APPLICATION NOTE

AlphaLISA *SureFire® Ultra*: elucidating TREM2/DAP12 signaling in neuroinflammation.

Authors

Antony Sheehan
Sheila Falcon

TGR BioSciences Pty Ltd

Juliette Seremak
Michelle Seng
Adam Carlson

Revvity, Inc.

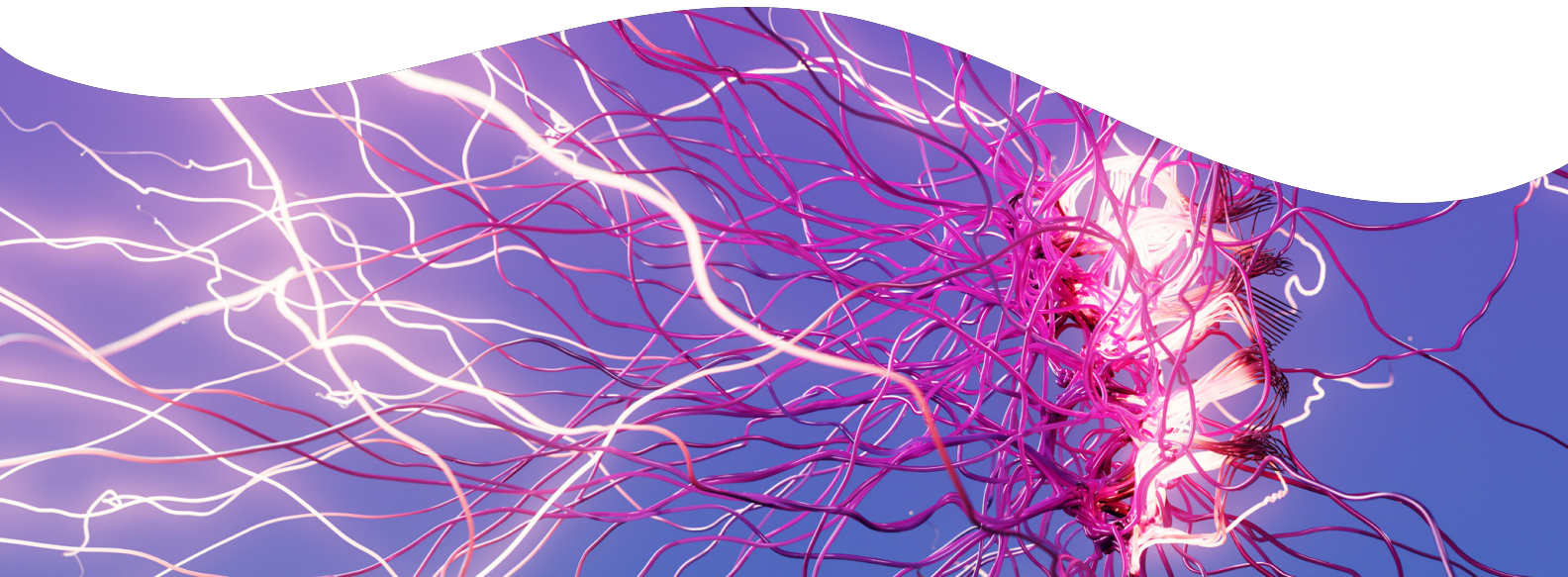
Overview

- Revvity's **AlphaLISA™ *SureFire® Ultra™*** technology provides a fast, robust, no-wash assay platform for studying protein interactions and post-translational modifications with high sensitivity.
- This application note demonstrates the utility of AlphaLISA technology in measuring key components of the TREM2/DAP12 signaling pathway using a small molecule TREM2 activator and a monoclonal antibody targeting the extracellular domain of TREM2.

Introduction

1.1. The role of TREM2 in neuroinflammatory diseases

Neuroinflammation involves a complex interaction of immune responses in the brain, primarily driven by the activation of microglia and astrocytes. In normal conditions, these specialized cells work to protect the brain from various pathogens and are beneficial for promoting tissue repair [1]. A sustained inflammatory state can be fatal and is a significant driver in the progression of neurodegenerative diseases, such as Alzheimer's Disease (AD) - the most common cause of dementia among older adults that is irreversible and debilitating [2]. This chronic state of inflammation exacerbates neuronal damage and cognitive decline, making it a significant topic in research to better understand the underlying signaling mechanisms and develop effective targeted therapies.



Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) is a receptor primarily expressed on the surface of microglia, the resident immune cells of the central nervous system (CNS) and has emerged as a key player in neuroinflammation and AD pathogenesis. In the context of AD, TREM2 confers a neuroprotective role by modulating microglial responses to neuroinflammatory stimuli, including amyloid-beta (Aβ) plaques, tau aggregation, and neurofibrillary tangles – hallmarks of AD progression. Specifically, TREM2's activation promotes the process of phagocytosis to facilitate the clearance of Aβ plaques [3], thus, helping to mitigate neuronal damage.

In contrast, deficiency or dysfunction of TREM2 has been linked to an increased risk of AD, with various studies demonstrating that specific mutations in the TREM2 gene are associated with a higher susceptibility to the disease [4]. In these studies, researchers concluded that TREM2 deficiency exacerbated neuroinflammation and impaired microglial phagocytosis of Aβ plaques, which can eventually lead to cell apoptosis and loss of neurons [5]. Mouse models of AD have further suggested that TREM2 deficiency accelerates its progression, highlighting the gene's importance in maintaining brain health and managing neurodegenerative processes [6]. These findings underscore the importance of TREM2-mediated signaling in the pathogenesis of AD and highlight its potential as a therapeutic target. Targeting TREM2 signaling pathways, including those involving DAP12 and SYK, holds promise for modulating microglial function and attenuating neuroinflammation in AD.

1.2. TREM2, DAP12, SYK cellular pathways

The signaling pathways associated with TREM2 involve interactions with two key adaptor proteins: DNAX-activating protein 12 (DAP12) and spleen tyrosine kinase (SYK) (Figure 1).

DAP12 is an adaptor protein that associates with the cytoplasmic tail of TREM2 upon ligand binding. Once activated, DAP12 undergoes phosphorylation, leading to the recruitment and activation of downstream signaling molecules, including SYK kinase.

SYK is a cytoplasmic tyrosine kinase that plays a central role in immune cell signaling. Activation of SYK downstream of the TREM2-DAP12 signaling cascade leads to the phosphorylation of several downstream targets, ultimately influencing microglial responses to pathological stimuli, such as Aβ aggregates in Alzheimer's disease. The resulting cascade of intracellular signaling events regulates various aspects of microglial function, including phagocytosis, cytokine production, and cell survival.

Understanding the complex interactions between TREM2, neuroinflammation, and AD pathology is essential for developing effective treatments. Revvity offers a full suite of first-in-class TREM2 immunoassays as part of its AlphaLISA *SureFire® Ultra* platform, renowned for its exceptional sensitivity and robustness. This platform features the most advanced, homogeneous, no-wash assays available on the market.

This study demonstrates the effectiveness of AlphaLISA *SureFire® Ultra* assays for measuring TREM2 activation, focusing on the following targets:

- TREM2/DAP12 Complex
- Phospho DAP12 (Y91)
- Total DAP12
- Phospho SYK (Y525/526)
- Total SYK
- Total TREM2
- TREM2 Aggregate
- DAP12 Aggregate

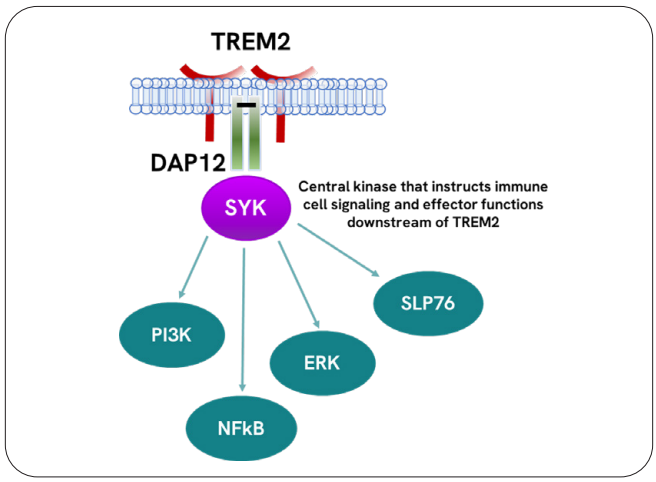


Figure 1. Schematic representation of TREM2/DAP12 signaling pathway. TREM2 activation leads to DAP12 phosphorylation and subsequent recruitment of SYK which promotes phosphorylation and activation of multiple downstream mediators such as PI3K, SLP76, ERK, and NFkB.

Materials and methods

2.1. Cell lines

THP-1 cells (passage 20, ATCC, Cat # TIB-202) were grown at 37°C with 5% CO_2 in RPMI medium (Gibco, Cat # 21870076) supplemented with 10% inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Cat # 15140122).

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll® Plaque Plus (Merck, Cat # GE17-1440-02). PBMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Cat # 11965) supplemented with 10% FBS and 1% penicillin/streptomycin. Monocytes were isolated by adherence and cultured for 6 days at 37°C with 5% CO_2 in complete DMEM supplemented with 20 ng/mL M-CSF (Sigma, Cat # M6518) to differentiate them into macrophages.

2.2. Activation of TREM2 signaling in THP-1 cells

THP-1 cells were harvested and seeded in fresh RPMI complete medium at a density of 600,000 cells/mL in a T75 flask. Cells were treated with 35 ng/mL of TGFβ (Abcam, Cat # ab84070) for 18 hours. Following treatment with TGFβ, approximately 30% of the cells had adhered to the flask. To detach the cells from the culture flask, adherent cells were gently scraped off and combined with the remaining suspension cells. The cells were washed via centrifugation at 300 RCF for 5 minutes in Hanks Balanced Salt Solution (HBSS; Gibco, Cat # 14175095) containing 0.1% bovine serum albumin (BSA, Jackson ImmunoDiagnostics, Cat # 001-000-173). Cells were then seeded at 400,000 cells/well in a 96-well plate (200 µL/well) in HBSS + 0.1% BSA and treated with 10 µM TREM2 Activator (MedChemExpress, Cat # HY-148095) for various time points (see Section 3.1.). Following treatment, cells were lysed with 50 µL of 5X Lysis Buffer. For the detection step, 10 µL of lysate was used in respective *SureFire® Ultra* assay, equivalent to approximately 16,000 cells/datapoint.

2.3. Activation of TREM2 signaling in primary macrophages

Primary macrophages were seeded at 50,000 cells/well in a 96-well plate and incubated overnight. Cells were treated with 35 ng/mL of TGFβ for 18 hours and further stimulated with 20 µM TREM2 activator for various time points (see Section 3.1.). Following treatment, cells were lysed with 50 µL of Lysis Buffer. For the detection step, 10 µL of lysate was used in respective *SureFire® Ultra* assays, resulting in approximately 8,000 cells/datapoint. For DAP12 Phospho (Y91) and DAP12 Aggregate assays, lysates were further diluted 1:4 in Lysis Buffer, resulting in approximately 2,000 cells/datapoint.

2.4. TREM2 Activator dose response in THP-1 cells

THP-1 cells were cultured and pre-treated with TGFβ as previously described (see Section 2.2.). Cells were seeded at 400,000 cells/well in a 96-well plate (200 µL/well) in HBSS + 0.1% BSA and treated with increasing concentrations of TREM2 Activator for 10 minutes. Following treatment, cells were lysed with 50 µL of 5X Lysis Buffer and lysates were analyzed using respective *SureFire® Ultra* assays. Equivalent to approximately 16,000 cells/datapoint.

2.5. Antibody-mediated activation of TREM2 pathway

THP-1 cells were cultured and pre-treated with TGFβ as previously described (see Section 2.2.). Cells were seeded at 400,000 cells/well in a 96-well plate (200 µL/well) in HBSS + 0.1% BSA. Cells were left untreated or treated with 5 µg/mL of an Anti N-terminal TREM2 Antibody (Cell Signaling Technology, Cat # 70551) for 5 minutes. Cells were lysed with 50 µL of 5X Lysis Buffer and 10 µL of lysate was used in respective *SureFire® Ultra* assays, resulting in approximately 16,000 cells/datapoint.

2.6. AlphaLISA *SureFire® Ultra* assay principle

AlphaLISA technology is a fast, highly sensitive and homogeneous no-wash assay platform with a broad dynamic range that can be performed in a microplate format. These features make AlphaLISA one of the best assays for measuring both very low and high levels of analytes in a myriad of matrix types. AlphaLISA assays utilize two types of beads: Donor beads and Acceptor beads. In an AlphaLISA *SureFire® Ultra* assay, Donor beads are coated with streptavidin to capture a biotinylated detection antibody, while Acceptor beads are coated with a proprietary Capt*Sure*™ agent to immobilize the other detection antibody labeled with a Capt*Sure* tag. In the presence of a target protein, the antibodies bring the Donor and Acceptor beads into proximity. Upon excitation at 680 nm, a photosensitizer within the Donor bead converts ambient oxygen into an excited singlet state, which diffuses up to 200 nm and produces a chemiluminescent reaction in the Acceptor bead, emitting light at 615 nm. If an Acceptor bead is not in proximity to a Donor bead, little to no signal is produced over the background. A schematic of the AlphaLISA *SureFire® Ultra* assay is shown in Figure 2.

AlphaLISA *SureFire® Ultra* kits offer the option for either 1 or 2-plate assay protocols, where the cell culture and assay can be conducted in a single plate or in two separate plates, respectively. For this study, the 2-plate assay protocol was followed for all assays as outlined in the AlphaLISA *SureFire® Ultra* manual. Briefly, 10 µL of prepared cell lysate was transferred from the 96-cell culture plate to a white 384-well OptiPlate (Revvity, Cat # 6007299), followed by the addition of 5 µL of Acceptor Mix from the respective AlphaLISA *SureFire® Ultra* assay kit. The plate was incubated for 1 hour at room temperature and then 5 µL of Donor Mix was added to each well. The plate was incubated for another hour at room temperature and protected from light. Plates were read on an EnVision 2105 Multimode Plate Reader using default AlphaLISA settings.

The following AlphaLISA *SureFire® Ultra* assay kits (Revvity) were used for each target as listed:

1. Human TREM2 Total (Cat # ALSU-TTREM2-A500)
2. Human TREM2 Aggregate (Cat # ALSU-ATREM2-A500)
3. Human DAP12 Total (Cat # ALSU-TDAP12-A500)
4. Human DAP12 Aggregate (Cat # ALSU-ADAP12-A500)
5. Human TREM2/DAP12 Complex (Cat # ALSU-TTRMDP-A500)
6. Human p-DAP12 (Tyr91) (Cat # ALSU-PDAP12-B500
7. Human and Mouse p-SYK (Tyr525/526) (Cat # ALSU-PSYK-A500)

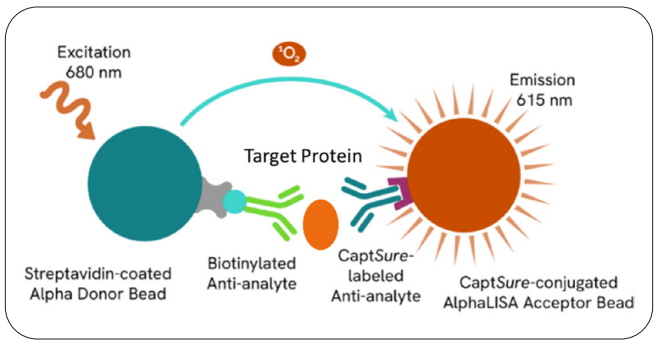


Figure 2. AlphaLISA *SureFire® Ultra* (ALSU) assay schematic demonstrating capture of a target protein. The target protein is detected in a sandwich assay using specific antibodies. One antibody is directed against a specific epitope on the analyte, while the other antibody is directed against another epitope on a distal part of the analyte. The resulting AlphaLISA signal in the immunoassay is directly proportional to the amount of target analyte present in the sample.

Results and discussion

3.1. TREM2 Activator rapidly induces TREM2 signaling in THP-1 and primary macrophages

The development of new drug-like small molecules targeting TREM2 has emerged as a promising therapeutic strategy for neuroinflammatory diseases. For instance, VG-3927, an oral small molecule TREM2 agonist developed by Vigil Neuroscience for AD, is currently being used in Phase I clinical trials [7, 8].

In this study, a small TREM2-activating compound, referred to as TREM2 Activator, was evaluated in THP-1 cells and macrophages isolated from PBMCs to assess its ability to activate the TREM2 signaling pathway. Pathway kinetics were analyzed by treating cells with a fixed dose of TREM2 Activator for up to 1 hour, followed by screening cell lysates with a range of specific AlphaLISA *SureFire® Ultra* assays. The TREM2 signaling profile was consistent across both immortalized THP-1 cells (Figure 3) and primary macrophages (Figure 4). The *SureFire® Ultra* TREM2/DAP12 Complex and TREM2 Aggregate assays, two novel approaches for measuring pathway activation, showed significant upregulation in both cell types following treatment with TREM2 Activator. Within just 2 minutes, levels of TREM2/DAP12 Complex and TREM2 Aggregate increased sharply, peaking at 10-30 minutes with a 30-40-fold increase in Alpha signal. In THP-1 cells, these elevated signal levels persisted for up to 1 hour.

Phosphorylation of DAP12 (Y91) and SYK (Y525/526) also peaked after 10 minutes of treatment, albeit to a lesser extent (Figures 3 and 4). In THP-1 cells, an 8–10-fold increase in phosphorylation was observed, while a 2–4-fold induction was detected in primary macrophages, with levels beginning to decline after 30 minutes. Similarly, the induction of DAP12 Aggregation was more subtle, with peak levels detected at 30 minutes. DAP12 Aggregate signal increased approximately 8-fold in THP-1 cells and 2-fold in primary macrophages.

No significant changes in Total DAP12 or Total TREM2 levels (data not shown) were seen, confirming that the increases in complex formation, phosphorylation, and aggregation were post-translational events specifically induced by TREM2 Activator.

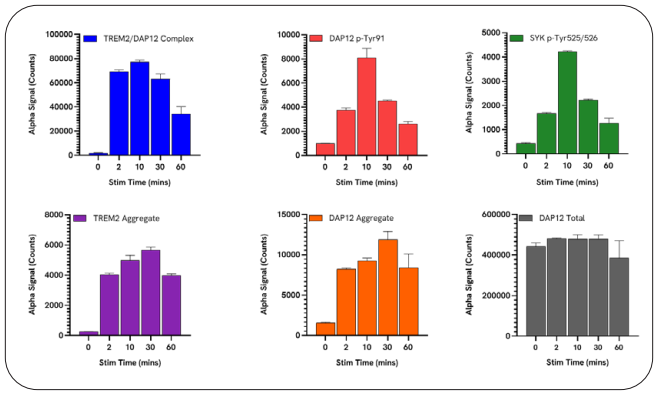


Figure 3. TREM2 Activator induces TREM2/DAP12 downstream cascade activation in THP-1. Cells were treated with TGFβ for 18 hours and further stimulated with 10 µM TREM2 Activator for different time points (0, 2, 10, 30, 60 minutes). Alpha Signal was measured for each target using the respective AlphaLISA *SureFire® Ultra* assay kit. Equivalent to approximately 16,000 cells/data point.

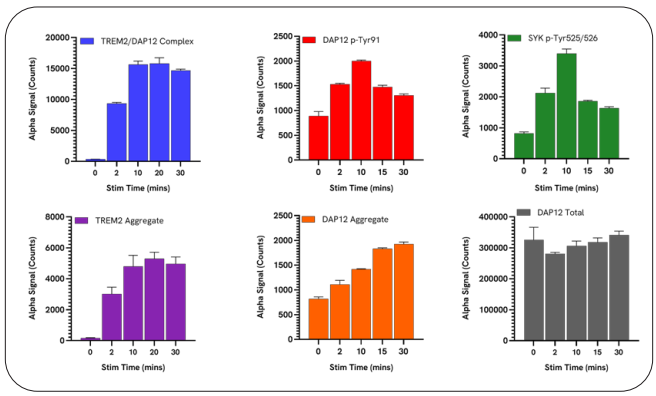


Figure 4. TREM2 Activator induces TREM2/DAP12 downstream cascade activation in primary macrophages. Cells were treated with TGFβ for 18 hours and further stimulated with 20 µM TREM2 Activator for different time points (0, 2, 10, 20, 30 minutes). Alpha Signal was measured for each target using the respective AlphaLISA *SureFire® Ultra* assay kit. Equivalent to approximately 8,000 cells or 2,000 cells/datapoint (DAP12 p-Tyr91 and DAP12 Aggregate).

3.2. TREM2 Activator induces TREM2 signaling in THP-1 cells in a dose-dependent manner

THP-1 cells were treated with a range of TREM2 Activator concentrations for 10 minutes to assess its potency (Figure 5). The TREM2 signaling cascade was upregulated in a dose-dependent manner, with the most pronounced effects observed in the TREM2/DAP12 Complex and TREM2 Aggregate cellular readouts. These two assays exhibited the largest signal windows and dynamic ranges across the concentration range tested. Similar EC_{50} values of 0.136 µM and 0.128 µM were determined for the TREM2/DAP12 Complex and TREM2 Aggregate, respectively. Downstream phosphorylation of DAP12 (Y91) and SYK (Y525/526) was also induced in a concentration-dependent manner, with estimated EC_{50} values of 0.40 µM and 0.22 µM. In contrast, DAP12 Aggregation showed a more modest induction, and no EC_{50} value was determined. Total DAP12 and Total TREM2 levels remained unchanged, confirming that TREM2 Activator selectively modulates specific TREM2 pathway signaling events.

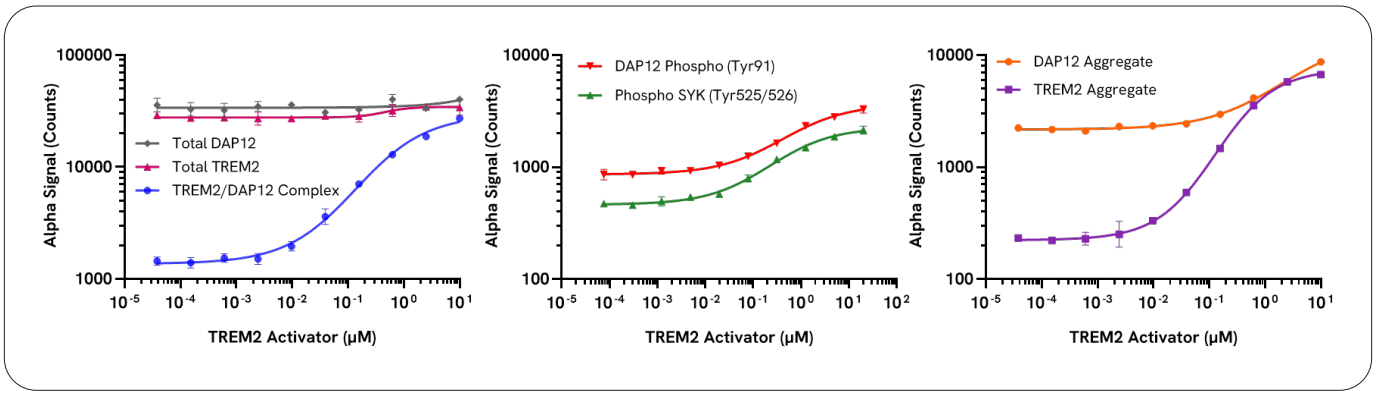


Figure 5. TREM2 Activator stimulates the TREM2/DAP12 pathway in a dose-dependent manner. THP-1 cells were treated with TGFβ for 18 hours and further stimulated with increasing concentrations of TREM2 Activator for 10 minutes. Alpha signal was measured for each target using the respective AlphaLISA *SureFire® Ultra* assay kit. Equivalent to approximately 16,000 cells or 1,600 cells/datapoint (Total TREM2 and Total DAP12).

3.3. Induction of TREM2 signaling in THP-1 cells using a TREM2-specific antibody

The use of biologics or therapeutic antibodies targeting TREM2 represents an alternative strategy for activating the TREM2 signaling pathway. For example, Alector and Abbvie have partnered to develop AL002, a humanized monoclonal antibody that binds to the TREM2 microglial receptor to activate signaling, including downstream SYK phosphorylation. AL002 is currently undergoing evaluation in a Phase II clinical trial for AD [9].

The AlphaLISA *SureFire® Ultra* assays effectively measured pathway activation in THP-1 cells treated with a TREM2-specific antibody targeting the extracellular domain of TREM2 (Figure 6). Stimulation of THP-1 cells for 5 minutes with the TREM2-specific antibody induced a 4-fold increase in TREM2 Aggregate and 2-fold and 4-fold increases in the downstream phosphorylation of DAP12 (Y91) and SYK (Y525/526), respectively. In contrast, DAP12 Aggregate levels remained unchanged. Although the TREM2-specific antibody evaluated was less potent compared to the TREM2 Activator in inducing signaling in THP-1 cells, these findings demonstrate the compatibility of AlphaLISA *SureFire® Ultra* technology with both biologics and small molecules for drug discovery research.

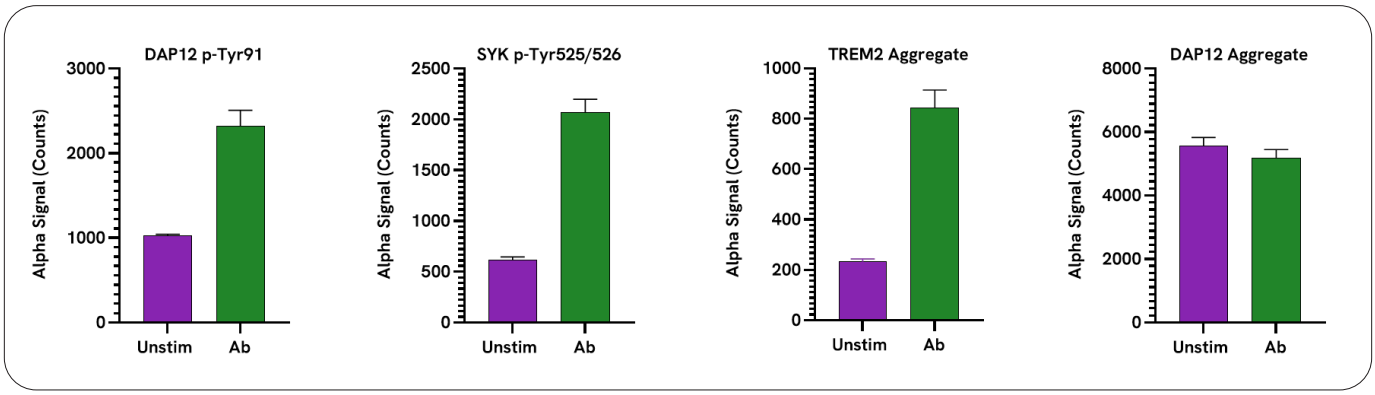


Figure 6. Anti-N-terminal TREM2 antibody induces TREM2/DAP12 cascade activation. THP-1 cells were treated with TGFβ for 18 hours. Cells were left untreated (Unstim) or treated with an Anti N-terminal TREM2 antibody (Ab) for 5 minutes. Alpha Signal intensity was measured for each target using the respective AlphaLISA *SureFire® Ultra* assay kit. Equivalent to approximately 16,000 cells/datapoint.

Conclusion

AlphaLISA *SureFire® Ultra* (ALSU) is a robust, no-wash, cell-based assay platform noted for its exceptional sensitivity, wide dynamic range, and ease of use. It serves as an invaluable tool for the quantitative detection of phosphorylated proteins, total proteins, and other post-translational modifications in both cellular and biological samples. This first-in-class technology offers fully developed and validated assay kits for detecting and analyzing key proteins involved in the TREM2 signaling pathway.

This study highlights the utility of multiple AlphaLISA *SureFire® Ultra* assays for measuring TREM2 signaling in both immortalized THP-1 cells and macrophages isolated from PBMCs. Pathway activation was demonstrated using a small molecule TREM2 Activator and a monoclonal antibody specifically targeting the extracellular domain of TREM2. The rapid induction of phospho-DAP12 (Y91) and SYK (Y525/526), well-characterized downstream effectors of TREM2 signaling, was observed within minutes of treatment.

For the first time, these results showcase novel functional assays for TREM2 activation, including TREM2/DAP12 Complex and TREM2 and DAP12 Aggregates. Depending on the biological context, these assays may provide more sensitive post-translational readouts than phosphorylation, offering new opportunities for deeper insight into TREM2 pathway dynamics. The exceptional sensitivity and specificity of these innovative AlphaLISA *SureFire® Ultra* assays were integral to the rapid detection of multiple key intracellular TREM2 signaling events in endogenous cell systems. These findings further highlight the potential of these assays for deployment in high-throughput screening programs aimed at identifying additional novel TREM2 activators.

Overall, AlphaLISA *SureFire® Ultra* is a powerful tool for advancing drug discovery and neuroinflammatory research, among other disease areas, underscoring Revvity's commitment to supporting research across all stages—from basic research to drug discovery, screening, preclinical, and clinical development.

References

1. Kwon, H.S. and S.-H. Koh, *Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes*. Translational Neurodegeneration, 2020. 9(1): p. 42.
2. Liu, G., et al., *The Pathogenesis in Alzheimer's Disease: TREM2 as a Potential Target*. J Integr Neurosci, 2023. 22(6): p. 150.
3. Li, Y., et al., *TREM2: Potential therapeutic targeting of microglia for Alzheimer's disease*. Biomed Pharmacother, 2023. 165: p. 115218.
4. Qin, Q., et al., *TREM2, microglia, and Alzheimer's disease*. Mech Ageing Dev, 2021. 195: p. 111438.
5. Simard, A.R., et al., *Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease*. Neuron, 2006. 49(4): p. 489-502.
6. Zhao, Y., et al., *TREM2 Is a Receptor for β-Amyloid that Mediates Microglial Function*. Neuron, 2018. 97(5): p. 1023-1031.e7.
7. Vigil Neuroscience, I. *Pipeline*. [cited 2024 November]; Available from: https://www.vigilneuro.com/pipeline.
8. Alzforum. *Therapeutics: VG-3927*. 2024 August 05, 2024 [cited 2024 November]; Available from: https://www.alzforum.org/therapeutics/vg-3927.
9. Long, H., et al., *Preclinical and first-in-human evaluation of AL002, a novel TREM2 agonistic antibody for Alzheimer's disease*. Alzheimer's Research & Therapy, 2024. 16(1): p. 235.