Pyroptosis Detection via Multiplexing Cell Death and Bioluminescent Caspase-1 Activity Assessments

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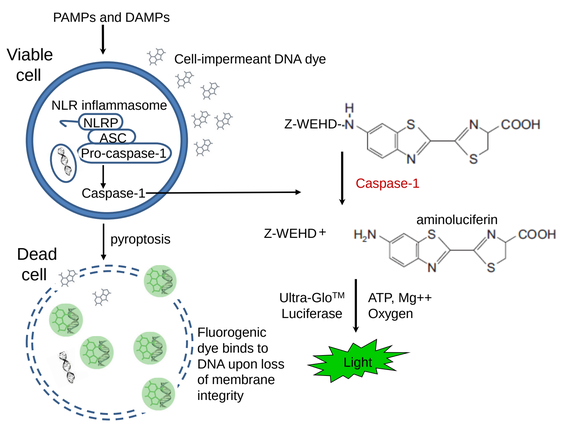
1. Introduction

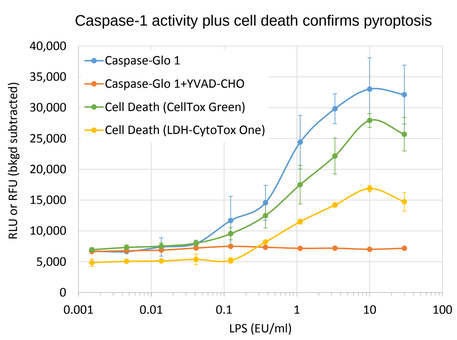
Pyroptosis is a unique immunogenic form of cell death dependent on inflammasome activation, provoked when pattern recognition receptors (PRRs) sense microbial infection or cellular stress. Caspase-1, an essential component of inflammasomes, mediates this innate immune response by processing cytokines and eliciting cell death. In addition to caspase-1, other inflammatory caspases (caspase-11 in mice and caspases-4, 5 in humans) can cleave gasdermin D and elicit pyroptosis in response to some intracellular microbial infections. However, caspase-1 is also activated under these circumstances, therefore caspase-1 activation coincident with cell death is a reliable indicator of pyroptosis. Understanding when PRR sensing leads to cell death and when it does not is essential to understanding the complexities of the innate immune response. We have developed a convenient method to monitor pyroptosis by multiplexing in the same assay well our homogeneous, bioluminescent caspase-1 activity assay with a membrane-impermeant, stable DNA dye for the detection of cell death. In contrast to endpoint assays for cell death such as lactate dehydrogenase (LDH) assays, the DNA dye can be used in real-time. Cell death can be monitored over time and when first detected, the caspase-1 assay can be performed confirming pyroptosis. Alternatively, culture medium can be removed for testing for caspase-1 release, leaving the cells available for measuring cell death by a variety of means. We show examples of pyroptosis in human THP-1 monocytes and mouse J774A.1 macrophages.

2. Simple Method for Determining Pyroptosis

We developed a bioluminescent assay that combines a substrate for caspase-1, Z-WEHD-aminoluciferin, with a thermostable luciferase in an optimized lytic reagent added directly to cultured cells. This assay can be combined with a fluorogenic, cell-impermeant DNA dye as a real-time monitor of cell death. The two assays together verify pyroptosis.



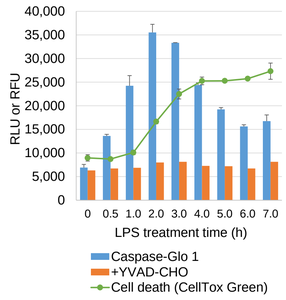
3. Pyroptosis in THP-1 Cells



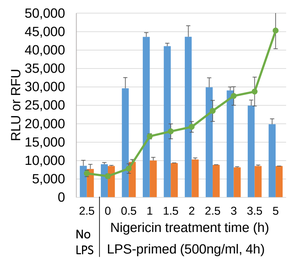
THP-1 cells (5 x 10^4/well) were differentiated (20nM PMA) for 3 days in 96-well plates. After differentiation, media was removed, DNA dye (CellTox™ Green) was added and cells were treated with a titration of LPS. Fluorescence was recorded after 2.5h just before adding Caspase-Glo® 1 reagent to some of the wells. In parallel wells, the Ac-YVAD-CHO inhibitor added to the lytic Caspase-Glo® 1 reagent confirms the specificity of the luminescent caspase-1 signal. The LDH reagent (CytoTox™ ONE) was added to the remaining wells and fluorescence recorded. All luminescence and fluorescence was recorded on a Glo-Max® unless noted otherwise.

4. Caspase-1 Activity is Induced Rapidly and Transiently in THP-1 and J774A.1 Cells

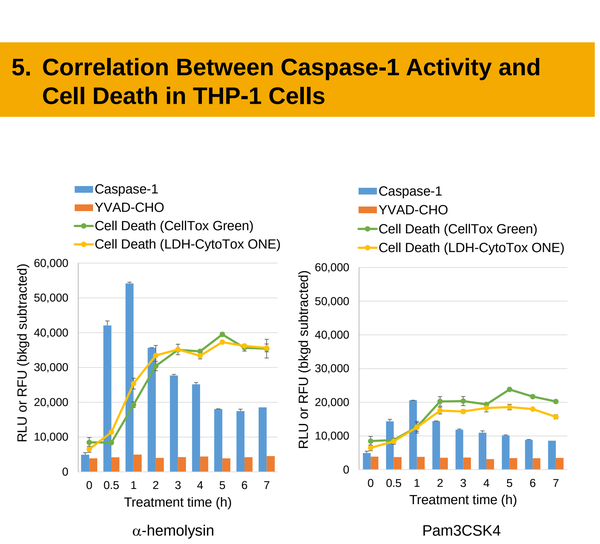
THP-1 cells (5 x 10^4/well) were differentiated (20nM PMA) for 2 days in 96-well plates. DNA dye was added and cells were treated with the TLR4 agonist LPS (1μg/ml) for various times. Fluorescence was recorded at the end of the time course just before adding caspase-1 reagent. **Only one stimulus is required for inflammasome activation in THP-1 cells.**



J774A.1 cells were added to 96-well plates at 5 x 10^4/well, primed with LPS or left unprimed, and treated with the K+ ionophore nigericin (20μM) for various times. Fluorescence was recorded just before adding caspase-1 reagent. **Two stimuli are required for inflammasome activation in J774A.1 macrophages.**

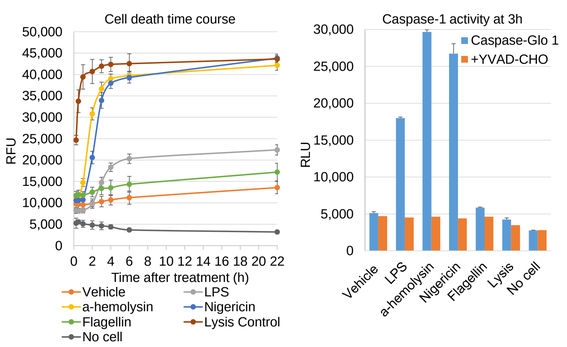


5. Correlation Between Caspase-1 Activity and Cell Death in THP-1 Cells



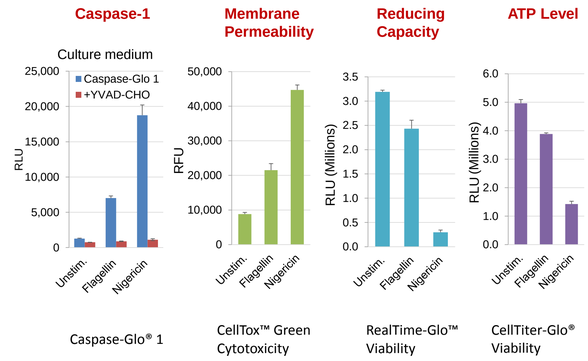
THP-1 cells (5 x 10^4/well) were differentiated (20nM PMA) for 3 days in 96-well plates. After differentiation media was removed, DNA dye was added and cells were treated at various times with the pore-forming toxin, α-hemolysin (1μg/ml), or the TLR 1/2 agonist, Pam3CSK4 (100ng/ml). Fluorescence was recorded just before adding the caspase-1 reagent. LDH reagent was added to a parallel plate and fluorescence was recorded.

6. Some Inflammasome Activators Trigger Pyroptosis in a Subset of the Cell Population



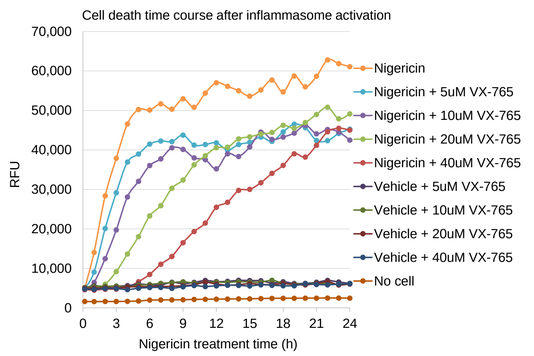
THP-1 cells (5 x 10^4/well) were differentiated (20nM PMA) for 2 days in a 96-well plate. After differentiation, media was removed, DNA dye was added and cells were treated with LPS (100ng/ml), α-hemolysin (1 μg/ml), nigericin (20μM), flagellin (1μg/ml), or a lytic control for cell death (0.18% Triton X-100). Fluorescence was recorded over time from the same wells. Just after the 3h read, the caspase-1 reagent was added to some of the wells. Fluorescence was monitored in the remaining wells out to 22h.

7. Monitoring Released Caspase-1 Activity Enables Multiplexing for Cell Death



THP-1 cells (5 × 10^4/well) in 96-well plates were differentiated with PMA (20nM, 3 days) followed by treatment with either flagellin (1μg/ml, 1h) or nigericin (20μM, 2h). Half of the culture medium (50μl) was transferred to a separate plate for a Caspase-1 Assay. The remaining cells and half the culture medium were tested for cell death in parallel by three parameters: membrane permeability, reducing capacity, and ATP decrease.

8. Inhibiting Caspase-1 Delays Cell Death



THP-1 cells (5 x 10^4/well) were added to a 96-well plate with CellTox™ Green and either VX-765 (caspase-1 inhibitor) at different doses or a DMSO control. Cells were incubated with the inhibitor for 1h before adding nigericin (20μM) or vehicle. Fluorescence was monitored every hour for 24h with a BMG Labtech CLARIOstar at 37°C in 5% CO_2.

9. Conclusions

Developed a convenient method to confirm pyroptosis
- Provides understanding of when inflammasome activation causes cell death
- Bioluminescent caspase-1 activity assay is multiplexed with a real-time, fluorescent cell death assay
- Confirmed pyroptosis in human THP-1 and mouse J774A.1 cells treated with several inflammasome inducers

Inflammasome activation triggers rapid pyroptosis
- Pyroptosis detected in cell population within 30-60min of caspase-1 activation
- Inflammasome-induced pyroptosis required 2 stimuli in J774A.1 cells, but only 1 stimulus in THP-1 cells
- Some inflammasome activators trigger pyroptosis in only a subset of the cell population

Preventing pyroptosis may shift form of cell death
- Inhibiting caspase-1 in THP-1 cells treated with a pyrogen delayed cell death but did not prevent it
- We are investigating the mechanism of this delayed cell death

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