APPLICATION NOTE

HTRF HMGB1, IL-1β and IL-18 assays discriminate between inflammasome-triggered pyroptosis, and necroptosis.

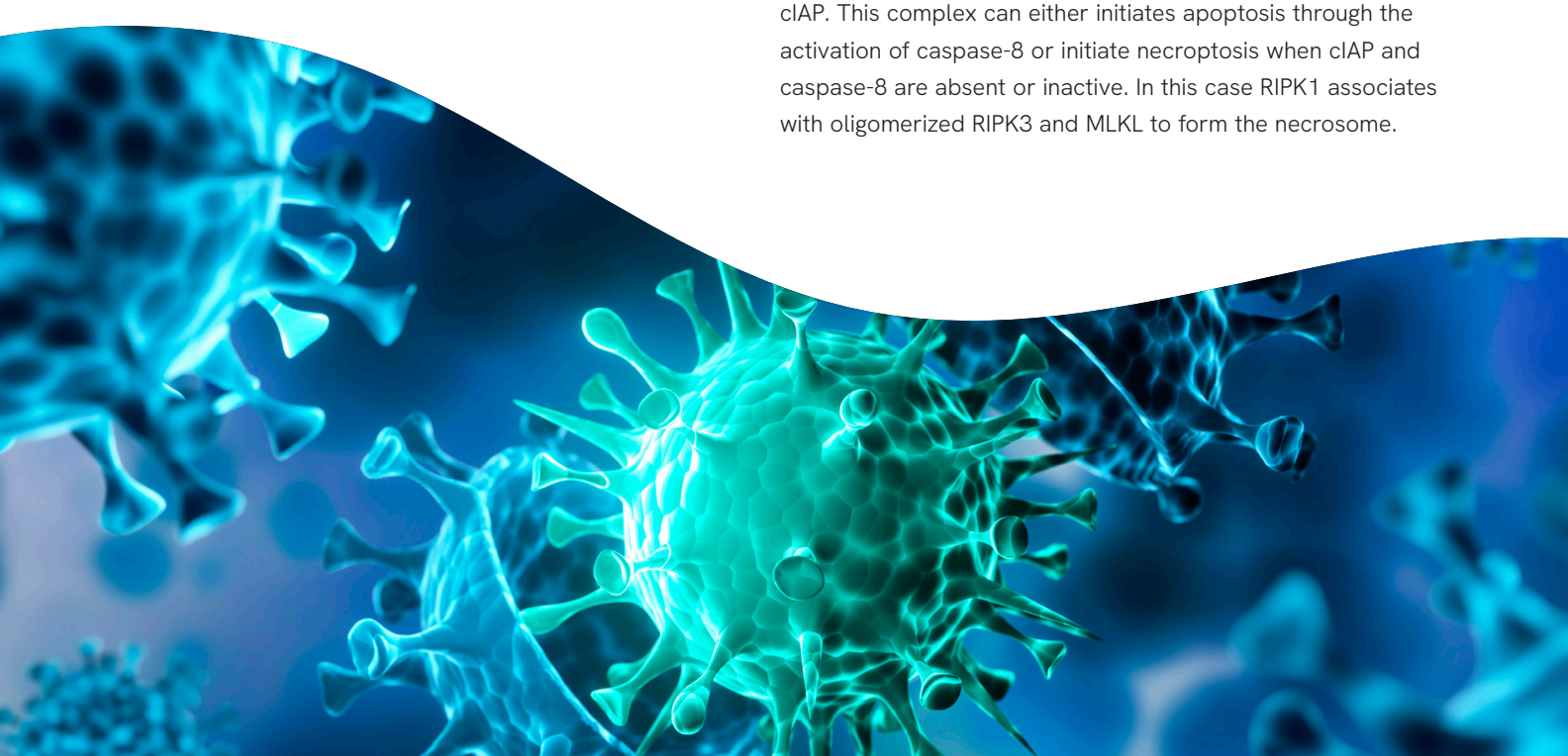
In this note is showed that the release of HMGB1 induced in the context of pyroptosis and necroptosis can be easily monitored with the HTRF® HMGB1 kit.

Introduction

Inflammatory responses are characterized by the release of endogenous DAMPs also known as alarmins. During non-apoptotic programmed or regulated cell death, such as pyroptosis and necroptosis, HMGB1 is released along with other pro-inflammatory molecules (1).

Pyroptosis relies on two steps. The first step is called “priming”. Following TLR stimulation (e.g LPS), the expression of NLRP3 and pro-IL-1β is upregulated through NF-kB transcription factor. In the second “activation” step, DAMPs such as extracellular ATP, or the bacterial toxin nigericin, are sensed by NLRP3 which oligomerizes, leading to the activation of the NLRP3 inflammasome. In turn, it induces the cleavage of pro-caspase-1 to its active form. Caspase-1 then cleaves the pro-forms of the inflammatory IL-1β and IL-18, that are released as their mature forms. Parallelly caspase-1 induces the cleavage of pro-GSDMD which forms pores at the cell membrane and the subsequent release of IL-1β, IL-18 and HMGB1.Therefore pyroptosis is characterized by the simultaneous secretion of HMGB1 together with mature IL-1β and IL-18.

Necroptosis involves the loss of membrane integrity and release of DAMPs. Necroptosis involves RIPK1 and/or RIPK3 and proceeds through a caspase-independent pathway (2,3). Necroptosis is promoted by death receptor activation. Upon TNFα binding, the cytosolic death domain of TNFR1 recruits a prosurvival complex, consisting of TRADD, RIPK1 and cIAP. This complex can either initiates apoptosis through the activation of caspase-8 or initiate necroptosis when cIAP and caspase-8 are absent or inactive. In this case RIPK1 associates with oligomerized RIPK3 and MLKL to form the necrosome.



RIPK3 phosphorylates MLKL which forms plasma membrane pores and triggers the release of HMGB1. Contrary to pyroptosis, necroptosis is associated with HMGB1 release but not mature IL-1β/IL-18.

Inflammasome dysregulation has been associated with pathologies, such as metabolic, autoimmune diseases, neurodegenerative or cardiovascular diseases. Thus inflammasome components, like NLRP3, are attractive drug targets. Whereas NLRP3 agonists are investigated in immuno-oncology to enhance immune function, antagonists display broader applications for autoimmune and chronic inflammatory diseases, metabolic disorders and ageing related pathologies.

In this note, we show that the release of HMGB1 induced in the context of pyroptosis and necroptosis can be easily monitored with the HTRF HMGB1 kit. In addition we provide data comparison with two other methods, luciferase and ELISA and establish well correlated pharmacological responses. Finally we demonstrate that the use of HTRF HMGB1 kit along with HTRF IL-1β and IL-18 represent a valuable approach to decipher between pyroptosis and necroptosis biological responses.

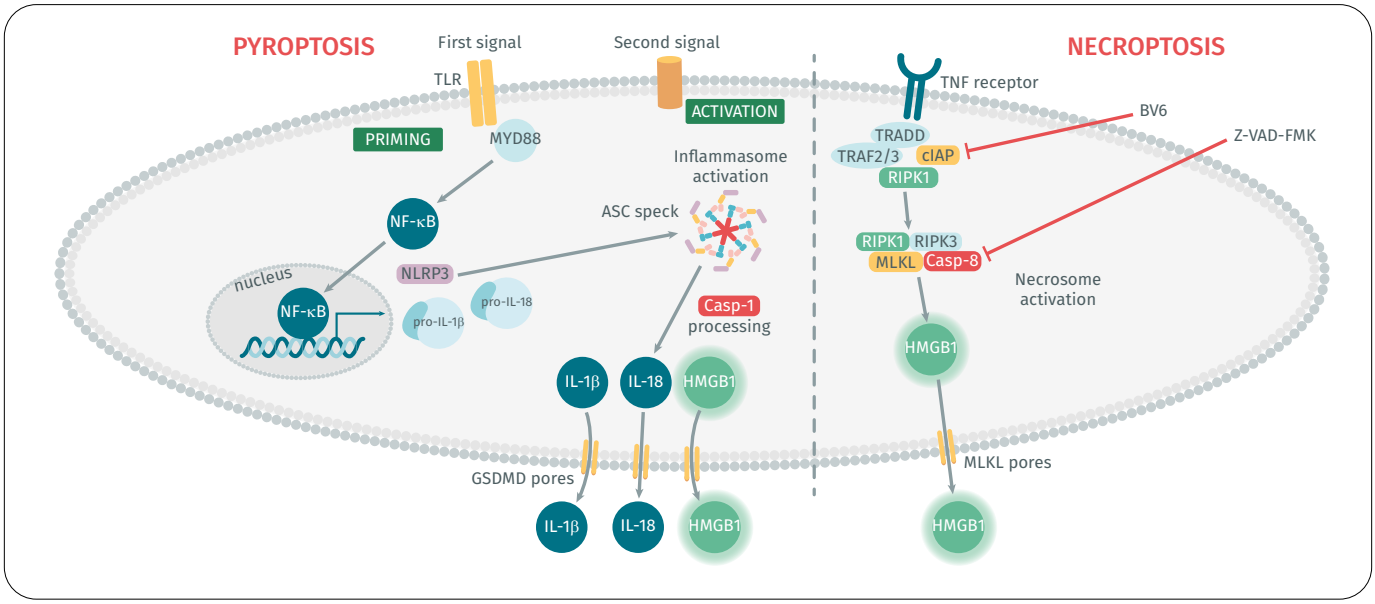


Figure 1: Pyroptosis and necroptosis description.

Principle of no-wash HTRF HMGB1 assay

After cell treatment, 16µL of cell supernatants are transferred to a HTRF detection microplate, followed by 4µL of pre-mixed HTRF antibodies. The HTRF signal is recorded after a 3h to 16-hour incubation at RT.

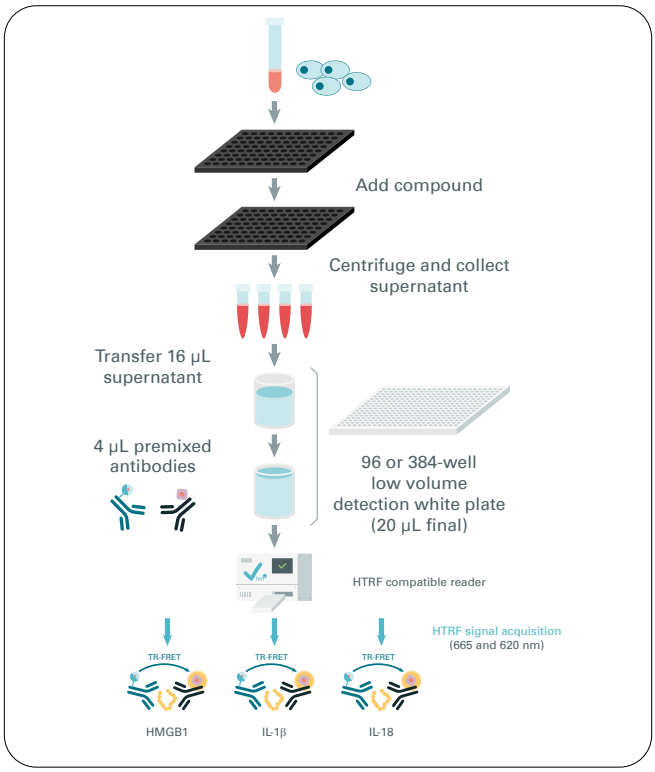


Figure 2: HMGB1, IL-1β and IL8 HTRF Assay protocol.

Experimental protocol

Cells stimulation

Experimental pyroptosis is obtained by submitting cultured THP1 cells to LPS priming followed by an activation step adding either ATP (mM range) or nigericin.

THP1-WT or THP1-HMGB1-Lucia™ cells were seeded at 400,000 cells per well in 190 µL of RPMI 1640, Glutamax, 25 mM HEPES, 10% heat-inactivated FCS. The cells were treated in parallel with the same reagent and concentrations.

After incubation at 37°C, 5% CO2, the cells were primed or not with 10µL LPS-EK (20µg/mL solution in medium prepared from a 1mg/mL DMSO frozen stock solution) 1µg/mL final.

After 3h incubation at 37°C, 5% CO2, the plate was centrifuged for 3 min at 400 RCF and 175 µL of medium were carefully removed and replaced by 165µL of medium, taking care not to aspirate cells.

Then the activation was done adding either 6µL of ATP (extemporaneously diluted in medium to 180mM) 5.5 mM final or 10µL of Nigericin (250 µM in medium) 12.5 µM final. Stimulation was performed for 20 hours.

Experimental necroptosis is obtained by stimulating cultured cell with TNFα while adding a CiAP inhibitor (BV6) and a pan-caspase inhibitor (Z-VAD-FMK), this combination refers as "TBZ" stimulation in the literature (3).

THP1-WT or THP1-HMGB1-Lucia cells (P4) were seeded at 400,000 cells per well in 190 µL RPMI 1640, Glutamax, 25 mM HEPES, 10% heat-inactivated FCS.

After incubation at 37°C, 5% CO2, the cells treated on not with 10µL Z-VAD-FMK (500 µM solution in medium prepared from a 10 mM DMSO frozen stock solution) 25µM final.

For the TBZ stimulation condition 20 µL of medium were carefully withdrawn and replaced by 10µL of BV6 (100µM in medium prepared from a 10 mM frozen stock in DMSO) 5µM final plus 10µL of TNFα (2µg/mL solution in medium prepared from a 100µg/mL frozen water solution) 100ng/mL final. (TBZ#1 and TBZ#2 are run under same conditions).

For the necrostatin inhibition condition (NS1) the same TBZ stimulation was applied, adding a further 10 µL of necrostatin (600 µM solution in medium prepared from a 50 mM DMSO frozen stock solution) 30µM final. Stimulation was performed for 20 hours.

The plates were centrifuged for 3 min at 400 RCF and 175 µL of supernatant were carefully withdrawn and transferred to polypropylene microtubes taking care not to aspirate cells. Supernatants can be frozen (-80°C) for further analysis taking care for the absence of cells which would release HMGB1 upon freeze/thaw step.

HTRF assays

THP1-WT supernatants (16µL) were transferred in triplicate to a detection plate prior to the quantification of HMGB1, IL-1β, and IL-18 according to the corresponding HTRF protocol. With the THP1-WT cells and conditions used herein, the concentrations in supernatants remain within the respective assay dynamic range, implying that no extra dilution steps were required. Concentrations were interpolated from the calibration curve (4PL fit) obtained from serial dilutions of the HMGB1, IL-1β or IL-18 standard in the test medium (RPMI 10% FCS). The HMGB1 fold change are expressed with respect to either LPS condition or Z-VAD condition.

HMGB1 LUCIFERASE assay

The supernatants from THP1-HMGB1-Lucia cells were transferred in white 96-well plates and the luminescence was measured on a BMG Pherastar reader (LUM plus module, 1s, gain 3600) after addition of QUANTI-Luc following the provider recommendations. The measured Relative Luminescence Units (RLU) were used to compute the HMGB1 fold change in respect to non-stimulated condition.

HMGB1 ELISA assay

The various THP1-WT supernatants collected from previous pyroptosis and necroptosis experiments were diluted in RPMI 10% FCS (1/2 for LPS+ATP ; 1/5 for LPS+NIGER ; 1/4 for TBZ) or undiluted for negative controls. The same batch of RPMI 10% FCS must be used and simultaneously tested for bovine HMGB1 content (average measured as 12.7 ng/mL). The measured OD450 values were interpolated on a calibration curve (4PL) established from serial dilutions of the porcine HMGB1 standard provided in the ELISA kit, from which the HMGB1 contribution from FCS was deduced.

Simultaneous htrf detection of hmgb1, il-1β and il-18 to distinguish pyroptosis and necroptosis

Pyroptosis experiment performed in THP1 WT cells

The HMGB1 concentration quantified by HTRF change relative to the LPS condition is around 10-fold for LPS+ATP and around 50-fold for LPS+Nigericin stimulations. As expected, upon ATP and nigericin activation, the IL-1β and IL-18 concentrations display an increase which follows the increase observed for HMGB1.

Figure 3: HMGB1, IL-1β and IL18 measurement under ATP and/or LPS stimulation.

Necroptosis experiment performed in THP1 WT cells

The HMGB1 concentration quantified by HTRF change relative to the Z-VAD condition is around 8-fold for TBZ stimulation. As expected, the IL-1β and IL-18 concentrations do not change upon TBZ stimulation, as opposed to HMGB1 which shows a significant increase upon TBZ stimulation.

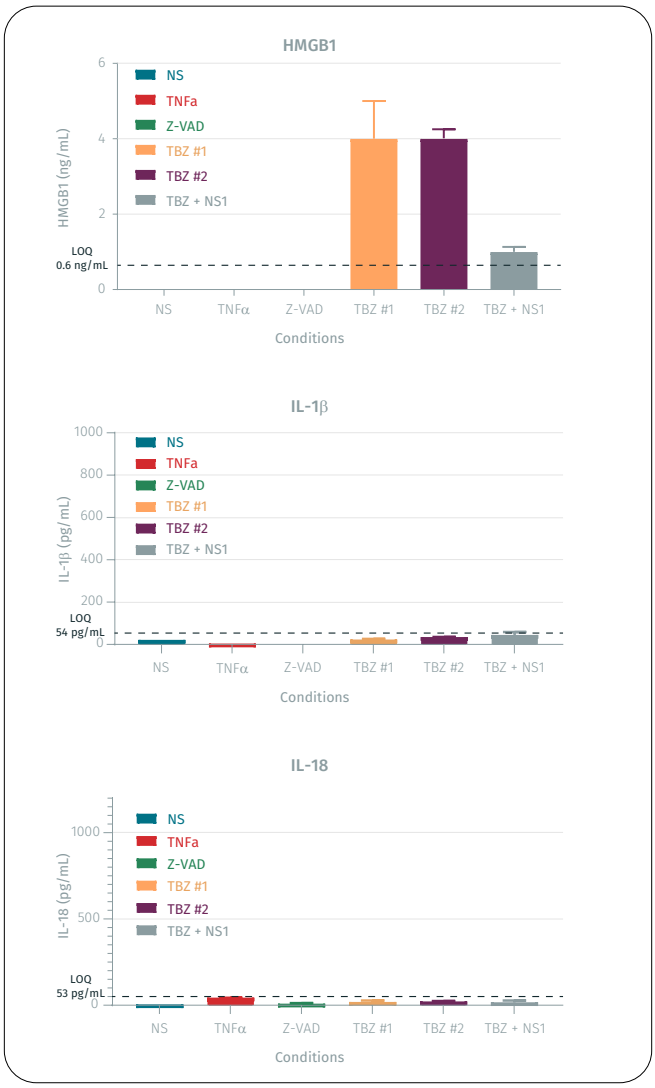


Figure 4: HMGB1, IL-1β and IL18 measurement under TNFα, Z-VAD or TBZ stimulation.

The above experiments show that pyroptosis is characterized by the simultaneous secretion of HMGB1 together with mature IL-1β/IL-18. In necroptosis, HMGB1 is released without mature IL-1β/IL-18 due to caspase and cIAP inactivation by TBZ cocktail. Thus the simultaneous HTRF detection of HMGB1 along with IL-1β and IL-18 can discriminate between pyroptosis and necroptosis.

HTRF HMGB1 measurement is well correlated with luminescence and elisa

Pyroptosis experiment performed in recombinant THP1 HMGB1- lucia cells and THP1 WT cells.

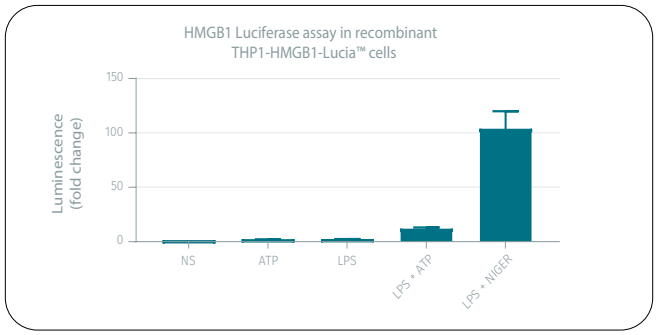


Figure 5: HMGB1 Luciferase assay in recombinant THP1-HMGB1-Lucia™ cells.

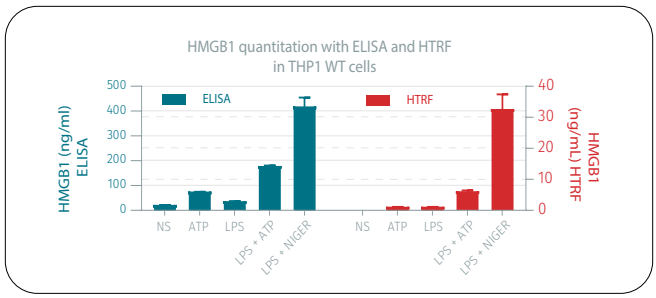


Figure 6: HMGB1 quantitation with ELISA and HTRF in THP1 WT cells.

Necroptosis experiment performed in recombinant THP1-HMGB1- Lucia Cells and THP1 WT Cells.

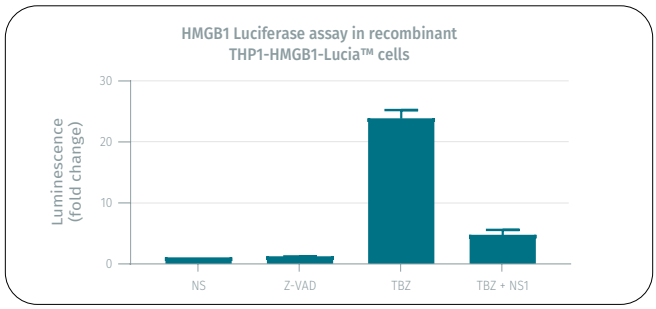


Figure 7: HMGB1 Luciferase assay in recombinant THP1-HMGB1-Lucia™ cells.

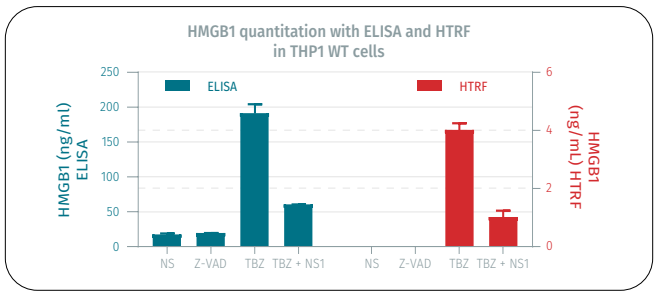


Figure 8: HMGB1 quantitation with ELISA and HTRF in THP1 WT cells.

The HMGB1 secretion profile obtained with the HTRF assay is well correlated both with the luciferase and the ELISA assays. We also observed a bias between HTRF and ELISA concentrations, which may be explained by a different HMGB1 recognition with the respective antibody's pairs used in each immunoassay, as well as the nature of the HMGB1 standard used (porcine for ELISA).

It should be noted that different biochemical forms of the released HMGB1 has been reported either in acetylated and reduced or oxidized states, depending of the biological mechanism (apoptosis, necrosis, pyroptosis). Pyroptosis has been shown to release HMGB1 as acetylated and both fully reduced and disulfide form (4) but the actual form released in necroptosis still remains to be elucidated.

Conclusion

The HMGB1 HTRF kit allows for the quantitation of the native HMGB1 released in pyroptosis and necroptosis and can distinguish between the two when used in combination with HTRF IL-1β/IL-18 kits. Contrary to luciferase assay, HTRF HMGB1 assay can be applied to unmodified cells, being more universal. Unlike the ELISA, this HTRF HMGB1 assay is insensitive to the bovine HMGB1 present in the FCS. Besides no wash steps required, the wider dynamic range of the HTRF kit makes it easier to use since no extra dilution of the supernatants were required, at least in this study.

References

Literature

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Vercammen *et al*. J. Exp. Med. (1998) 187, 1477

Yoon *et al*. Cell Death and Differentiation (2016) 23(2):253

Magan *et al*. Mol Med. (2014) 20:138

Magan *et al*, Nat Rev Drug Discovery (2018) 17:588-606

Reagents

HTRF kits : 62HMGPEG, 62HIL-1βPEG, 62HIL-18PEG (Revvity)

ELISA : HMGB1 ELISA kit (IBL ST51011)

THP1-HMGB1-Lucia Cells (Invivogen, thp-gb1lc)

THP1-WT (Wild Type) (ATCC, TIB202)

LPS lipopolysaccharide from E. coli K12 (Invivogen, tlrl- peklps)

Nigericin (Sigma, N7143)

ATP (Invivogen, tlrl-atpl)

Z-VAD-FMK : VAD-FMK (Invivogen, tlrl-vad)

Recombinant human TNF-α (Invivogen, rcyc-hTNFα)

BV6 (Euromedex SE-S7597)

Necrostatin-1 (Sigma, N9037)

QUANTI-Luc (Invivogen, rep-qlcg1)

Acronyms

HMGB1: High Mobility Group Box 1 protein; PAMPS: Pathogen-Associate Molecular Pattern; DAMPS: Danger-Associate Molecular Pattern; TLR: Toll-Like Receptors; NLRP3 : NOD-like receptor family, pyrin domain containing; GSDMD: Gasdermin D; RIPK1 / 3 : Receptor- Interacting Protein Kinases 1 and 3; TRADD: TNF-Receptor-Associated Death Domain; cIAP: cellular Inhibitor of Apoptosis; MLKL : Mixed Lineage Kinase-Like

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