APPLICATION NOTE

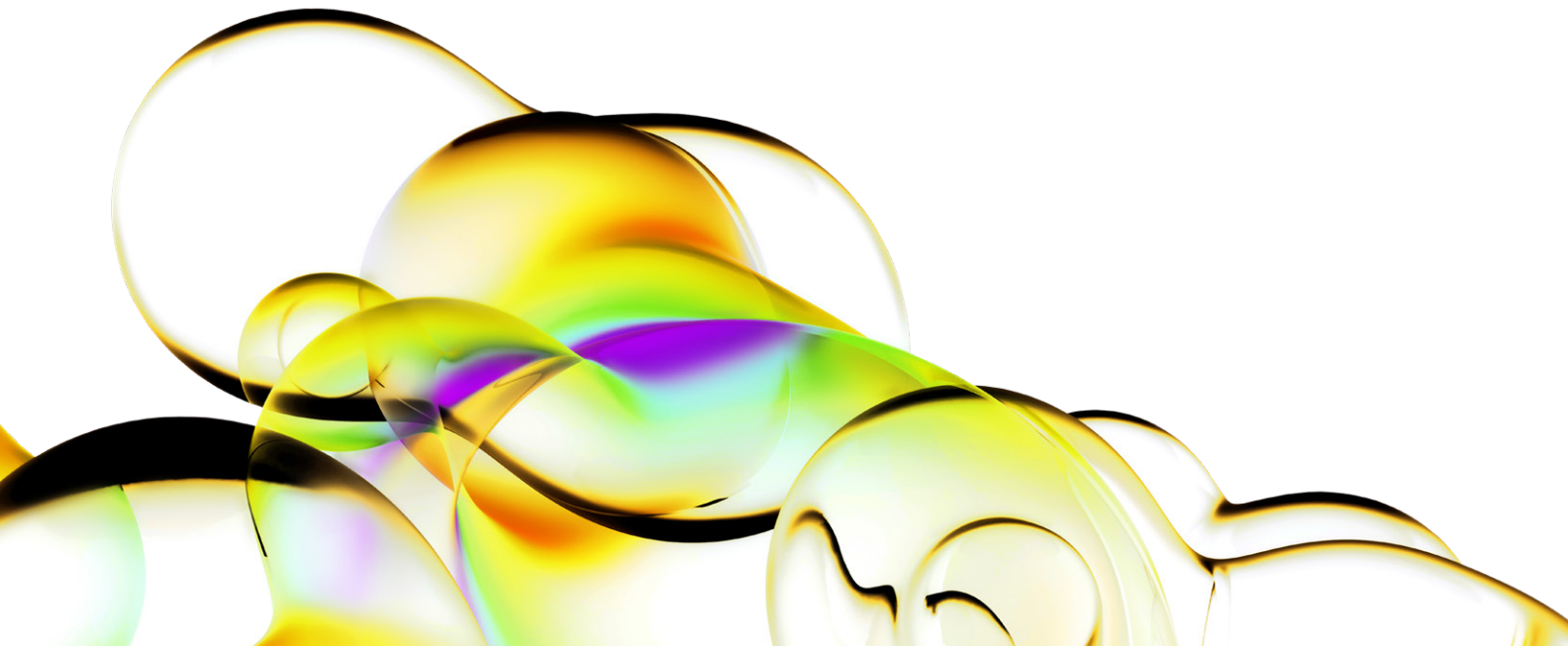
HTRF measurement of cytokine release from fresh blood samples.

Abstract

Revvity offers a comprehensive line of HTRF cytokine assays to investigate the functional response of immune cells. These versatile, homogeneous assays are suitable for a variety of sample types, such as cell culture supernatants, using immortalized cell lines or more physiological cell models such as PBMC.

Blood Assay Solutions is specialized in the development of assays performed in whole blood. Investigating cytokine release in whole blood gives access to a complex biological environment where all blood components are represented (PBMC and granulocytes including neutrophils, autologous plasma, red blood cells, platelets) thus allowing the investigation of important physiological immune responses like cytokine storm, inflammation and immunogenicity.

This application note, in collaboration with Blood Assay Solutions, illustrates the use of Revvity's HTRF™ cytokine assays for the analysis of immunological response in samples directly obtained from donors' whole blood.



It is clear today that the evaluation of potential adverse effects of drugs on the human immune system should be incorporated throughout their standard development. Drug immunotoxicity can be defined as unintended immunosuppression or immunomodulation. Whereas immunosuppression can lead to decreased host resistance to infectious agents or tumor cells, enhancement of the immune system can exaggerate autoimmune diseases or allergic reactions. Drug-induced systemic immune activation can lead to severe reactions associated with an excessive cytokine release, also termed cytokine storm. Because the function of the immune system is highly species-specific, cytokine release in humans is hard to predict through animal studies.

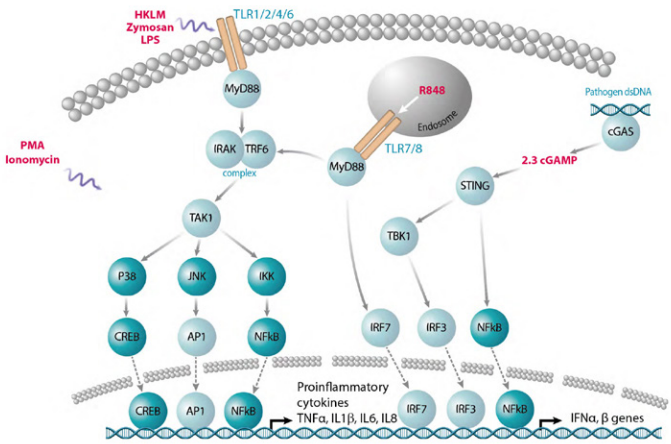
Whole blood assays have been described in drug development as an easy-to-do *in vitro* solution that could mimic the human situation. Such assays represent a fast and easy solution to monitor cytokine release in humans, enabling a prediction of efficacy as well as safety of the drug.

This approach is faster and cheaper than the more established PBMC based assay, since no purification and isolation of cells is required. In addition, small volumes of blood (i.e. <5 mL) are sufficient to test multiple experimental conditions.

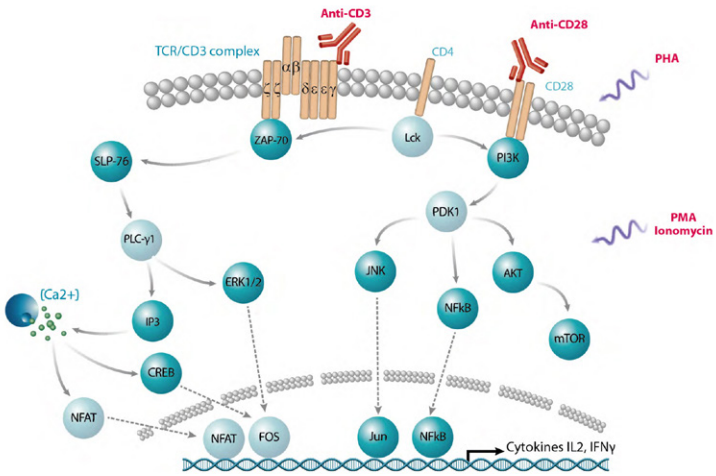
In order to further improve both sample studies and time savings, along with precise and accurate interpretation of biological responses, HTRF cytokine immunoassays were used for cytokine quantification.

This study demonstrates the capability of HTRF cytokine assays to measure cytokine release in whole blood samples. Relevant immune pathways and related agonists were selected to target either the Toll-Like receptor pathways, the STING pathway (scheme 1) or the TCR activation pathway (scheme 2). Finally human IL1β, IL2, IL6, IL8, TNFα and IFNγ were quantified by HTRF. Furthermore, HTRF data were compared with data obtained with ELISA reference kits to evaluate the correlation between the two techniques.

Scheme 1



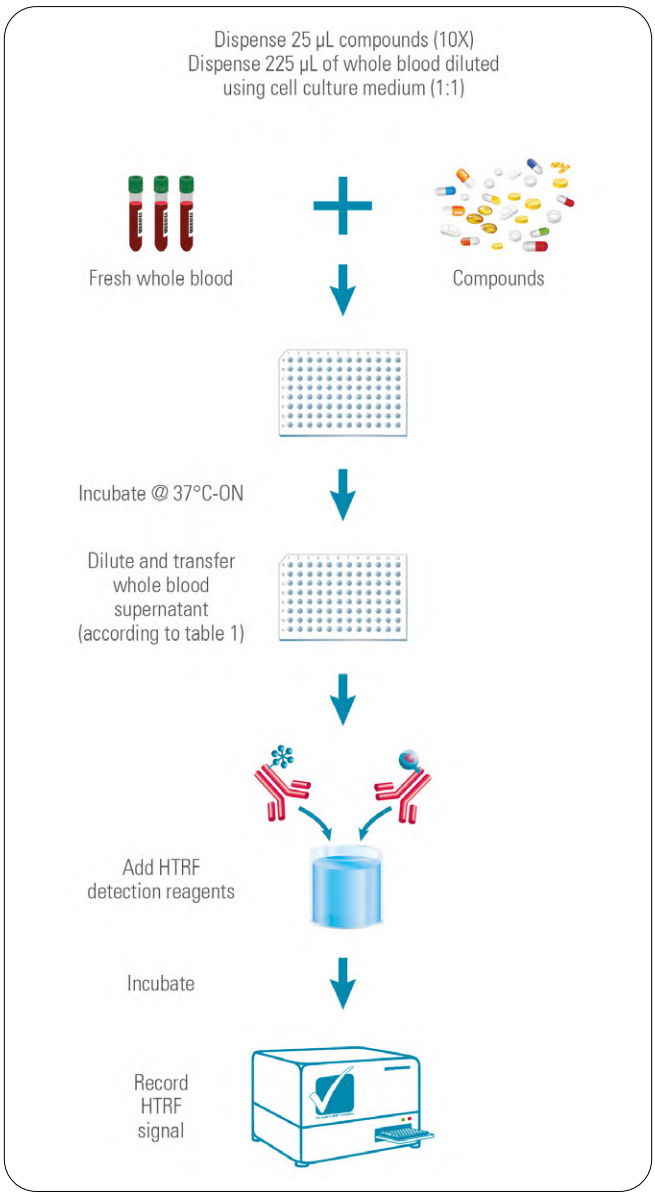
Scheme 2



Revvity cell signaling assays available

Materials and methods

Flowchart



Fresh blood from 3 different donors was collected in heparin-tubes, and whole blood samples were diluted in RPMI after homogenization (final dilution blood:RPMI was 1:1). 25 µL of compounds concentrated 10X were dispensed into 96 well plate followed by the addition of 225 µL of pre-diluted whole blood samples. Whole blood samples were incubated overnight at 37 °C. Supernatants were collected and frozen at -80 °C. Prior to cytokine measurements either by HTRF or ELISA, whole blood supernatants were diluted as indicated in Table 1.

Biological models and reagents

Whole blood samples were obtained from French National Blood Service (EFS Pyrénées-Méditerranée)

HTRF Cytokine assays: Revvity Bioassays, Human IL1β [62HIL1BPEG], Human IL2 [64HIL02PEG], Human IL6 [62HIL06PEG], Human IL8 [62HIL08PEG], Human TNFα [62HTNFAPEG], Human IFNγ [62HIFNGPEG].

ELISA kits: Human IL-8 (CXCL8) ELISA development kit (HRP) (Mabtech# 3560-1H-6)

Agonists: Heat killed Listeria monocytogenes (HKLM,invivogen #tlrl-hklm), LPS (invivogen #tlrl-3pelps), Zymosan (invivogen #tlrl-zyn), PHA (ebioscience #00-4977-93), R848 (invivogen #tlrl-r848), PMA+ Ionomycin (ebioscience #00-4970-93), 2,3 cGAMP (invivogen #tlrl-nacga23), OKT3+Anti CD28 (ebioscience #16-003781-BD#347690 respectively).

Antagonist: Dexamethasone (Sigma #D4902)

Experimental conditions

Given the quantification range of each assay and expected concentrations for each cytokine, a dilution step of whole blood supernatant was performed, using the diluent buffer included in each kit, prior to the detection procedure.

Table 1: Experimental conditions: U : Untreated; H : HKLM; L : LPS; Z : Zymosan; R: R848; C: cGAMP; PI: PMA/ionomycine; PHA: Phytohemagglutinin; O/C: Okt3/ antiCD28 antibody

HTRF assays	Assay range (pg/mL)	Expected concentrations	Dilution factors								
			U	H	L	Z	R	C	PI	PHA	O/C
IL1β	4.7 - 6 500	1 000 - 23 000	6	6	6	6	6	6	6	6	6
IL2	7- 8 000	2 000 - 170 000	6	6	6	6	6	6	38	6	6
IL6	7.6 - 7 500	24 000 - 118 000	6	25	25	25	25	13	13	25	13
IL8	6.1 - 4 000	8 000 - 426 000	6	128	50	50	25	13	128	50	50
TNFα	5 - 2 500	600 - 13 000	6	6	6	6	6	6	6	6	6
IFNγ	14.1 - 4 000	600 - 354 000	6	6	6	6	25	13	128	25	13

Htrf cytokine assays

All HTRF cytokine assays are no-wash sandwich immunoassays where the fluorescent signal intensity is proportional to the analyte concentration in the sample. The detection is performed via analyte-specific antibodies coupled to donor and acceptor dyes.

To perform the HTRF assays, 16 µL of pre diluted whole blood supernatant were dispensed in duplicate into a white 384-well small volume microplate and 4 µL of pre-mixed HTRF reagents were added. A negative control was included in each assay by replacing the supernatant with diluent buffer. After incubation at room temperature, the HTRF signal was recorded on a Tecan infinite 200pro reader with flash lamp excitation.

ELISA ASSAYS

In parallel to HTRF human IL8, whole blood supernatants were analyzed by ELISA according to the manufacturer's instructions.

Data handling

✓ **HTRF assays:**

- The HTRF Ratio was calculated for each well of standard or samples independently, using the following formula:

$$\text{HTRF Ratio} = \frac{(\text{signal 665 nM})}{(\text{signal 620 nM})} \times 10^4$$

- Generation of the standard curve
  The standard curve was obtained by data reduction using the four parameter logistic (4PL plus 1/ y^2) curve fit
  Learn more about HTRF data reduction and curve fitting at www.revvity.com.
- Means and Standard Deviations (SD) are shown on the graphs.
- Y axis is represented either in linear or logarithmic scale, depending on the range of cytokine concentrations measured.
- Multiple comparisons were performed on GraphPad using the parametric one-way Anova Dunnett post-test or the parametric two way Anova.

✓ **ELISA assays:**

The Optical Density at 450 nM (OD450) was measured using a Tecan infinite 200pro microplate reader.

Results

Inter donor variability

The inter-donor variability regarding the magnitude of immune responses measured using whole blood assays is a well-known phenomenon that has to be taken into account when assessing a compound's effect (Mueller doi: 10.1186/1471-2288-12-112, Duffy doi: 10.1016/j.immuni.2014.03.002).

Data previously obtained by Blood Assay Solutions (poster ECI 2015, "Whole-blood assays reveal inter-individual variations of innate and adaptive immune cell responses") confirmed this phenomenon.

All the results presented in the following sections, were obtained from 3 independent experiments performed on whole blood from 3 healthy donors.

Determination of the optimal agonist concentration

Preliminary dose response experiments enabled us to select a dose (referred 1X) for each agonist inducing a strong response in the majority of healthy donors.

As shown in Figure 1 this step is crucial since it shows that, contrary to other stimuli (see zymosan as an example below), PMA/ionomycin when used at 5X concentration, induces a reduction of IL1β and TNFα level that could potentially reflect a cytotoxic effect.

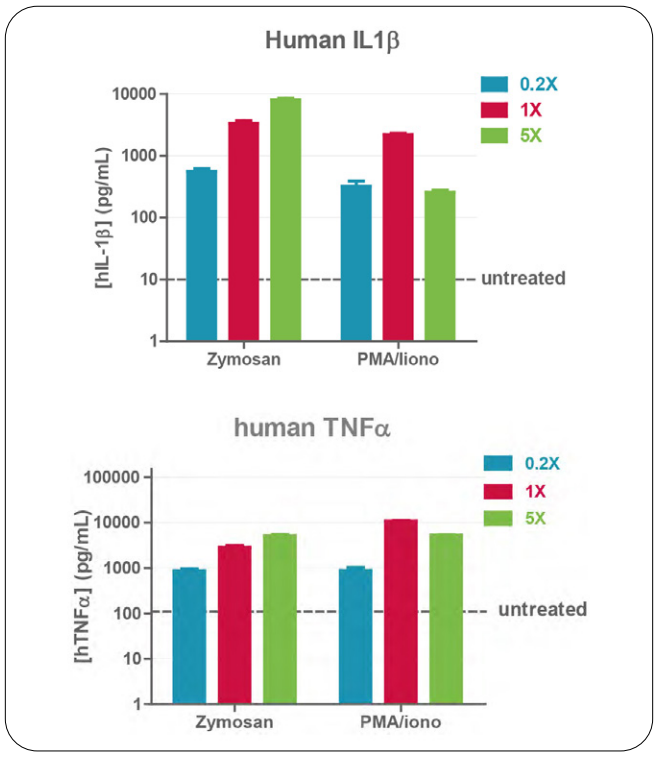


Figure 1: quantification of IL1β and TNFα release after whole blood stimulation with Zymosan or PMA/Iono at 3 different concentrations.

Cytokine release upon various immune pathway engagements

Activation of the Toll-Like 1, 2, 4, 6 Receptors

TL1, 2, 6 or TLR4 pathways were stimulated with HKLM, LPS or Zymosan.

Figure 3A shows the concentration of each measured cytokine in untreated versus treated samples. HTRF solutions enable the determination of basal cytokine levels (dotted lines) in all samples as well as the measurement of the increase in cytokine secretion.

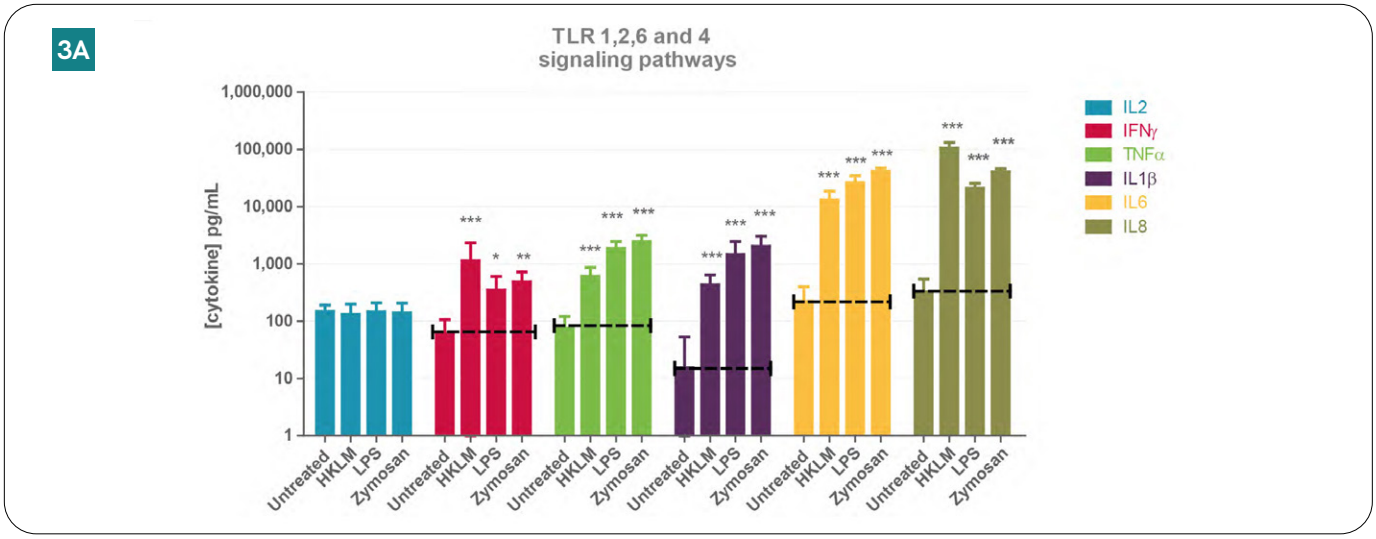


Figure 3 A: Expression in pg/mL of cytokine profiles after stimulation of the Toll-Like 1, 2, 4, 6 Receptors.

Figure 3B displays a representation in fold of induction of cytokines released calculated from the untreated condition. These results clearly indicate that, even though IL6 and IL8 reach the highest cytokine concentrations upon stimulation, IL1β shows the best mobilization with a 350 fold increase upon zymosan treatment.

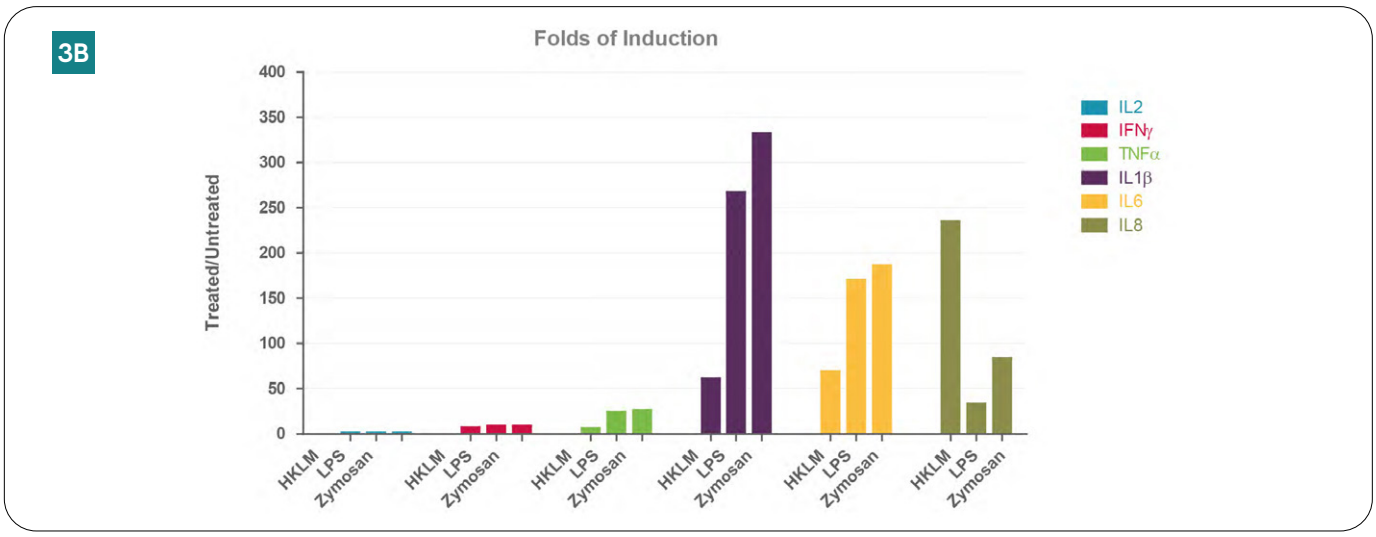


Figure 3 B: Expression in folds of induction over untreated condition for cytokine profiles after stimulation of the Toll-Like 1, 2, 4, 6 Receptors.

Activation of the Toll-Like Receptors 7 and 8 pathway

As seen in figure 4, treatment with R848, a known activator of the TLR7-8 pathway, significantly induces a strong IL6 secretion compared to the other cytokines.

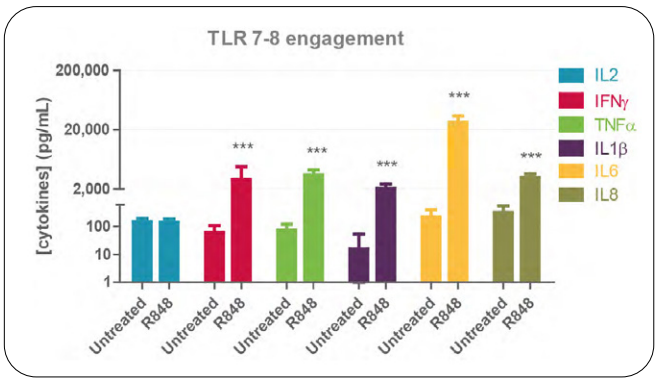


Figure 4: Cytokine profiles after stimulation of the Toll-Like Receptors 7 and 8 pathway

Activation of the STING pathway

2,3cGAMP was used as a specific activator of the STING pathway.

Cytokine secretion profiles upon activation display a marked increase in TNFα, IL1β and IL6. On the contrary, no significant response on IFNγ, IL2 or IL8 was evidenced in the same experimental conditions (figure 5).

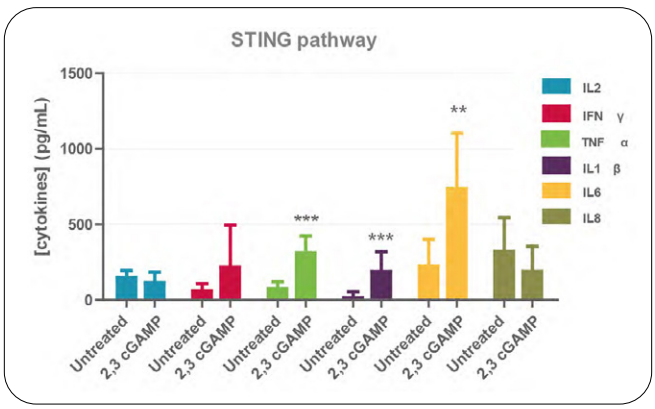


Figure 5: Cytokine profiles after stimulation of the STING pathway

Activation of the T-Cell Receptor pathway

Figure 6 shows that stimulation of T cells in whole blood by OKT3/anti CD28 induced a slight IFNγ release and failed to induce any IL2 production. Nevertheless the control conditions PHA or PMA/ionomycin induced a strong increase of both IL2 and IFNγ cytokines, as measured by HTRF assays. Thus these data indicate that the OKT3/CD28 stimulation was inefficient to promote a strong T-cell specific response on these 3 different whole blood donors.

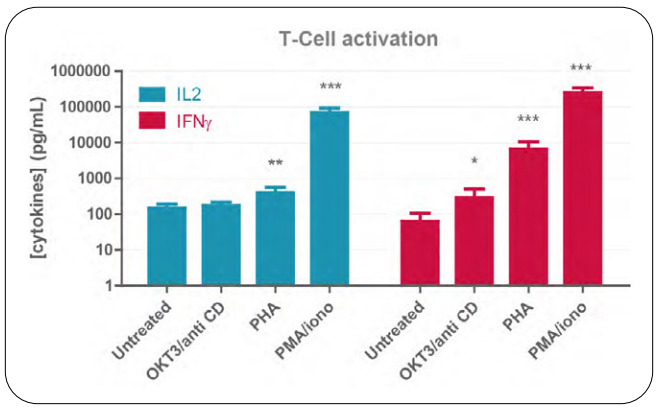


Figure 6: Cytokine profiles after stimulation of the TCR pathway

Comparison of cytokine response between whole blood and pbmc

We next addressed the differences between cytokine releases when investigating biological response in whole blood and PBMC. Figure 7 shows the results obtained on IL6 secretion, from whole blood samples versus the corresponding isolated PBMC cultured in medium containing 10% autologous plasma, after LPS stimulation in the presence or not of the antagonist dexamethasone. The concentration of IL6 is much more elevated in whole blood samples compared to PBMC. In both biological samples, dexamethasone treatment clearly impairs IL6 secretion induced by LPS.

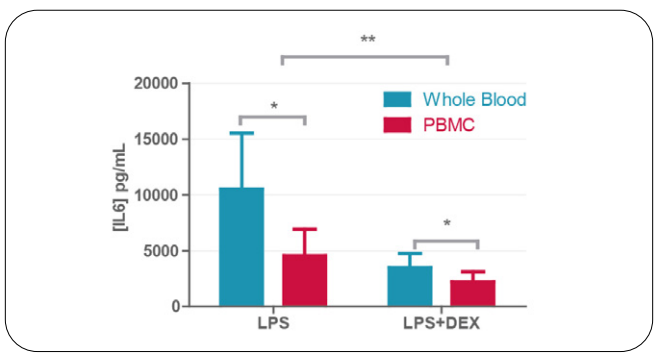


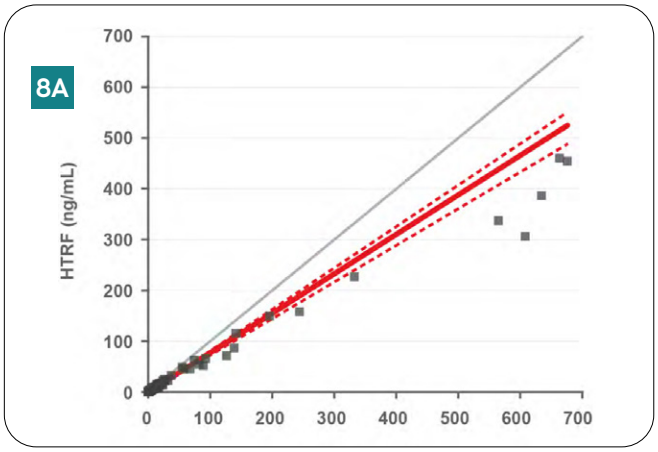
Figure 7: Comparison of IL6 concentrations (pg/mL) between whole blood and PBMC samples after LPS and dexamethasone treatment.

Correlation between htrf and elisa assays

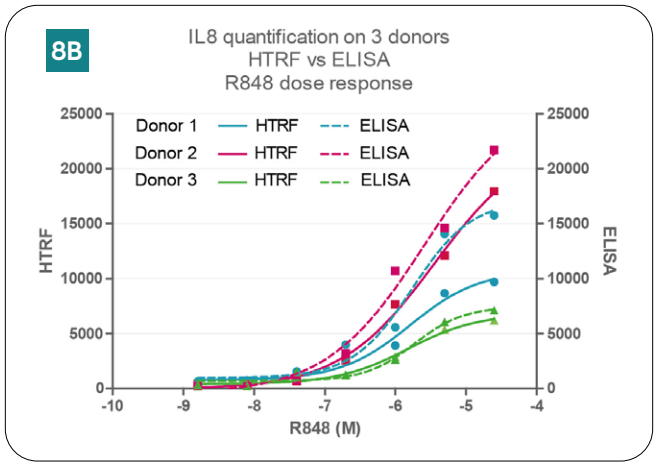
Since the ELISA method still remains the gold standard for cytokine measurements in biological fluids, we next wanted to compare the cytokine concentrations obtained using our HTRF assays to those from commercially available ELISA assays. As previously described, whole blood samples from 3 different donors were first stimulated with R848 (TLR 7/8 activation) in the presence or not of dexamethasone.

The measurement of IL8 release upon R848 +/- dexamethasone treatment was performed either by HTRF or ELISA, and the EC_{50} or IC_{50} of both compounds established.

Figure 8A shows the correlation obtained on 164 samples between HTRF and ELISA human IL8 assays. Here we demonstrate a very good correlation between the two assays, with a correlation value r=0.993 and a proportional bias of 0.77.



Then we compared the R848 and dexamethasone pharmacological dose responses obtained on 3 different donors by HTRF and ELISA (figures 8B and 8C). Once again, the same profile of responses was established between HTRF and ELISA, as evidenced by the values of EC_{50} for R848 or IC_{50} for dexamethasone which are very similar (see table 2).



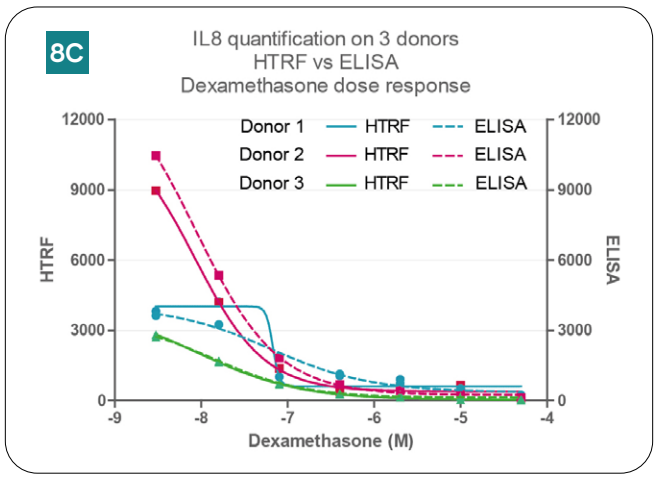


Table 2: Determination of R848 EC_{50} and dexamethasone IC_{50} on the release of IL8 induced by R848, as measured by HTRF or ELISA from whole blood samples of 3 donors

		Donor #1	Donor #2	Donor #3
EC_{50} R848	HTRF	1.63E-06	3.6E-06	1.518E-06
	ELISA	1.74E-06	2.59E-06	1.875E-06
IC_{50} Dexamethasone	HTRF	6.733E-08	8.45E-09	0.918E-08
	ELISA	6.405E-08	8.51E-09	1.424E-08

Conclusion

With the fast evolution of Research and Drug Discovery in fields like immuno-oncology or metabolic disease, monitoring the response of the immune system to treatment is more important than ever.

Whole blood assays are a simple and powerful *in vitro* tool to assess normal and/or unwanted immune responses to therapeutics. The new HTRF cytokine assays with their unique no-wash protocols, low sample consumption, and extended range, enable an increase in the number of tested compounds compared to ELISA and obtain relevant data on cytokine secretion in whole blood.

The combination of Blood Assay Solution's experience on fresh whole blood and immune response with Revvity cytokine kits' robustness and ease-of-use creates the ideal platform to test any compound in a physiological environment.

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