

February 2025

Keywords or phrases:MLR, T Cell, Flow Cytometry, Checkpoint Inhibitor, APCs, DCs, IFN γ , Cancer

Utilizing Mixed Lymphocyte Reaction (MLR) to Evaluate Checkpoint Inhibitor Therapies Using High-throughput Screening by Cytometry

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Introduction

Immune checkpoints are regulatory signals that control the synapse between T cells and antigen presenting cells (APCs), such as dendritic cells (DCs). The balance between these stimulatory and inhibitory signals plays an important role in the tumor microenvironment, ensuring T cells can be 'switched-on' to increase tumor cell killing, but also 'switched-off' to prevent over activation of the T cells and attacking of healthy cells. Immunotherapies, termed checkpoint inhibitors, have been developed that block the immune checkpoints that usually 'switch off' T cells. This block tips the balance of the checkpoint regulatory signals, increasing T cell activation and resulting in enhanced tumor cell killing. Currently approved checkpoint inhibitor therapies include monoclonal antibodies that target PD-1, PD-L1 and CTLA4 whilst the continued exploration of additional immune checkpoint targets is a hot topic in the drug discovery field.¹

Mixed lymphocyte reaction (MLR) assays mimic dendritic cell activation of T cells *in vitro* to provide a model for investigation of potential checkpoint inhibitor therapies. Immune cells from two individuals are cultured together and the detection of 'non-self' antigens presented by the DCs triggers a T cell activation response. This relies on differences in the donors' HLA haplotypes. There are two versions of MLR assay: one-way and two-way MLR. One-way MLR involves co-culturing of CD4+ T cells from one donor with DCs from another donor, resulting in unidirectional T cell activation. Two-way MLR is a co-culture of PBMCs from two different donors, and results in stimulation of T cells from both donors.

A potential therapeutics' ability to potentiate T cell response in an MLR assay can be evaluated through measurements of cell marker expression, proliferation and cytokine release. Conventional techniques for analysis of MLR assays, such as traditional flow cytometry and ELISA are often limited because they:

- Require separate assays to measure cytokines, proliferation and activation marker expression. This means readouts for each treatment are taken from different sample populations and analysis timepoints which can introduce variance in data.
- Necessitate manual correlation of data for a single treatment from multiple assay platforms, such as a plate reader and an ELISA assay.

- Use instrumentation with low-throughput acquisition and large sample volume requirements.
- Are laborious and time-consuming

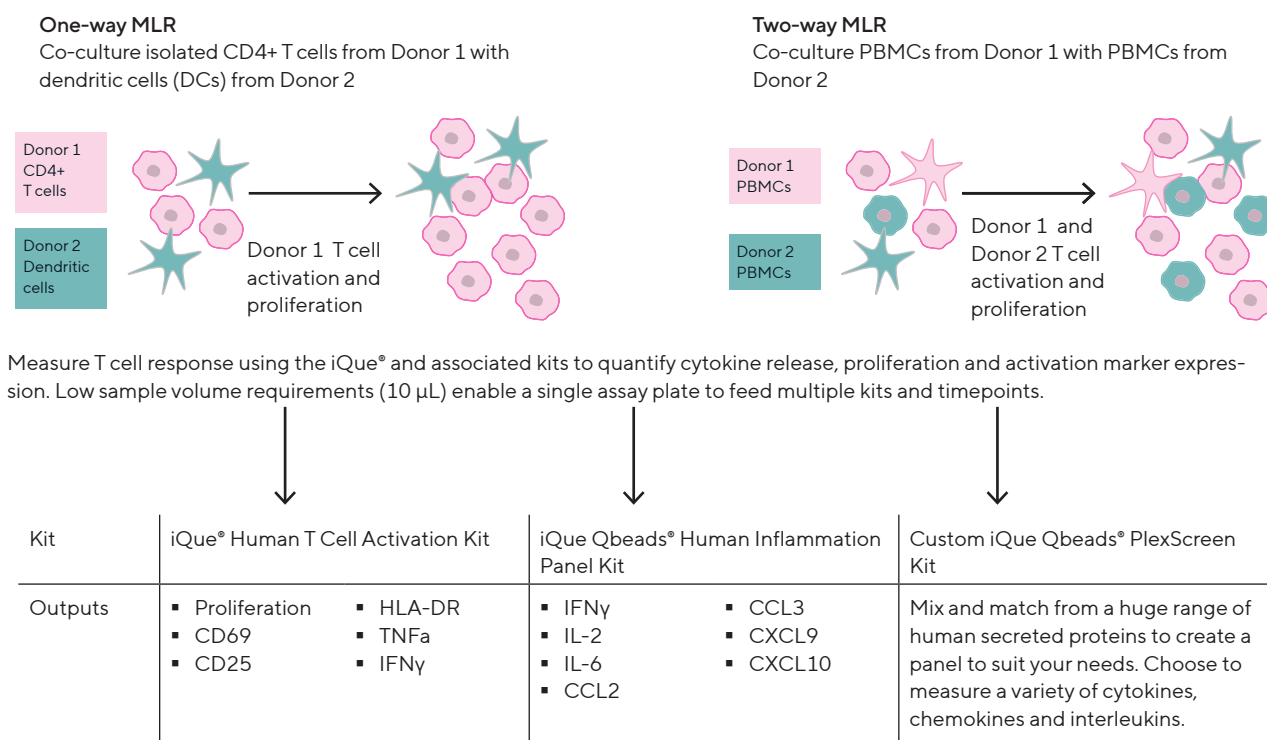
To address these challenges, we have developed a simple, high-throughput workflow for quantifying T cell response in an MLR assay. Marker expression, proliferation and cytokine release data are acquired from a single assay plate, collapsing the workflow into concurrent data analysis, using the iQue® High-Throughput Screening (HTS) Cytometry Platform. The integrated iQue Forecyt® software provides instantaneous pharmacological readouts for T cell activation response to checkpoint inhibitor drugs.

Assay Concept

Immune cells from two different donors were co-cultured in one-way (CD4+ T cells and DCs) or two-way (PBMC mix) MLR formats. Samples (10 µL) were collected and analyzed using a range of validated reagents from compatible iQue® kits, including the iQue® Human T Cell Activation Kit, the iQue Qbeads® Human Inflammation Panel Kit and a Custom iQue Qbeads® PlexScreen Kit (Figure 1). Low sample volume requirements to feed the kits mean they

can be used alone or in combination to measure proliferation, marker expression and cytokine release as desired, all from a single assay plate. Data acquisition was performed using the iQue® HTS Cytometer, which facilitated rapid sampling of cell and supernatant samples from 96 and 384 well plates. Each kit is supplied with a pre-set gating template that can be imported into the iQue Forecyt® software enabling an instant readout of the T cell phenotype and | or cytokine response.

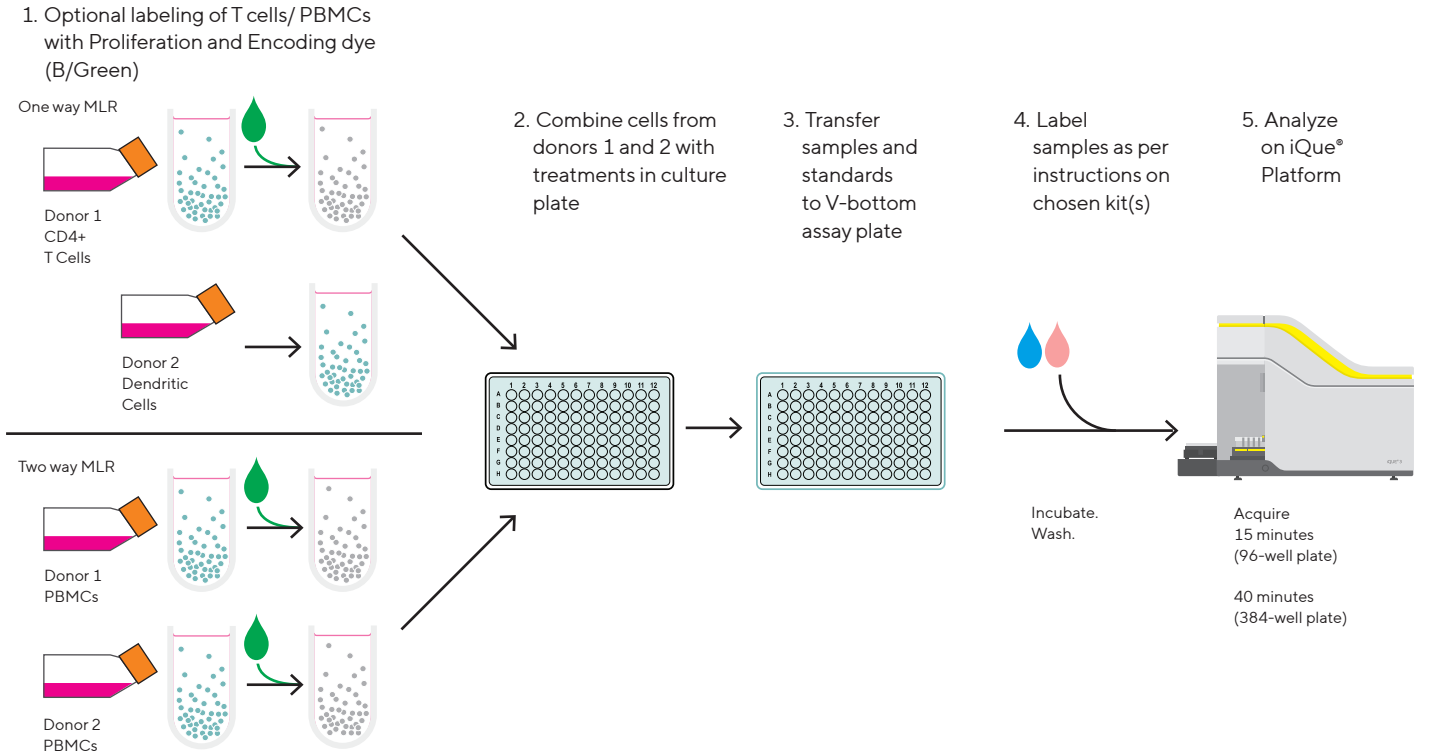
Figure 1. Illustration of the MLR assay principles



Analysis of T cell response from one or two-way MLR can be performed using the iQue® and a range of associated kits, including the iQue® Human T Cell Activation Kit, the iQue Qbeads® Human Inflammation Panel Kit and a Custom iQue Qbeads® PlexScreen Kit. These kits can be used alone or in combination to measure proliferation, marker expression and cytokine release as required to suit user needs, all from a single assay plate).

Figure 2. Schematic of the simple, one-wash protocols for the one-way and two-way iQue® MLR assays.

MLR Assay Workflow



- Optional: If performing analysis of cell populations (as opposed to cytokine only analysis), CD4+ T cells (one-way MLR) or PBMCs (two-way MLR) are labelled using the iQue® Proliferation and Encoding (B/Green) Dye provided with the iQue® Human T Cell Activation Kit.
- Prepare desired concentrations of treatments (i.e. checkpoint inhibitors) in cell culture media and add to either a 96 or 384 well plate.
- Resuspend cells from each donor to an appropriate density and combine with treatments in the cell culture plate. Incubate for 3-7 days.
Note: We recommend a 3:1 ratio of CD4+ T cells to DCs in a one-way MLR assay. In two-way MLR, PBMCs from each donor should be combined at a 1:1 ratio.
- Optional:** 10 µL samples of cells and/ or supernatant can be taken at defined timepoints to give temporal marker expression data and/ or cytokine concentration.
- Assay cells and/or supernatants using the reagents and protocol from the iQue® kit(s) of your choice. This assay was validated using the iQue® Human T Cell Activation Kit, the iQue Qbeads® Human Inflammation Panel Kit and a Custom iQue Qbeads® PlexScreen Kit (see Figure 1 for the outputs of each kit).
- Resuspend labelled cells in wash buffer and acquire samples using the iQue® Platform.
- Import the kit(s) gating template into the iQue Forecyt® software for automated analysis of T cell response.

Activation response in MLR is stronger when HLA surface antigens on T cells and DCs are more heterogenous

HLA antigen profiling is most commonly used in selecting donors for organ or stem cell transplantation as donors with similar HLA haplotypes are less likely to induce an immune response and trigger graft rejection.² In MLR we use HLA typing for the opposite effect, to select for donors most likely to induce allogenic T cell activation. This allows us to mimic the tumor microenvironment in which tumor antigens are over-expressed on APCs, which induces T cell activation. Initial experiments aimed to verify the importance of T cell and DC donor HLA haplotypes in an MLR reaction by quantifying differences in response between two donor pairs using the iQue® Human T Cell Activation Kit.

Two T cell donors were selected based on profiling of 8 key HLA antigens and how they compared to the chosen DC donor. The first T cell donor (Low T cells) had a low number of HLA antigens that were mismatched with the DC donor, i.e. they had similar HLA profiles, whilst the second T cell donor (High T cells) had a high number of HLA allele mismatches. The CD4+ T cells and DCs were co-cultured at a range of T cell-to-DC ratios (9:1, 4.5:1 and 3:1) for 5 days in a one-way MLR assay. Results demonstrate that when the donors’ HLA alleles were highly mismatched, CD25 activation marker expression on T cells increased when increasing numbers of DCs were included in the co-culture (i.e. when the T:DC ratio was decreased) (Figures 3A and B).

At the 9:1 T:DC ratio, CD25 expression was very similar to the T cell only control, with values of $2.9 \pm 0.5\%$ and $2.8 \pm 0.4\%$, respectively. This increased 5-fold to $15.8 \pm 2.6\%$ CD25 expression at the 4.5:1 ratio and increased further to $33.9 \pm 0.9\%$ with the 3:1 ratio. CD25 expression at the 4.5:1 ratio and increased further to $33.9 \pm 0.9\%$ with the 3:1 ratio. Comparatively, there was very little CD25 expression on T cells in the low mismatch MLR with a maximum expression of $7.9 \pm 0.4\%$ at the lowest T:DC ratio (3:1). These data support the notion that the activation response in MLR is induced by the recognition of 'non-self' HLA antigens on DCs by T cells. Notably, CD25 expression in the 'Low T cell' only control was higher than in the MLR ($14.2 \pm 3.5\%$), perhaps indicating a level of non-APC related auto activation by this donor in the absence of DCs. CD4+ T cell proliferation displayed a very similar profile to the CD25 expression across the conditions tested which reinforces the allogenic activation response (Figure 3C). CD3/CD28 Dynabeads or Immunocult were added to T cell monocultures as positive controls and induced very high levels of both activation and proliferation.

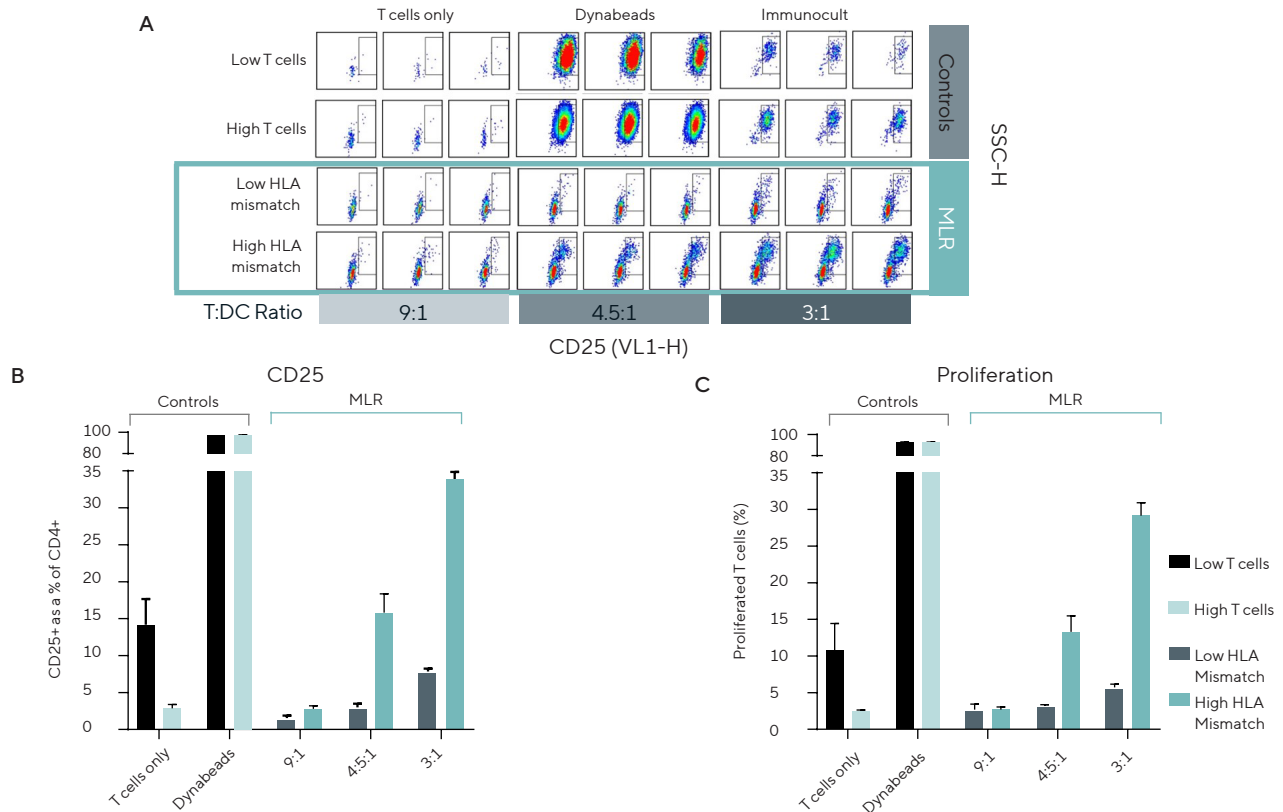
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T cell activation in MLR is enhanced in the presence of the checkpoint inhibitor Pembrolizumab

The iQue® MLR assay was used to investigate the effects of a checkpoint inhibitor drug on the T cell activation response. T cells and DCs from donors with highly mismatched HLA alleles were incubated with varying concentrations of Pembrolizumab, which is an anti-PD-1 monoclonal antibody indicated for treatment of cancers such as non-small cell lung cancer (NSCLC) and melanoma.^{3,4} The PD-1 receptor, PD-L1, is highly expressed on DCs and together these proteins represent a major checkpoint for immune regulation. Blocking this interaction with drugs such as Pembrolizumab 'takes the brake off' T cells and enhances their activation and anti-tumor activity.

Figure 3. Heterogeneity in HLA haplotype of donors in MLR induces higher levels of T cell activation and proliferation



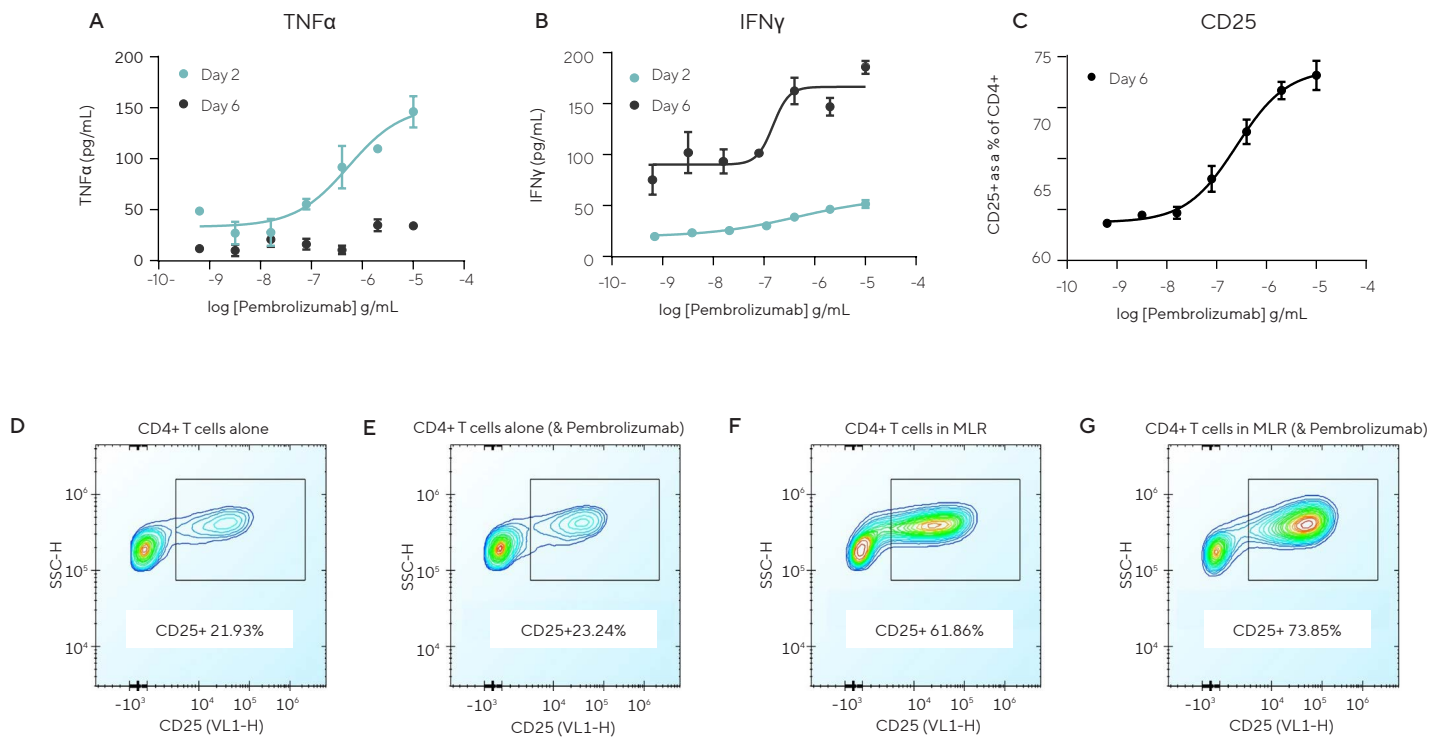
B/Green Proliferation and Encoder Dye labelled CD4+ T cells (45K/well) from two donors were separately co-cultured with DCs from a single donor at a range of T:DC ratios. The T cell donors were selected based on the level of HLA mismatch with the DC donor (Low T cell donor = low mismatch, High T cell donor = high mismatch). Controls included T cells cultured alone (negative) or with CD3/CD28 Dynabeads or Immunocult (positive). Analysis was performed on day 5 using the iQue® Human T Cell Activation Kit. (A) Plate view shows well by well differences in CD25 expression within the CD4+ population. (B) and (C) Bar charts quantifying the percentage of CD25+ and proliferated cells in the CD4+ population.

Cytokine samples were analyzed on days 2 and 6 for IFN γ and TNF α concentrations using Qbeads from the iQue[®] Human T cell Activation Kit. Pembrolizumab induced a concentration dependent increase in the release of both cytokines, indicating an increase in T cell activation, but the temporal profile of production of each cytokine was different (Figure 4A and 4B). TNF α release increased in a drug dependent manner on day 2 (EC_{50} = 0.54 μ g/mL) then decreased by day 6. Conversely, IFN γ release was low on day 2 and increased by day 6, with an EC_{50} of 0.44 μ g/mL; very similar to the EC_{50} for day 2 TNF α .

In monoculture, the checkpoint inhibitor did not affect the level of T cell activation, with day 6 CD25 expression of $21.9 \pm 9\%$ and $23.2 \pm 5\%$ in the absence and presence of

10 μ g/mL Pembrolizumab (Figure 4D and E). In the MLR co-culture, CD25 expression was $61.9 \pm 2\%$; about 3-fold greater on CD4+ T cells in monoculture (Figure 4F). Pembrolizumab induced a further concentration dependent increase in CD25 expression, with a maximum of a 12% increase in the presence of drug compared to the co-culture alone (Figure 4C and 4G). This increase in activation is less stark than the increase due to the addition of DCs, which may reflect the clinical situation, where the drug is designed to delicately tip the balance towards enhanced T cell activation, without inducing over-activation. If T cells become too highly activated, an inflammatory response can be triggered, leading to a loss of specificity of immune cell killing of cancer cells and resulting in killing of healthy cells.

Figure 4. Pembrolizumab induces a concentration dependent increase in activation markers and cytokines by T cells in the presence of DCs



DCs were thawed and activated overnight (with IL-4, granzyme B and LPS) prior to plating at 40K/well in a 96 well plate. CD4+ T cells were added at a 3:1 T cell-to-DC ratio. Checkpoint inhibitor Pembrolizumab was added to enhance T cell activation. Cytokine samples (day 2 and 6) and marker expression (day 6) were analyzed using the iQue[®] Human T Cell Activation Kit. (A) and (B) Concentration response curves show day 2 and 6 release of cytokines, TNF α and IFN γ , in response to Pembrolizumab. (C) Day 6 CD25% expression in CD4+ T cells from MLR. (D-G) Contour plots show % CD25 expression in T cell monoculture controls and MLR co-cultures, both with and without 10 μ g/mL Pembrolizumab.

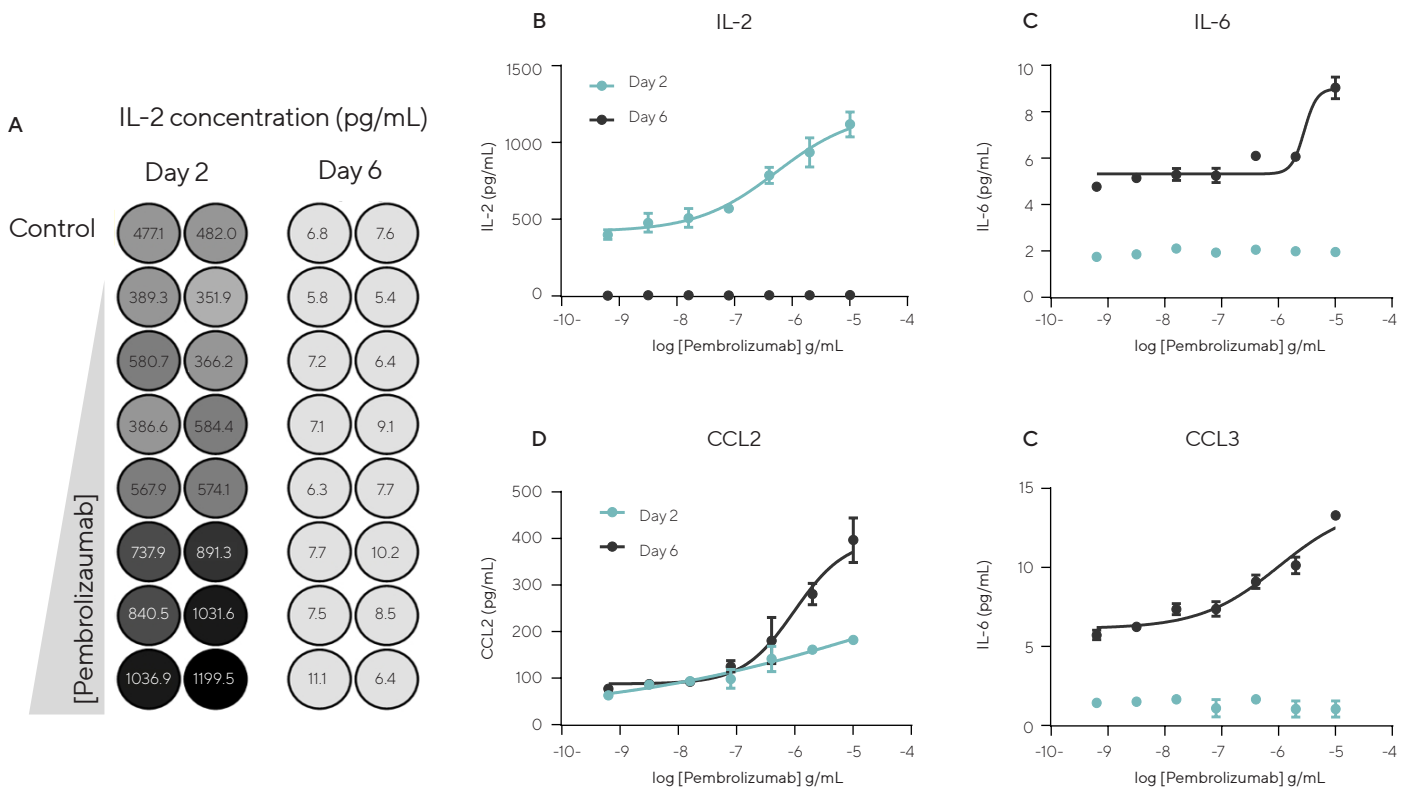
Pembrolizumab induces inflammatory cytokine release in MLR

Common side effects of checkpoint inhibitors relate to the increase in T cell response causing an attack on healthy body cells, resulting in adverse inflammatory responses.⁵ Signals that propagate these adverse responses include pro-inflammatory cytokines. To examine the secreted protein release profile in response to Pembrolizumab in MLR, the iQue Qbeads[®] Human Inflammation Panel Kit was used to measure the concentration of a combination of 7 inflammatory cytokines and chemokines in a multiplex assay (full list in Figure 1). Pembrolizumab induced a concentration dependent increase in 6 out of the 7 secreted proteins measured (representative data for IL-2, IL-6, CCL2 and CCL3 shown in Figure 5). The only signaling molecule for which this effect was not observed was CXCL9 (data not shown), however by day 6, release of this chemokine had exceeded the maximum range of this assay across the entire Pembrolizumab concentration range used, so the results for drug concentration dependent effects were inconclusive.

IL-2 provided an indication of early stage inflammatory response, with an EC₅₀ for day 2 IL-2 release in response to

Pembrolizumab of 0.53 µg/mL. By day 6 all IL-2 production has ceased. IL-6, CCL2 and CCL3 levels all increased from day 2 to 6, with day 6 EC₅₀ values for CCL2 and CCL3 release very similar at 0.98 and 1.01 µg/mL, respectively. Many of these inflammatory molecules are produced by helper T cells (CD4+ T cells) and dendritic cells in a normal, productive immune response, however, their prolonged and elevated release can lead to inflammatory disease.⁶ In the cases where checkpoint inhibitor induced inflammation has progressed to inflammatory disease, a common first line therapy is treatment with anti-inflammatory drugs such as corticosteroids.⁵ To explore this effect in vitro we compared the levels of secreted protein release in MLR in the presence and absence of the corticosteroid Dexamethasone (1 µM) using the iQue Qbeads[®] Human Inflammation Panel Kit. The impact of Pembrolizumab was also explored with the addition of a single concentration of Pembrolizumab (10 µg/mL) to the MLR. Figure 6 shows IFN γ , CCL3 and IL-2 release which demonstrates a general increase in the production of inflammatory cytokines and chemokines with Pembrolizumab, and a similar temporal profile as was observed in Figures 4 and 5. Cytokine release with CD4+ T cells in monoculture was minimal.

Figure 5. Release of inflammatory cytokines fluctuates temporally and increases in the presence of Pembrolizumabs

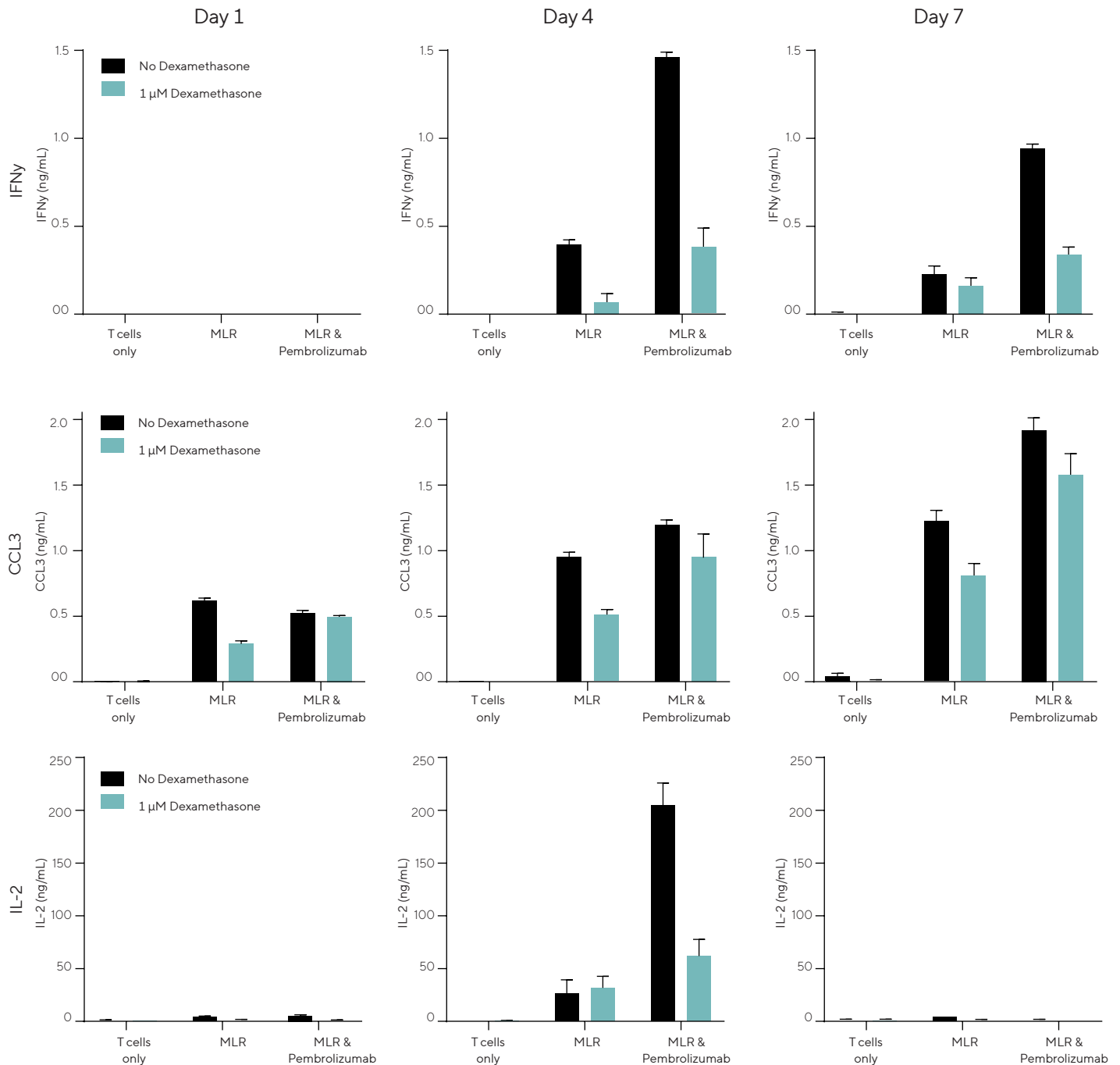


Pre-activated DCs were seeded (40K/well) with CD4+ T cells (3:1 T cell-to-DC ratio) and treated with Pembrolizumab. 10 µL samples were analyzed on Days 2 and 6 using the iQue Qbeads[®] Human Inflammation Panel Kit. (A) Heat map showing IL-2 (pg/mL) release per well. Control wells contained T cells and DCs in the absence of drug. (B-E) Curves highlight temporal and Pembrolizumab concentration dependent release of inflammatory cytokines and chemokines: IL-2, IL-6, CCL2 and CCL3.

Both in the presence and absence of Pembrolizumab, the inclusion of Dexamethasone induced a decrease in the level of inflammatory cytokines, IFN γ and CCL3 and IL-2 in MLR. IFN γ and IL-2, (in the presence of Pembrolizumab) saw a 3- and 4-fold decrease in production with the addition of Dexamethasone, respectively. In comparison, the effects of Dexamethasone on CCL3 release were more subtle with a maximum 2-fold decrease (day 4, MLR alone). Dexamethasone generally caused a decrease in production of inflammatory cytokines, however, across the panel of 7

secreted proteins tested, there were two chemokines which saw an increase in concentration in the presence of Dexamethasone: CCL2 and CXCL10 (data not shown). This draws the conclusion that whilst Dexamethasone clearly impacts inflammatory cytokine release in this in vitro MLR model, it is not clear whether the summative impact of changes to the cytokine release profile would cause an overall increase or decrease in inflammation in vivo and further experiments are needed to fully profile the effects of corticosteroids in this model.

Figure 6. Production of IFN γ , CCL3 and IL-2 in MLR were reduced in the presence of Dexamethasone



DCs were thawed and activated overnight with cytokines prior to plating at 30K/well in a 96 well plate. CD4+ T cells were added at a 3:1 T cell-to-DC ratio. Cells were incubated with or without Dexamethasone (1 μ M) and/or Pembrolizumab (10 μ g/mL). Supernatant samples were analyzed for inflammatory cytokine concentrations using the iQue Qbeads[®] Human Inflammation Panel Kit on days 1, 4 and 7.

Sensitivity of response to an anti-CTLA4 antibody differs between PBMC donor pairs

Aside from PD-1 targeting therapies, the most common target for checkpoint inhibitor drugs is CTLA4. Approved therapies include Ipilimumab, a monoclonal antibody used for the treatment of advanced or unresectable melanoma.⁷ To investigate this, the two-way MLR assay model was used to interrogate the effects of an anti-CTLA4 antibody *in vitro*. Unlike in previous experiments, PBMC donors were not pre-selected based on their HLA typing information, instead PBMCs were mixed to create 8 donor pair combinations and compared their IFN γ and TNF α release in a 384 well assay format.

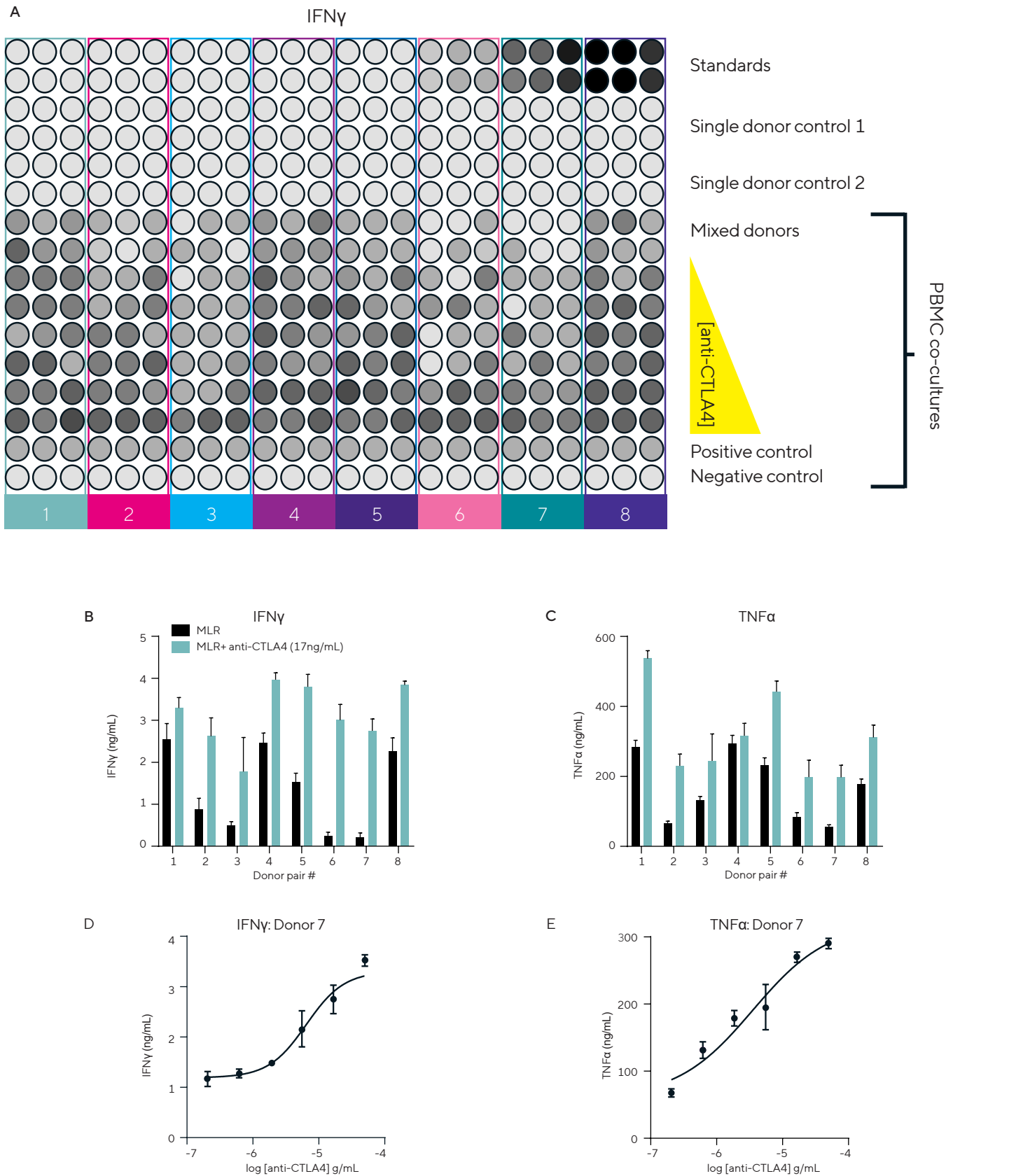
As seen in previous experiments, there was an increase in the release of IFN γ when the PBMC donors were mixed compared to the single donor controls (Figure 7A). Both IFN γ and TNF α release were further enhanced with the addition of the anti-CTLA4 checkpoint inhibitor (Figure 7). There was a clear difference in the sensitivity of the response to anti-CTLA4 between each of the 8 donor pairs (Figure 7A-C). For example, IFN γ release by donor pair 7 was low at the lowest concentrations of anti-CTLA4 but increased with antibody concentration with an EC₅₀ of 6.5 μ g/mL (Figure 7D). Conversely with donor pair 8, the level of IFN γ release is consistently high in the absence of antibody and across the concentration range used, indicating greater non-drug specific activation and reduced drug induced enhancement of response. A similar release profile as for IFN γ was seen for TNF α release with donor pairs 7 and 8. The large differences in drug response

between donor pairs highlights the need to test a potential therapeutics' efficacy with a range of donors to build a full profile of its effects on T cell activation. This demonstrates why high-throughput, quantitative techniques are essential to speed up drug discovery processes.

Checkpoint inhibitor drugs used in combination can induce synergistic effects on immune response

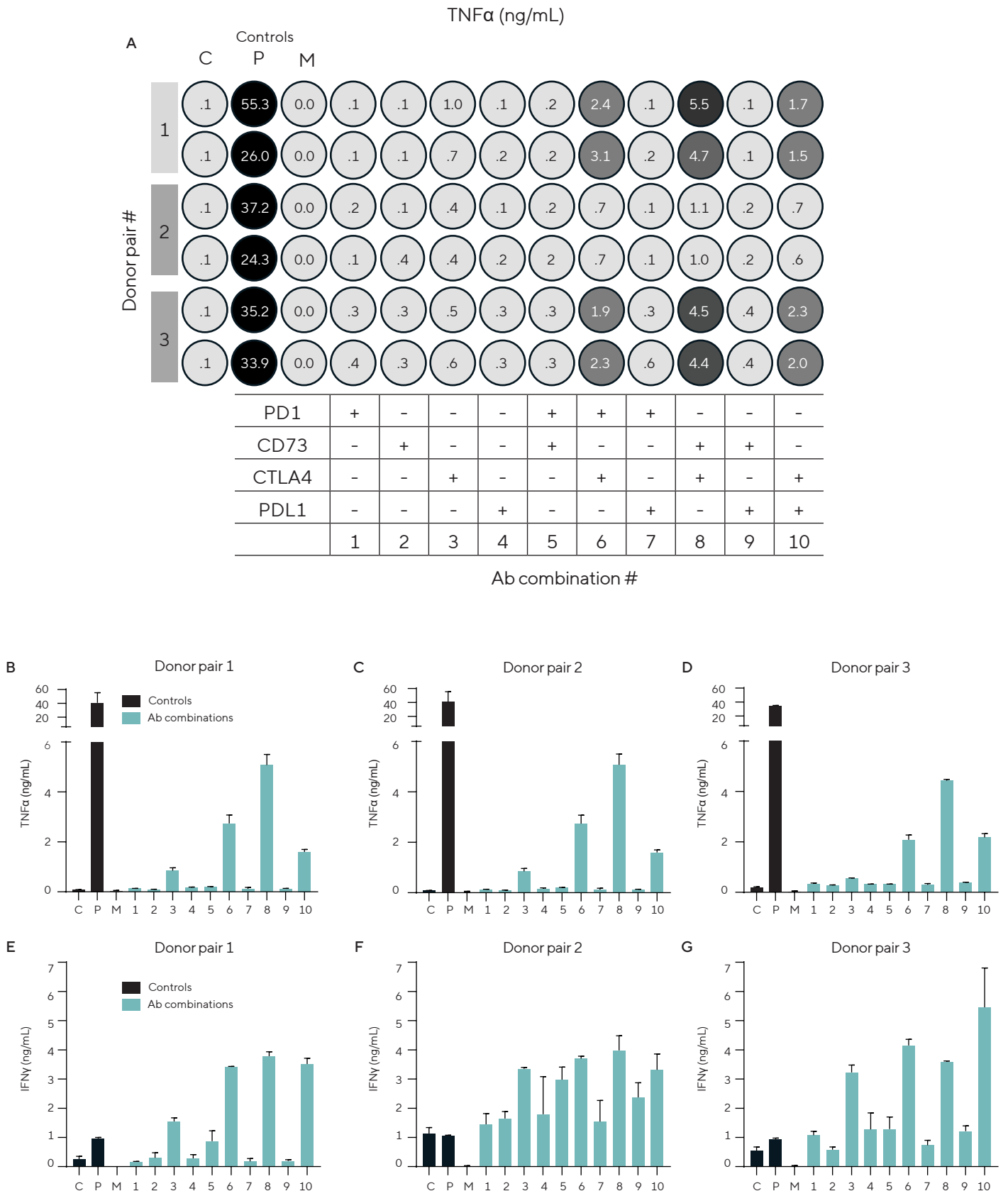
Checkpoint inhibitor drugs with different checkpoint targets can be used in combination to increase T cell activation and therefore increase the anti-tumor response.⁸ For example, anti-CTLA4 antibody Ipilimumab and anti-PD-1 antibody Nivolumab have been approved as a combination therapy for treatment of metastatic melanoma. To examine the combinatory potential of checkpoint inhibitor antibodies using the iQue[®] MLR assay model, we added 4 monoclonal antibodies: anti-PD-1, anti-CD73, anti-CTLA4 and anti-PD-L1, either alone or in combination, to three PBMC co-cultures and measured the response using IFN γ and TNF α iQue Qbeads[®]. The heat map shown in Figure 8A highlights 3 'hits' for antibody combinations that stimulated high levels of TNF α release: combinations 6, 8 and 10. These hits all contained the anti-CTLA4 antibody in combination with each of the other three antibodies. Across all 3 donor pairs, maximal TNF α release was observed with combination 8, which included anti-CTLA4 alongside anti-CD73. The addition of the anti-CD73 antibody alongside the anti-CTLA4 induced a 6-, 3- and 8- fold increase compared to the CTLA4 antibody alone in donor pairs 1, 2 and 3, respectively (Figure 8).

Figure 7. Cytokine release differs between PBMC donor pairs in co-culture and is enhanced with an anti-CTLA4 antibody



PBMCs (80K/well) from multiple donors were seeded alone (single donor controls) or in combination (8 donor pairs). Cells were treated with several concentrations of an anti-CTLA4 checkpoint inhibitor antibody. Mixed PBMCs with PHA and MMC were included as positive and negative controls, respectively. After 3 days, samples were analyzed for cytokine concentrations using multiplex IFN γ and TNF α iQue Qbeads[®] and concentrations calculated using a standard curve. (A) Heat map highlights differences in anti-CTLA4 induced IFN γ release between PBMC donor pairs. (B) and (C) Bar charts compare IFN γ and TNF α release from MLR in the absence and presence of 17 ng/mL anti-CTLA4. (D) and (E) Representative concentration response curve showing donor pair 7 IFN γ and TNF α release in response to antibody.

Figure 8. Activation of T cells by anti-CTLA4 Ab is enhanced when used in combination with other checkpoint inhibitor Abs



PBMCs from 3 pairs of donors were co-cultured at 80K/well in the presence of a range of combinations of checkpoint inhibitor antibodies: anti-PD-1, anti-CD73, anti-CTLA4 and anti-PD-L1 (n=2). After 3 days, samples were analyzed for cytokine concentrations using multiplex IFN γ and TNF α iQue Qbeads[®]. (A) Heat map shows TNF α release (ng/mL) per well. Columns 1-3 include: no antibody negative control (C); PHA (P) positive control and an MMC (M) negative control. Columns 4-13 (highlighted by pink box) include MLR co-cultures with each antibody combination (1-10 listed in the table below). (B) to (D) Bar charts show TNF α release by donor pairs 1-3 in the presence of antibody combinations 1-10 compared to controls. (E) to (G) As in B-D but showing IFN γ release from the same wells.

Antibody combinations 6, 8 and 10 also induced the greatest release of IFN γ . In donor pair 1, anti-CTLA4 paired with the three other antibodies induced a 2 to 2.5- fold increase in IFN γ compared to anti-CTLA4 alone, although unlike with the TNF α , there was little difference between the three combinations. Donor pair 3 released similar levels of IFN γ whether the anti-CTLA4 antibody was added alone or in combination with anti-PD-1 or anti-CD73, meaning unlike with donor 1, the synergistic effect of the antibodies in combination was not observed. Donor pair 3 appeared to have the highest IFN γ release with Ab combination 10, although these data points showed high variability and therefore the mean was within 1 standard deviation of the next highest releasing antibody pair (#6).

Donor pair 2 showed much lower sensitivity to the checkpoint inhibitor antibodies compared to donor pairs 1 and 3. Donor pair 2 saw minimal TNF α release with any of the antibody combinations used, whilst IFN γ release was high across all of the antibody combinations tested, showing little specificity in response in relation to the checkpoint inhibitors. This further exemplifies the importance of testing potential therapeutics with a range of donor pairs to gain a full understanding of their influence on T cell activation.

Conclusions

The iQue[®] HTS Platform used in conjunction with a validated suite of reagent kits provides a simple and flexible workflow for measuring T cell response in an MLR assay. Combining fast sample acquisition by the iQue[®] Platform with pre-set gating and analysis using the inbuilt iQue Forecyt[®] software means libraries of potential checkpoint inhibitor therapeutics can be profiled for their activity in minimal time. The experiments in this note have highlighted the advantages of this workflow, including:

1. Cell markers and cytokines are measured in multiplex; speeding up time to actionable results and negating the need to correlate data from multiple platforms. This improves data coherence with all readouts for each treatment provided by the same population of cells at a single time point.
2. Small sample volume (10 μ L) requirements mean a single assay plate can feed multiple different kits and time points; allowing the user to generate a wide range of assay outputs with minimal usage of precious sample.
3. Pharmacological readouts, such as EC₅₀ values, generated using the Forecyt software, can be used to rank checkpoint inhibitor drugs based on their activity in inducing T cell response.

4. High throughput data acquisition by the iQue[®] Platform means that a 96 well immune assay can be read in 15 minutes or a 384 well in 40 minutes. This facilitates rapid screening of multiple different drugs for their activity with cells from multiple donor pairs.

Together, these benefits make this technique a powerful tool with potential applications in research and drug discovery.

Acknowledgement

The authors would like to acknowledge Clare Szybut for contributing to this work while employed at Sartorius.

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