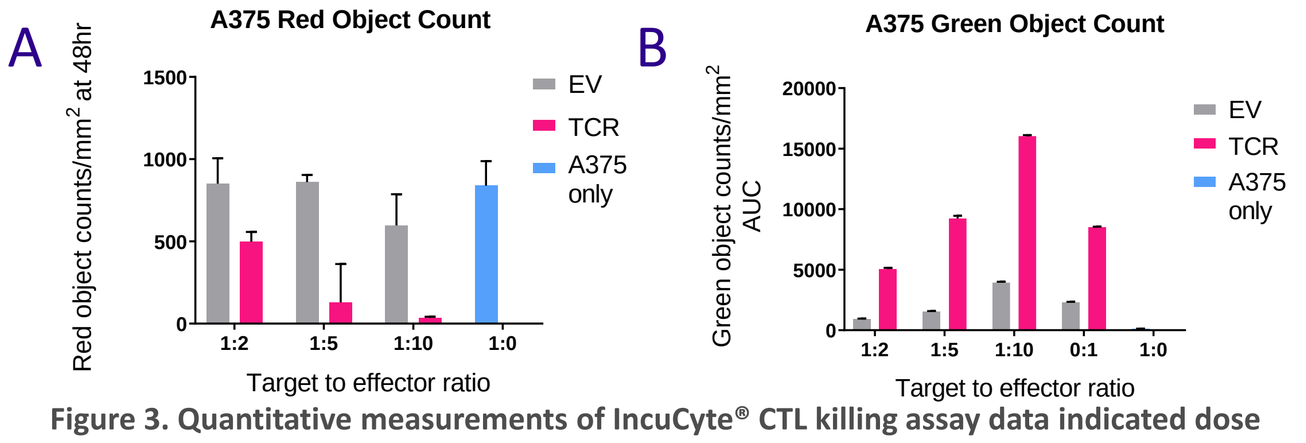


Figure 3. Quantitative measurements of IncuCyte® CTL killing assay data indicated dose dependent killing of A375 with increasing ratios of TCR+ CTLs. (A) The total red object counts/mm^2 of A375 tumour cells at 48 hours. **(B)** Green object counts/mm^2 at 48-hour time point reflects Caspase-3/7 mediated apoptosis. This included dead CTLs, however a clear dose-dependent response was still evident. The smaller dose-dependent killing observed by EV+ CTLs was likely due to an allogenic response and increasing competition for nutrients.

CONCLUSIONS

The transduced anti-NY-ESO-1 TCR+ CTLs showed targeted killing of the NY-ESO-1+ A375 cell line.

Future directions involve development into a 3D spheroid killing assay to increase physiological relevance.

REFERENCE

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. *Molecular Biology of the Cell*. 4th ed. New York: Garland Science