APPLICATION NOTE

Profiling compound effect on immune cells by combining ImmuSignature assays and LEGENDplex bead-based multiplex protein quantification.

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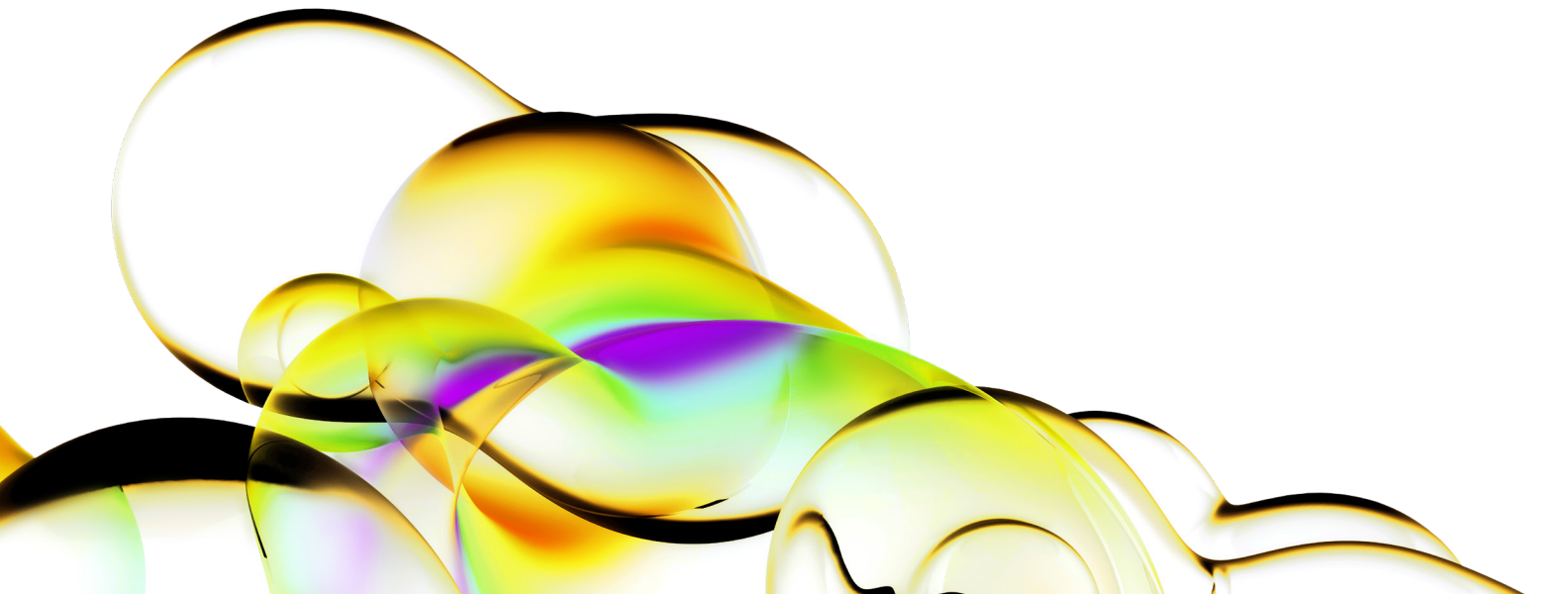
Introduction

From target identification to clinical trial, the drug discovery process involves several approaches to help decipher compounds' modes of action. A crucial step before clinical applications is assessing the therapeutic candidate´s capability to interact with the immune system. During this process, *in vitro* immune cell screening can be used to uncover novel drug targets, reveal disease mechanisms, prioritize targets, support mechanism of action studies, help unearth biomarkers, and explore the realm of immunotherapy. Our ImmuSignature™ assays provide rapid, robust, and sensitive results on compounds' immunogenicity in a miniaturized semi-automated format, using both flow cytometry and HTRF™ as standard readouts.

To obtain a deep understanding of the immunogenicity of tested therapeutic candidates, we successfully implemented LEGENDplex™ multi-analyte quantification as a new readout to our ImmuSignature portfolio. We miniaturized this bead-based multiplex protein quantification assay to study up to 14 different targets and validated this format for cytokine quantification of the Mixed Lymphocyte Reaction (MLR) assay supernatant. This new multiplexed readout can be a powerful supplementary tool to gain more insights into the potential effects of the compounds on the immune system.



Methods

ImmuSignature MLR assay screen of five immunomodulatory compounds was performed as described previously using three independent T cell donors (1). Assay supernatants were transferred into storage plates at two different time points (Day 1 and Day 4) and stored at -20°C until LEGENDplex and HTRF analysis. Human Essential Immune Response Kit (BioLegend #740930) was used on neat or diluted supernatant according to the manufacturer´s instructions or in a miniaturized format. Acquisition and primary data analysis were performed with Sartorius iQue3 HTS (High Throughput Screening) flow cytometer and associated software, and data analysis was done with GraphPad Prism.

Results

Adaptation of LEGENDplex protocol for HTS screening

LEGENDplex (LPX) technology is a flow cytometry bead-based immunoassay that allows the quantification of up to 14 different targets simultaneously with great sensitivity and dynamic range. It utilizes the same basic principles of a sandwich immunoassay, whereby a soluble analyte is captured onto a bead between two antibodies. The biotinylated detection antibody is then labeled with a streptavidin PE molecule to allow for quantification. Individual beads are distinguishable by size and varying levels of internal APC fluorescence (2).

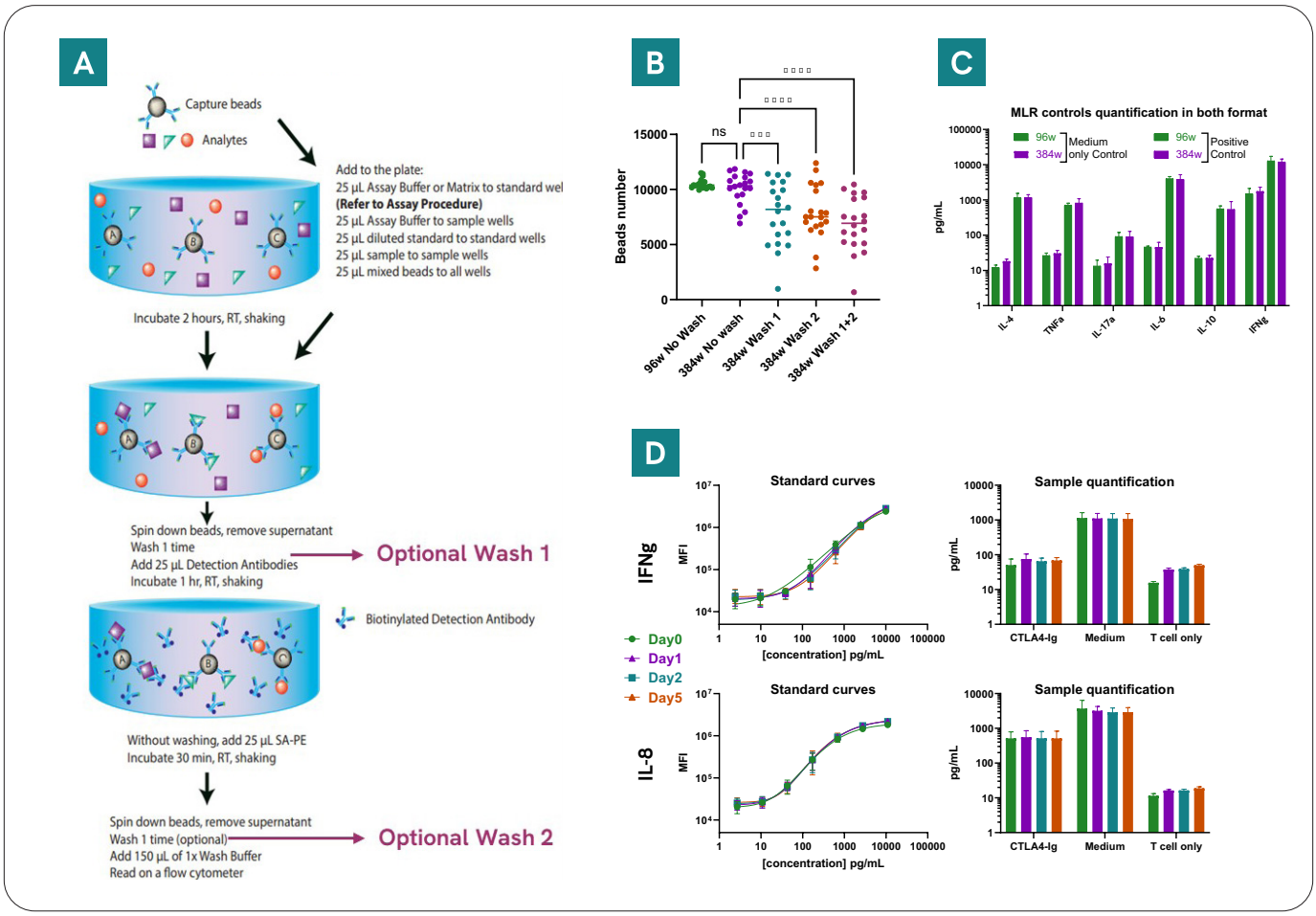


Figure 1: **Miniaturization of LPX Kit protocol for 384w plates.** (A) BioLegend LPX 96w plate protocol and optional additional washing steps have been adapted to the 384w plate by lowering the volume of the reagents by 2.5-fold. (B) Number of beads acquired by sample and standard displayed higher CV% in 384w plates with additional washing steps (median, multiple one-way ANOVA comparison with p>0.05). (C) Quantification of MLR controls in both 96w and 384w plate formats. Results are shown for six analytes as mean ± STDEV of 8 replicates. (D) MLR control samples are quantified over time (up to 5 days) on their respective standard curves. Results for IFNγ and IL-8 are shown as examples and represented as mean ± STDEV of 8 replicates.

Our ImmuSignature assays are performed in a miniaturized 384w plate format. Therefore, we needed to adapt the LPX 96w plate kit protocol to this format to avoid supernatant plate reformatting. To do so, we reduced reagents volume by 2.5-fold for each protocol step and evaluated the need to add 1 or 2 optional washing steps during staining before detection antibodies addition, or final resuspension of beads in wash buffer (e.g. optional wash 1 and 2, Fig.1A). Incubation time was kept as described in the original protocol but shaking speed was increased to 1500 rpm. For supernatant removal and reagent addition, semi-automated devices (384 channel pipettes and reagent dispenser, or plate washer) were used after optimizing aspiration height and speed. This 384w plate protocol was performed alongside the original 96w plate protocol, and results and performance were compared after the acquisition of 20 µL of standard or samples. When looking at the number of acquired beads per well, no statistical differences were found between 96w and 384w plate no-wash protocols, even if the dispersion was higher in 384w (Fig.1B). Addition of wash steps increased CV%, probably because of bead loss during repeated addition/removal of reagents, even when using automated devices. Interestingly, the background signal was only decreased by the additional wash after detection antibodies incubation (e.g. Wash 2), but without improving the global assay window. These results, combined with ease of use and throughput, favor our 384w plate protocol without additional washes, which also displayed better standard errors for most of the thirteen proteins. When MLR inter-plate controls were quantified in both formats (Fig.1C), consistent results were obtained for all the panels, with similar standard errors for both 96w and 384w formats. Furthermore, we also assessed the signal stability over time. Examples are shown for two analytes in 384w plates (Fig.1D). Even if the standard curves can slightly differ between times, and only for four analytes out of 13, as illustrated for IFNγ in top left graph, we still observed a reliable reproducibility of the quantified results at all acquisition time points, even five days post-staining. These results confirm that our miniaturized 384w plate protocol is suitable for quantifying MLR supernatant, with good reproducibility and stability over time.

Multi-analyte quantification of ImmuSignature assays compound screen supernatant

To assess LPX performance in quantifying ImmuSignature supernatant cytokines, we used MLR supernatants from a 5-compound screening of antibodies or small molecules in a 7-fold dose response. Supernatants were harvested after 1 and 4 days of co-culture, whereas cells were analyzed by flow cytometry for activation and proliferation at the assay endpoint (Day 4), and results are shown in Figure 2. First, LPX quantification allows visualization of cytokine release profiles throughout the MLR assay in the presence of controls or compound top doses (Fig.2A)

Indeed, after 24h co-culture of cells with compounds, we already detect the production of CCL2, CXCL10, IFNγ, IL-2, and IL-8 at various levels (Fig.2A). After four days, the production of IL-6 and TGFβ is higher, while IL-2 is not strongly expressed in most of the treatment. On the other hand, LPX profiling shows that IL-10, IL-12, IL-17A, IL-1β, or IL-4 are not strongly modulated by co-culture of T cells with moDCs, nor by any of the tested compounds.

If we look more closely at the compound dose-response, we confirm their modulatory effect on T cell activation and proliferation that have been found by analyzing T cell response. Durvalumab and pembrolizumab antibodies are respectively targeting PD-L1 and PD-1 immune receptors, preventing immune system down-regulation by these co-receptors. They induce T cell activation (Fig.2B top graph), and production of IFNγ (middle graph) and IL-8, but pembrolizumab has a stronger effect on IL-6 induction than durvalumab (Fig.2B, middle and bottom graph).

Adalimumab and galunisertib target two cytokines: TNFα and TGFβ and induce the opposite effect in MLR reaction: adalimumab has a small inhibitory effect. In contrast, galunisertib small molecules slightly induce T cell activation (top graph) and IFNγ production (middle graphs). Furthermore, adalimumab also inhibits CXCL10, while galunisertib strongly induces it. Interestingly, they both induce CCL2 production, but adalimumab is three times more effective.

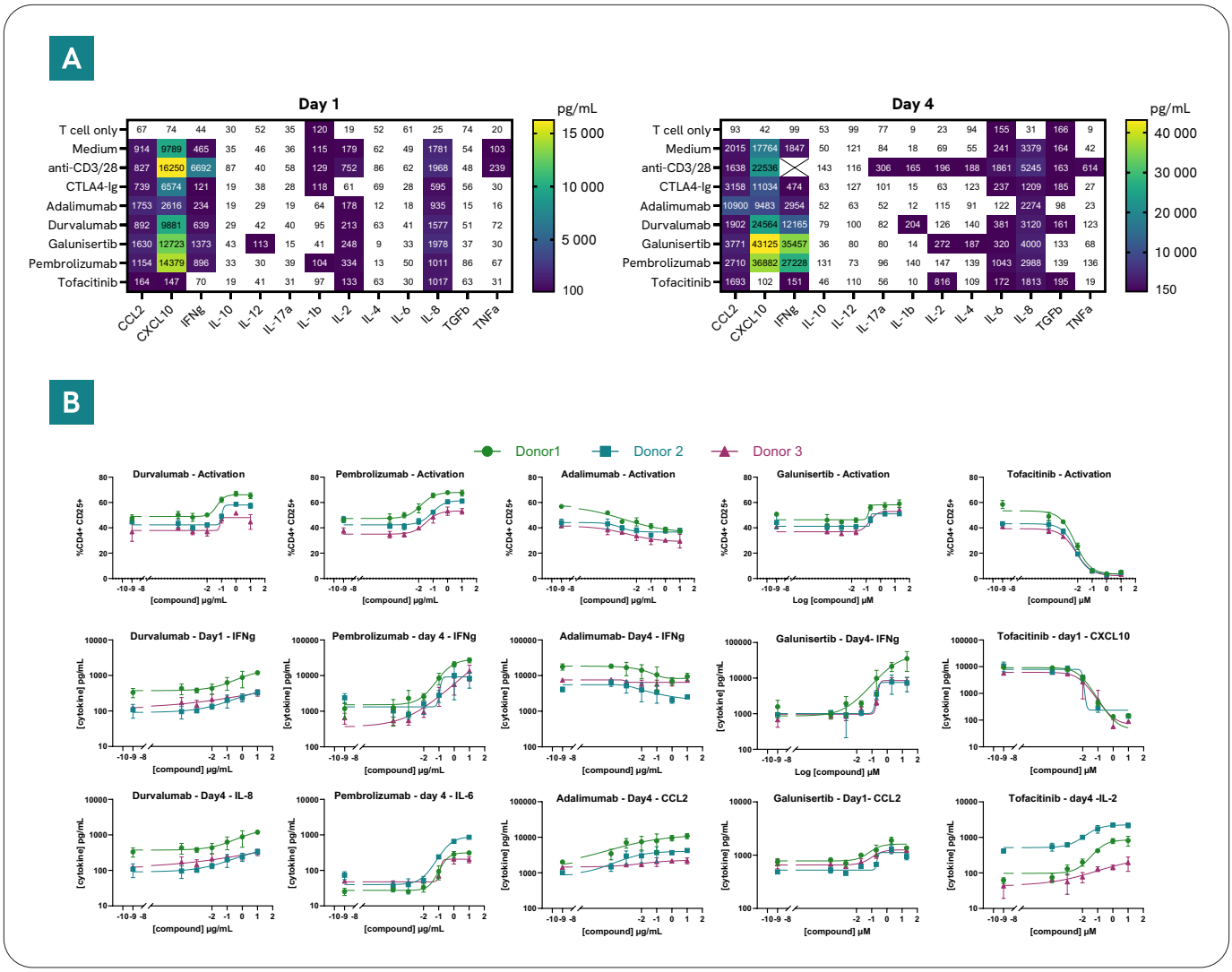


Figure 2: **Profiling cytokine release in MLR assay compound screen.** (A) Heatmap of quantification of each cytokine by LPX in 1 donor out of 3, treated or not with compound top dose or MLR inter-plate controls (T cell only, Medium, anti CD3/28, CTLA4-Ig). (B) Dose-response curves for each compound in the three donors. Top graphs show T cell activation assessed by flow cytometry at day 4 (% of CD4+CD25+), while the middle and bottom graphs display LPX results for two different cytokines out of 13, as an example. All graphs are represented as mean ± STDEV of 4 replicates for each dose and each donor.

Finally, tofacitinib is a strong JAK/STAT pathway inhibitor that leads to complete inhibition of T cell activation and proliferation in MLR assay (Fig.2B, top graph). This compound also totally or partially abrogates CXCL10, IFNγ, and IL-8 production, which can be seen as soon as Day 1 (Fig.2B, middle graph). On the other hand, by inhibiting T cell activation and proliferation, this compound allows IL-2 accumulation in the medium to be quantified by LPX (Fig.2B, bottom right graph).

Besides providing an overview of the compound's effect on 13 different analytes, LPX allows us to decipher the compound's mode of action on different pathways by examining the differential expression of those 13 cytokines.

Conclusion

Understanding the compound mode of action is critical to evaluate its impact on the immune system, to favor or impair an immune reaction, and to evaluate any side-effect if used as a treatment. To accelerate the hit-to-lead process, we successfully implemented BioLegend LEGENDplex multiplex cytokine quantification in a miniaturized format as a new readout for our ImmuSignature suite of immune cell screening assays (3). This new feature will help decipher compound effects on multiple pathways using a low amount of supernatant, increase throughput and cost-effectiveness, allow more in-depth exploration of compound effects on the immune system, and therefore help our partners identify potential immunotherapies, evaluate safety, and ultimately contribute to more effective treatments.

References

1. ImmuSignature™ MLR: Rapid high-throughput assessment of therapeutic immunogenicity. Application Note. *Revvity Inc.,* 2022

2. Introduction to LEGENDplex™, Blog Article. *BioLegend,* 2022.

3. Immune Cell Compound Screening Compendium, *Revvity Inc.,* 2023