Antigen-stimulated PBMC cytokine release measured by AlphaLISA bovine cytokine kits.

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Introduction

Leptospirosis is an infectious disease that can spread across different species of mammals. It is caused by bacteria known as *Leptospira*. Twenty-one different species of *Leptospira* have been identified and thirteen are known to infect animals and humans. Leptospirosis can spread through contact or consuming food sourced from infected domestic animals, such as milk and meat.

Leptospira infection in cattle is one of the leading causes of reproductive failure. Abortions, poor fertility, weak calves, and reduced growth and milk production all affect commercial productivity and profitability. In the dairy and beef industries it is extremely important to keep animals healthy not only for food production and profitability, but also to prevent the spread of the disease to humans. The epidemiology of Leptospirosis in humans is well known, and it is estimated that about 10 million people are infected annually worldwide. Vaccinations against *Leptospira* bacteria are widely used to prevent infection in domestic animals and to help reduce transmission from animals to humans.

To investigate immune responses to vaccination and to better understand the mechanisms of cell-mediated immune responses, the United States Department of Agriculture (USDA) has conducted a year-long study in cattle using two commercial vaccines and an experimental vaccine. The study measured cytokine release from antigen-stimulated Peripheral Blood Monocytes (PBMCs) isolated from the blood of vaccinated cattle. Cytokine secretion by cultured PBMCs was measured using AlphaLISA™ bovine cytokine kits developed by Revvity.

AlphaLISA is a highly sensitive, homogeneous, user-friendly immunoassay platform that allows for the reproducible detection and quantification of compounds in buffer, cell culture supernatant, serum, plasma, and other biological matrices. In an AlphaLISA assay, a biotinylated anti-analyte antibody binds to Streptavidincoated Alpha Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in a sharp peak of light emission at 615 nm. This light emission can then be detected on an Alphaenabled reader, such as the EnVision™ multilabel plate reader. Figure 1 shows an example of an AlphaLISA Bovine Cytokine kit in which bovine cytokine, the analyte of interest, is recognized by two antibodies, a biotinylated anti-analyte antibody and antianalyte-conjugated AlphaLISA Acceptor bead.
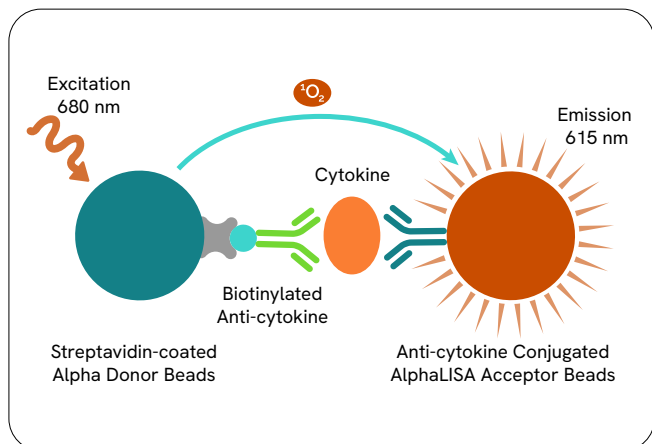

Figure 1. Schematic of AlphaLISA bovine cytokine assay principle.

This application note demonstrates the performance of AlphaLISA bovine cytokine kits (IL-6, IL-10, IL-12, IL-13, IL-17A, IL-18, IFNγ, and TNFα) by measuring the cytokine release from antigenstimulated PBMCs isolated from cow blood and shows how cytokine measurements are critical for understanding cellmediated immune responses during vaccine development.

Materials and methods

Reagents, instruments, and data analysis

Cytokine levels in PBMC culture supernatant samples were measured using the following Revvity AlphaLISA bovine cytokine kits: IL-6 (Revvity #AL538F), IL-10 (Revvity #AL546F), IL-12/p40 (Revvity #AL553F), IL-13 (Revvity #AL557F), IL-17A (Revvity #AL547F), IL-18 (Revvity #AL558F), IFNγ (Revvity #AL535F) and TNFα (Revvity #AL534F). RPMI 1640 (Cat #30-2001) was obtained from ATCC and FBS was obtained from Hyclone (Cat # SH30071.03). TopSeal™-A Plus Adhesive Sealing Film (Revvity #6050185) and OptiPlate™-384, white opaque 384-well microplates (Revvity #6007290) were used.

AlphaLISA assay plates were read on a Revvity EnVision multilabel plate reader (Figure 2) equipped with the Alpha option using the AlphaScreen™ standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%). Data were summarized, analyzed, and graphed using GraphPad Prism (GraphPad Software, La Jolla CA) and Microsoft Excel®.

Figure 2. Revvity EnVision Multilabel Plate Reader.

PBMC cell culture supernatant samples

USDA provided two sets of PBMC culture supernatant samples. The first set of 42 samples derived from PBMCs was isolated from two cows in the USDA blood donor pool by density centrifugation. Cells were then cultured in RPMI supplemented with 10% FBS and stimulated with various antigens including positive controls (Concanavalin A (Con A) and Pokeweed Mitogen (PWM)), negative control (No Stim), bacterial antigen *Leptospira borgpetersenii* Hardjo strain 203 (Lepto 203), lipopolysaccharides from

Escherichia coli O111:B4 (LPS), *Porphyromonas levii* (PLevii), and *Treponema phagedenis* strain 4A (tPhage). Cell culture supernatants were collected at 24, 48, and 72 hours post stimulation and frozen at -20°C until assayed.

The second set of 936 samples included antigen-stimulated PBMC culture supernatants. These samples were collected during a year-long study to investigate vaccine efficacy b measuring cytokine release from antigen-stimulated PBMCs. In this study (Figure 3), 24 cows (>18 months in age) were divided into four groups. Three groups were vaccinated: one vaccine per group (two commercial *Leptospira* serovar Hardjo vaccines and an experimental vaccine), while one group received an adjuvant control. 33 weeks after vaccination, cows were then infected with live *Leptospira borgpetersenii* serovar Hardjo bacteria (infection or challenge). Whole blood samples were collected at 0, 13 and 33 weeks post vaccination and at 1, 4 and 8 weeks post infection (challenge). PBMCs were isolated by density centrifugation at each time-point and cultured in RPMI supplemented with 10% FBS and stimulated with various 3 antigens including *L. borgpetersenii* Hardjo strain 203, *L. borgpetersenii* Hardjo strain 197, *L. borgpetersenii* Hardjo strain RZ33, *Leptospira interrogans* Pomona strain RM211, *L. interrogans* Fiocruz, *Leptospira biflexa* strain Patoc, Concanavalin A (Con A), and Pokeweed Mitogen (PWM). Cell supernatants were collected at 48 hours post stimulation and frozen at -20°C. The cell culture supernatant samples were shipped to and tested by Revvity, Hopkinton, MA, where the cytokine concentrations were measured using AlphaLISA bovine cytokine kits.
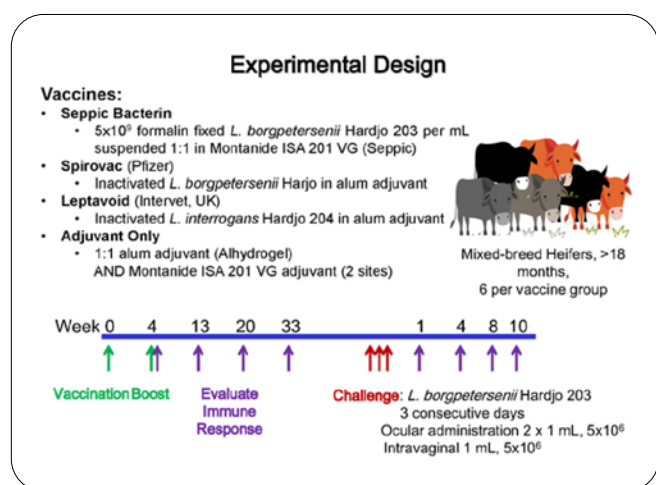

Figure 3. Experimental Design of USDA Vaccine Immune Response Study. This figure is used in this application note with permission from USDA. It is owned by USDA and was presented at the Conference for Research Workers in Animal Disease, December 4-6, 2016.

Reagent preparation and bovine cytokine assays

Eight cytokine assays (IL-6, IL-10, IL-12/p40, IL-13, IL-17A, IL-18, IFNγ, and TNFα) were performed according to the assay protocols provided in Technical Data Sheets (TDS) of each kit. The standard curves (STD) were prepared in RPMI supplemented with 10% FBS. All other components were prepared in the buffers recommended in the TDS. Cell culture supernatant samples received from USDA were initially stored at -80°C. Samples were placed in a 4°C refrigerator overnight and then allowed to warm up to room temperature for one hour before running the assays. All samples were provided in 12 plates (96-well sample storage plate). Each sample was run in duplicate with the appropriate 12 point standard curve(s) run in triplicate and 12 point blank samples (no analyte). Four 384-well assay plates (two kits/plate) were used to complete 42 samples and 48 384-well assay plates (6 plates/kit) were used to complete 936 samples.

For the two-step protocol (bovine IL-6 and IL-10 kits), assays were performed by adding the prepared reagents to 384-well plates: 5 µL of standard in triplicate or 5 µL of cell culture supernatant sample in duplicate was added, followed by 20 µL of anti-cytokine Acceptor beads and biotinylated anti-cytokine antibody mix to all the wells. The plates were then incubated 60 minutes at 23°C. After the incubation, 25 µL of streptavidin Donor beads was added and incubated 30 minutes at 23°C in dark. Plates were read on an EnVision multilabel plate reader.

For the 3-step protocol (bovine IL-12/p40, IL-13, IL-17A, IL-18, TNFα, and IFNγ kits), assays were performed by adding the prepared reagents to 384-well plates: 5 µL of standard in triplicate or 5 µL of cell culture supernatant sample in duplicate, followed by 10 µL of anti-cytokine Acceptor beads. Plates were incubated for 30 minutes at 23°C, and then 10 µL of biotinylated anti-cytokine antibody was added and the plate incubated for 60 minutes at 23°C. After the incubation, 25 µL of streptavidin Donor beads was added and incubated 30 minutes at 23°C protected from light. Plates were read on an EnVision multilabel plate reader.

Prior to each incubation step, the plates were sealed with TopSeal-A, tapping gently to ensure that samples settled to the bottoms of their respective wells. All pipetting was done using electronic multi-channel pipettes with multi-dispensing functions, with the exception of streptavidin Donor beads, which were added using a JANUS® automated workstation (Revvity) equipped with WinPREP™ 4.8 software, an eight-tip Varispan™ arm with Versatip® adaptors and Modular Dispense technology arm (Figure 4).

Figure 4. A JANUS Automated Workstation (Revvity) equipped with WinPREP™ 4.8 software, an 8-tip Varispan arm with Versatip adaptors.

Results

Antigen-stimulated cellular cytokine release in 42 samples

The results of cytokine measurements secreted in 42 cell culture supernatant samples collected from PBMCs that were stimulated with six antigens are summarized in Figure 5. IFNγ secretion was increased dramatically by PBMCs stimulated with PWM, LPS, tPhage, *P. levii*, Lepto 203, and Con A compared to that of non-stimulated cells. This was most evident when the cells were cultured and stimulated for 48 and 72 hours (Figure 5A). In Figure 5B, IL-17A secretion was stimulated by all antigens except *P. levii* at 24 and 48 hours. A time-dependent increase in IL-17A release was observed for all antigens.

Changes in IL-6 release were not as dramatic (Figure 5C). LPS, Lepto 203, and *P. levii* appeared to increase the secretion of IL-6 compared to the non-stimulated control. Con A did not stimulate the IL-6 secretion. Interestingly, IL-10 secretion was stimulated by only LPS and PWM in a time-dependent manner. All other antigens appeared to increase IL-10 at 24 hours, but not at 48 and 73 hours (Figure 5D).

IL-12/p40 secretion was stimulated by all antigens compared to the non-stimulated control. The highest amounts of IL-12/p40 released were observed when cells were stimulated by LPS, PWM, or Con A at all three time points (Figure 5E). Similar to IL-12/p40 secretion, IL-13 release was also increased mostly by LPS, PWM, and Con A. However, the secretion was enhanced by increasing the time of stimulation (Figure 5F).

IL-18 secretion (Figure 5G) did not display any specific pattern. IL-18 release appeared to be stimulated by *P. levii* at 24 hours and by LPS, Lepto 203, or *P. levii* at 72 hours. The secretion of TNFα was enhanced by LPS, PWM, and Con A at three time points (Figure 5H).

Antigen-stimulated cellular cytokine release in 936 Samples

The results of antigen-stimulated cellular cytokine release measured by AlphaLISA bovine cytokine kits in 936 cell culture supernatant samples are shown in Figure 6. Data from six cytokines were analyzed from PBMCs stimulated with *L. borgpetersenii* Hardjo strain 203 by vaccine treatment groups (six cows/group).
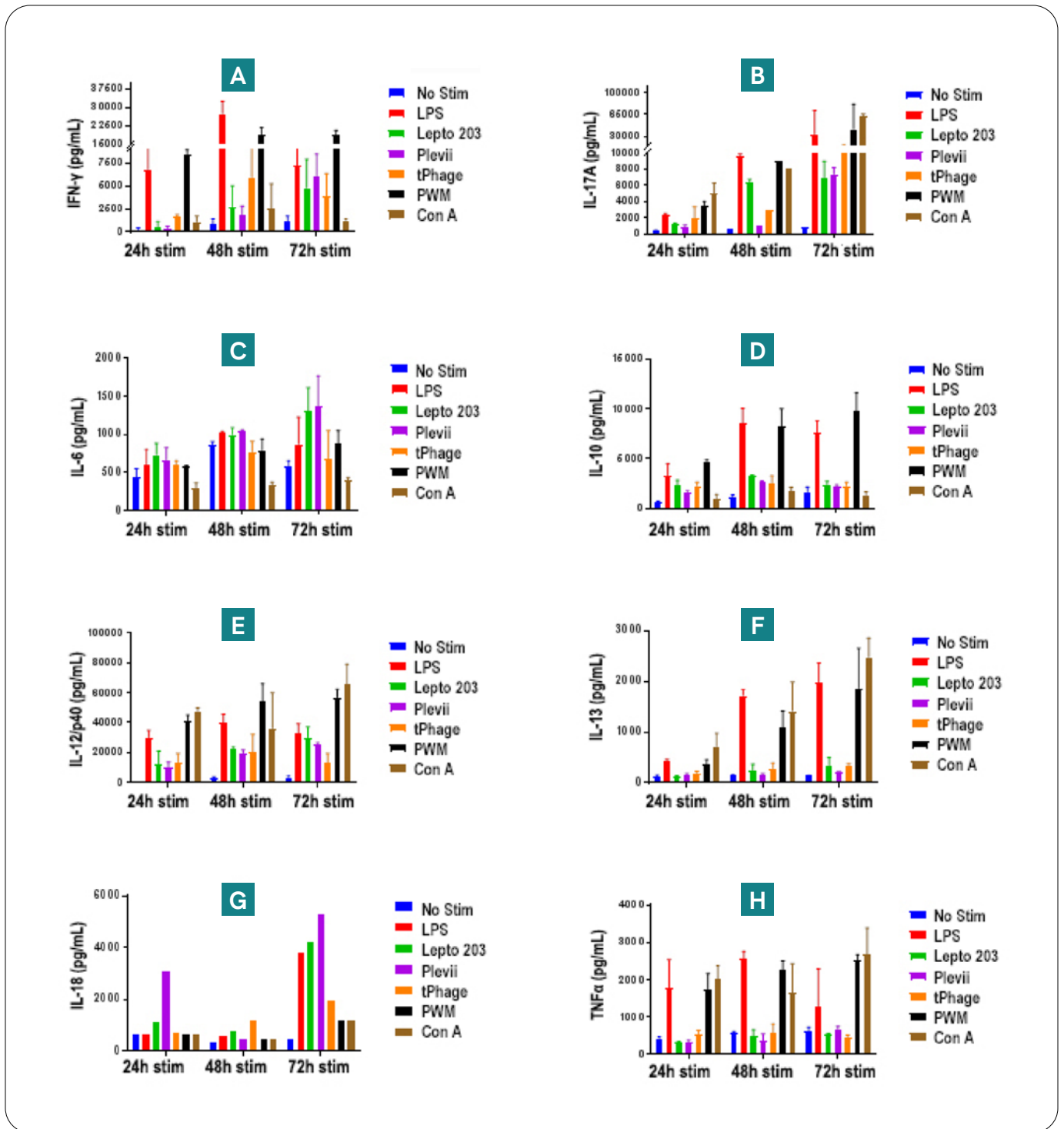
Antigen-stimulated PBMC cytokine release measured by AlphaLISA bovine cytokine kits.

Figure 5. Antigen-stimulated PBMCs cytokine release quantified by AlphaLISA bovine IFN∴ (A), IL-17A (B), IL-6 (C), IL-10 (D), IL-12/p40 (E), IL-13 (F), IL-18 (G), and TNFα (H) kits. PBMCs were isolated from whole blood of two cows, cultured, and stimulated with Lipopolysaccharides (LPS), *L. borgpetersenii* Hardjo strain 203 (Lepto 203), *Porphyromonas levii* (PLevii), Treponema 4A (tPhage), Pokeweed Mitogen (PWM), and Concanavalin A (Con A). "No Stim" indicates no stimulation (background). Error bars represent the standard deviation.

Regardless of timepoint, vaccinations, and infection, cytokine levels were low and often below the limit of detection in the cell culture supernatant collected from PBMCs that were not stimulated ("No Stim"). In contrast, all six secreted cytokines (IL-17A, IFNγ, IL-12/p40, IL-13, IL-6, and IL-10) were increased significantly in cell culture supernatant samples collected from PWM-stimulated PBMCs that were isolated from all groups (including the adjuvant only group).

In response to *L. borgpetersenii* Hardjo strain 203 stimulation, cytokine secretion was varied in PBMCs harvested from different vaccine groups at different timepoints following vaccination and after the challenge. IL-17A secretion was increased in cows vaccinated with Seppic and Spirovac vaccines at week 33 after vaccination and at weeks 1, 4, and 8 post-challenge. For Leptavoid vaccine group, the increases were seen at week 3, and weeks 1 and 4 post-challenge, but not at week 8 post-challenge. The cells from the adjuvant-only group secreted very a little IL-17A at all timepoints measured.

IFNγ secretion was low at week 0 following vaccination, started to increase at week 13, and increased significantly at weeks 4 and 8 post-challenge in cows vaccinated with Seppic. In Spirovac and Leptavoid groups, IFNγ secretion was higher at week 33 after vaccination and at weeks 1, 4, and 8 post-challenge compared to the week 0, week 13, and adjuvant group.

Patterns of IL-12/p40 secretion in different groups at various timepoints were similar to the patterns in IL-17A release. IL-13 release was higher in all groups at week 0 and week 33 compared to the other timepoints (week 13 after vaccination, and weeks 1 and 8 post-challenge).

IL-6 release was increased at week 13 and week 33 after vaccination and at weeks 1, 4, and 8 post-challenge in all three vaccine groups compared to the week 0 and adjuvant group. In the adjuvant group, higher amounts of IL-6 secretion were observed at week 13 and afterwards compared to week 0. IL-10 release was increased at week 13 and week 33 after vaccination and weeks 1, 4, and 8 post-challenge in all groups compared to week 0.
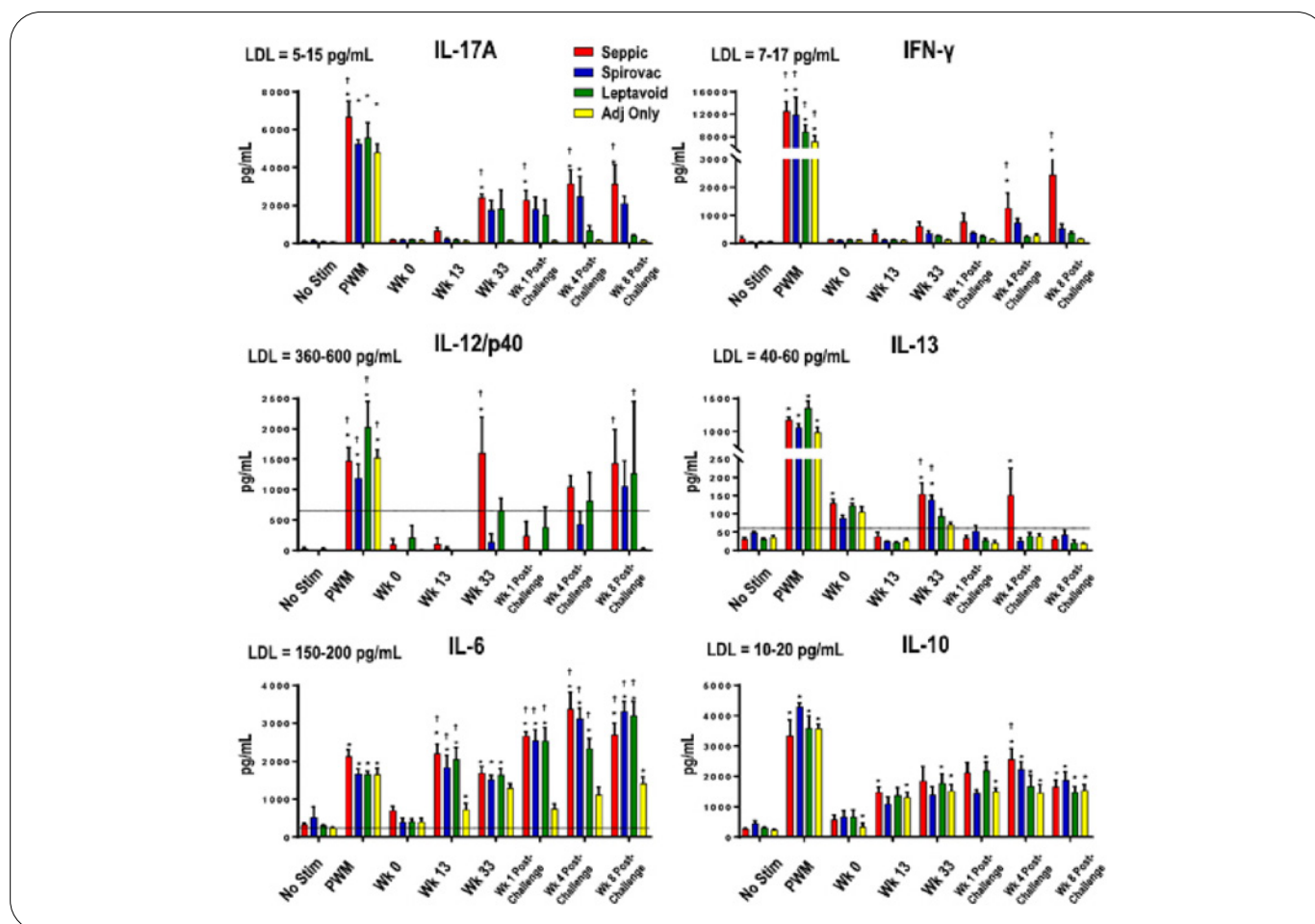

Figure 6. Antigen-stimulated cellular cytokine release measured by AlphaLISA bovine cytokine kits. The cytokine assays were performed using 936 cell culture supernatant samples and the data are summarized as means of six animals/vaccine group by USDA National Animal Disease Center at Ames Iowa. Data from PBMCs stimulated with *L. borgpetersenii* Hardjo strain 203 antigen at various timepoints following vaccination and after infection (challenge) are presented. PWM and "No stim" (background) data are averaged from all timepoints. Stimulating PBMCs with other *Leptospira* strain antigens such as *L. borgpetersenii* Hardjo strain 197, *L. borgpetersenii* Hardjo strain RZ33, *L. interrogans* Pomona strain RM211, *L. interrogans* Fiocruz, and *L. biflexa* strain Patoc generated nearly identical results (data not shown). Significant increases (P<0.05) were observed in comparison with adjuvant only group value at identical time intervals (†) as well as in comparison to the group's no Stim valves (*). Note USDA owns these data that were presented at Conference for Research Workers in Animal Disease, December 4-6, 2016. This figure is used in this application note with permission from USDA.

Bovine cytokine assay performances

Cytokine assay kit performance is summarized using the following assay specifications: LDL (Lower Detection Limit); LLOQ (Lower Limit of Quantitation); EC_{50} , and S/B ratio (Table 1 and Figure 7). Standard curves of eight cytokine assays were generated during the measurement of cytokine concentrations in culture media (Figure 7). The standard curves were prepared in RPMI with 10% FBS and all other components of the assays were prepared in the recommended buffer for each kit. Sensitivities (LDL) for each cytokine are referenced in Table 1. Signal to background (S/B) ratio was > 42 for all assays (Table 1). The assay range (Dynamic Range) was three to five logs. Overall, excellent performance of each assay was observed (Figure 7).

Table 1. Summary of assay performance. Excellent performance of all eight cytokine assay kits was observed.

Assay Performance	IFNγ	IL-17A	IL-6	IL-10	IL-12p40	IL-13	IL-18	TNFα
LDL[1] (pg/mL)	6	16	111	14	193	23	324	154
LLOQ[2] (pg/mL)	20	49	396	47	819	111	1397	585
EC_{50} (ng/mL)	16	9	21	130	264	26	102	90
S/B Ratio	592	323	43	1910	190	56	43	89

[1] The LDL is calculated by interpolating the average background counts ((12 wells) without analyte) + 3 x standard deviation value (average background counts + (3 x SD)) on the standard curve.

[2] The LLOQ as measured here is calculated by interpolating the average background counts ((12 wells) without analyte) + 10 x standard deviation value (average background counts + (10 x SD)) on the standard curve.
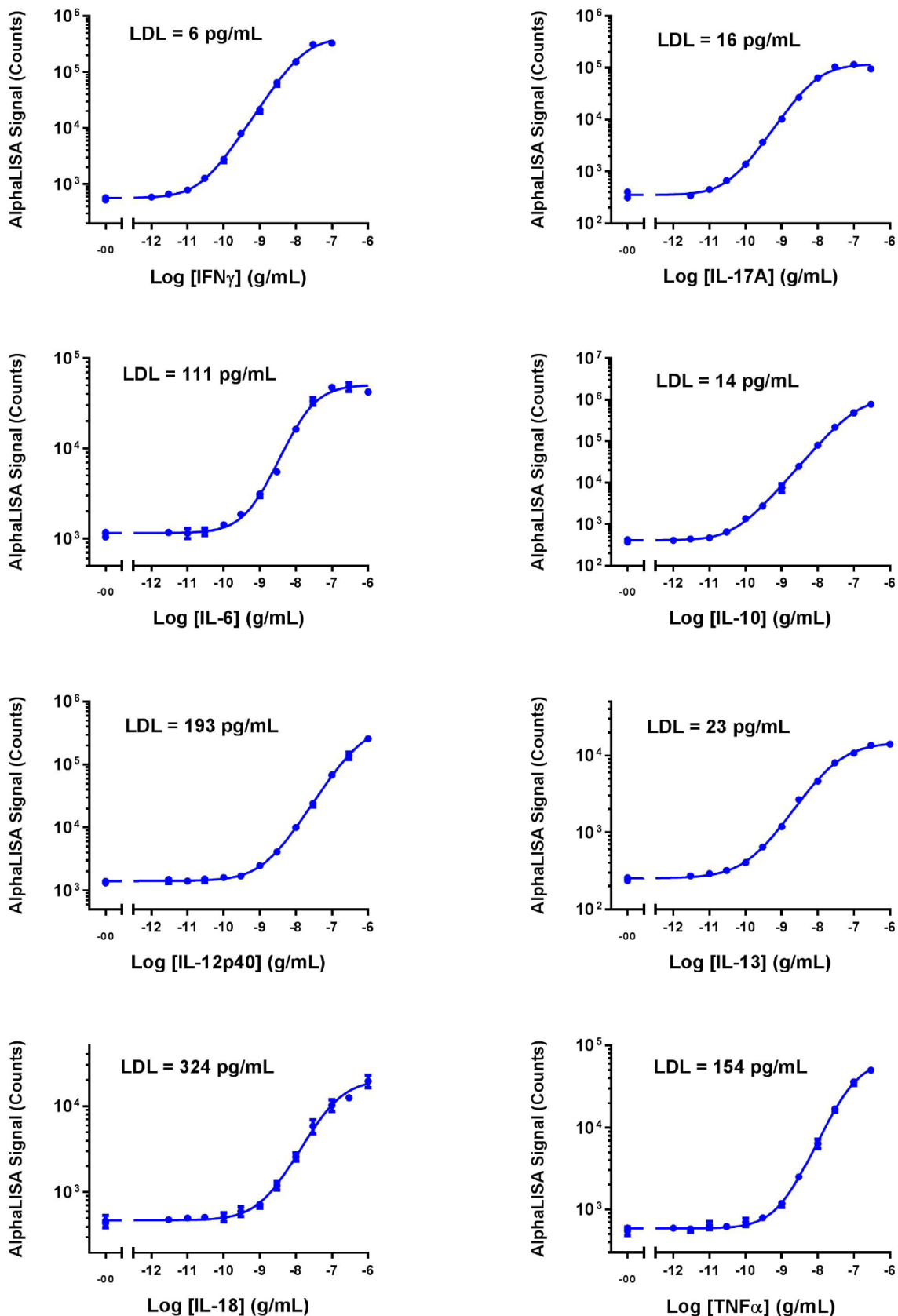
Antigen-stimulated PBMC cytokine release measured by AlphaLISA bovine cytokine kits.

Figure 7. Standard curves for interpolating cytokine concentration in PBMC culture supernantants. The sensitivity (LDL) of each assay is shown within the graph. These curves were generated when the first set of 42 samples was assayed. Four additional standard curves (not shown) were generated for each assay kit when measuring the cytokine concentrations in the second set of 936 samples.

Discussion

In this application note, we demonstrated the performance of eight AlphaLISA bovine cytokine detection kits. AlphaLISA bovine cytokine assay kits were validated and used to measure cytokine release in cell culture supernatants of bovine PBMCs stimulated with various antigens, together with the EnVision multilabel plate reader.

For the validation of cytokine assays, 42 cell culture supernatant samples were derived from PBMCs stimulated with various bacterial antigens or lymphocyte-cell mitogens for up to 72 hours. Pokeweed Mitogen (PWM), a T and B cell stimulator, and Concanavalin A (Con A), a T-cell stimulator, were used to stimulate cytokine release. Some cytokine secretion was stimulated by both mitogens, while others were stimulated by either PWM or Con A alone. PWM stimulated seven of eight cytokines that were tested in the current study, while Con A stimulated four of eight cytokines. These results suggest differences in the ways PWM and Con A act to stimulate PBMCs for specific cytokine secretion. Similarly, stimulation by the bacterial antigens, including the antigen for experimental vaccine (Lepto 203) produced greater cytokine secretion. For example, lipopolysaccharides (LPS) derived from the outer membranes of gram-negative bacteria induced all of the cytokines that were measured, supporting other findings that LPS elicits strong immune response.[1-6] Lepto 203, an experimental antigen for vaccine development, increased the release of IFNγ, IL-17A, IL-6, IL-10, and IL-12/p40, and slightly increased IL-13. The amounts of IFNγ, IL-17A, IL-6, and IL-12/p40 released in antigen-stimulated PBMCs appeared to correlate with the length of culture time. These cytokine release data demonstrate the utility of the AlphaLISA bovine cytokine assay kits in cell culture supernatant samples collected from the PBMCs stimulated with various antigens and T-cell mitogens.

The utility of AlphaLISA bovine cytokine assay kits was further confirmed by testing 936 cell cultures of supernatant harvested from PBMCs. The PBMCs were isolated, cultured, and stimulated *in vitro* with various antigens of experimental and commercial vaccines. The data generated using AlphaLISA bovine cytokine assay kits, together with the EnVision plate reader, provided new information about cell-mediated immune responses to vaccination and infection in cattle.
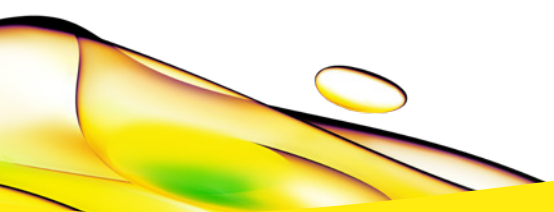
Cytokine assays have demonstrated that cell-mediated responses in to the presence of antibody are necessary for development of effective immune responses to *Leptospira borgpetersenii* serovar Hardjo. Seppic and Spirovac vaccine induced IL-17A and IFNγ release by PBMCs in culture supernatants, suggesting that in addition to known Th1-type cell-mediated immune responses (e.g., IFNγ), Th17-type (additional T cell subsets) cell-mediated response and other inflammatory cytokines may be involved. Therefore, the original thought that Leptospirosis vaccination induces Th1-type immune responses[7] requires re-visitation as this may no longer represent a complete immunological picture.

Conclusion

AlphaLISA bovine cytokine assay kits offer new tools for vaccine research and development, and new insight into assessing immune responses due to vaccination in livestock. Current results indicate that IL-17A is involved in immune response to the Leptospirosis vaccine in cattle and thus could be a new biomarker for vaccine development.

Antigen-stimulated PBMC cytokine release measured by AlphaLISA bovine cytokine kits.

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