Quantifying changes in CD28 and CTLA-4 levels in peripheral blood mononuclear cells with AlphaLISA technology.

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Introduction

Immunotherapy harnesses the immune system to enhance and direct anti-tumor responses in cancer treatments. One approach to immunotherapy is the modulation of immune checkpoints that are critical in regulating the degree and duration of immune system responses and preventing autoimmunity. An immune checkpoint of significance is the inhibitory (anti-inflammatory) transmembrane protein cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4, or CD152). It competes with another transmembrane protein, CD28, which is a co-stimulatory (pro-inflammatory) receptor present on T-lymphocytes. CD28 and CTLA-4 compete for the same ligands, CD80 and CD86, present on antigen presenting cells. Monitoring the levels of CD28 and CTLA-4 on T-cells allows for precise recognition of any changes to the balance of this immune checkpoint. In a quiescent state, levels of CD28 on T-cells are known to be detectable and present, while CTLA-4 levels are very low or undetectable[1, 2, 3]. After activation, T-cells express CTLA-4 to regulate the immune system response. Thus, monitoring CD28 and CTLA-4 levels can provide an approach to studying the effect different immunostimulants and immunosuppressants have on the balance of CD28 and CTLA-4. CTLA-4's importance in maintaining immune system balance presents itself as a strong candidate to be monitored to better understand immune system responses that may lead to the development of better immunotherapy treatments.
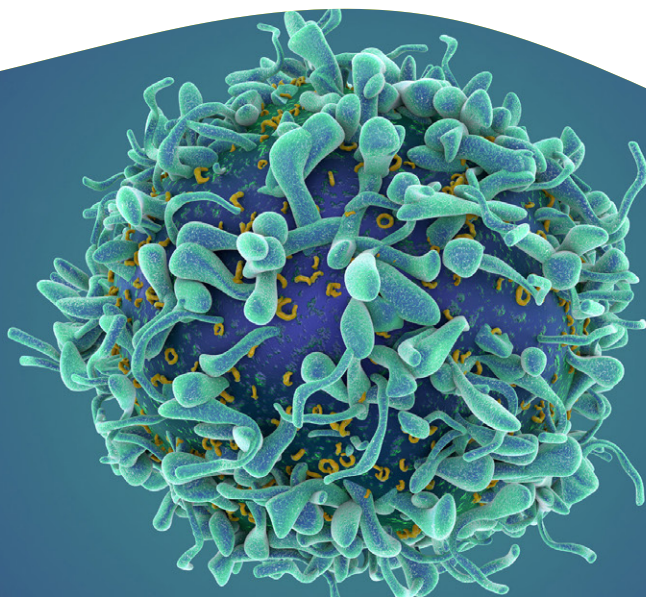
Amplified luminescence proximity homogeneous assay (Alpha) technology is a highly useful tool to quickly and precisely detect and quantify different biomarkers such as CD28 and CTLA-4. Alpha technology allows for the robust detection of the molecule of interest using a simple and homogeneous no wash format. Figure 1 provides an example of AlphaLISA™ technology, where streptavidin-conjugated Donor beads associate with a biotinylated anti-analyte antibody and an anti-analyte antibody is directly conjugated to Acceptor beads. When the analyte is present, the antibodies sandwich the analyte and bring the Donor and Acceptor beads within close proximity. Upon excitation with a 680 nm laser, the Donor beads generate singlet oxygen molecules that transiently diffuse in solution to activate nearby Acceptor beads, which through a series of reactions emits light at 615 nm. The light emission (AlphaLISA signal) is then detected on an Alpha-enabled instrument.
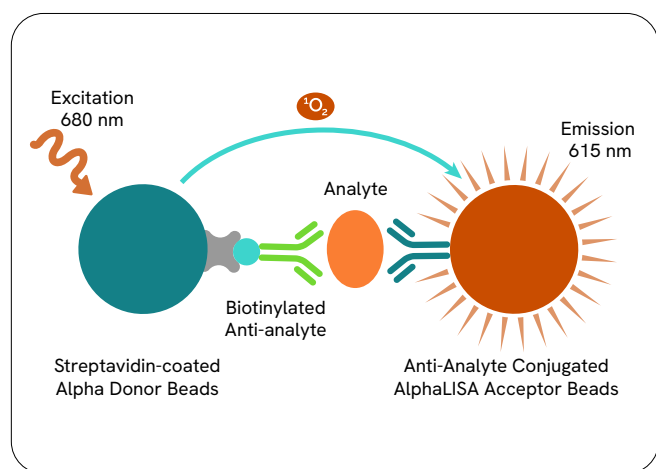

| Figure 1: AlphaLISA detection assay schematic.

Herein, we demonstrate how AlphaLISA technology can be used to detect and quantify CD28 and CTLA-4 levels in peripheral blood mononuclear cells (PBMCs). First, to illustrate AlphaLISA assay performance, standard curves were generated for both CD28 and CTLA-4 in several buffers and cell culture media. Second, levels of CLTA-4 and CD28 were assessed in unstimulated PBMCs across different cell seeding densities and tested at 0, 24, and 48 hour time points. To further assess the levels of CD28 and CTLA-4 expression in PBMCs, T-cells were stimulated using CD3/CD28 Dynabeads™. Finally, PBMCs were exposed to known immunostimulant (phytohemagglutinin) and immunosuppresants (Orthoclone OKT3, rapamycin, and cyclosporin A) in a dose-response fashion to attempt to modulate CD28 and CTLA-4 levels in PBMCs.

Materials and methods

Materials

AlphaLISA detection kits for CD28 (Revvity #AL3044) and CTLA-4 (Revvity #AL3050) were used to quantify expressed levels of these proteins from T-cells. AlphaLISA Immunoassay buffer (Revvity #AL000) was used to dilute all Alpha reagents, and AlphaLISA Lysis buffer (Revvity #AL003) was used to lyse cells. All AlphaLISA assays were performed in 384-well white OptiPlates (Revvity #6007290). Normal human primary Peripheral Blood Mononuclear cells (PBMC) were purchased from ATCC (#PCS-800-011). Human T-activator CD3/CD28 Gibco™ Dynabeads™ were purchased from Thermo Fisher (#11131D) and used to activate T-cells. DMEM (ATCC #30-2002) and RPMI (ATCC #30-2001) cell culture media was supplemented with 10% culture grade fetal bovine serum (ThermoFisher #26140-079). For all cell based assays, ½ Area 96-well ViewPlates (Revvity #6005760) were used to incubate cells. The following immunosuppressors were purchased from Millipore-Sigma: Orthoclone OKT3 (#SAB4700041), Rapamycin (#R8781), Cyclosporin A (#C662), and Dimethyl sulfoxide (DMSO; #D2650). Ipilimumab (#hhtcla4-mab1) and immunostimulant phytohemagglutin (#inh-phap) were purchased from InvivoGen. Mouse IgG2a control (#MAB003) was purchased from R&D Systems. Human IgG1 Isotype control (#403102) was purchased from BioLegend.

CD28 and CTLA-4 AlphaLISA detection assays

AlphaLISA immunoassays for CD28 and CTLA-4 were performed according to the recommended protocols provided with each kit. Each kit supplies the necessary Acceptor beads, biotinylated antibody, Donor beads, analyte, and the recommended assay buffer to perform the assay. Standard analytes and samples were prepared in the appropriate diluent according to the protocols.

For CTLA-4 assays, 5 µL of prepared standard analyte or samples were added to a 384-well white OptiPlate (Revvity #6007290), followed by the addition of 10 µL of an Acceptor bead mix. The plate was sealed with TopSeal A-PLUS (Revvity #6050185) and incubated for 30 minutes at room temperature. TopSeal-A PLUS is recommended for preventing evaporation during incubations. Next, 10 µL of biotinylated antibody was added to each well of the plate. The plate was sealed and incubated for 60 minutes at room temperature.

Afterwards, 25 µL of streptavidin Donor beads were added under subdued light, in the dark or under green filters (Rosco #389), to prevent photobleaching. The plate was sealed, covered with a black lid (Revvity #6000027), and incubated at room temperature for 30 minutes prior to measuring AlphaLISA signal.

For CD28 assays, 5 µL of prepared standard analyte or samples were added to a 384-well white OptiPlate followed by the addition of 5 µL of an Acceptor bead and biotinylated antibody mix. The plate was sealed with TopSeal A-PLUS, and incubated for 60 minutes at room temperature.

Next, 40 µL of streptavidin Donor beads were added under subdued light. The plate was sealed, covered with a black lid, and stored at room temperature for 30 minutes prior to measuring AlphaLISA signal. A plate can be read with or without the TopSeal A-PLUS with no measurable difference. If solutions do not appear to be settled at the bottom of the well (i.e. droplets on well wall), gently tapping the plate flatly on a surface may be sufficient to settle solutions. If this is not sufficient, briefly centrifuge the plate at 500 x g for about 10 seconds prior to reading.
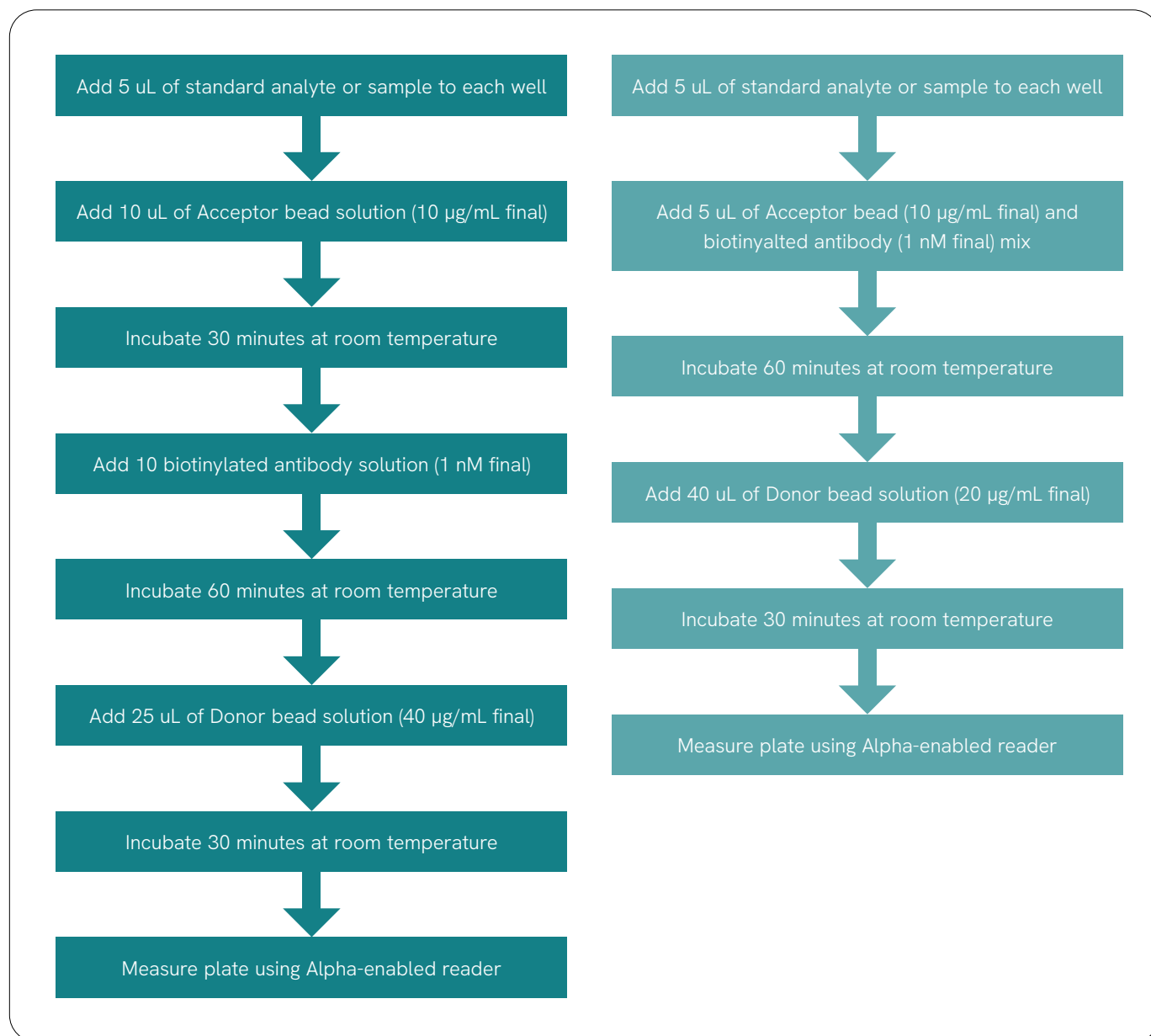
CTLA-4 (left)

- Add 5 uL of standard analyte or sample to each well
- Add 10 uL of Acceptor bead solution (10 µg/mL final)
- Incubate 30 minutes at room temperature
- Add 10 biotinylated antibody solution (1 nM final)
- Incubate 60 minutes at room temperature
- Add 25 uL of Donor bead solution (40 µg/mL final)
- Incubate 30 minutes at room temperature
- Measure plate using Alpha-enabled reader

CD28 (right)

- Add 5 uL of standard analyte or sample to each well
- Add 5 uL of Acceptor bead (10 µg/mL final) and biotinyalted antibody (1 nM final) mix
- Incubate 60 minutes at room temperature
- Add 40 uL of Donor bead solution (20 µg/mL final)
- Incubate 30 minutes at room temperature
- Measure plate using Alpha-enabled reader

Figure 2: Assay workflow for AlphaLISA CTLA-4 (left) and CD28 (right) detection assays.

Preparation and culture of peripheral blood mononuclear cells for cell-based assays

A vial of liquid nitrogen frozen PBMCs was placed in a 37 °C water bath until just thawed. The vial was inverted several times to gently mix. In a cell culture hood, PBMCs were slowly transferred to a 15 mL conical tube and the vial was rinsed with 1 mL of ice-cold Hanks Buffered Salt Solution (HBSS) and added dropwise to the conical tube. The volume of the conical vial was slowly adjusted to 10 mL with ice-cold HBSS. A small sample of cells was taken and used to determine cell concentration, while the remaining cells were centrifuged at 300 x g for 5-7 minutes to form a pellet. HBSS was carefully aspirated without disturbing the cell pellet. Cells were resuspended in warmed (37 °C) RPMI + 10% FBS. Next, 12 µL of cells were transferred to a 96-well ½ Area ViewPlate, followed by the addition of 6 µL of drug solution or cell culture media (if no drug is added) and 6 µL of CD3/CD28 Dynabeads (at a 1:1 bead to cell ratio) or cell culture media for unstimulated conditions. Cells were incubated for the desired duration (0, 24, or 48 hours) in a humidity controlled 37 °C incubator with 5% CO_2. After the incubation period, 6 µL of 5X AlphaLISA Lysis buffer was added to each well of the plate, covered with TopSeal A-PLUS and incubated for 15 minutes on a plate shaker set to 750 rpm at room temperature. If plates were not tested immediately, they were stored at -20 °C until use.

Instruments

All Alpha assays were measured on standard Alpha EnVision™-2103 multimode plate reader (Figure 3) using the 640as mirror module (#444) and the M570w emission filter (#244). Standard Alpha measurement settings were used: total measurement time 550 ms and excitation time 180 ms. For optimal performance, white 384-well OptiPlates were used for all assays.

Figure 3: EnVision multilabel plate reader.

Data analysis

Standard curves were plotted in GraphPad Prism version 7.0 using nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. Sample concentrations were determined by interpolating counts onto a standard curve. Lower limit of detection (LDL) was calculated by averaging the blank wells (wells without analyte) and adding three times the standard deviation then interpolating the concentration from the standard curve.

Results and discussion

CD28 and CTLA-4 AlphaLISA assays are simple, quick and offer exceptional assay performance

AlphaLISA technology is a sensitive, straightforward and robust bead-based immunoassay technology that utilizes a homogenous format without washing or additional signal development steps. Furthermore, AlphaLISA detection kits are available for many different biomarkers including other immuno-oncology markers. To demonstrate some of the benefits of Alpha technology to assess immuno-oncology markers, standard curves were prepared for CD28 and CTLA-4 AlphaLISA kits using AlphaLISA Immunoassay buffer (IAB), AlphaLISA Lysis buffer (ALB), RPMI supplemented with 10% fetal bovine serum (FBS) and 1x ALB, and DMEM supplemented with 10% FBS and 1x ALB. Figure 4 shows standard curves for CD28 and CTLA-4 performed in white 384-well OptiPlates. Each assay demonstrated exceptional performance characteristics as shown in the table in Figure 4 where low pg/mL sensitivities, wide dynamic ranges spanning more than 4-logs, and high signal-to-background ratios were observed. It is important to note the assay can perform differently in some diluents, highlighting the importance of preparing standard curves in the same solution used to prepare samples for the most accurate results.

CD3/CD28 Dynabeads™ effectively stimulated peripheral blood mononuclear cells to alter CD28 and CTLA-4 levels

AlphaLISA technology can be used to precisely measure differences in protein levels in immune cells. To demonstrate, CD3/CD28 Dynabeads™ were utilized to stimulate peripheral blood mononuclear cells (PBMCs) to alter the levels of CD28 and CTLA-4. For this experiment, cells were plated from 120,000 cells/well down to 938 cells/well (diluted in two-fold increments) in a 96-well ½ Area ViewPlate.

Cells were incubated for 0, 24, and 48 hours with or without CD3/CD28 Dynabeads™ (added at 1:1 bead-to-cell ratio). In unstimulated conditions (without CD3/ CD28 Dynabeads™), CD28 was observed to increase slightly with time and was detectable in as few as 15,000 cells/well after 48 hours of incubation (Figure 5A). In the presence of CD3/ CD28 Dynabeads™, CD28 levels increased approximately 2-fold and 3.5-fold after 24 and 48 hours respectively compared to unstimulated conditions. Furthermore, CD28 levels increased significantly between 24 and 48 hours

of stimulation (Figure 5B). For CTLA-4, levels were observed to be at or below the lower detection limit in unstimulated conditions at any time point tested (Figure 5C). However, in the presence of CD3/CD28 Dynabeads™, CTLA-4 levels were easily detected and observed to increase with the longer incubation period. Between 24 and 48 hours of stimulation, CTLA-4 levels were observed to increase as much as four-fold (Figure 5D). Further, it is important to note that increased ratio of total CTLA-4:CD28 was observed between the 0, 24, and 48 hour time points (Figure 5E).

CD28	LDL, pg/mL	Dynamic range	S/B
IAB	19.7	19.7 pg/mL to 1 µg/mL	1297
ALB	12.1	12.1 pg/mL to 1 µg/mL	1306
RPMI	455	455 pg/mL to 1 µg/mL	103
DMEM	45.8	45.8 pg/mL to 1 µg/mL	1049

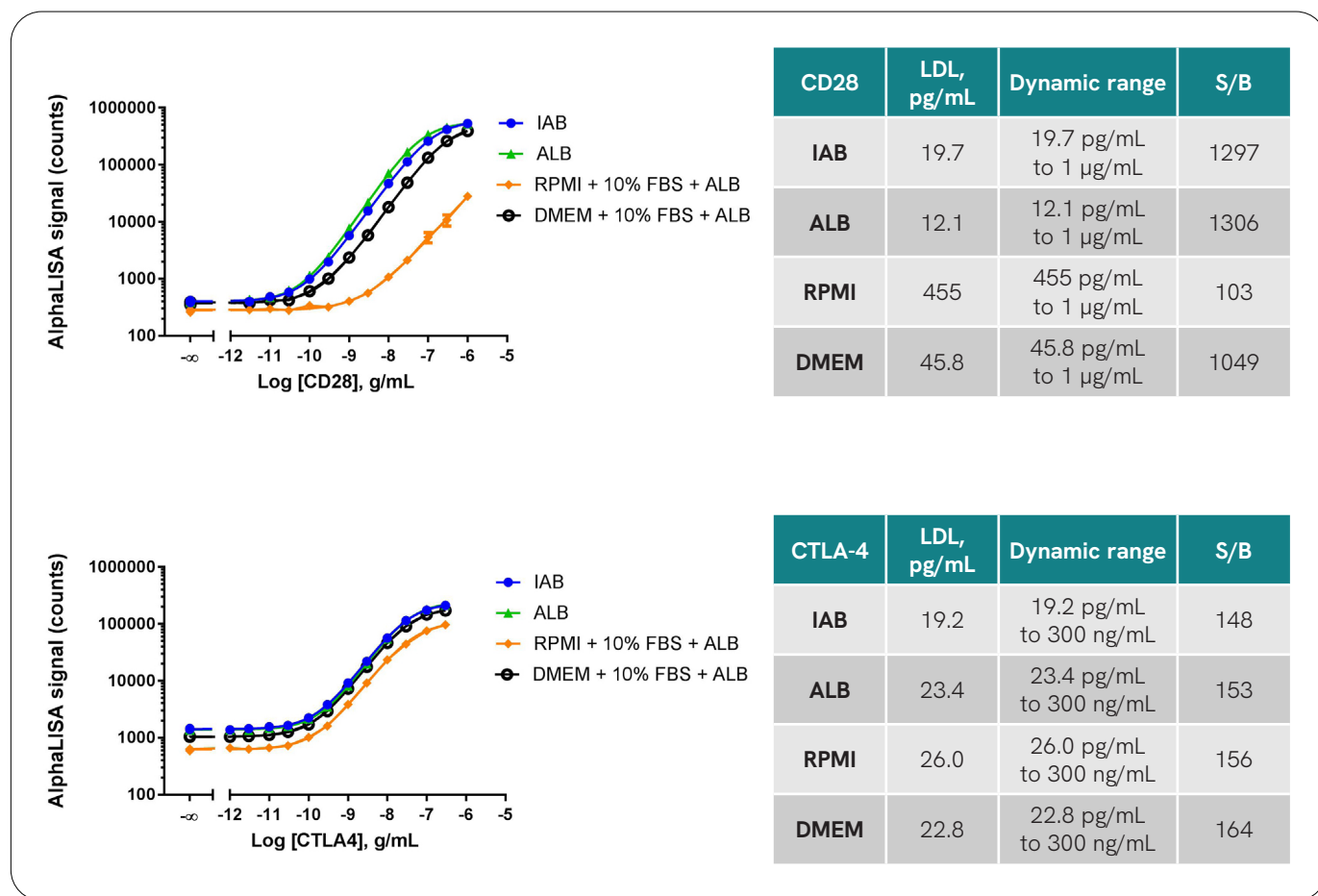| CTLA-4	LDL, pg/mL	Dynamic range	S/B
IAB	19.2	19.2 pg/mL to 300 ng/mL	148
ALB	23.4	23.4 pg/mL to 300 ng/mL	153
RPMI	26.0	26.0 pg/mL to 300 ng/mL	156
DMEM	22.8	22.8 pg/mL to 300 ng/mL	164

Figure 4: Performance of CD28 (top row) and CTLA-4 (bottom row) AlphaLISA immunoassays. Standard curves are shown for each assay performed in AlphaLISA Immunoassay buffer (IAB), AlphaLISA Lysis buffer (ALB), RPMI + 10% FBS + 1x AlphaLISA Lysis buffer, and DMEM + 10% FBS + 1x AlphaLISA Lysis buffer. The tables on the right display the performance characteristics for each assay.
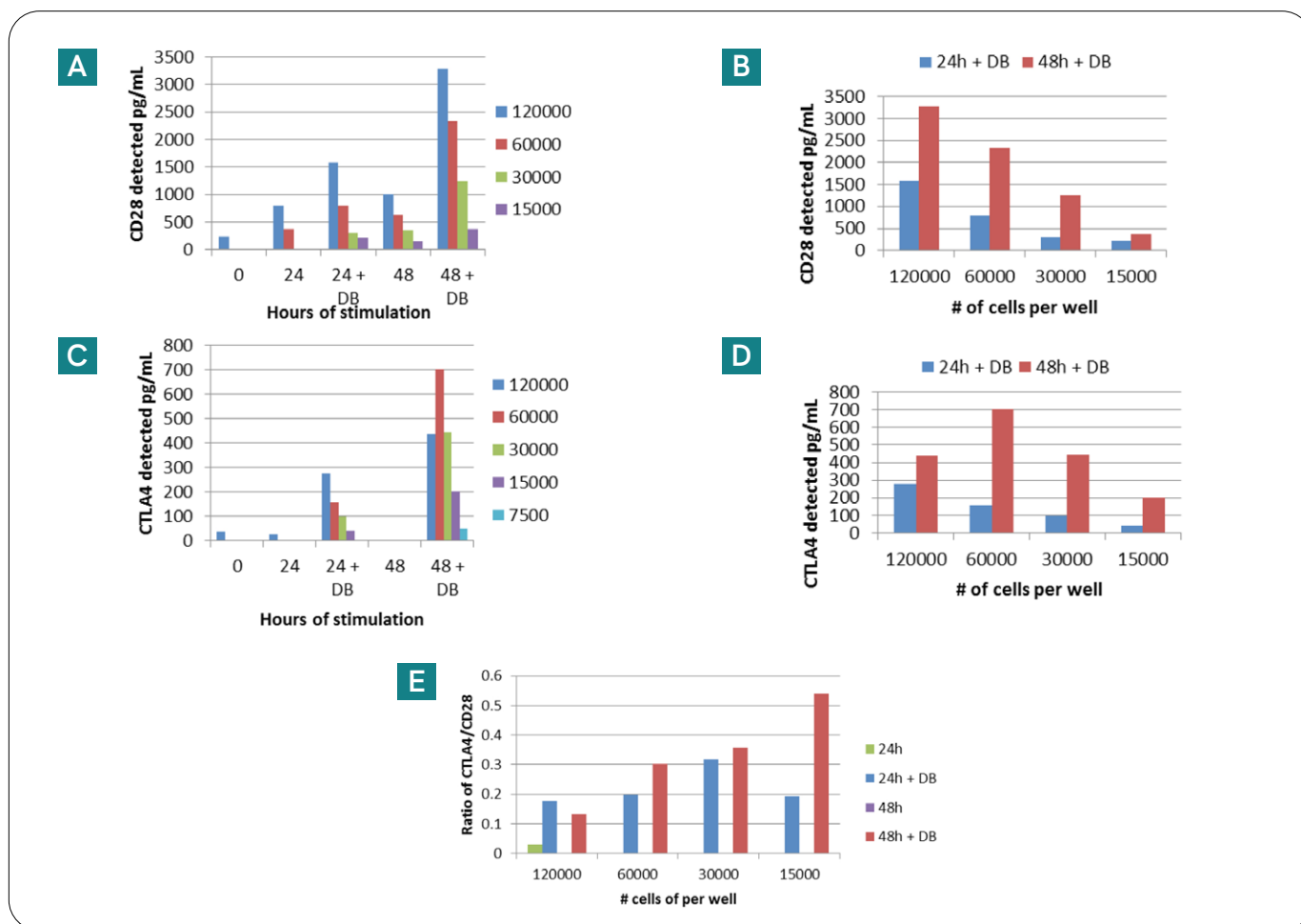
Figure 5: Changes in CD28 and CTLA-4 levels were detected by AlphaLISA detection kits after stimulation with CD3/CD28 Dynabeads™ (DB). PBMCs at varying cell numbers per well were tested unstimulated and stimualted with CD3/C28 Dynabeads™ (1:1 bead-to-cell ratio). CD28 levels were measured after 0, 24, and 48 hours (A, B). CTLA-4 levels were measured after 0, 24, and 48 hours (C, D). CTLA-4/CD28 ratio was computed for each at 24 and 48 hours in both unstimulated and stimulated conditions (E).

Immunostimulants and immunosuppresants modulate levels of CD28 and CTLA-4 in peripheral blood mononuclear cells

To further assess the utility of the CD28 and CTLA-4 AlphaLISA detection kits, several known immune system modulators were tested. For all experiments, 60,000 PBMCs were added to each well and either stimulated or suppressed with the modulators for 48 hours. Only PBMCs exposed to immunosuppressants were incubated with CD3/CD28 Dynabeads™ to better observe alterations in CD28 and CTLA-4 levels. First, phytohemagglutinin (PHA), a mitogenic lectin, was tested to stimulate PBMCs. A dose-response was performed and compared to cells incubated only with RPMI + 10% FBS, the media used to prepared the PHA dilutions. As expected, the presence of PHA increased CD28 and CTLA-4 levels compared to controls (Figure 6A, 6B). The greatest increases for both CD28 and CTLA-4 were observed at 10 µg/mL of PHA.

Next, rapamycin, cyclosporine A, and Orthoclone OKT3, all known immunosuppressors, were tested in a dose-response fashion and compared to the appropriate controls.

As expected, rapamycin was observed to suppress CD28 and CTLA-4 levels compared to DMSO controls from the range of 30 to 3 ng/mL (Figure 6C, 6D). The highest DMSO concentration tested was 5% and reduced in two-fold increments with RPMI + 10% FBS. Interestingly, cyclosporin A appeared to have no effect on CD28 levels, but did affect CTLA-4 levels (Figure 6E, 6F). Additionally, Orthoclone OKT3 appeared to increase both CD28 and CTLA-4 levels (Figure 6G, 6H). Although this result does not appear to show the typical immunosuppressor response where T-cells were activated and CTLA-4 levels increased compared to controls, the result does correlate with observations in the literature[4]. It is known that Orthoclone OKT3 interferes with a T-cell's ability to recognize foreign antigens by blocking the T-cell receptor, specifically CD3, but consequently activates the immune cell through the T-cell receptor which helps explain why increased CTLA-4 levels were observed here. Ipilimumab, an FDA approved CTLA-4 inhibitor, was also tested but the antibody was observed to interfere with the detection of CTLA-4.

Quantifying changes in CD28 and CTLA-4 levels in peripheral blood mononuclear cells with AlphaLISA technology.
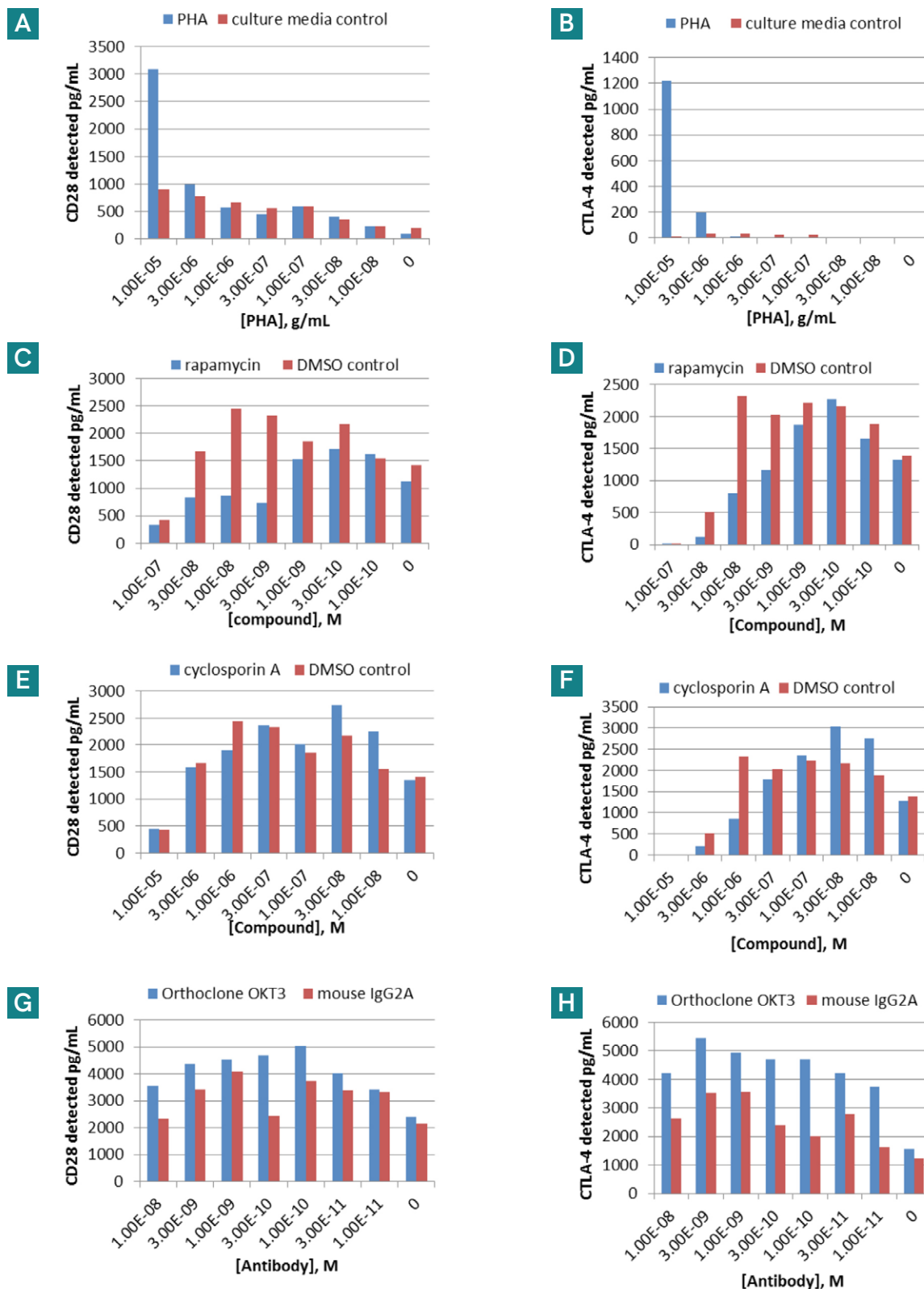

Figure 6: AlphaLISA CD28 and CTLA-4 detection kits can detect altered CD28 and CTLA-4 levels caused by exposure to immunosuppressants and immunostimulants. In each case, 60,000 PBMCs were added to each well. Immunostimulant PHA was tested without CD3/CD28 Dynabeads™ from 10 µg/mL to 10 ng/mL (A,B). Immunosuppressor rapamycin was tested in the presence of CD3/CD28 Dynabeads™ from 100 nM to 100 pM (C, D). Immunosuppressor cyclosporin A was tested in the presence of CD3/CD28 Dynabeads™ from 10 µM to 10 nM (E, F). Immunosuppressor Orthoclone OKT3 was tested in the presence of CD3/CD28 Dynabeads™ from 10 nM to 10 pM (G, H).
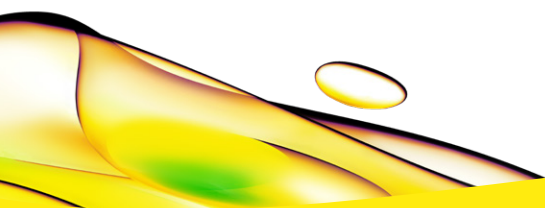
Conclusions

To demonstrate the benefits of Alpha to assess immuno-oncology markers, AlphaLISA was used to measure changes in CD28 and CTLA-4 levels in PBMCs. The sensitivity and versatility of the CD28 and CTLA-4 AlphaLISA detection assays were demonstrated by performing standard curves in several different solutions. This showed the exceptional sensitivity and amenability of AlphaLISA assays to a variety of different solutions. Then using CD3/CD28 T-cell activation Dynabeads™, PBMCs were stimulated at various cell numbers and for varied durations of time. It was observed that CD28 is detectable in unstimulated cells and at increasing levels over time, while CTLA-4 was at or below the detectable limit in unstimulated cells. When the cells were stimulated, a significant increase in CD28 and CTLA-4 levels was observed. Next, PBMCs were either immunosuppressed or immunostimulated with known immune system modulators. The mitogenic lectin, PHA, was observed to stimulate T-cells in the absence of CD3/ CD28 Dynabeads™ and a significant increase in CD28 and CTLA-4 levels was detected compared to a culture media control. Rapamycin and cyclosporin A both were observed to inhibit CTLA-4 in PBMCs stimulated with CD3/CD28 Dynabeads™, while only Rapamycin reduced the level of CD28 compared to a DMSO control. Finally, Orthoclone OKT3, a known immunosuppressor, was observed to stimulate the immune cells. This observation follows observations published in the literature where mouse Orthoclone OKT3 prevents the immune cells from rejecting transplants by blocking foreign antigen recognition by blocking the T-cell receptor, but activates immune cells[4, 5].

It was demonstrated that AlphaLISA technology is a sensitive, straightforward and robust bead-based immunoassay technology that can be used to detect and quantify important immuno-oncology biomarkers for immunotherapy development.

References

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