APPLICATION NOTE

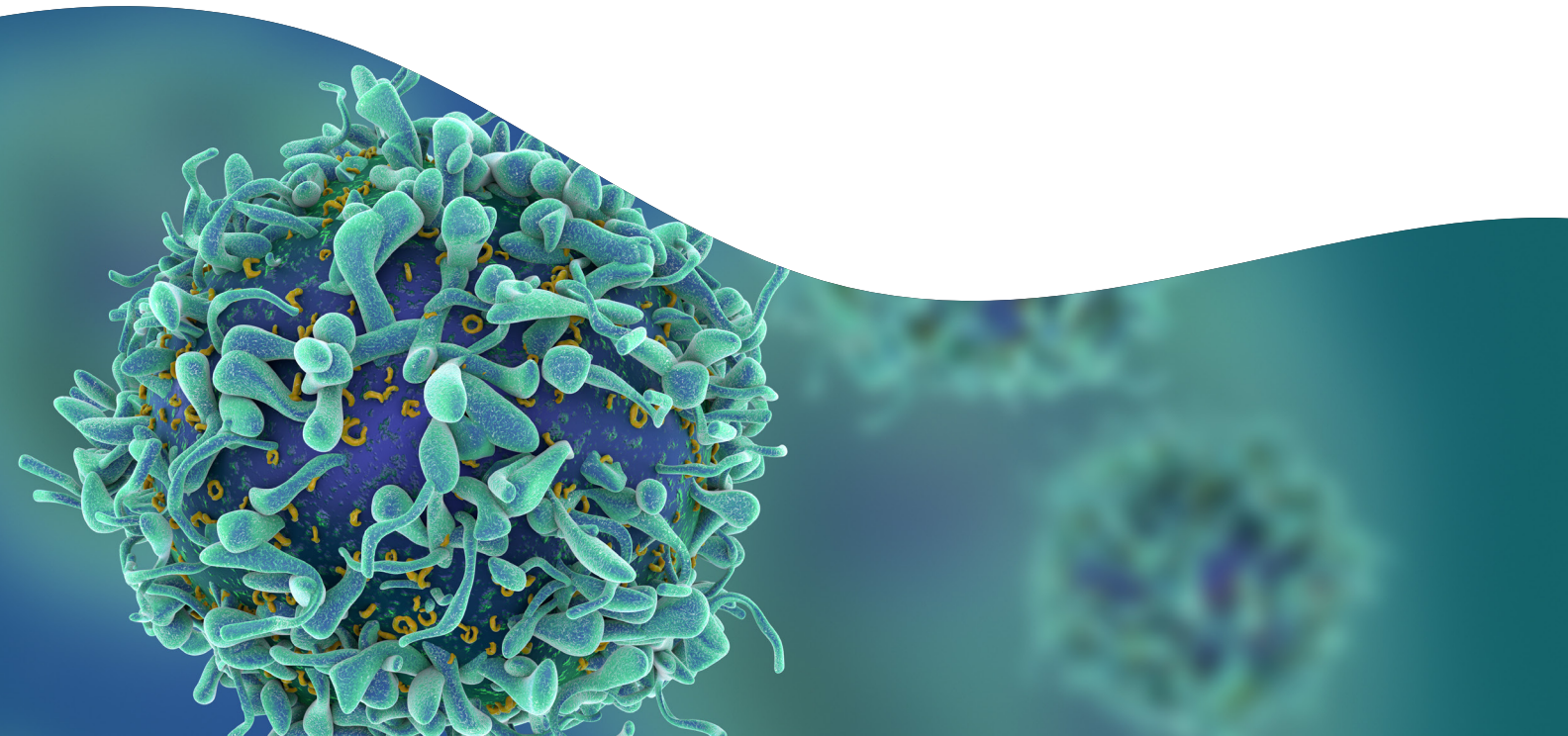
TCR signaling investigation with HTRF phospho assays.

This application note shows that phosphorylation modulation of proteins present in the TCR pathway can be monitored with HTRF® assays both in PBMCs and isolated T-CD8 cells.

Abstract

It is now clear that in some tumors, inhibitory checkpoint proteins are over-expressed by the tumor cells to escape the immune system. An important line of research is thus checkpoint inhibitors able to block that interaction and restore T cell efficiency. Today, research is also turning to the activation of stimulatory checkpoints to boost T cell mediated tumor death. These potential treatments, among others, all target T cells and TCR intra-cellular signaling, so knowing how this pathway is modified is crucial to understanding any treatment's mechanism of action.

In this Note, we used HTRF phospho assays to study specific and ubiquitous proteins in the TCR pathway (ZAP70, SLP76, ERK ...) in Peripheral Blood Mononuclear Cells (PBMC) and isolated T cells both under stimulation and in checkpoint inhibition conditions.



TCR Pathway

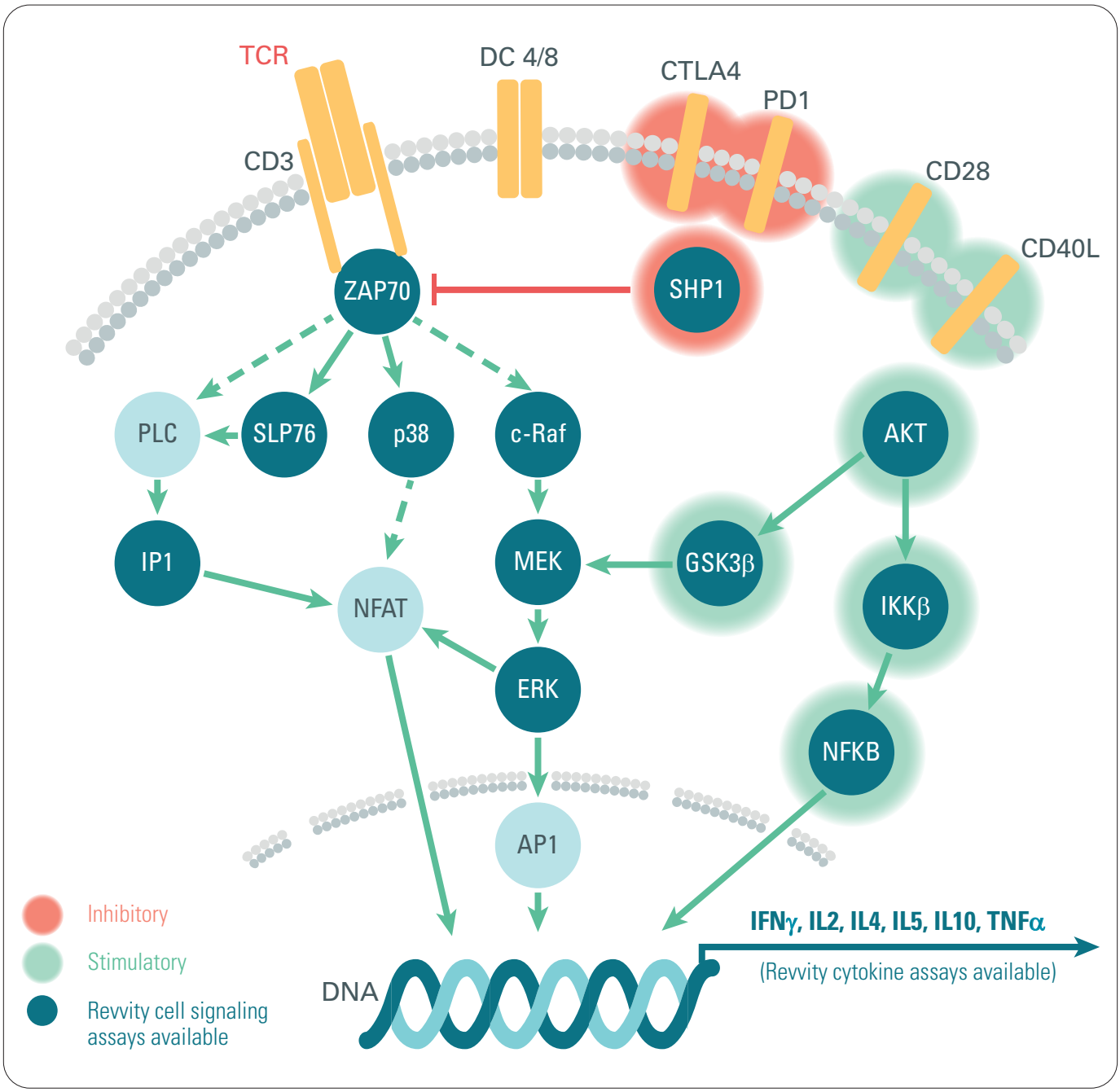


Figure 1: TCR pathway

Flowchart/workflow

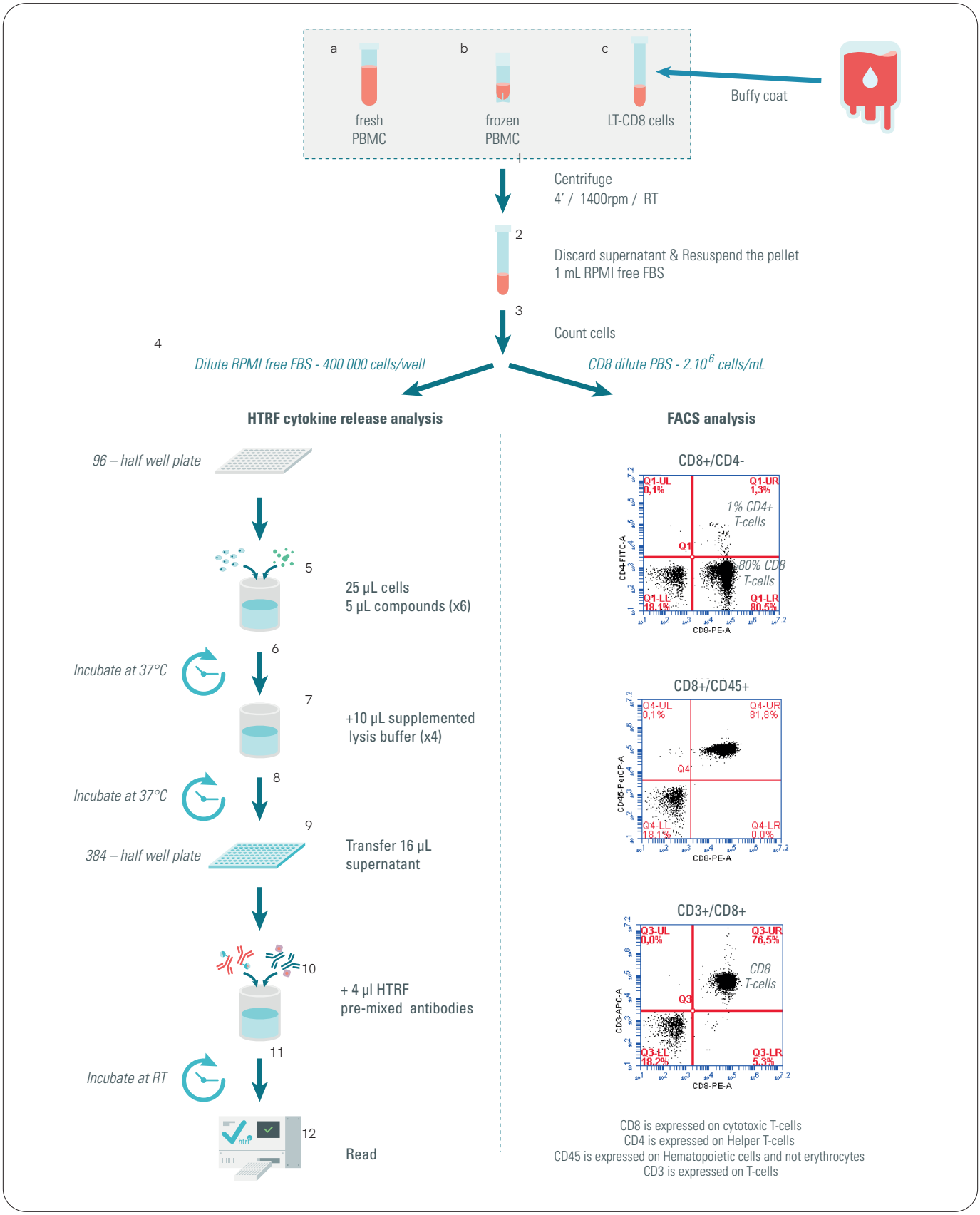


Figure 2: Assay flowchart

Cells sources:

a. Fresh PBMC were isolated from a buffy coat, following the protocol described in tech note « Guidelines from PBMC isolation to cytokine assay optimisation ».

b. Frozen PBMC were isolated from a buffy coat, following the same protocol and were frozen and thawed, following the protocol described in tech note « Guidelines for PBMC freezing ».

c. LT-CD8 cells were purified from a buffy coat using the StraightFromTM Buffy Coat CD8 MicroBead Kit, human (130-114-978) from Miltenyi Biotec. After centrifugation as described below, LT CD8 cells were diluted in PBS at 2 million cells per mL to control the purity of the cells by flow cytometry.

Protocol:

1. Cells were centrifuged at 1400 rpm for 4 minutes to room temperature.
2. The supernatant was discarded and the pellet resuspended with 1mL of RPMI, FBS free, warmed at room temperature.
3. Cells were counted to determine the cell density in 1mL.
4. Cells were diluted with RPMI FBS free to reach the expected cellular density (cellular density is given for each experiment).
5. Cells were plated in 96 half well plate (Greiner#675075) under 25 µL. Cell treatment: 5 µL of compound were added to the cells.(6X)
6. The plate was incubated at 37°C (incubation time is given for each experiment).
7. 10 µL of lysis buffer (4X)+ blocking reagent (25X) were added to the cells.
8. The plate was incubated at room temperature for 30 minutes under shaking.
9. 16 µL of the mix were transferred into a 96 well small volume plate (Revvity#66PL96001).
10. 4 µL of HTRF pre-mixed antibodies were added.
11. The plate was incubated at room temperature (incubation time depending on the HTRF kit).
12. The HTRF signal was recorded on a HTRF-compatible reader.

Negative is technological background (medium + detection reagents) and indicated as a dotted line in all graphs. Basal signal corresponds to constitutive activation of unstimulated cells.

Data reduction:

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

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

Résultats

1. Monitoring TCR pathway in PBMC

1.1 Optimization of PBMC density

We first determined the optimal cell density to ensure a proper quantification fitting with the linearity range of the HTRF phospho assays. Of note, the linearity of HTRF signals may vary from one HTRF phospho-assay to another, and biological responses may vary from one PBMC donor to another. Thus optimal cell density must be carefully determined for each assay and PBMC batch. Four different densities of PBMC were seeded ranging from 100,000 to 800,000 cells in 96 well microplates. Increasing doses of PMA or anti-CD3 were applied for 15 minutes in order to trigger cell signaling activation. The results shown below represent the effect of PMA or anti-CD3 on the phosphorylation of ERK and SLP76 respectively, which are both involved in the TCR signaling pathway.

Whereas phosphorylation of ERK increases proportionally between 100,000 and 200,000 (x 2) and between 200,000 and 400,000 cells/well (x 2), the linearity of HTRF signal decreases above 400,000 cells per well (graph 1A). This result indicates that 400,000 PBMC is optimal in these experimental conditions.Graph 1B displays the phosphorylation of SLP76 after after anti-CD3 stimulation at different cell densities. As evidenced here, 200,000 PBMC/well is the optimal cell density to assess SLP76 phosphorylation.

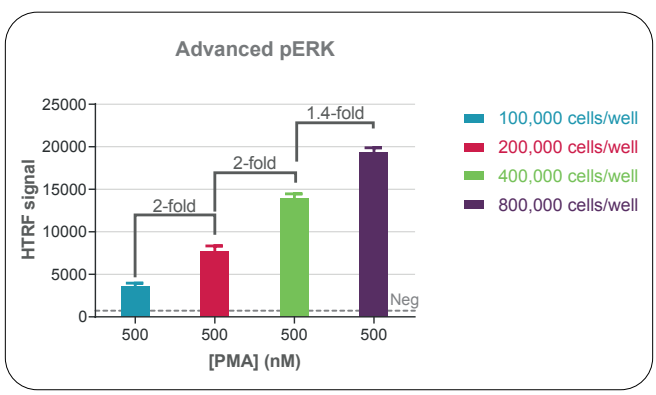


Figure 3A: ERK phosphorylation under PMA stimulation on several PBMC cell densities.

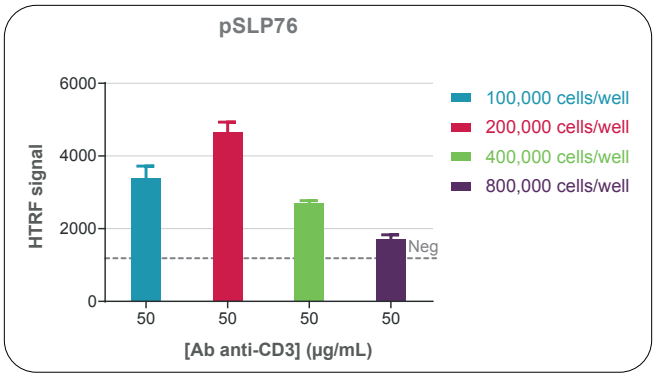


Figure 3B: SLP76 phosphorylation under anti-CD3 stimulation on several PBMC cell densities.

1.2 Comparison of phospho-ERK response on fresh and cryopreserved PBMC

As presented in the technical note : Guidelines for PBMC freezing, frozen PBMC allow the utilization of cells from a single donor in several experiments dedicated to cytokine quantification. Here, we aimed to validate their use for studying the intra cellular signaling pathway.

Briefly: fresh or frozen PBMC from the same donor were dispensed at 100,000 cells/well, then stimulated with increasing concentrations of PMA for 15 minutes.

As illustrated in figure 4, PMA stimulation induces a similar phospho-ERK response in fresh and frozen PBMC, thereby confirming that cryopreserved PBMC can be utilized to address cell signaling pathways.

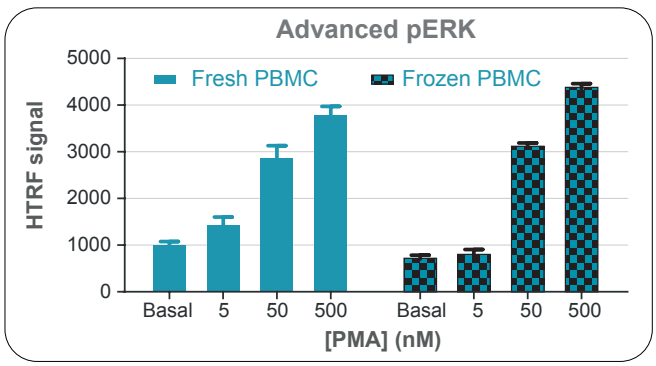


Figure 4: Comparison of ERK phosphorylation on fresh or frozen PBMC.

1.3 Investigating the TCR signaling pathway with HTRF phospho-protein assays

1.3.1 Nonspecific PBMC stimulation

PMA leads to PKC activation which drives NFKB activation involved in the transcriptional regulation of IL2 and IFNγ genes. In addition, PMA also induces the phosphorylation of other effectors such as ERK, AKT or CREB.

Here we provide data showing that nonspecific PMA stimulation efficiently activates ERK, AKT and CREB pathways in a dose dependent manner (Figure 5), without affecting the total ERK protein level. Even though basal phosphorylation levels of CREB are high, HTRF robustness enables a significant effect of increasing doses of PMA to be uncovered.

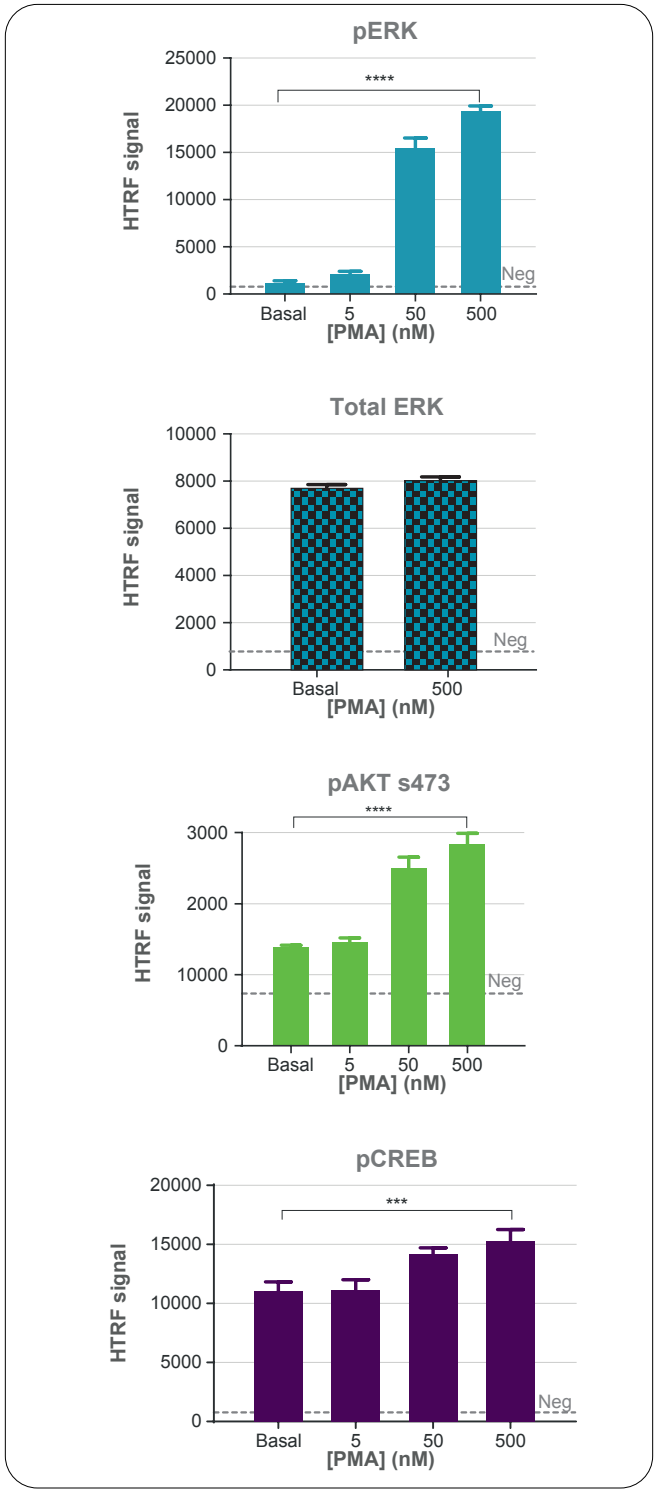


Figure 5: Phosphorylation of TCR signaling pathway proteins, in PMA-stimulated PBMC.

1.3.2 Specific PBMC stimulation by anti-CD3 antibody

CD3 is expressed by circulating peripheral T cells forming a complex with the T cell receptor (TCR), and constitutes a specific activation signal for the pathway. A treatment with anti-CD3 antibody was used to stimulate PBMC and trigger TCR signaling cascades, involving ZAP70 and SLP76 phosphorylation.

Anti-CD3 stimulation was performed for 5 minutes on 800,000 and 100,000 PMBC per well for phospho ZAP70 and SLP76 respectively. As shown in Graph 4A, these experimental conditions failed to induce any detectable levels of phosphorylated ZAP70. In contrast, the results reported in Graph 4B clearly indicate a constitutive SLP76 phosphorylation which is not potentiated by the anti-CD3 antibody.

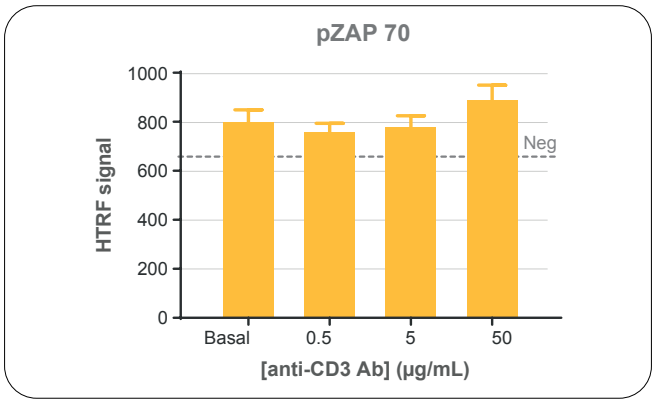


Figure 6A: ZAP70 phosphorylation under anti-CD3 stimulation on PBMC.

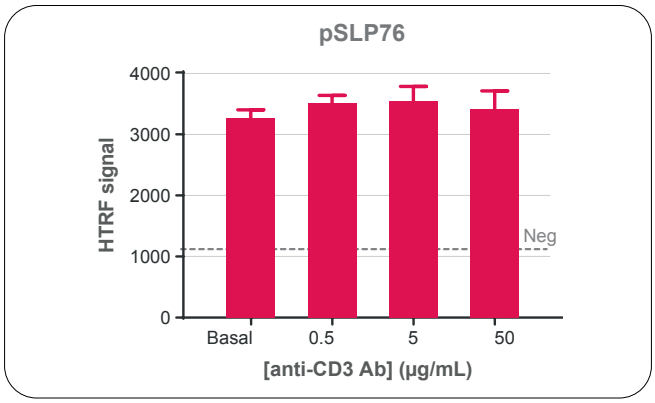


Figure 6B: SLP76 phosphorylation under anti-CD3 stimulation on PBMC.

1.3.3 Modulation of the TCR signaling pathway by Immune Checkpoint Inhibitor

To challenge pathway functionality, we tested PDL1 inhibition on the activation of SLP76 induced by anti-CD3 antibody. As shown in Figure 7, 40 µg/mL of recombinant PD-L1 significantly reduced SLP76 phosphorylation. This result demonstrates that HTRF phospho-SLP76 is perfectly adapted to improving understanding o the mechanism of actions of Immune Checkpoint Inhibitor.

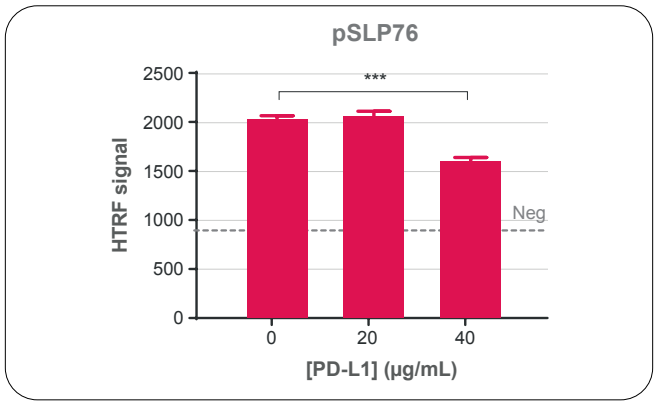


Figure 7: Inhibition of SLP76 phosphorylation with PD-L1 on PBMC.

1.3.4 PBMC stimulation by hIL6 or hIFNa cytokines

To assess phospho-STAT3 signaling pathway functionality, 300,000 PBMC per well were stimulated with increasing concentrations of IL6 or IFNα for 30 minutes. Our data show that both IL6 and IFNα activate the Jak/Stat pathway and induce Stat3 phosphorylation (Figure 8).

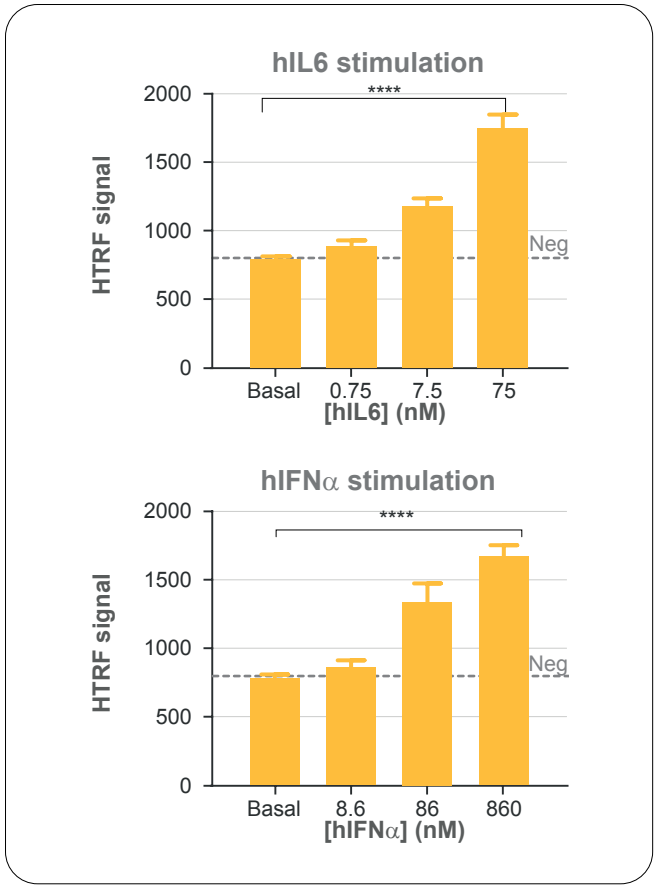


Figure 8: STAT3 phosphorylation under hIL6 and hIFNα stimulation, on PBMC.

2. Monitoring the TCR pathway in purified CD8 Tcells

2.1 Specific CD8+ Tcells stimulation by anti-CD3 antibody

CD8+ T lymphocytes were isolated from a buffy coat and the TCR signaling pathway was assessed by monitoring the phosphorylation of key effectors.

Figure 9 displays the results obtained on 300,000 cells per well, stimulated with anti-CD3 antibody for 5 minutes.

Anti-CD3 stimulation significantly induces the phosphorylation of ERK, SLP76, AKT, ZAP70 and NFKB. Contrary to the results obtained with PBMC (Fig.5) where we were unable to monitor ZAP70 phosphorylation modulation, the same experiment in isolated T cells shows a significant increase in ZAP70 phosphorylation.

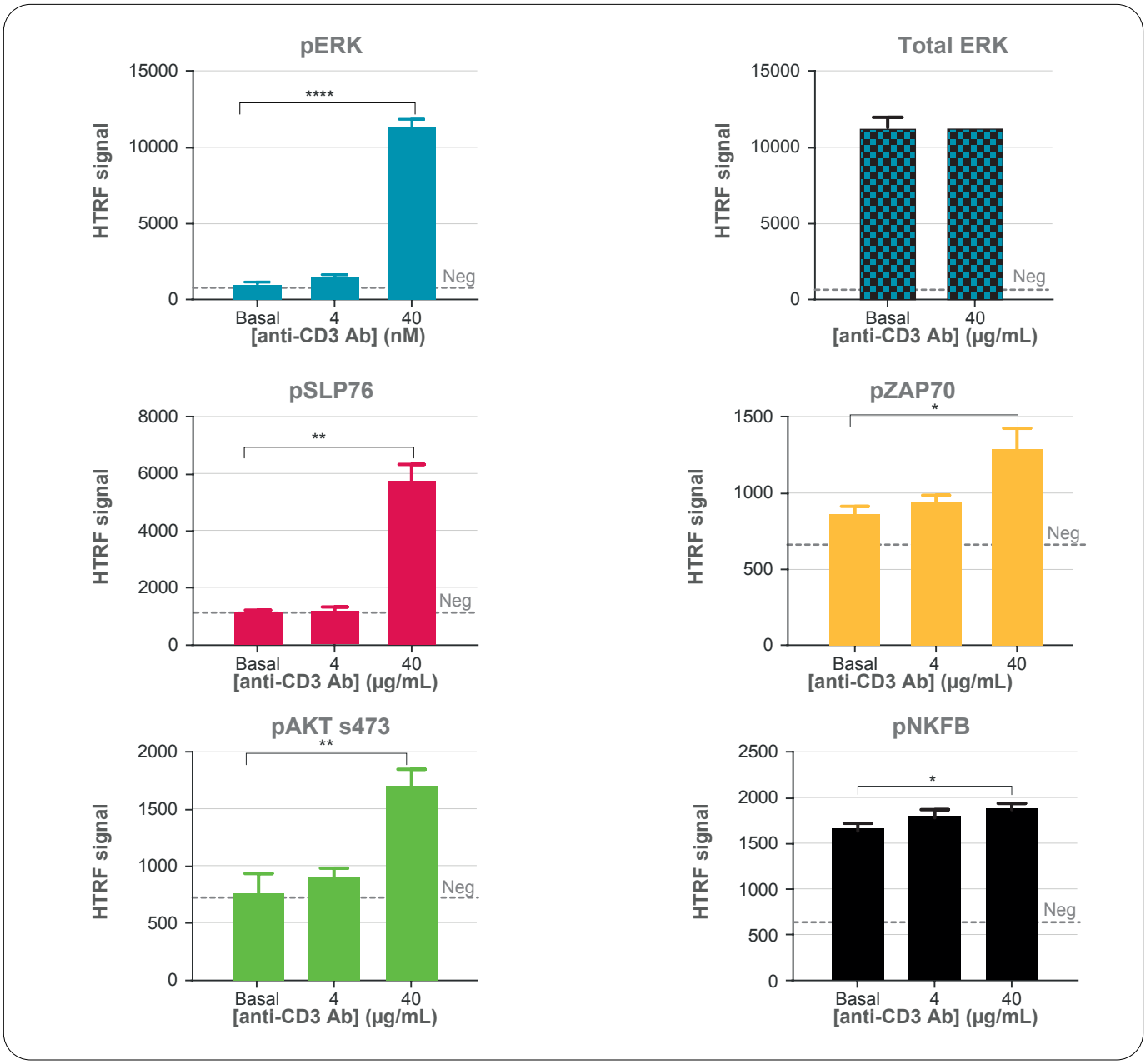


Figure 9: Phosphorylation of TCR signaling pathway proteins, under anti-CD3 stimulation on LT-CD8.

2.2 Stimulation of isolated CD8+ T cells by hIFNα

To further assess T cell functionality, 800,000 cells/well were plated and stimulated for 15 minutes with IFNα, and then STAT3 phosphorylation was assessed.

As observed in PBMC, IFNα efficiently activates STAT3 as revealed by the increased HTRF signal (Fig. 10).

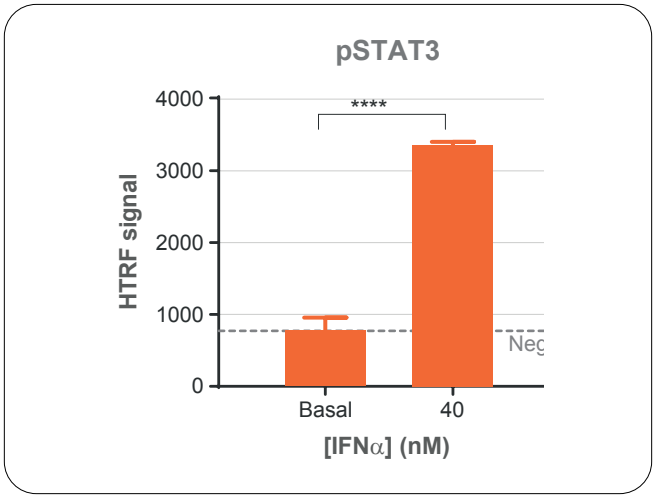


Figure 10: STAT3 phosphorylation under hIFNα stimulation on LT-CD8.

Conclusion

This note shows that phosphorylation modulation of proteins present in the TCR pathway (SLP76, ZAP70, ...) can be monitored with HTRF assays both in PBMCs and isolated T-CD8 cells. It enables an easy and reliable study of the TCR intra-cellular signaling pathway in relevant models, for better efficiency in the choice of checkpoint inhibitors or TCR agonists.

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