# Cell signaling pathways in autoimmune diseases and inflammation disorders with HTRF assays


## Abstract

Autoimmune diseases and inflammation disorders result from failures in the coordination, regulation and aiming of inflammatory and immune responses. These mechanisms are complex and monitored by significant intracellular pathways. An approach to address those disorders consists in understanding and modulating the signaling cascades that trigger their pathogenesis, which can be achieved in several ways.


### Monitoring inflammation pathways

The targeted disruption of known central inflammation pathways to prevent a signal transduction to the nucleus has proven effective in previous publications and therapies. This is usually achieved by inhibiting a kinase or keeping interacting partners from forming a phosphorylation complex.

### Deciphering inflammatory signal transduction - case of the sting pathway

*Gaidt M.M, et al. (2017) Cell 171(5):1110-1124.e18*

In a 2017 Cell-published study, Gaidt *et al.* thoroughly investigated the cGAS-STING-LCD-NLRP3 axis, which is well known as a key transduction signal in the expression of type 1 IFNs and high-inflammatory cellular death (Fig 1).

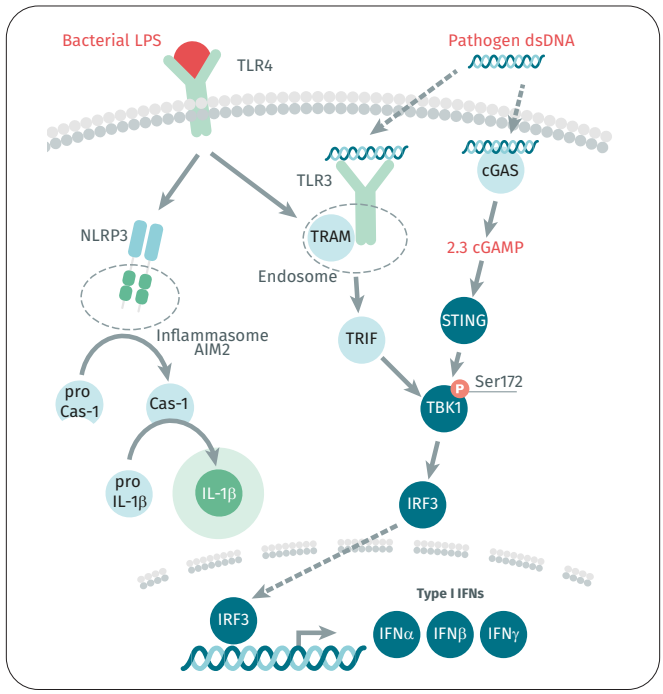


Figure 1: cGAS-STING-NLRP3 pathway. Green highlight on IL-1β. Dark blue highlight on Revvity available kits

Among a collection of detection technologies and targets, they especially focused on the expression and maturation of IL-1β (cleavage of its precursor by caspase-1) following the axis stimulation, which they interpreted and used as a direct measure of AIM2 inflammasome activation.

The inflammasome AIM2 is usually activated via the stimulation of its paired protein NLRP3, whose classical agonist is nigericin. In this study, the IL-1β results obtained in various situations allowed the authors to better understand this activation trigger (fig. 2).

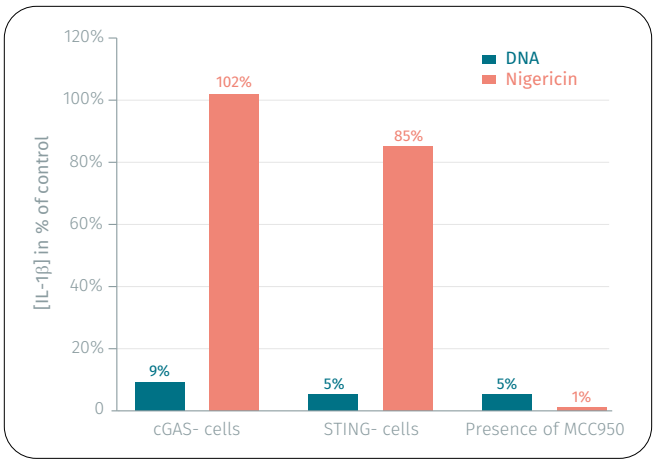


Figure 2: % of control IL-1β expression in presence of DNA or nigericin, in cGAS- and STING- cells or in presence of NLRP3 inhibitor MCC950. IL-1β expression levels were compounded against the control associated to their respective experiment. Adapted from gaidt M.M, *et al.* (2017) Cell 171(5):1110-1124.e18.

They observed that inflammasome AIM2 activation was not dependent from NLRP3 binding to an agonist, but could also result from cytosolic DNA and induce the same caspase-1 and IL-1β maturation.

It was noted, however, that these DNA-induced IL-1β and caspase-1 activation were significantly diminished in cGAS and STING-deficient cells, suggesting that inflammasome activation by DNA is not only potentially triggered by the cGAS-STING axis, but requires it to perform to its full extent. In contrast cGAS-STING was shown non-necessary for the inflammasome activation by NLRP3 stimulation with nigericin (fig. 2).

Finally, the use of a previously described NLRP3 inhibitor (MCC950) was shown to neutralize IL-1β expression in both classical agonist nigericin presence and in response to transfected DNA, suggesting that contrary to the cGAS-STING axis, NLRP3 remains necessary to the proper activation of the inflammasome AIM2 (fig. 2).

## Characterizing known inhibitors - case of the map kinase pathway

*Garrido Montalban A, et al. (2010) Eur J Pharmacol 632(1-3):93-102*

In 2010, Garrido Montalban *et al.* studied the MAP kinase pathway, a network involved in cell division, growth and stress reaction, whose end result triggers the expression of cytokines including TNFα, a key player in the pathogenesis of rheumatoid arthritis and inflammatory bowel diseases. The authors were looking to pharmacologically characterize inhibitors of the p38 MAP kinase and used the TNFα HTRF® kit to assess the inhibition of that kinase when incubated with such compounds (fig. 3).

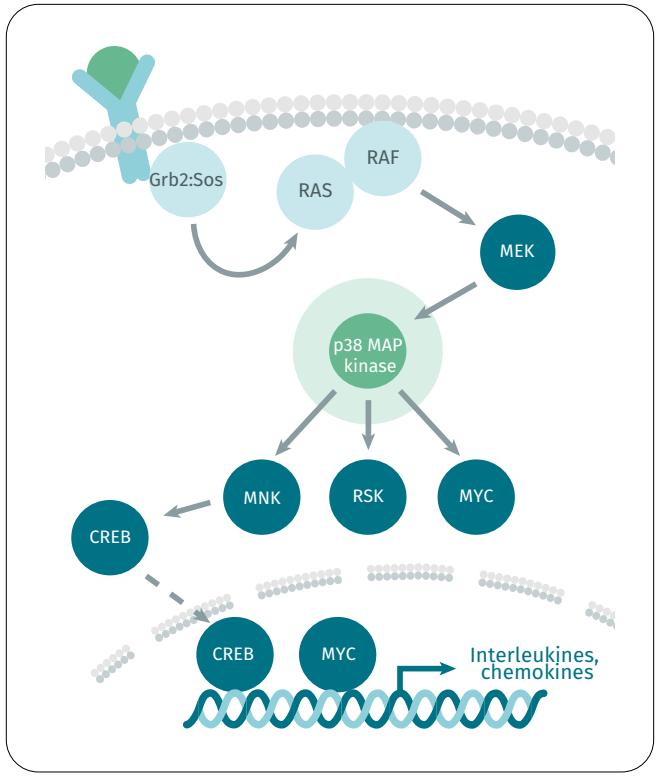


Figure 3: MAP kinase pathway. Green highlight on p38 MAP kinase.Dark blue highlight on Revvity available kits

A human monocytic cell line THP-1 was exposed to LPS and incubated with one these three inhibitors: KR-003048, SB203580, and BIRB796.

The assay allowed to determine the IC50 for each inhibitor and concluded that BIRB796 was the most potent compound in this cellular context, with an IC50 of 12 nM. SB203580 and KR-003048 displayed an apparent potency in this assay that was roughly one half-log higher, with IC50s of 45 and 49 nM, respectively (table 1).

Table 1: Inhibition of TNFa secretion - IC50 of three p38 MAP kinase inhibitors. Adapted from Garrido Montalban A, *et al.* (2010) Eur J Pharmacol 632(1-3):93-102

| Inhibitor | IC50 (nM) |
|---|---|
| SB203580 | 45 |
| BIRD796 | 12 |
| KR-003048 | 49 |

## Screening for novel drug candidates - case of the NF-KB pathway

*Gotoh Y, et al. (2010) Anal Biochem 405(1):19-27*

In 2010, Gotoh *et al.* took this pathway inhibition approach a step further and implemented an HTRF assay for the screening of inhibitors of the NF-kB pathway, a crucial signaling cascade which promotes the expression of many cytokines, enzymes and adhesion molecules that are strongly suspected to be part of the pathogenesis of autoimmune diseases such as rheumatoid arthritis.

The step of this pathway the authors targeted is the association of IKKβ and IKKγ in the assembly of the phosphorylation complex IKK (fig 4).

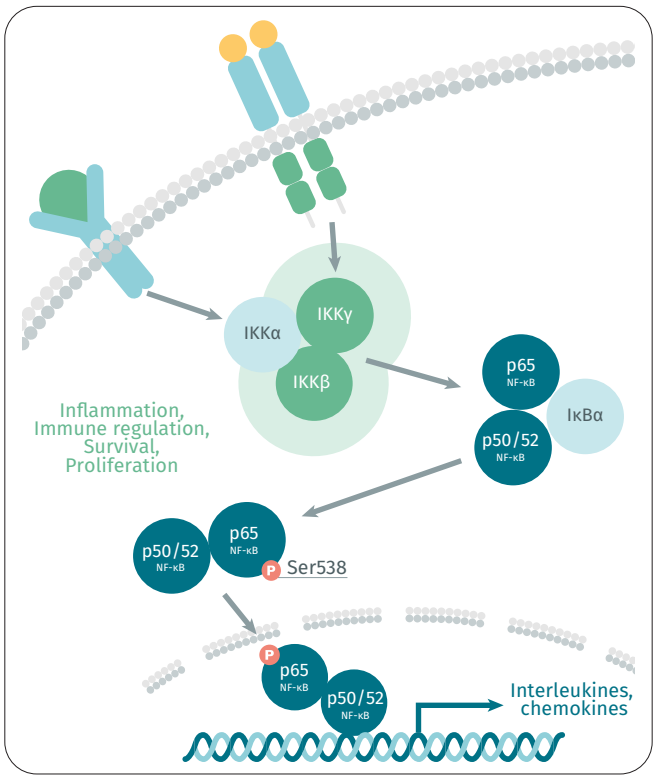


Figure 4: NF-κB pathway. Green highlight on IKKβ:IKKγ complex. Dark blue highlight on Revvity available kits

They used an HTRF protein-protein interaction assay to monitor this interaction (fig. 5) and screen over 15,000 compounds, which allowed for the identification of 7 hits exhibiting inhibitory effects over 39%, 2 of which were over 70%.

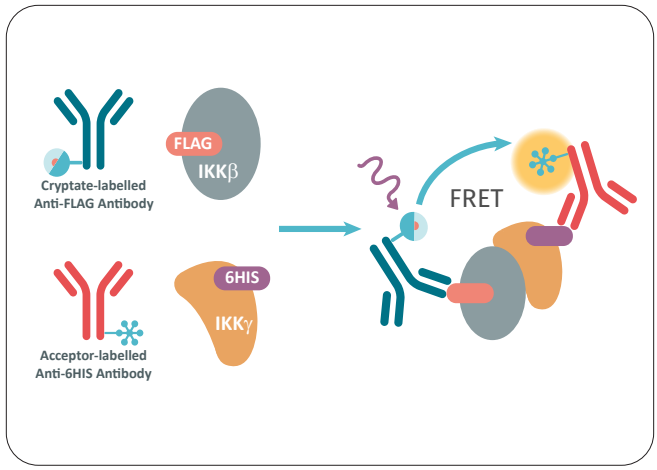


Figure 5: Assay principle - IKKβ and IKKγ are respectively tagged with FLAG and 6HIS. Anti-FLAG and anti-6HIS antibodies are respectively labelled with HTRF donor (cryptate) and acceptor. When IKKβ and IKKγ interacts, the HTRF donor and acceptro are brought in close proximity which triggers FRET.

The assay displayed a sound to background ratio of 21.5 (standard deviation of 3.9) and a Z' factor of 0.93, leading the authors to conclude on the satisfactory robustness and consistency of this HTS format approach.

## Monitoring immune cells activation

Another approach to autoimmune diseases and inflammation consists in directly targeting immune cell activation to prevent the co-stimulatory amplifications that usually take place in inflammatory and immune responses.

## CD4+ T-cells activation - SRC kinases in TCR activation pathway (Zap-70)

*McRae B.L, et al. (2005) Int Immunopharmacol 5(4):667-77*

The Src family kinases, and especially Lck, intervene in the activation pathway of CD4+ T-cells via TCR stimulation (Fig. 6). Back in 2005, McRae and his team were looking to design an inhibitor suited to restrict the activity of these kinases and came up with A-420983.

They used an HTRF format to perform kinase assays and determine the IC50 of that compound against various enzymes of interest (fig. 7). They extended their experiment to other known inhibitors to provide comparison between A-420983 and them.

The results of their study allowed to conclude on the stronger inhibitory potency of the new A-420983 compound compared to the usual Src family kinase inhibitors. When tested on CD4+ T-cells, the compound showed the expected inhibition of differentiation. The authors latter observed that IL-15 induced T-cell differentiation was unafected by A-420983, which led them to suggest that this inhibitor's potency is specific to TCR-induced differentiation.

This had the authors conclude on the probable implication of Src kinases in the sole TCR-induced activation pathway, making them especially attractive targets to monitor TCR signaling in CD4+ T-cells.

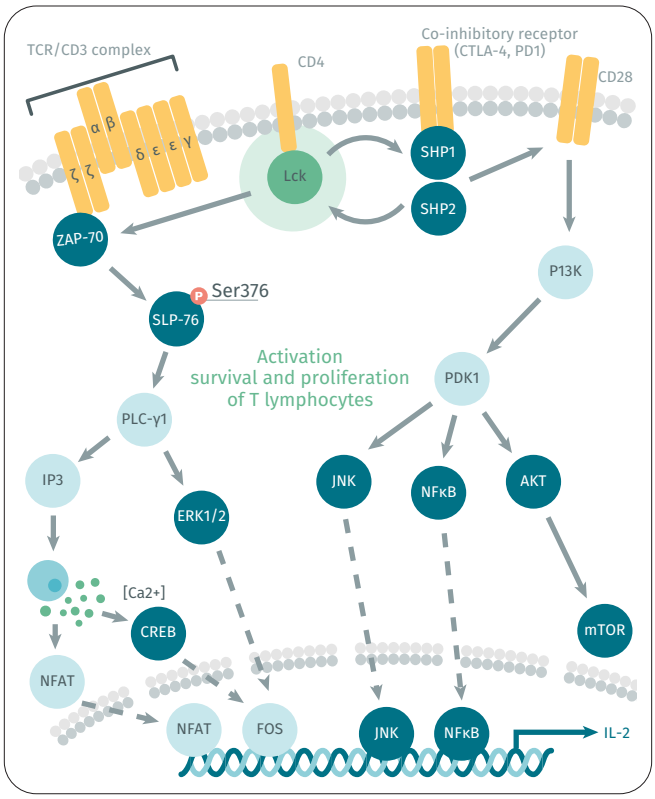


Figure 6: ZAP-70 pathway.Dark blue highlight on Revvity's available kits

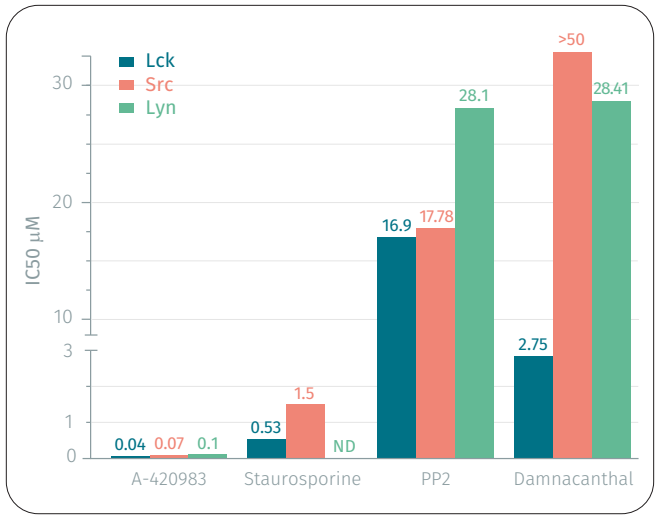


Figure 7: Inhibition of Src family kinases. IC50 of A-420983 and 3 inhibitors for Lck, Src and Lyn kinases. Adapted from McRae B.L, *et al.* (2005) Int Immunopharmacol 5(4):667-77.

## B-Cells activation - SYK kinase in BCR activation pathway (BTK)

*Harbert C, et al. (2008) Current Chem Genomics 1: 20–26*

Syk kinase is an enzyme that was shown to be essential in the transduction of signal following antigen presentation to B-cells, and BCR signaling. It is required for the differentiation of B-cells into lymphocytes (Fig. 8).

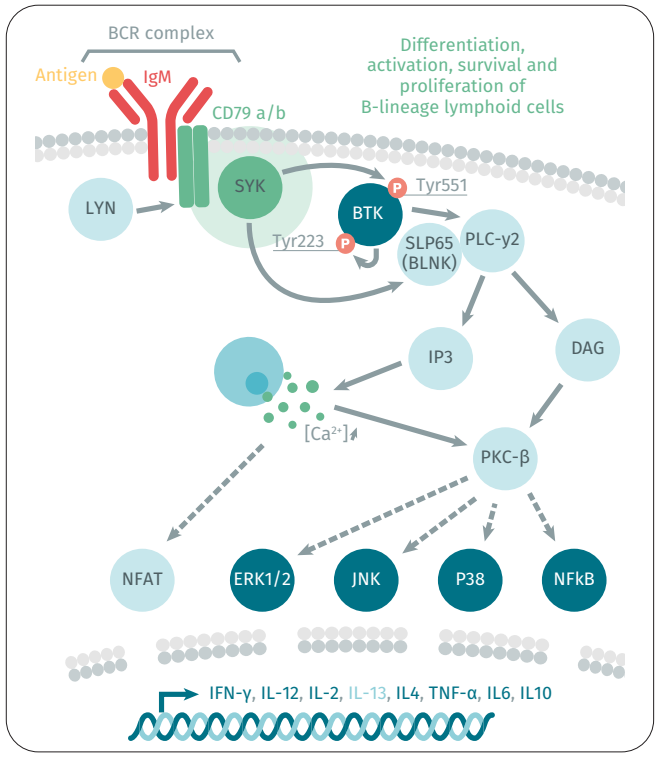


Figure 8: BTK pathway. Green highlight on Syk kinase. Dark blue highlight on revvity available kits

In 2008, Harbert *et al*. aimed to implement an HTS-compatible HTRF assay for monitoring the activity of Syk Kinase. It was validated using Staurosporine, one of its known inhibitors.

Using this assay, they ran various concentrations of Syk enzyme against different concentrations of TK substrate-biotin. The resulting Michaelis-Menten analysis of fluorescence led to a Km apparent value of about 0.1µM TK substrate-biotin and the assay proved effective to address such topics said the authors. They especially noted an assay window of 30.7 for a substrate concentration of 3.4nM (fig. 9)

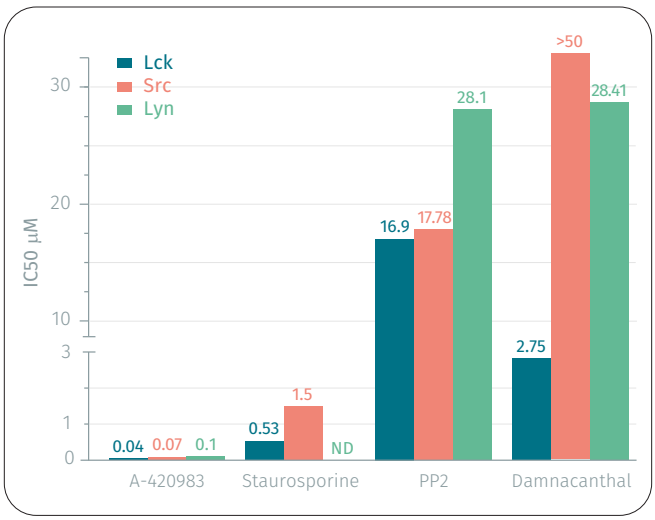


Figure 9: Michaelis-Menten analysis of Syk tyrosine kinase activity.

These results allow for a better understanding of the signaling partners in B-cells activation and provide a valuable insight into the potential druggable targets of this signal transduction.

## Conclusion

Inflammatory and immune regulation/activation pathways offer drug discovery targets that have been investigated for years and HTRF assays have been successfully used to that end for over a decade. They have demonstrated an acute relevance for the various requirements and formats of drug discovery.

The publications featured in this note report the effective implantation of such assays for pharmacological characterization and screening, as well as the significant robustness and consistency they provided to HTS steps.

Moreover, these publications exhibit a range of formats including sandwich assays, enzyme activity monitoring and protein-protein interaction assays, which were all successfully matched by HTRF.

## Works cited


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