APPLICATION NOTE

Homogeneous detection of cytokines from PBMC supernatants using AlphaLISA High Performance assays.

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Introduction

Cytokines are molecular messengers secreted by many cell types that play an essential role in inflammatory processes. They consist of interleukins, interferons, chemokines and tumor necrosis factors (TNFs) and are involved in both physiological and pathological processes. Most notably, cytokines are an important mediator of both the intensity and duration of the immunological response to stimuli such as viral and bacterial infection, but also play a role in the pathology of many diseases including cancer, autoimmune conditions, neurodegenerative disorders, and atherosclerosis. As such, cytokines are often used either directly or indirectly as targets of drug discovery for the development of new immunomodulatory therapies.[1]

Peripheral blood mononuclear cells (PBMC) are a mixture of mononuclear immune cells containing monocytes, dendritic cells, and lymphocytes such as T cells, B cells, and NK cells. PBMC are often used as an *in vitro* model system for the screening and identification of new drug candidates aiming to target immune pathways. The ability to measure cytokine secretion from such primary cell models requires assays that are both sensitive and robust.

AlphaLISA™ technology is a bead-based, highly sensitive homogenous assay platform that allows for the detection of small amounts of endogenous analytes in a microplate-based format. AlphaLISA assays require two bead types: Donor beads and Acceptor beads. The Donor beads generate singlet oxygen upon illumination at 680 nm. The singlet oxygen can then diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead,

resulting in light production. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. AlphaLISA High Performance cytokine assays use two unique monoclonal antibodies specific for the cytokine of interest. One monoclonal antibody is biotinylated and binds to the Streptavidin-coated Donor bead whereas the Acceptor bead is conjugated directly with an additional monoclonal antibody. In the presence of a cytokine the monoclonal antibodies in the assay bind to the cytokine, bringing both the Donor and Acceptor beads into close proximity. Excitation of the Donor bead leads to emission from the nearby Acceptor bead. AlphaLISA assay signal generated is proportional to the amount of cytokine present in the sample. An assay schematic for AlphaLISA assays is shown in Figure 1.

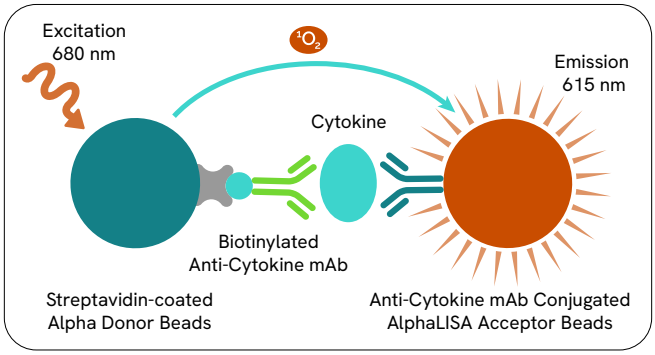

Figure 1: Example of an AlphaLISA High Performance cytokine assay

In this application note, AlphaLISA High Performance cytokine kits were used to detect and quantify interferon gamma (IFNγ), interleukin 2 (IL-2), and tumor necrosis factor alpha (TNFα) in cell culture media secreted by human PBMCs in the presence or absence of drug treatment. The AlphaLISA assay detection procedure followed a simple workflow that is illustrated in Figure 2.

Materials and methods

Cell culture and treatment

PBMC isolated using Ficoll gradient (Cytiva / #17-5442-02) for Buffy Coat (French National Blood Service (EFS Pyrénées-Méditerranée) /#B3111) were cultured in RPMI media (Gibco / #61870-010) supplemented with 10% FBS (Eurobio / #CVFSVF00-01) and plated in 96-well black plates (Greiner / #655090) at a cell density of 200,000 cells per well. Optimal cell density was determined by a cell seeding experiment testing from 25,000 to 400,000 cells per well for each cytokine to select the best density for further experiments (data not shown). For more information on PBMC preparation, please see our technical note "Guidelines From PBMC Isolation to Cytokine Assay Optimization" available on our website.

After cell seeding, cells were immediately treated with compounds. For cell stimulation experiments, increasing concentrations (0 - 20 ng/mL) of lipopolysaccharides (LPS, Sigma Aldrich / #L4391) or (0 - 50 ng/mL) of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich / #L1585) and 1 µg/mL of ionomycin (Sigma Aldrich / #I0634) were incubated with PBMC for 24 hours prior to removal of cell supernatants for testing in AlphaLISA assays. The timepoint of 24 hours was determined after testing timepoints from 2 - 24 hours post-treatment, with 24 hours of treatment time determined to be optimal using this PBMC preparation (data not shown). Please note that the optimal conditions for detection may vary by both the type of cytokine and by PBMC preparation.

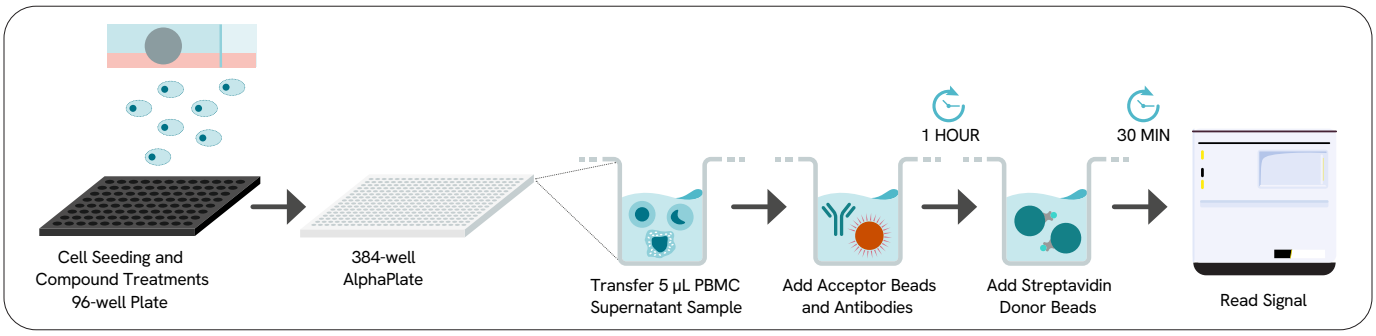

Figure 2: Experimental flowchart for testing PBMC cell supernatant samples with AlphaLISA.

For inhibition experiments, PBMC were treated with increasing concentrations (0 - 50 µg/mL) of dexamethasone (Sigma Aldrich / #D2915) or a fixed concentration (220 µg/ mL) of Rituximab (Selleckchem / #A2009) and co-treated with a fixed concentration either LPS (0.8 ng/mL) or a combination of PMA (2 ng/mL) and ionomycin (1 µg/mL) for 24 hours prior to removal of cell supernatants for testing in AlphaLISA assays. Optimal concentrations of LPS and PMA for inhibition studies were determined from running a dose response curve of these compounds and using the values of compound at the curve EC_{80}.

AlphaLISA high performance cytokine measurements

To assay concentrations of cytokines secreted from PBMC, 5 µL of each cell supernatant sample was tested using the AlphaLISA High Performance Human TNFα Detection Kit (AL3157C), the AlphaLISA High Performance Human IFNγ Detection Kit (AL3153C), and/or the AlphaLISA High Performance Human IL-2 Detection Kit (AL3155C) using the protocols listed in each kit's respective technical data sheet. Recombinant proteins provided in each AlphaLISA High Performance Detection Kit were titrated in RPMI + 10% FBS to produce standard curves that were used for interpolation of sample data to quantify concentrations of cytokines in culture supernatants.

Data analysis

All experiments were performed on an EnVision™ 2103 multimode plate reader using standard Alpha settings. Standard curves for each AlphaLISA cytokine assay, compound stimulation, and compound inhibition dose response curves were plotted in GraphPad Prism® using nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/ Y^2$ weighting. Cytokine concentrations in each cell supernatant sample were determined by interpolating the counts measured from the standard curve. All data points shown are the result of biological duplicate samples each assayed in triplicate wells.

Results

Standard curves for each AlphaLISA were prepared in cell culture media (RPMI +10% FBS) and each representative standard curve is shown in Figure 3. The AlphaLISA assays demonstrate superb performance with lower detection limits less than 2 pg/mL for each of the cytokine targets tested.

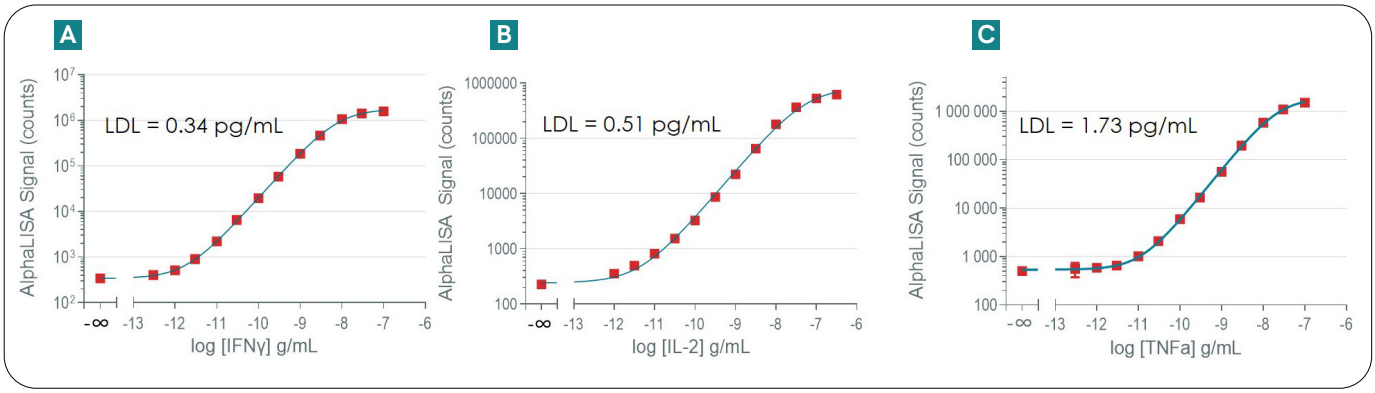

Figure 3: AlphaLISA High Performance standard curves for detection of IFNγ (A), IL-2 (B), and TNFα (C) in cell culture media (RPMI + 10% FBS).

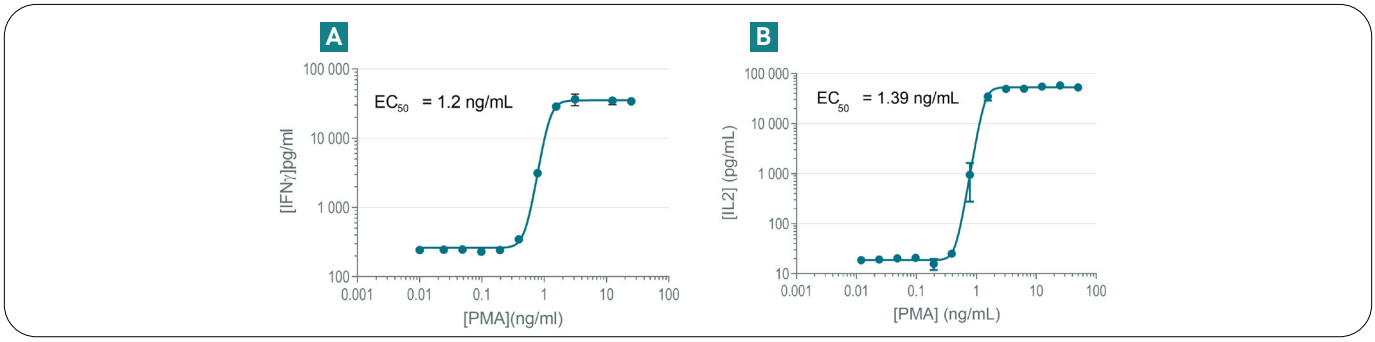

Figure 4: Stimulation of cytokine release: IFNγ (A) and IL-2 (B) secretion post-PMA/ionomycin treatment.

Stimulation of PBMC cytokine release

Full activation of PBMC cytokine release was stimulated using a mixture of PMA, a small organic compound, and ionomycin, a calcium ionophore which increases the uptake of PMA by cells. This treatment has been shown to activate the nuclear factor of activated T cells (NFAT) pathway, and the secretion of many cytokines, particularly Th1-type cytokines IFNγ and IL-2.[2] The concentrations of IFNγ and IL-2 cytokines secreted from PMA/ionomycin treated PBMC were quantified by interpolating the amount of AlphaLISA signal generated from each sample to a corresponding AlphaLISA standard curve for each cytokine. Figure 4 demonstrates the dose response curves obtained for both IFNγ and IL-2 secretion post-treatment.

In order to specifically activate B-cells, PBMC were treated with LPS, a component of the cell wall of gram-positive bacteria which has been shown to stimulate the production of the cytokine TNFα.[3] Samples of PBMC treated with LPS were tested in the AlphaLISA assay. A dose response of LPS treatment results in increasing concentrations of TNFα secretion, as shown in Figure 5. The EC_{50} for LPS treatment was calculated to be 0.34 ng/mL and the EC_{80} was calculated to be 0.8 ng/mL.

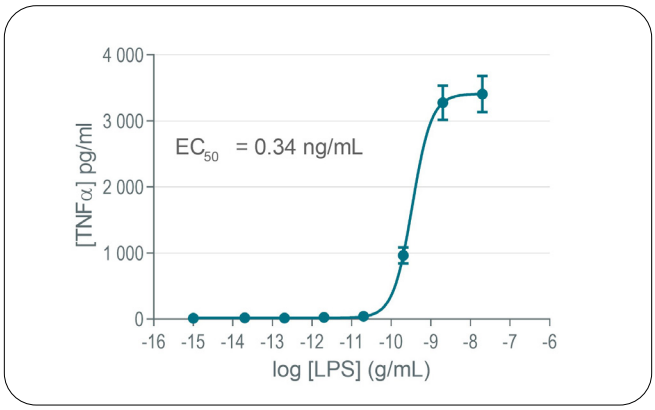

Figure 5: B-cell induced cytokine release of TNFα post-LPS treatment.

Dexamethasone treatment of PBMC

Dexamethasone is a corticosteroid that inhibits the inflammatory response and down regulates cytokine expression.[4] PBMC were co-treated with either PMA/ionomycin or LPS at each respective EC_{80} concentrations (as calculated in Figures 4 and 5) and increasing concentrations of dexamethasone. Figure 6 presents the dexamethasone dose response curves for each of the cytokines tested with AlphaLISA. IC_{50} measurements obtained for each of the three cytokines tested were all about 3 ng/mL.

Rituximab treatment of PBMC

The chimeric monoclonal antibody Rituximab specifically targets CD20 protein commonly found on the surface of B cells. It is marketed under several different brand names as FDA approved treatments for many types of cancer including chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL). We tested co-treatment of PBMC with both stimulating compounds and increasing concentrations of Rituximab and monitored the effect of cytokine release with AlphaLISA. In the presence of Rituximab, a 7.7-fold reduction of IFNγ and a 3.6-fold reduction of IL-2 was observed (Figure 7A and 7B), in agreement with a recent study.[5] A 2-fold increase in TNFα secretion was measured post-treatment (Figure 7C). Recent publications have shown that Rituximab treatment on PBMC samples may have little to no effect, if not a slight increase in secretion of TNFα.[5,6] Our results here align with these studies, and more investigation may be needed to better elucidate the mechanisms associated with Rituximab-induced cytokine secretion.

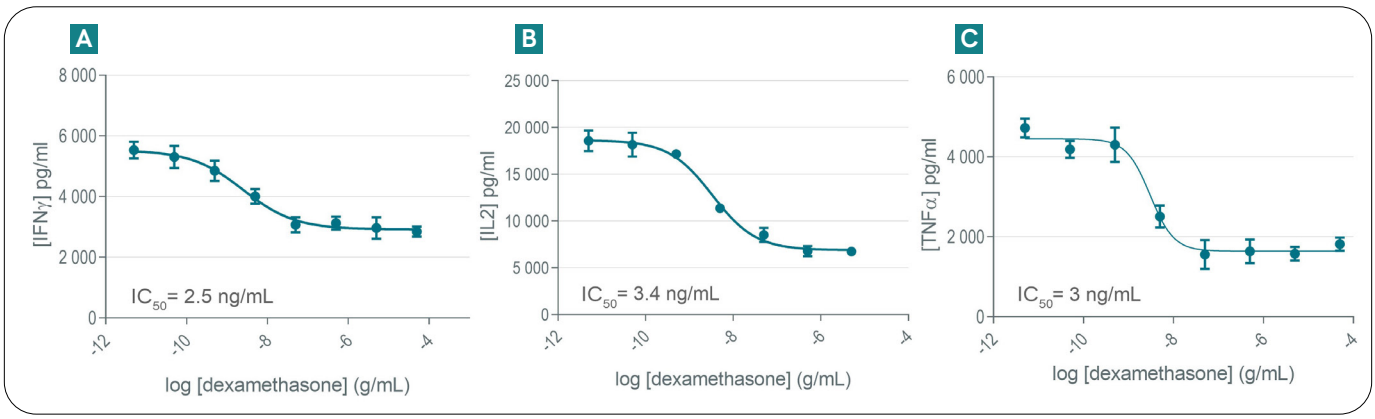

Figure 6: Inhibition of cytokine release of IFN (A), IL-2 (B), and TNF (C) secretion post-dexamethasone treatment.

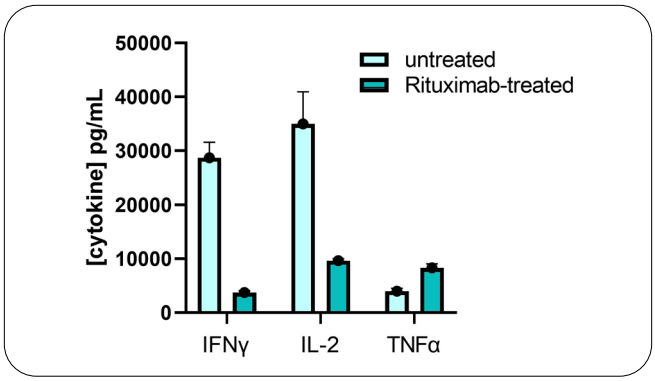

Figure 7: Cytokine release after Rituximab treatment. Concentrations of IFNγ, IL-2, and TNFα secreted into cell culture supernatants post stimulation and inhibition with the monoclonal antibody Rituximab.

Conclusion

Here we have demonstrated how the AlphaLISA High Performance Cytokine Detection kits can be utilized to quantitate IFNγ, IL-2, and TNFα from PBMC cell supernatant samples after various compound treatments that both stimulate and inhibit the production of cytokines, including both a small molecule inhibitor and a biologic drug. The AlphaLISA assays require the use of just 5 µL of sample and provide results in less than three hours making it an ideal platform to identify, characterize, and determine the efficacy of immunomodulatory drugs.

References

1. Wallner FK, Hopkins M, Woodworth N, Bark tL, Olofsson, P, Tilevik, A. Correlation and cluster analysis of immunomodulatory drugs based on cytokine profiles. Pharmalogical Research. 2018; (128):244-251.
2. Ai W, Li H, Song N, Li L, Chen H. Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. Int J Environ Res Public Health. 2013;10(9):3834-3842.
3. Leelawat S, Leelawat K. Cytokine Secretion of Peripheral Blood Mononuclear Cells by Hydnocarpus anthelminthicus Seeds. J Trop Med. 2018;2018:6854835.
4. Creed TJ, Lee RW, Newcomb PV, di Mambro AJ, Raju M, Dayan CM. The Effects of Cytokines on Suppression of Lymphocyte Proliferation by Dexamethasone. The Journal of Immunology. 2009; 183(1) 164-171.
5. Djaldetti M, Leibovitch C, Ganelin _Cohen E, Bessler H. Rituximab Modifies Peripheral Blood Mononuclear Cells Immune Responses. Int J Immunol Immunother. 2019; 6:037.
6. Barahona Correa JE, Cortés MAF, Ángel Uribe J, Rodriquez Camacho, LS. Comparison of Plasma Cytokine Levels before and after Treatment with Rituximab in Patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus- Associated Polyautoimmunity. Universitas Médica. 2018; 59 (3).

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