APPLICATION NOTE

ImmuSignature™ MLR: Rapid high-throughput assessment of therapeutic immunogenicity

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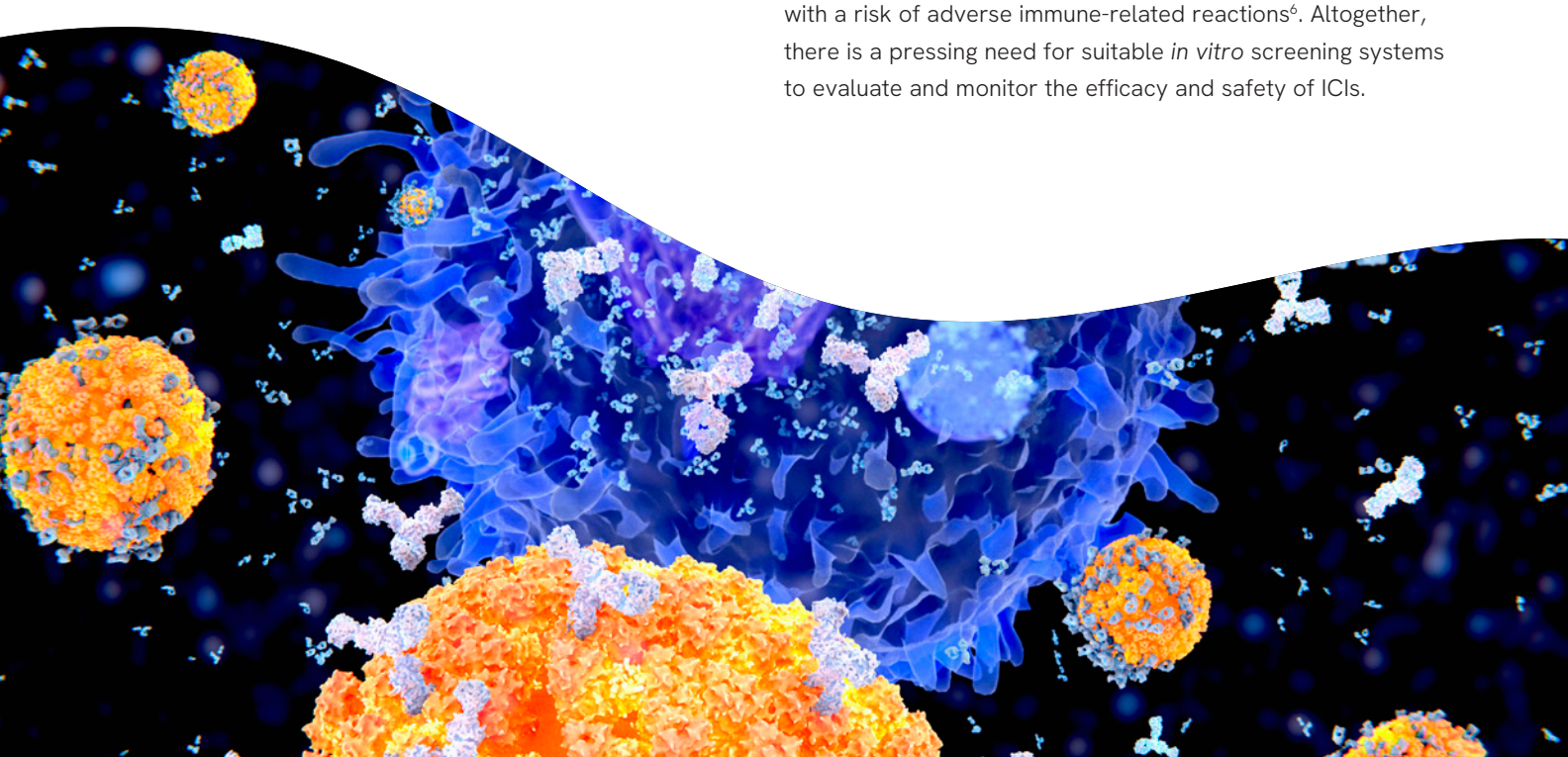

Introduction

Immune checkpoints are regulatory signals that act as 'on and off switches' for T cells[1], a mechanism tumor cells commonly deploy to evade immunosurveillance[2]. Immune checkpoints are engaged when a T cell binds to partner proteins on an antigen-presenting cell (APC) or tumor cell. Interaction of these receptor and ligand pairs results in dysfunction and/or exhaustion characterized by impaired effector function such as reduced cytotoxicity or cytokine production, lack of response to stimuli, and altered transcriptional and epigenetic states[3]. Immune checkpoint inhibitors (ICIs) target this co-inhibitory synapse between T cells and APCs/tumor cells to elicit an anti-tumor response[4-5]. These therapies aim not to kill cancer cells directly but to release blocks that shield tumor cells from immune destruction.

Immunomodulators are now used as single agents or combination therapies for various cancer types and represent about two thirds (~3000) of active clinical oncology trials[1]. With the success of existing ICI therapies and substantial clinical progress in treating certain aggressive cancers, ICIs continue to be an area of focus for intensive research and development for bringing newer therapies to market.

Given the nature of these drugs, characterizing their mechanism of action requires complex assays reliant upon the interaction between primary T cells and APCs. Additionally, although ICIs show immense promise, they have historically been associated with a risk of adverse immune-related reactions[6]. Altogether, there is a pressing need for suitable *in vitro* screening systems to evaluate and monitor the efficacy and safety of ICIs.

This application note focuses on the properties of our mixed lymphocyte reaction (MLR) assay as a rapid, relevant, and reliable platform to characterize the functionality of molecules of interest and address safety considerations.

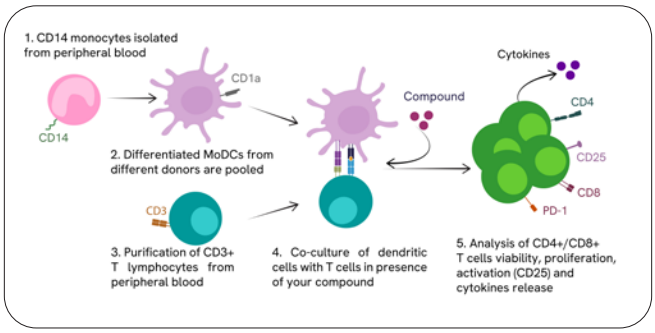

Figure 1. **One-way MLR assay setup**. MoDCs derived from CD14+ monocytes are co-cultured with CD3+ T cells over several days. Effect of compounds addition to the coculture is assessed by a multiplexed readout that measures the activation and proliferation of CD3+ T cells in response to the MoDC donor mismatch. Flow cytometry measures CD25 expression as a marker of activation and proliferation in the total CD3+ T cell fraction and CD4+ and CD8+ T cell subsets. Activation of T cells is determined by cytokine release using HTRF (Homogenous Time-Resolved Fluorescence) technology.

Mixed lymphocyte reaction

The interaction between T cells and APCs is critical in mounting an effective immune response, with dendritic cells (DCs) widely recognized as the most efficient class of APCs[7]. The mixed lymphocyte reaction (MLR) mimics this immunological synapse and is helpful as an *in vitro* model to explore the modulatory effects of drugs and facilitate drug discovery processes. In an MLR assay, T cells (responders) are co-cultured with APCs (stimulators) in an allogeneic manner. In the *in vitro* microenvironment, the T cells scan the surface of the stimulator cells. The recognition of 'non-self' antigen resulting from Human Leukocyte Antigen (HLA) donor mismatch leads to potent T cell activation that can be evaluated through increased production and cytokine production. Conventionally, MLR assays can be conducted as either one-way or two-way reactions. In a one-way MLR, T cells from one donor are cultured with APCs from an alternative donor. For a two-way MLR, Peripheral Blood Mononuclear Cells (PBMCs) isolated from two distinct donors are co-cultured with a bidirectional activation of T cells within both populations. Due to the unidirectional nature of stimulation, a one-way MLR is advantageous as a cleaner system for measuring T cell activation in a background-free model. Screening of compounds and lead identification can be a lengthy investment process.

Our standard MLR assay, described in Figure 1, offers a rapid solution for screening agents with a multiplexed readout, with data delivery in as little as four weeks. A miniaturized 384-well format combined with a semi-automated approach enables the screening of up to 18 compounds in three independent T cell donors in a single run, including dose-response controls. Additionally, to further shorten the process, we utilize cryopreserved cells in our assay with ample evidence through optimizations and comparisons that cryopreserved cells perform equally well as freshly isolated cells.

Our MLR assay is a robust standard offering several quality control (QC) assessment stages. First, isolated and differentiated cells undergo a thorough purity check before cryopreservation and assay set-up. Second, assay performance is a prerequisite to further data analyses, evaluated through our resolute MLR bioinformatic pipeline and the use of relevant positive and negative controls. This assay performance QC also informs on the existence of plate-to-plate variations, if any. Figure 2 overviews our assay performance QC criteria and the use of internal assay controls (CD4+ T cells shown as representative). The top panel reflects proliferation in T cells as measured by CellTrace™ Violet (CTV), and the bottom panel indicates CD25 expression as a surface marker of T cell activation. CTV is a proliferation tracker dye that diminishes in intensity upon cell division, with each division representing a distinct peak. CD3+ T cells cultured alone do not proliferate and are not activated, as evidenced by a single CTV peak (no loss in CTV intensity) and lack of CD25 staining. In comparison, CD3+ T cells co-cultured with MoDCs (medium) undergo cell division and express high levels of CD25 in an allogeneic reaction.

As internal assay performance controls, we use a single dose of recombinant cytotoxic T-lymphocyte antigen 4 (CTLA4) or anti-CD3/28 antibodies as negative and positive controls, respectively. Interactions of APC ligands with CTLA4 result in T cell anergy/serve to inhibit T-cell responses[8]. True to this, CTLA4 protein (CTLA4-Ig) acts as a negative regulator of T cell proliferation and activation in our assay (Figure 2). Anti-CD3/28 antibodies, on the other hand, serve as a positive control and provide a co-stimulatory signal that engages the T cell receptor resulting in robust proliferation and increased CD25 expression.

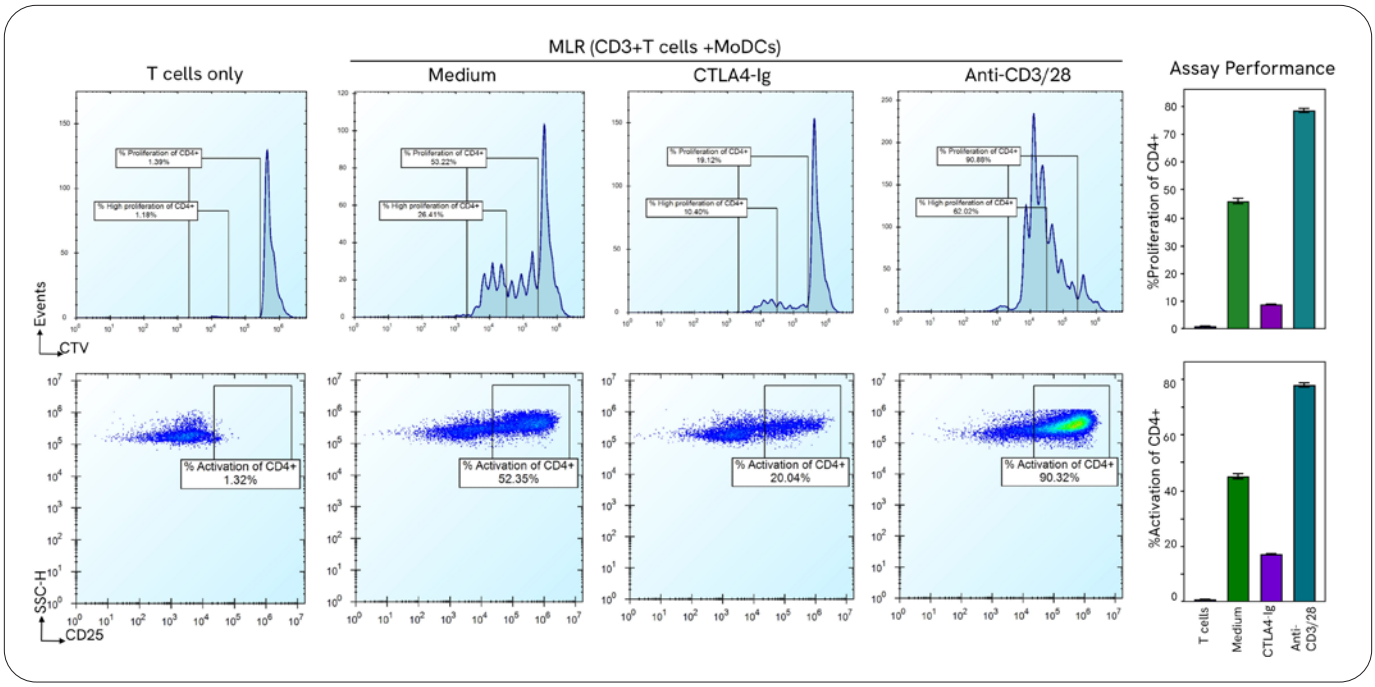

Figure 2. MLR assay performance QC measure using internal assay controls. Representative flow cytometric measure of proliferation (top panel) and activation (bottom panel) in CD4+ T cell fraction of CD3+ T cells cultured alone or in the presence of MoDCs. Co-culture conditions comprise no treatment (medium) or treatment with a single dose of CTLA4-Ig (negative control) or anti-CD3/28 (positive control). The reduction of the fluorescent tracker dye CellTrace™ Violet (top panel) shows proliferation levels and CD25 expression as a T cell activation surface marker (bottom panel). Cumulative assay performance data obtained from quadruplicates and standard deviation of a single donor data are depicted in the graphs on the right for proliferation (top panel) and activation (bottom panel), respectively.

Each condition is run in quadruplicate (per plate), and the overall assay performance is depicted in the graphs on the far right in Figure 2 for both proliferation (top panel) and activation (bottom panel).

Robust assay window and multiplexed readout for screening activators and inhibitors

T cells play a critical role in shaping the immune landscape in the context of the tumor microenvironment and in autoimmune and inflammatory diseases. In this context, the MLR assay makes for a highly relevant *in vitro* model to understand the effects of biologics and/or small molecules that modify the interaction between APCs and T cells to activate, deactivate or repolarize the lymphocyte response. It can also provide critical information for undesired immunological responsiveness from a drug safety perspective. The assay window becomes critical to address the impact of test agents, keeping all these considerations in mind.

Achieving proliferation and activation of T cells in the 40-60% range is ideal for a reliable assay window for screening activators and inhibitors. Furthermore, this assay window must be consistently reproducible using primary immune cells and potential donor variability as a by-product. To control donor dependencies without impacting physiological relevance, our MLR assay considers several factors: 1) To maximize the chance of 'non-self' recognition, we utilize MoDCs pooled from multiple individual donors. These are then co-cultured in a fixed ratio to the number of T cells in the assay to achieve consequent T cell activation and proliferation in a controlled manner. 2) A minimum of three independent T cell donors are assessed in the assay to account for both donor reproducibility and variability. 3) The semi-automated process controls the overall assay variability and reduces manual error.

Our optimized MLR offers a reliable assay window that assesses outcomes linked to either activation or inhibition of T cell function. To assess the suitability of our assay window as fit for purpose, we conducted a 20-compound alpha screen that included a mix of biologics and small molecules. This panel included the monoclonal antibodies, nivolumab and daclizumab, and their respective isotype controls, depicted in Figure 3 as representative examples. Nivolumab is a clinically approved humanized IgG4 antibody that blocks

the immune checkpoint PD-1 and can restore antitumoral response by abrogating PD-1 pathway-mediated T-cell inhibition[9]. On the other hand, daclizumab is a humanized IgG1 antibody that inhibits effector T cell activation by blocking CD25, a critical subunit of the interleukin-2 receptor (IL-2R) required for T cell maintenance[10].

A nine-point dose range (eight doses plus vehicle control) was tested using a three-fold semi-logarithmic serial dilution series and the highest final concentration of 10 µg/mL. Using a multiplexed readout, we assessed T cell activation, proliferation, and viability through flow cytometry and IFN-g production by HTRF. Beyond total CD3+ T cells, the flow cytometric readout also provides granularity on CD4+ and CD8+T cell subsets. Donor reproducibility is essential when screening test agents for their ability to modulate responses. To this end, we observed in Figures 3A-B and E-F the respective inhibitory and activating effects of daclizumab and nivolumab on the activation and proliferation of these T cell subsets in all three donors. While panels 3A and 3E show the impact of these compounds on CD25 expression (activation) and CellTrace Violet staining (proliferation) in CD4+ helper T cells, panels 3B and 3F show their equivalent in cytotoxic CD8+ T cells. Figures 3C and G depict the effect of these test agents on T cell viability. As anticipated, daclizumab targets IL-2R critical for T cell maintenance and thus affects the viability of all three donors (Figure 3C). On the contrary, viability upon nivolumab treatment did not have significant changes (Figure 3G).

In summary, this data reiterates the strength and robustness of our MLR assay in terms of an assay window that allows for the screening of inhibitors/activators and its ability to capture donor reproducibility and variability, including T cell subset granularity.

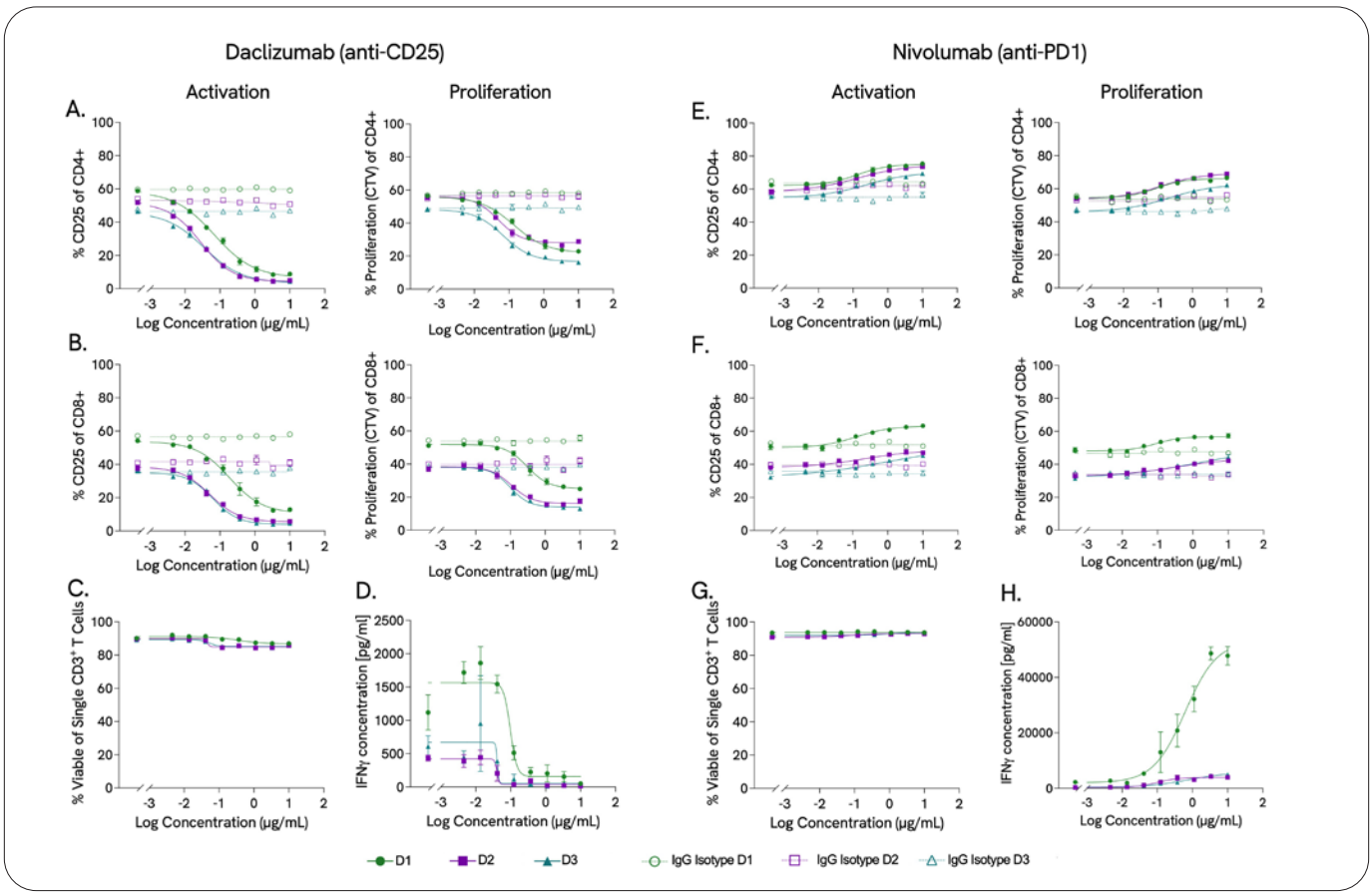

Figure 3. **Screening of inhibitory and activating immunomodulators using the MLR assay multiplexed readout**. Treatment of CD3+ T cell and MoDC co-cultures with anti-CD25 monoclonal antibody daclizumab (Panels A-D) and anti-PD1 immunotherapy nivolumab (Panels E-H) and the relevant isotype controls. Nine-point dose range (eight points + vehicle) and 3-fold semi-logarithmic dilution. A & E. Flow cytometric measure of activation (CD25) and proliferation (CTV) in CD4+ T cells. B & F. CD25 expression (activation) and proliferation (CTV) in CD8+ T cells as measured by flow cytometry. C & G. Flow cytometric assessment of total CD3+ T cell fraction percentage viability upon drug treatment. D & H Quantification of IFN-γ production by T cells using HTRF technology. D1-3 represent three independent CD3+ T cell donors. Data plotted as mean with standard deviation.

Capturing subset-specific effects

As detailed above, one of the advantages of multiparameter flow cytometry is that it allows the distinction of different subsets within a cell population, enabling the teasing apart of subset-specific effects of compounds that may otherwise get masked. Another outcome from our alpha screen was basiliximab (biosimilar)[10], a chimeric mouse-human monoclonal antibody that, like daclizumab, targets the α chain (CD25) of the IL-2R of T cells. Although the basiliximab biosimilar results in a reduction in CD25 expression in both CD4+ and CD8+ T cells in line with its target (Figure 4, A and C), interestingly, the outcome on the proliferation of these cells is converse (Figure 4, B and D).

While CD4+ T cells exhibit a reduction in proliferating cells (as measured by the percentage of cells positive for CTV), the co-culture in the presence of basiliximab increases the percentage of proliferating CD8+ T cells. CD4+ T cells comprise ~70% of total CD3+ T cells and are the dominant population in culture. In the absence of this subset granularity, the impact of this biologic on CD8+ T cell proliferation would have been disguised, with potentially significant implications *in vivo*. Overall, this data emphasizes the importance of utilizing an assay that screens for subset specific and/or potentially undesirable effects in the early stages of drug discovery and would offer end-users a good indication of the research focus.

A platform for biologics and small molecules

To test the applicability and relevance of our MLR assay in a greater context, we included a variety of compounds in our 20-compound alpha screen. Adalimumab is a monoclonal TNF-a blocker currently administered for several autoimmune conditions such as rheumatoid arthritis and Crohn's disease to dampen the immune response[11]. Our assay in Figures 5A and 5C reflects the resultant inhibition of proliferation in CD4+ and CD8+ T cells upon treatment with an adalimumab biosimilar in all three donors (identical impact on activation; data not shown). Emerging evidence suggests that small molecule targeted inhibitors can promote an antitumoral immune response in combination with immunotherapy. Counterintuitive to their immunosuppressive properties, mechanistic target of rapamycin (mTOR) inhibitors can promote or repress the immune response. Based on this reasoning, we also included the dual mTOR1/2 inhibitor KU-0063794 in our alpha screen to support small molecule drug discovery. Data from Figures 5B and 5D exhibit the negative impact of KU-0063794 on CD4+ and CD8+ T cell proliferation. Once again, this effect is consistent across three independent T cell donors. Altogether, these data showcase the suitability of our MLR assay for screening different types of compounds ranging from biologics to small molecules in various contexts.

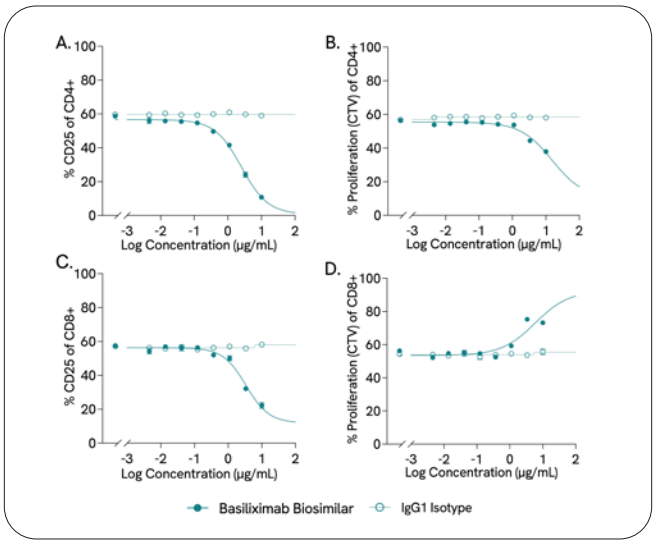

Figure 4. **Subset specific effects in T cells upon treatment with basiliximab biosimilar.** Flow cytometric measure of CD25 expression (activation) and proliferation (CTV intensity) in CD4+ (A and B) and CD8+ T cells (C and D). CD3+ T cell and MoDC co-cultures were treated with either a basiliximab biosimilar or IgG1 isotype control. Single donor depicted for representative purposes; data plotted as mean with standard deviation.

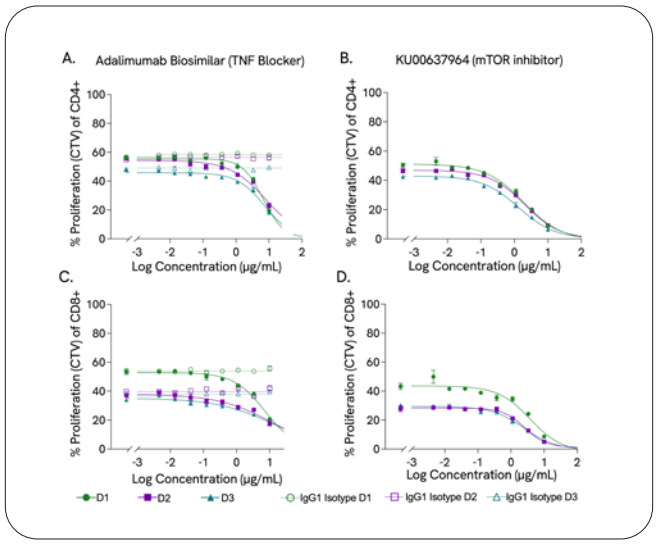

Figure 5. **Suitability of the MLR assay for screening biologics and small molecules**. CD3+ T cell and MoDC co-cultures were treated with either A&C. Adalimumab biosimilar or IgG1 isotype control or B&D. the small molecule mTOR inhibitor KU00637964. Percentage proliferation (CTV intensity) in CD4+ (A-B) and CD8+ T cells (C-D) as measured by flow cytometry. D1-3 represent three independent CD3+ T cell donors. Data plotted as mean with standard deviation.

Decipher compound effect with additional readouts

In addition to IFN-γ, other cytokines can be quantified in the supernatant of MLR assay, comprising TNF-α and IL-2. TNF-α is a major pro-inflammatory cytokine produced in response to toll-like receptor stimulation, and IL-2 is an early-produced cytokine that promotes T cell growth. The release of those three cytokines in the supernatant has been assessed by HTRF every day, for all duration of the assay, with or without the addition of control compounds (Figure 6A).

As expected, quantities of the three cytokines are not significantly modulated over time in the assay supernatant without compound treatment (medium condition). The addition of Anti CD3/28 TCR activators induced strong production of the three tested cytokines compared to baseline but with different kinetics. IFN-γ and TNF-α release in the supernatant increases over time and is maximal after 3-4 days. On the other hand, anti-CD3/CD28 leads to potent induction of IL-2 as soon as day 1, but that decreases over time, probably because of T cells activation and proliferation that consumes the IL-2 present in the medium.

The addition of daclizumab induces a slight increase of IL-2 in the supernatant over time. This observation can be explained by the fact that this antibody targets a subunit of the IL-2 receptor and therefore blocks IL-2 uptake by T cells[12]. Nivolumab antibody induces T cell activation in MLR assay, like anti-CD3/28, but with slower kinetics for IFN-γ and TNF-α, and the effect on IL-2 cannot be seen. Early cytokines assessment does not impact final readouts (data not shown), showing our MLR assay robustness and customizable readouts.

In addition to secreted protein, other markers can be induced by MoDC-induced T cell activation, like the immune checkpoint PD-1 (Figure 6B). PD-1 plays a critical role in the induction and maintenance of immune tolerance and is a target for cancer immunotherapies. Therefore, we added its detection as an optional readout in our flow cytometry panel.

As shown in Figure 6C, adding nivolumab, an anti-PD1 antibody, leads to decreased PD-1 detection in our MLR assay, as expected. This readout allows us to detect the compound effect on this specific immune checkpoint, and it can be used to verify the efficiency of antibodies targeting this receptor at the surface of T cells.

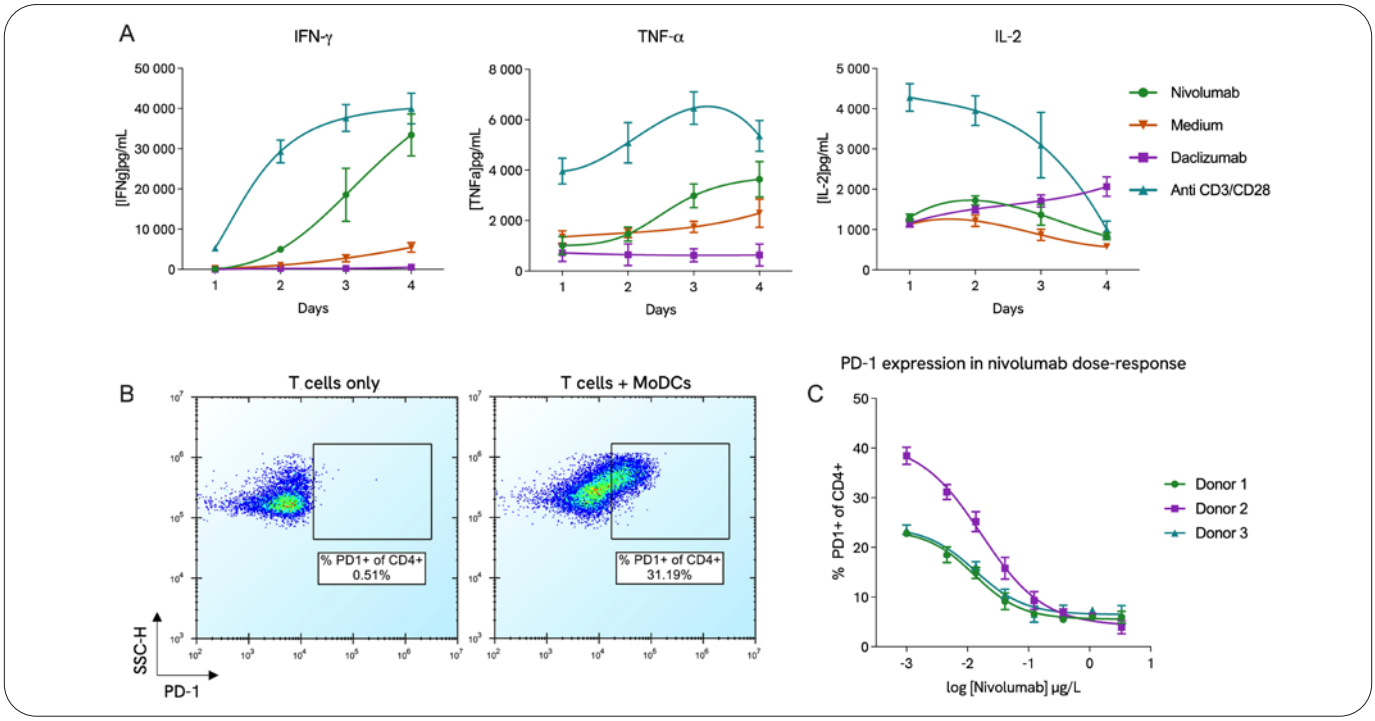

Figure 6. **Optional readout available in MLR assay**. A. CD3+ T cells from 3 different donors, in co-culture with pooled MoDCs, were treated with different compounds at day 0, and the supernatant was analyzed every day for cytokines release. Graphs show results in 1 representative CD3+ T cells donor as the mean of 4 replicates ± CV% for each time point. B. Flow cytometry representative dot plot for PD-1 expression in T cells only or cocultured with MoDcs. C. PD-1 blockade assessment by nivolumab in MLR assay. Data are plotted as the mean 4 replicates of the CD4+ T cells subset results with standard deviation, in three different T cell donors.

Summary

Boosting or suppressing the immune system to overcome disease has revolutionized therapy. Testing the efficacy and safety of potential therapeutics in a relevant and timely manner comes with challenges. With a semi-automated workflow and rapid turnaround time, we demonstrate how our standard MLR assay offers a powerful approach to furthering biological research applications and drug discovery processes. This application note exhibits its suitability, scalability, and flexibility for screening biologics and small molecules. Our multifunctional MLR assay is a highly relevant primary immune co-culture *in vitro* model to assess drugs for various conditions. It enables the understanding and monitoring of any significant immunomodulatory effects of drug candidates, not only for oncology but also for autoimmunity, inflammation, and host/graft rejection applications.

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