A FACS assay to measure activation of human cytotoxic T-lymphocytes and CD73-dependent suppression by AMP

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1 ABSTRACT

CD73 is an immuno-oncology drug target of significant interest based on its key role in the generation of immunosuppressive adenosine within the tumour microenvironment. Adenosine suppresses T-cell functions including cancer cell killing. By targeting the CD73 enzyme which converts AMP to adenosine, T-cell function can be restored. Alternatively the conversion of ATP to AMP can be targeted by inhibiting the enzyme CD39. Both CD39 and CD73 are widely expressed on immune cell types, including cytotoxic CD8+ T-cells depending on activation status.

Compounds which inhibit CD73 are described in the literature, including two molecules which entered phase 1 clinical trials: AB-680 and LY-3475070. Earlier molecules are AMPCP and the derivative with improved properties PSB-12379.

We show a 96-well FACS assay for testing the restorative effects of CD73 inhibitor compounds on T-cell activation. The main readout is proliferation by dye dilution method using the covalent dye CFSE. Alternative readouts of T-cell activation tested were upregulation of CD25 surface expression and release of interferon gamma into culture medium.

2 MATERIALS AND METHODS

PBMCs are isolated from human blood anti-coagulated with Na Citrate, by centrifugation at 800g over Histopaque (Sigma). CD8+ cell isolation is done with anti-CD8 beads (Miltenyi Biotech). Labelling with 3µM CFSE (Fisher) is done in PBS/ 0.1% BSA 37ºC 10 min. Cells are washed x2 in Immunocult XF medium (Stem Cell Technologies) and seeded into U bottom 96 well culture plates (Costar) at 35k cell/ well in a final volume of 200µl Immunocult XF medium with 100 IU/ml IL-2 (R&D systems), 25µ/ml Immunocult Human CD3/CD28/CD2 T-cell activator and 5µM EHNA (Sigma) adenosine deaminase inhibitor to stabilize adenosine derived from metabolised AMP. For testing CD73 inhibitors cells are initially pre-incubated for 30 min with a final DMSO concentration of 0.2%. AMP is added at the same time as CD3/CD28/CD2 T-cell activator. Assay plates are incubated at 37ºC for 4 days. Cells are transferred to V bottom plates for FACS staining. Cell culture medium is collected following centrifugation and measured for interferon gamma (MesoScale Detection kit). FACS staining is done with the following antibodies: CD25 (BD 555434), CD3 (Miltenyi 130-113-697), CD8 (Miltenyi 130-110-815), CD73 (Miltenyi 130-112-061), CD39 (Miltenyi 130-110-789), Isotype control (Miltenyi 130-113-434). Stained cells are resuspended in 100µM FACS stain buffer (BD) containing 0.625 µg/ml 7-AAD (BD) and analysed with an Accuri C6 flow cytometer. Live cells are gated using 7-AAD and FSC/SSC. Proliferation is calculated as the % of cells with at least one cell division. CD73 inhibitor IC50 values are derived from curve fits generated using XLFit 5 software (IDBS).

3 RESULTS

1) Isolation of CD8+ T-cells

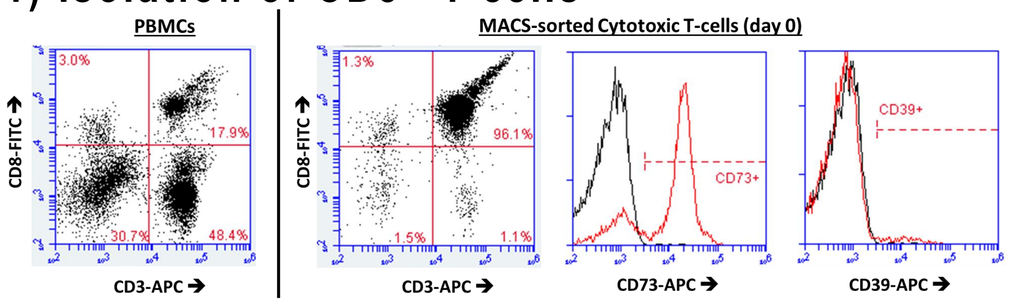

Figure 1. FACS analysis shows >96 % purity for CD8+ lymphocytes isolated from human PBMCs by magnetic bead positive selection. Purified CD8+ cells initially express high levels of CD73 but not related enzyme CD39, black line = isotype control. During T-cell activation CD73 is downregulated and CD39 upregulated (data not shown).

2) Proliferation assay principle (CFSE)

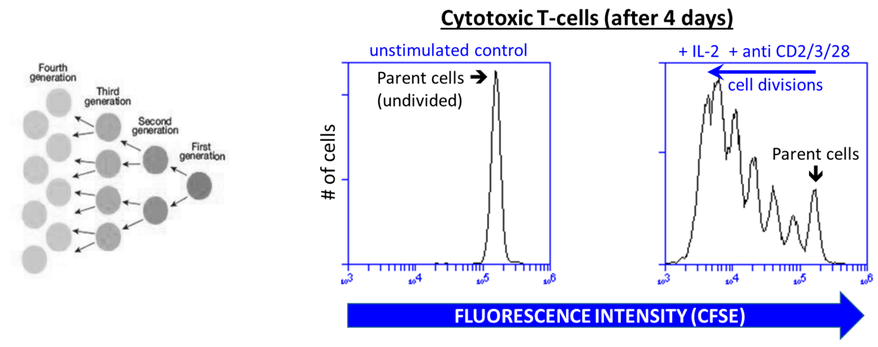

Figure 2. CD8+ cytotoxic T-cells are labelled with CFSE dye, activated with IL-2 + anti-CD2/CD3/CD28 and cultured for 4 days. Control unstimulated T-cells give a single population with uniform high fluorescence intensity. T-cell proliferation results in multiple cell populations with reduced fluorescence. As cells divide the daughter cells receive approximately half of the original dye per division.

3) AMP suppresses T-cell proliferation

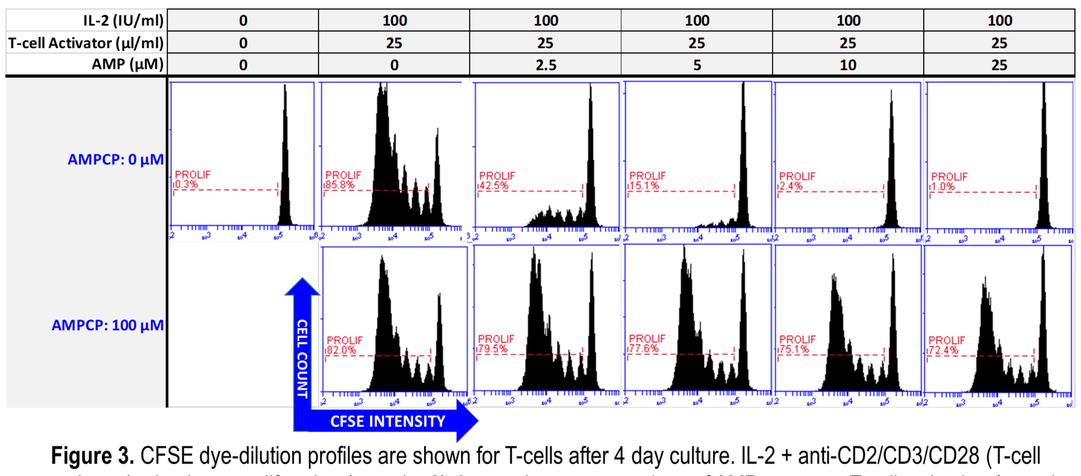

Figure 3. CFSE dye-dilution profiles are shown for T-cells after 4 day culture. IL-2 + anti-CD2/CD3/CD28 (T-cell activator) stimulates proliferation (top, plot 2). Increasing concentrations of AMP suppress T-cell activation (top, plots 3-6). AMPCP inhibits the enzyme CD73 which converts AMP to immunosuppressive Adenosine (bottom row)

4) AMP suppresses T-cell CD25 upregulation

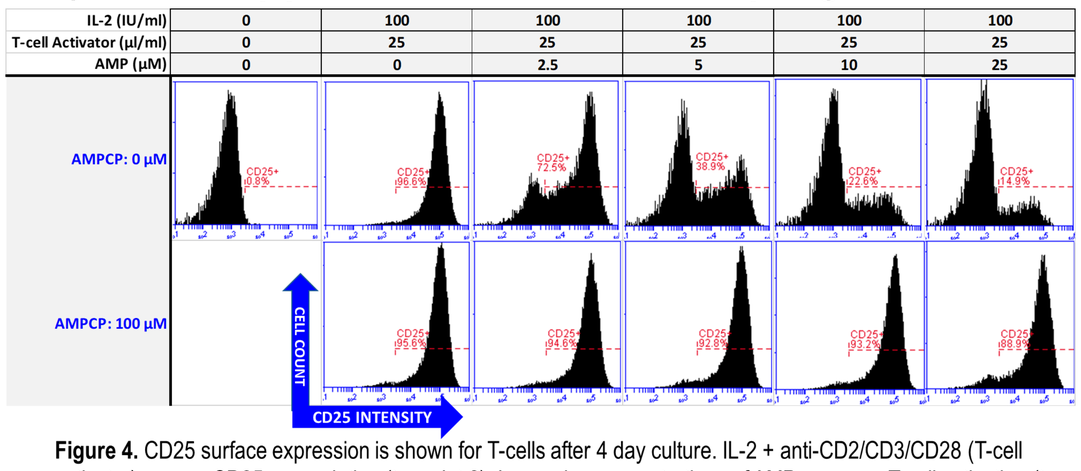

Figure 4. CD25 surface expression is shown for T-cells after 4 day culture. IL-2 + anti-CD2/CD3/CD28 (T-cell activator) causes CD25 upregulation (top, plot 2). Increasing concentrations of AMP suppress T-cell activation (top, plots 3-6). AMPCP inhibits the enzyme CD73 which converts AMP to immunosuppressive Adenosine (bottom row)

5) CD73 inhibitors restore T-cell function

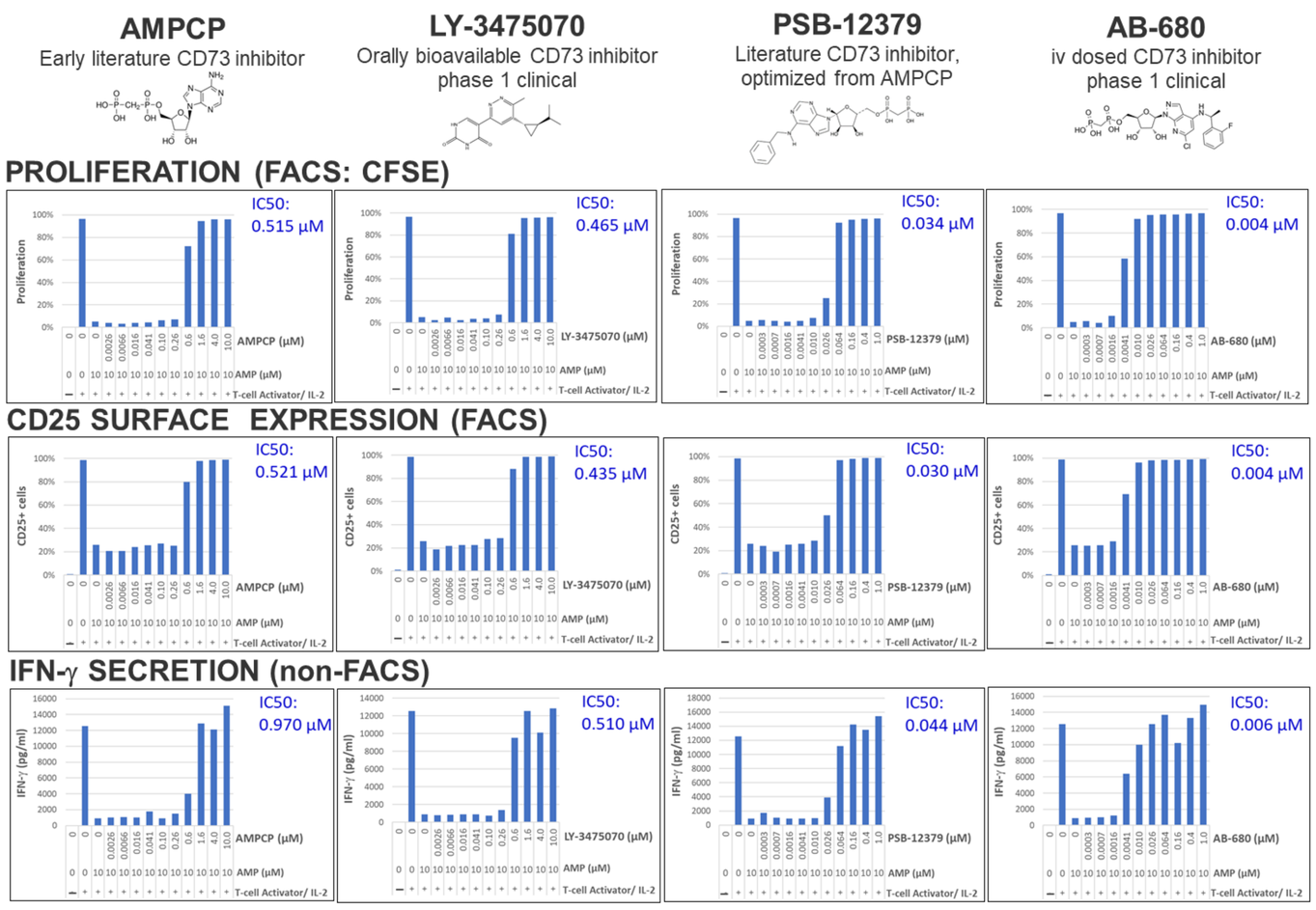

Figure 5. After 4 days culture cells were harvested for FACS (row 1: CFSE proliferation, row 2: CD25 expression) and culture medium was collected for measuring secretion of IFN-γ (row 3). Addition of the CD2/3/28 T-cell activator yields ~95% proliferation, suppressed by AMP to ~5%, and restored by CD73 inhibitors. Compound ranking is consistent across the readouts of T-cell activation in rows 1-3, and release of lactate (not shown).

4 CONCLUSIONS

We have shown how a 96-well CFSE dye-dilution T-cell proliferation assay using flow cytometry, can be used to measure the immunosuppressive effects of AMP and rescue with CD73 inhibitors. CFSE dye benefits from cost-effectiveness, high intrinsic fluorescence intensity and compatibility with any flow cytometer with a 488 nm laser. Toxicity of CFSE is avoided by optimization of labelling conditions and inclusion of 0.1% BSA in the labelling reaction. Assay parameters were also optimized to maximize assay window, robustness and sensitivity to detecting AMP-suppression of T-cell activation, whilst remaining sensitivity to reversal with CD73 inhibition.

Assay parameters optimized included CFSE labelling concentration, cell culture medium, cell seeding density, DMSO tolerance, inclusion of adenosine deaminase inhibitor, concentration of anti-CD2/CD2/CD28 reagent, duration of culture period and AMP concentration. We found the ImmunoCult reagents from Stem Cell Technologies gave robust T-cell activation and growth. Data shown is representative from single blood donors, with some variation in sensitivity being seen between experiments using different blood donors. Subsequent work has focussed on generating large batches of cryopreserved T-cells and adaptation of the assays for these cells.

AMP was found to dose-dependently and profoundly suppress T-cell activation as detected by FACS (proliferation by CFSE and CD25 surface expression) or release of interferon gamma into the culture medium. The AMP effect was reversed by either of four selective CD73 inhibitor compounds tested, consistent with the AMP effect requiring metabolism by CD73 present on the surface of CD8+ cytotoxic T-cells.