Understanding the Role of the Inflammasome in Inflammation

Martha O’Brien, Ph.D.
Assay Design Group
Innate Immune Response in Inflammation

- Flagellin and LPS activate TLR-4 and TLR-5.
- TLR-1/2/6 activate TLR-4 and TLR-5.
- Viral nucleic acids activate TLR-3/7/9.
- Adaptor proteins include PYD and ASC-CARD.
- NLR inflammasome includes LRR, NBD, ASC, and CARD.
- Pro-caspase-1 is activated.
- Caspase-1 cleaves Pro-IL-1β to IL-1β, promoting inflammation.
- Endolysosome and nucleus are involved in the response.
- PAMPs, DAMPs stimulate the immune system.

©2015 Promega Corporation.
Inflammasome Multiprotein Complex

1. Agonist
   - NLRP3
   - PYD
   - CARD
   - CASP

2. ASC

3. Active Caspase-1

4. Pro-IL-1β → IL-1β
   - Pro-IL-18 → IL-18

Santos, G. et al. (2012)
*Lung Cellular and Molecular Physiology* 303: L627

http://www.umassmed.edu/fitzgeraldlab
Different Stimuli Induce Different Inflammasome Complexes

DAMPs: MSU, PPD, Silica, alum, Asbestos, amyloid-β

PAMPs: LPS, peptidoglycan, viral and bacterial RNA, DNA

anthracis toxin
MDP

Flagellin
dsDNA

NLRP1b
inflammasome

NLRP3
inflammasome

Naip/NLRC4
inflammasome

AIM2
inflammasome

www.a-star.edu.sg
Inflammasome Engagement Activates Caspase-1

Current Methods for Monitoring Inflammasome Activation

Western Blots for processed IL-1β, IL-18 or Caspase-1
- Cumbersome, time consuming
- Need to make lysates or use serum-free supernatants
- Variable Ab quality
- Activity can be missed because oligomerization but not cleavage of caspase-1 required for activity (Broz, et al., 2010)

ELISAs for Released IL-1β, IL-18, or Caspase-1
- Cumbersome, time consuming
- Ab exhibit some cross-reactivity with pro-IL-1β
- Pro-IL-1β can also be released complicating analysis
- IL-1β can be processed extracellularly by other proteases (van de Veerdonk, et al. 2011)
- Dynamic Range is limited

FLICA (fluorescently-labeled inhibitor of caspase, e.g., FAM-YVAD-FMK)
- Cells need to be washed, very problematic since caspase-1 is released from cells
- FLICA is not blocked by unlabeled inhibitor leading to questions of specificity

HTRF (IL-1β), AlphaLISA (IL-1β)
- Similar to the ELISA, Abs exhibit some cross-reactivity with pro-IL-1β
- Requires special instrumentation
Bioluminescent Caspase-1 Substrates

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrapeptide caspase-1 substrate similar to YVHD endogenous IL-1β cleavage site

Optimal caspase-1 tetrapeptide substrate determined from recombinant peptide library

Known tetrap
Substrate Performance in Cell Model for Inflammasome Activation

Cell Model

α-hemolysin-treated THP-1 cells

α-hemolysin is a pore-forming toxin virulence factor from Staphylococcus aureus that elicits inflammasome activation and pyroptosis in THP-1 monocytic leukemia cells
Substrate Performance in Cell Model for Inflammasome Activation

**Cell Model**

α-hemolysin-treated THP-1 cells

α-hemolysin is a pore-forming toxin virulence factor from Staphylococcus aureus that elicits inflammasome activation and pyroptosis in THP-1 monocytic leukemia cells
Substrate Performance in Cell Model for Inflammasome Activation

**Cell Model**

α-hemolysin-treated THP-1 cells

α-hemolysin is a pore-forming toxin virulence factor from *Staphylococcus aureus* that elicits inflammasome activation and pyroptosis in THP-1 monocytic leukemia cells

- Inhibition of proteasome with MG-132 required to see inflammasome signal
- Z-WEHD-aminoluciferin is the better substrate
Coupled-Enzyme Assay System

Z-WEHD-H N
\[ \begin{array}{c}
  & \text{Caspase-1}
  \\
  \text{Z-WEHD + H}_2\text{N} & \text{aminoluciferin}
  \\
  \text{Ultra-Glo}^\text{TM} & \text{Thermostable Luciferase}
  \\
  \text{ATP, Mg}^{++} & \text{Oxygen}
  \\
\end{array} \]

Light

THP-1 cells

nigericin

K+ ionophore triggers inflammasome

RLU

0 20,000 40,000 60,000 80,000 100,000 120,000

0 30 60 90 120 150 180

Time after reagent addition (min)

No cell
Vehicle
Nigericin
### Ac-YVAD-CHO is Caspase-1 Selective Inhibitor

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Aldehyde $K_i$ YVAD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase-1</td>
<td>0.76</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>362</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>163</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase-6</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>352</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>970</td>
</tr>
<tr>
<td>Caspase-10</td>
<td>408</td>
</tr>
</tbody>
</table>

Ac-YVAD-CHO (1µM) Completely Inhibits Cell Signal

Assay is linear

R² = 0.9954
R² = 0.9997
R² = 0.9934
Ac-YVAD-CHO can Distinguish Caspase-1 from Cross-reacting Caspases

Ac-YVAD-CHO inhibitor at 1µM in the reagent knocks down 99% of the caspase-1 activity.

Ac-YVAD-CHO does not substantially inhibit cross-reacting caspases.
Ac-YVAD-CHO Confirms that the Luminescent Signal is Due to Caspase-1 in THP-1 cells

Signal inhibited by YVAD-CHO = caspase-1!

Signal not inhibited by YVAD-CHO is not caspase-1!
Apoptosis Activity in THP-1 cells is Inhibited by VEID-CHO

XIAP (endogenous inhibitor of caspases 3, 7, and 9) partially inhibits signal

VEID-CHO completely inhibits the apoptotic signal

Although VEID-CHO is not completely selective for caspase-6, it is consistent with our in vitro data that caspase-6 contributes to the apoptotic signal

YVAD-CHO does not inhibit the apoptosis signal providing the assay specificity
Caspase-Glo® 1 Assay Cell-based Protocol

1. Resuspend lyophilized Z-WEHD Substrate with Caspase-Glo® 1 Buffer.
2. Add MG-132 Inhibitor to inhibit nonspecific activity.
3. Transfer up to half of the Caspase-Glo® 1 Reagent to a separate tube, and add Ac-YVAD-CHO Inhibitor to prepare Caspase-Glo® 1 YVAD-CHO Reagent.
4. Add an equal volume of Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent to the sample, and incubate at room temperature.
5. Measure luminescence.
Caspase-1 Activation Detected with Several Inflammasome Inducers in THP-1 cells

THP-1 cells PMA-differentiated and treated in 384-well plates

- Caspase-Glo 1
- Caspase-Glo 1+YVAD-CHO

RLU

- No cells
- Unstim
- MSU
- a-hemolysin
- Nigericin
- LPS
- R848
- Pam3CSK4
Assay Enables High Throughput Screening for Inflammasome Activation

384-well plate with automated dispensing
Testing high, medium, and low inducer

<table>
<thead>
<tr>
<th>Inducer</th>
<th>S/B</th>
<th>Z'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigericin</td>
<td>7.3</td>
<td>0.469</td>
</tr>
<tr>
<td>LPS</td>
<td>4.0</td>
<td>0.494</td>
</tr>
<tr>
<td>Flagellin</td>
<td>2.4</td>
<td>0.252</td>
</tr>
<tr>
<td>Nigericin +YVAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS +YVAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flagellin +YVAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstim.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stable signal in 384-well
Macrophages Require Two Signals for Caspase-1 Activation

Unprimed cells

LPS-primed J774A.1 cells
Two Signal Hypothesis for NLRP3 Inflammasome Activation and Cytokine Processing

Macrophages require two signals for processing and release of IL-1β and IL-18 mediated through the NLRP3 inflammasome

Signal 1:
Pathogen associated molecular patterns (PAMPs) bind to TLRs and upregulate pro-IL-1β/IL-18 and NLRP3 via the NF-κB pathway

Signal 2:
ATP, crystals, and other damage associated molecular patterns (DAMPs) trigger inflammasome formation, caspase-1 activation, and IL-1β, IL-18 processing through a common mechanism thought to be K+ efflux, ROS, or lysosomal damage

Yang et al. (2012) Int. Neurourology J. 16:2
Caspase-1 Activated by One Inflammasome Stimulus in THP-1 Cells

In THP-1 cells, Signal 2 (nigericin, α-hemolysin) alone can activate caspase-1 via K+ efflux.
Caspase-1 Activated by One Inflammasome Stimulus in THP-1 Cells

In THP-1 cells, Signal 2 (nigericin, α-hemolysin) alone can activate caspase-1 via K+ efflux.

Signal 1 (TLR agonists) alone can activate caspase-1 in differentiated THP-1 cells.

![Diagram of inflammasome activation in THP-1 cells]

- **Signal 1**: TLR agonists
- **Signal 2**: nigericin, α-hemolysin

**RLU (Relative Light Units)**

- **No cell**: Undifferentiated
- **Unstim.**: Undifferentiated
- **LPS**: PMA-differentiated
- **Pam3CSK4**: PMA-differentiated

**Graph showing RLU values**

- No significant differences observed between treatments.
Caspase-1 Activated by Two Inflammasome Signals in J774A.1 Macrophages

J774A.1 macrophages require both Signal 1 and 2 for NLRP3 inflammasome engagement and caspase-1 activation
Caspase-1 Activation is Rapid and Transient

PMA-differentiated THP-1 cells

LPS-primed J774A.1 cells

RLU vs Time of treatment (h)
Caspase-1 is Released into Culture Medium

Caspase-1 is released with its substrates, IL-1β and IL-18

A simple transfer of half the culture medium to a 2nd plate can be done to detect released caspase-1

**Advantages**
- Although the RLUs are lower, better S/B (*greater sensitivity*) is achieved due to lower background
- Leaves the cells available for multiplexing
Culture Medium can be Frozen at -20°C Before Monitoring for Caspase-1 Activity

![Graph showing the comparison of S/B values for fresh culture medium and culture medium frozen at -20°C for 2 days for Flagellin and YVAD-CHO at 1h, 2h, and 3h.](image-url)
Released Caspase-1 Activity from Mouse Primary BMDMs and Human Primary PBMCs

Culture medium from mouse bone marrow-derived macrophages

Assay validated in human PBMCs and mouse BMDMs

Carlene Petes and Dr. Katrina Gee
Queen’s University

Drs. Sivapriya Kailasan Vanaja and Vijay Rathinam
University of Connecticut

©2015 Promega Corporation.
Caspase-1 Induced Pyroptosis is Protective

Pyroptosis (in contrast to apoptosis) is lytic and inflammatory releasing IL-1β, IL-18 and other cytokines.

Recent research demonstrates that pyroptosis can also limit pathogen replication by releasing the pathogen before replication and exposing them to neutrophil uptake and clearance.
Multiplexing to Monitor Caspase-1 Activity and Pyroptosis

Cell death with caspase-1 activation = pyroptosis
Cell death, no caspase-1 activation = no pyroptosis
# Multiplexing to Monitor Caspase-1 Activity and Cell Viability or Cell Death

<table>
<thead>
<tr>
<th>Caspase-Glo® 1</th>
<th>CellTox™ Green Cytotoxicity</th>
<th>RealTime-Glo® Viability</th>
<th>CellTiter-Glo® Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Culture Medium</strong></td>
<td><strong>Cell permeability</strong></td>
<td><strong>Reducing capacity decrease</strong></td>
<td><strong>ATP decrease</strong></td>
</tr>
</tbody>
</table>
Multiplexing to Monitor Caspase-1 Activity, IL-1β Release, and Pyroptosis

Caspase-1 Activity in Culture Medium

- Caspase-Glo 1
- Caspase-Glo 1+YVAD-CHO

IL-1β Release (ELISA on Culture Medium)

- PMA 20nM 2 days
- Undifferentiated
Multiplexing to Monitor Caspase-1 Activity, IL-1β Release, and Pyroptosis

Caspase-1 Activity in Culture Medium

- Caspase-Glo 1
- Caspase-Glo 1+YVAD-CHO

CellTox Green Cytotoxicity (Pyroptosis)

- No cell
- Vehicle
- LPS
- Nigericin

PMA 20nM 2 days
Undifferentiated
Multiplexing Summary in THP-1 Cells

• In differentiated THP-1 cells, nigericin induces caspase-1 activation, pyroptosis, and IL-1β release.

• In undifferentiated THP-1 cells, nigericin induces caspase-1 activation and pyroptosis, but not IL-1β release.

• The lack of IL-1β release in undifferentiated THP-1 cells is expected since differentiation or priming is required for pro-IL-1β up-regulation.

• The caspase-1 assay has enabled a demonstration of caspase-1 activation and pyroptosis separate from IL-1β/IL-18 release in THP-1 cells.

• This led to the question: Is caspase-1 activation directly driving pyroptosis in the IL-1β independent cell model?
Inhibition of Pyroptosis with Cell Permeable Caspase Inhibitors

Our results suggest that caspase-1 is driving pyroptosis in these cell models, but without complete inhibition with YVAD-FMK, further work will be needed to confirm this.
Understanding the Outcomes of Inflammasome Activation

Summary

We have developed a bioluminescent assay to directly monitor activated caspase-1 for assessing inflammasome activation.

Caspase-1 activity can be measured directly in cells in the lytic assay or from culture medium when caspase-1 is released from cells.

The assay has enabled determining the kinetics of caspase-1 activation and we have shown that it is typically rapid and transient in our cell models.

The assay can be multiplexed with other assays to monitor IL-1β/IL-18 release or cell death to assess pyroptosis.

The assay demonstrated that two signals are not required for caspase-1 activation in THP-1 cells, whereas two signals are required in macrophages.

In the THP-1 model we were able to separate the distinct outcomes of caspase-1 activation—namely IL-1β/IL-18 release and pyroptosis—by testing undifferentiated and PMA-differentiated cells.

The high throughput capability of this convenient caspase-1 assay will enable screening for modulators of inflammasome activation.
Acknowledgments

Danielle Moehring  Jeri Culp
Gedi Vidugiris  Jackie Jackson
Dan Lazar  Stuart Forsyth
Jim Cali  Hélène Benink
Troy Good  Shikha Gupta
Laurent Bernad  Pam Guthmiller
Lauren Hongo  Sally Floyd
Tenaya Noce  Terry Riss

Thank you for your attention!

Collaborators
Justin Callaway and Jenny Ting (U. North Carolina)
Raúl Muñoz-Planillo and Gabriel Núñez (U. Michigan)
Carlene Petes and Katarina Gee (Queen’s University)
Sivapriya Kailasan Vanaja and Vijay Rathinam (U. Connecticut)