Comprehensive HTRF solutions to improve research on neutralizing antibodies in autoimmune diseases.

The data presented here demonstrates that HTRF biochemical and cellular assays offer a comprehensive and easy-to-implement platform for the identification of potent biotherapeutics to address autoimmune pathologies.

Abstract

Today, biopharmaceuticals account for a steadily increasing proportion of all approved drugs and play a pivotal role in research projects as they can be applied to a wide variety of diseases, including cancers, autoimmune diseases, infectious diseases, and cardiovascular diseases.

The identification of innovative lead antibodies in the drug discovery process requires the use of assays that not only measure binding of the antibody to the target molecule, but also assess a wide range of other properties reflective of their mechanism of action (MOA).

Revvity offers HTRF® (Homogeneous Time-Resolved Fluorescence) biochemical and cell-based assay kits dedicated to biologics research. These assays enable the rapid selection of the most potent binders based on affinity ranking, and their ability to inhibit relevant protein-protein interactions and provide cell-based solutions to delineate their mechanism of action.

This study illustrates the successful application of HTRF in the drug discovery process of biologics drugs by studying anti-IL6R therapeutic antibodies (Tocilizumab and Sarilumab), as well as anti-TNFα antibodies (Infliximab and Adalimumab) which are used in the treatment of autoimmune diseases. These therapeutic antibodies were characterized through multiple approaches from affinity selection to functional cellular modulation.
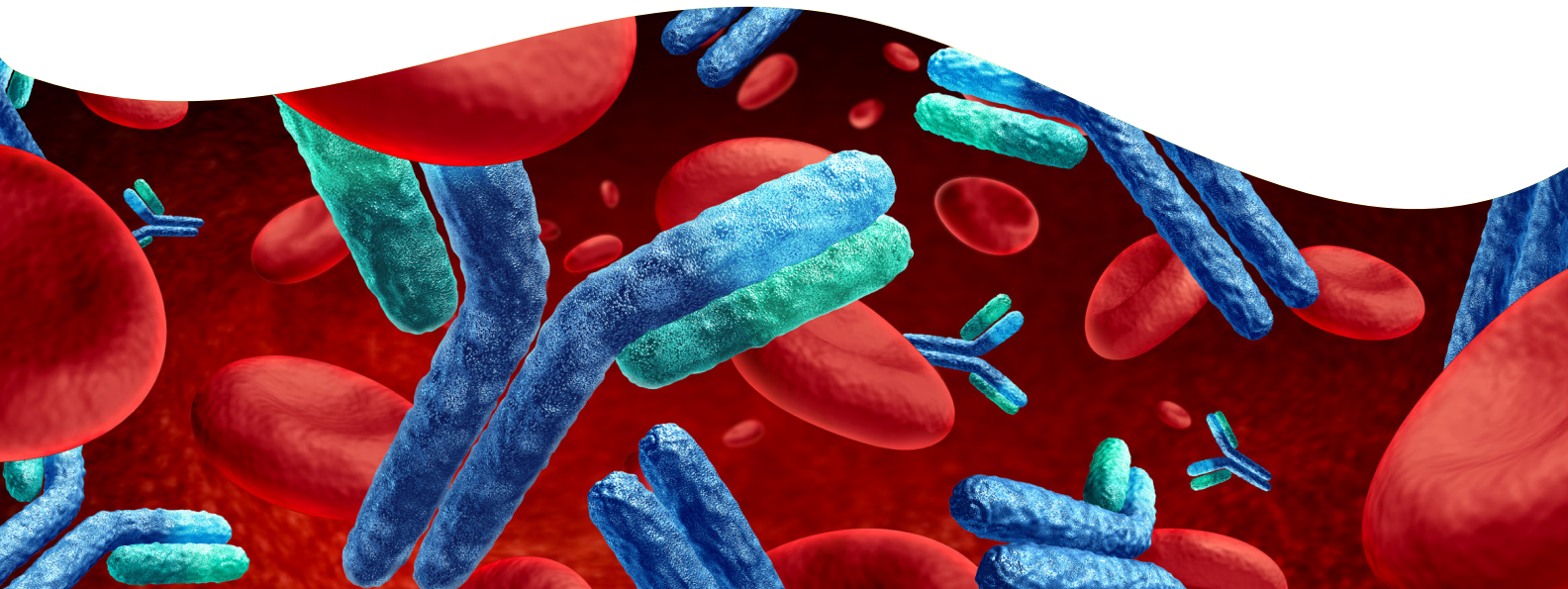
Assay workflow

Inflammatory cytokines are key regulators of immune responses in fighting against infections. However uncontrolled production of inflammatory cytokines is associated with the development of autoimmune diseases. Therefore, by disrupting intracellular signaling pathways, monoclonal antibodies neutralizing inflammatory cytokines or their corresponding receptor represent a therapeutic strategy to treat autoimmune diseases.

TNFα pathway

TNFα is a master regulatory cytokine produced by several cell types such as macrophages, dendritic cells, neutrophils, or T lymphocytes, and plays an essential role in inflammation and autoimmune diseases, including Rheumatoid Arthritis (RA) or Inflammatory Bowel Disease (IBD). TNFα induces its biological responses through two distinct receptors, TNFR1 and TNFR2, which are coupled to intracellular signaling cascades such as NFκB. As they inhibit TNFR signaling pathways, neutralizing Anti-TNF antibodies such as infliximab and adalimumab, as well as etanercept, are used in the management of autoimmune diseases.
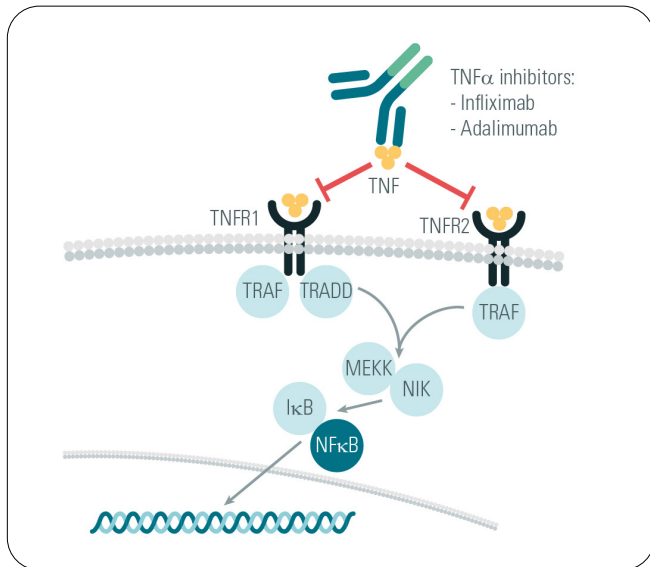

Figure 1: TNFα simplified pathway

IL-6 pathway

Like TNFα, IL-6 is a pleiotropic cytokine produced by various hematopoietic and non-hematopoietic cells. It exerts a wide range of biologic activities in immune regulation, hematopoiesis, and inflammation, as well as metabolic processes.

IL-6 utilizes two different pathways to mediate its biological effects:

1. The classical pathway where IL-6 binds to IL6R inducing the formation of a heterohexameric complex, and

2. The trans-signaling pathway where circulating IL-6 initiates signaling by binding to soluble IL6R (sIL6R) complexes. Finally, the formation of the heterohexamer results in the activation of the JAK/STAT3 signaling pathway, leading to the transcription of STAT3 target genes.

IL-6 also plays a key role in the development of autoimmune models such as RA, IBD, and psoriasis. Therefore neutralizing antibodies targeting IL-6 or its receptor, such as tocilizumab (humanized IL-6R-specific MAb) or sarilumab (fully human IL-6Rα MAb), have been approved in the treatment of Autoimmune Diseases such as RA.
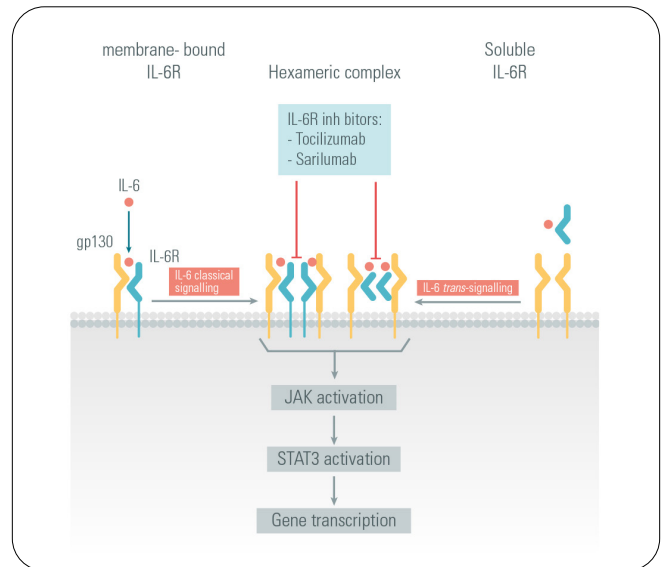

Figure 2: IL6 simplified pathway
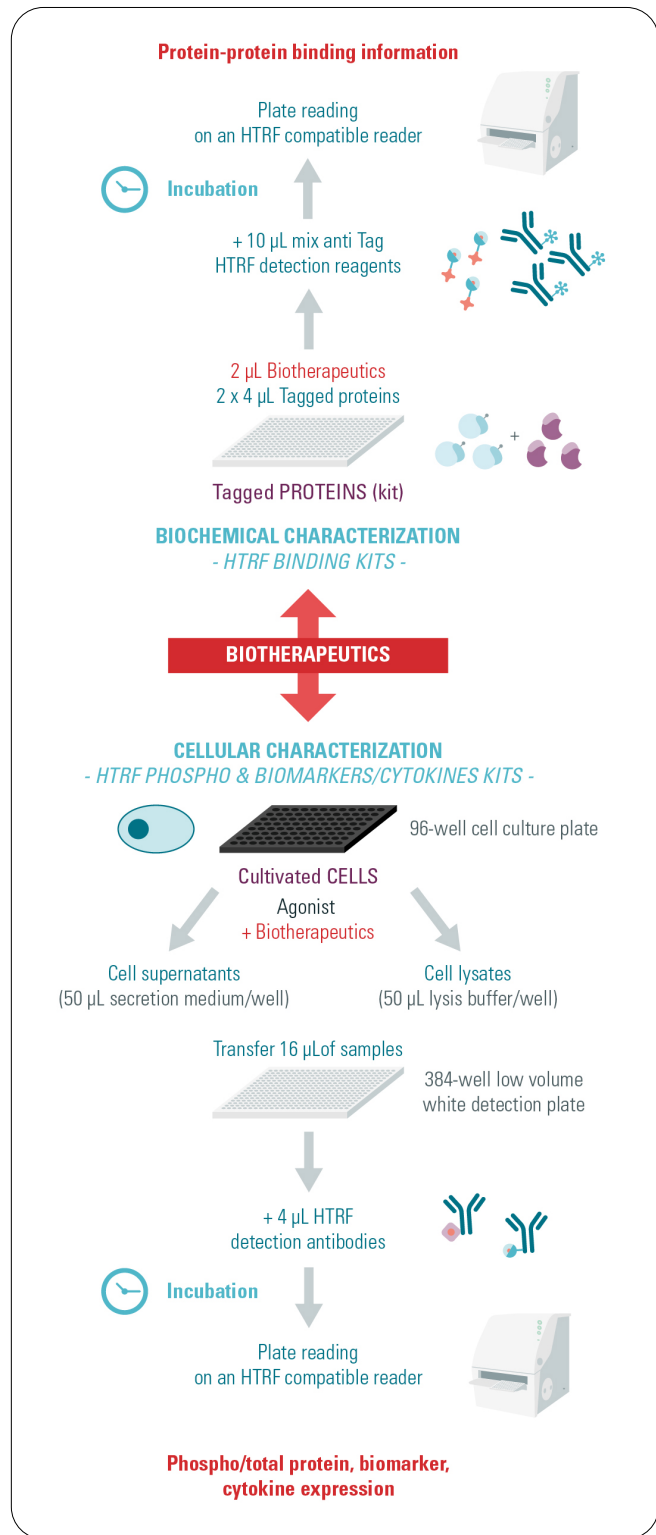
Materials and methods

Figure 3: Experimental flowchart

Cell models and reagents

THP-1 cells (ATCC® TIB202™) were cultured in RPMI medium supplemented with 10% FBS and 1% antibiotics. Cells were plated with phenol red free medium.

Adalimumab and Infliximab are from NovusBio (NBP2-62567 and NBP2-52655), Tocilizumab and Sarilumab from Seleckchem (A2012 and A2011). An IgG1 K (Millipore AG502) was used as a negative control. Cytokines TNFα and IL6 are from Sigma, references SRP3177 and SRP3096.

The HTRF cellular assay kits (Revvity-Revvity) used for this study were:

- Human TNFα/TNFR1 binding kit part number #64BDTNFPEG

- Human IL6/IL6R binding kit part number #64BDIL6PEG

- Phospho-STAT3 (Tyr705) part number #62AT3PEG

- Phospho-NFκB (Ser536) part number #64NFBPEG

- Human IL8 part number #62HIL08PEG

Experimental conditions cell treatments and sample generation

To assess optimal stimulation conditions, cytokine doseresponse curves were tested at various cell densities. Once the optimal cell density and cytokine concentration were determined, biotherapeutic antibodies were tested to measure their capacity to inhibit the cytokine-receptor interaction.

Depending on whether the antibody was targeting the cytokine or the receptor, the antibody was incubated with its target for 2 or 3 hours. After a 10 min stimulation with the cytokine, lysis buffer was added to the cells for the detection of intra-cellular proteins. For secreted protein, the incubation was pushed to overnight before supernatant collection.

HTRF binding assays

HTRF protein-protein binding kits are designed to measure the interaction between 2 proteins in a biochemical assay. Without treatment, proteins bind to each other, and the binding of each detection antibody to its respective tagged protein generates an HTRF signal. In the presence

of a specific compound targeting one of the partners, for example a biotherapeutic antibody, the interaction is disrupted and the HTRF signal decreases.

To perform these assays, 2 μL of compounds were mixed with 4 μL of each tagged protein. Then 5 μL of each anti-tag detection antibody were added. After incubation following the manufacturer's instructions, the signal was recorded on an HTRF compatible microplate reader.

HTRF cellular assays

HTRF cellular assays are no-wash sandwich immunoassays based on TR-FRET, where the fluorescent signal intensity is proportional to the analyte concentration in the sample. The detection is achieved using analyte-specific antibodies conjugated to donor and acceptor fluorophores. In the particular case of a phospho-protein, one of the antibodies specifically recognizes the phosphorylated residue(s) of interest.

To perform these HTRF assays, 16 μL of sample (cell lysate or supernatant) were transferred into a 384-well low volume white plate, and 4 μL of pre-mixed HTRF detection reagents were added. After incubation following the manufacturer's instructions, the signal was recorded on an HTRF compatible microplate reader.

HTRF data handling

The measurement of a protein-protein interaction in a biochemical assay is a relative quantification where the HTRF signal intensity (HTRF ratio) is directly proportional to the binding of each protein to the other. The measurement of human IL8 in cell supernatants is an absolute quantification done by interpolating the HTRF specific signal (delta ratio) from the assay standard curve performed in the same matrix as the samples (cell culture medium).

The measurement of intracellular proteins is a relative quantification where the HTRF signal intensity (HTRF Ratio) is directly proportional to the concentration of the phosphorylated or total protein in the cell lysate.

Visit our website for more details about HTRF data reduction, standard curve fitting, signal treatment and signal analysis.

Statistical analyses were performed with GraphPad Prism software and pharmacological dose-response curves were fitted using the log(compound) vs response curve with four parameters. Histobars represent the mean +/- SD (Standard Deviation) of each experimental triplicate.

Results

anti-IL6R Antibodies

The effect of anti-IL6R antibodies was assessed with the HTRF IL6/IL6R binding kit and by monitoring the phosphorylation of STAT3 in tyrosine 705.

The binding of Tocilizumab and Sarilumab were confirmed by their ability to prevent IL6 interaction to IL6R, with an affinity of respectively 220 pM and 132 pM (Figure 4).
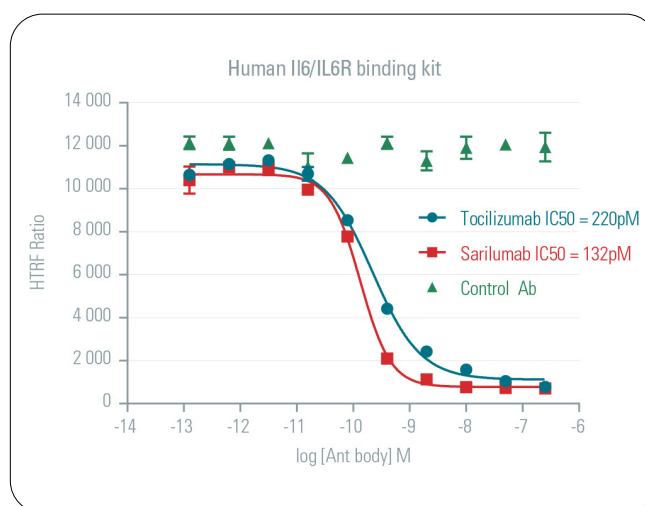

Figure 4: anti-IL6R antibody binding with Human IL6/IL6R binding kit

For cellular *in vitro* characterizations, THP-1 cells were first cultured at different cell densities in a 96 well plate with increasing concentrations of IL6 for 10 min. As shown in Figure 5, 100k cells/well were enough to generate a robust signal. Under these conditions a 1nM IL6 concentration close to the EC90 was considered optimal, and chosen to start cellular inhibition tests.
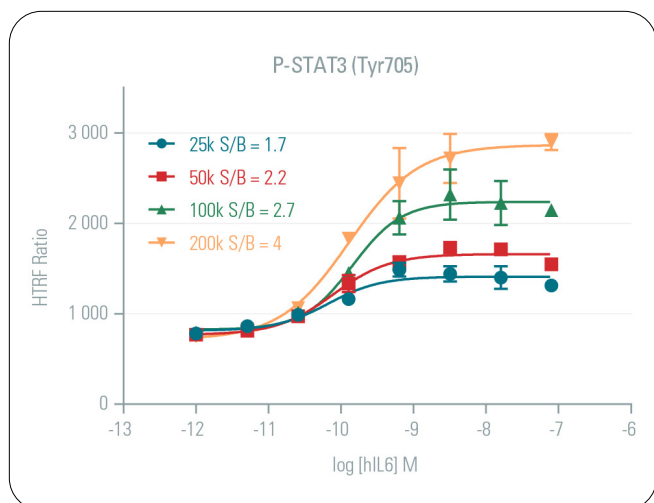
Figure 5: IL6 dose response curve in THP-1 cell densities

Tocilizumab and Sarilumab were then incubated for 3 hours with THP1 cells at a density of 100k cells/well. During the last 10 minutes, cells were incubated with 1nM IL6. As shown in Figure 6, Tocilizumab and Sarilumab successfully inhibited STAT3 phosphorylation induced by IL6, with IC50 of 133 pM and 164 pM respectively.
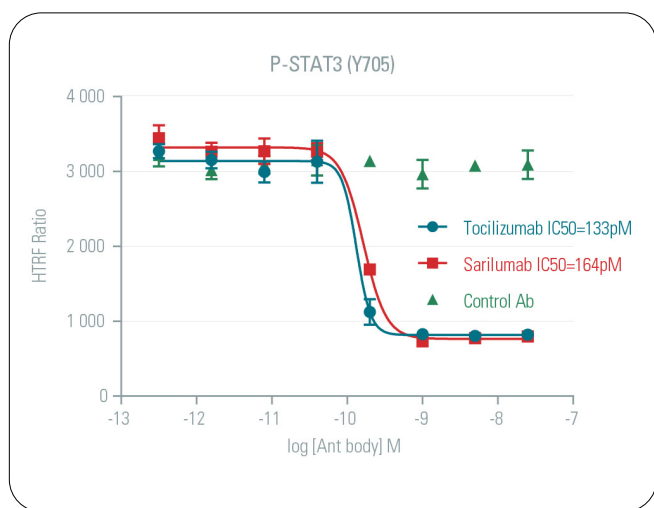

Figure 6: anti-IL6R antibody inhibition of IL6-induced STAT3 phosphorylation

anti-TNFα Antibodies

The effect of anti-TNFα antibodies was assessed at different levels: biochemically using the HTRF TNFα/TNFR1 binding kit, and in a cellular context either by monitoring the phosphorylation of NFKB on Serine 536 or by the secretion of human Interleukin 8.

The HTRF binding kit revealed that Adalimumab and Infliximab were able to prevent TNFα interaction with TNFR1, with an affinity of 224 pM and 144 pM (Figure 7).
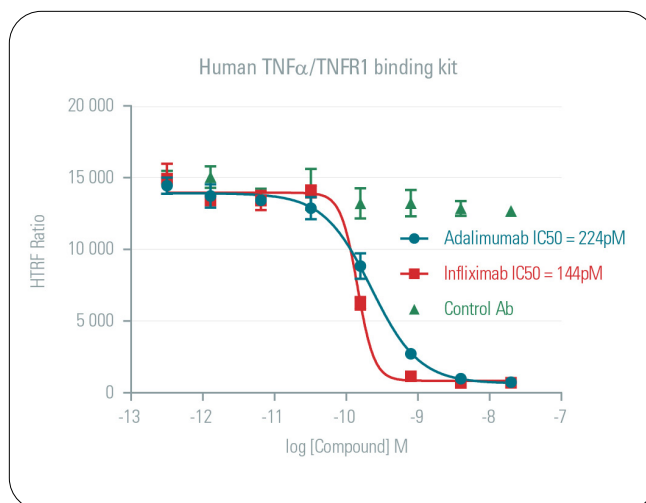

Figure 7: anti-TNFα antibody binding with Human TNFα/TNFR1 binding kit

Optimizations were done on THP1 cells with TNFα stimulation yields to select appropriate conditions using the HTRF Phospho-NFKB (Ser536) kit (not shown). A density of 100k cells/well was enough to obtain a pharmacological induction fold of 4.2. A 1 nM TNFα concentration was also close to the EC90, and was selected to assess antibody inhibition.

In order to assess cytokine targeting, the antibody was pre-incubated for 2 hours with the cytokine. This mix was then added to cells for 10 minutes. As shown in Figure 8, Adalimumab fully inhibited the TNFα induced phosphorylation, with an IC50 of 283 pM, whereas Infliximab inhibited it only partially with an affinity of 599 pM.
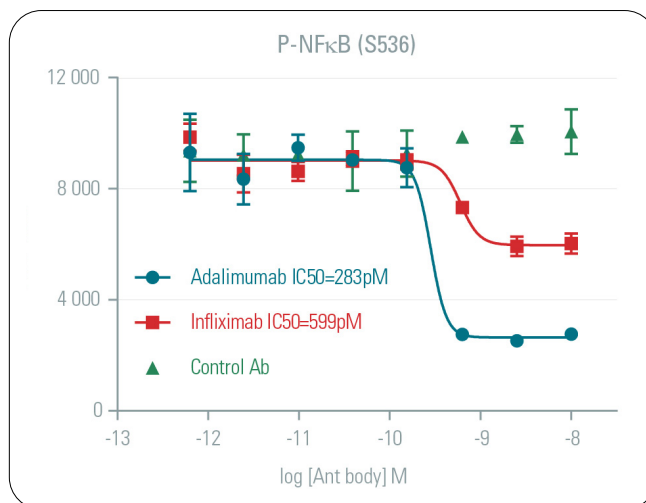

Figure 8: anti-TNFα antibody inhibition of TNFα induced phosphorylation of NFKB

Finally, to complete the phenotypic reading, human IL8 secretion was measured after an overnight stimulation. Supernatants were interpolated with the standard curve.

The results shown in Figure 9 clearly demonstrate that, in line with the phosphorylation status of NFκB transcription factor, Adalimumab fully inhibited IL8 secretion, while Infliximab was less potent.

Figure 9: anti-TNFα antibody inhibition of TNFα induced secretion of IL8

Conclusion

Therapeutics antibodies such as Tocilizumab and Adalimumab are biologic drugs prescribed for the treatment of adults with moderate to severe rheumatoid arthritis (RA), giant cell arteritis, and polyarticular and systemic juvenile idiopathic arthritis. Those types of drugs work by blocking the cytokine ligand (TNFα or IL6) and functionally inhibiting the pro-inflammatory signaling pathway.

The data presented here demonstrate that HTRF biochemical and cellular assays offer a comprehensive and easy-to-implement platform for the identification of potent biotherapeutics to address autoimmune pathologies.

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