Apoptosis, Necrosis, Necroptosis & Pyroptosis

By Sally Hed Dahlquist, President, BS, MBA
October 20, 2020

© ImmunoChemistry Technologies LLC 2020
Agenda

- Cell death is vital for life
- Cells die differently
- Accidental vs programed cell death
- Classification of cell death
- Morphology
- Caspases
- TNFR1 death receptor
- Key regulatory molecules
- Oncosis to necrosis
- Necrosis
- Necroptosis
- Apoptosis
- Pyroptosis
- Detection Kits from ICT

Sally Hed Dahlquist, President
BS Genetics & Cell Biology, MBA
Cell death is vital for life

Good  
Controlled

Bad  
Uncontrolled
Cells die differently
Pathways of cell death

Necrosis & Oncosis

Necroptosis

Apoptosis

Pyroptosis

Others:
Anoikis
Entosis
Ferroptosis
Methuosis
Paraptosis
Mitoptosis
Parthanatos
NETosis
And more…
Programmed
Regulated Cell Death (PCD or RCD) such as Apoptosis, etc; Good or Bad

Not Programmed
Accidental Cell Death (ACD) Necrosis & Oncosis; Bad; Uncontrolled

Programmed Apoptotic cell death

Not regulated Cell death

Accidental

Non-programmed

Necrosis (Oncosis)

Programmed Apoptotic cell death

Necroptosis

Apoptosis

Morphology
Scanning electron microscope

- Plasma membrane explosion
- RIPK1 and RIPK3
- Mediated by mixed lineage kinase domain-like (MLKL) lipophilic oligomers in the plasma membrane
- N-terminal sequence important
- MLKL forms channels
- Influx of ions
- Osmotic swelling
- Necroosome
- Inflammation

- Dismantled (not ruptured)
- Caspase 8,9,10 activation
- Cellular contents packaged
- Apoptotic bodies
- Taken up by phagocytic cells
- Membrane blebbing
- Shrinkage
- ATP-dependent
- Apoptosome
- Not inflammatory

- Plasma membrane rupture
- Mediated by gasdermin-D (GSDMD) after its cleavage by caspase-1 or caspase-11
- Generates the N-terminal, GSDMD-N, lipophilic
- GSDMD-N forms non-selective pores that do not rely on osmolarity
- Pyroptotic bodies protrude
- Cell flattens
- Inflammasome NLRP3
- Inflammation

Adapted from Figure 1 of Xin Chen et al. 2016. Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. Cell Research. 26:1007-1020
Caspases

- Cysteine-dependent aspartate-directed proteases
- Active caspase enzymes cleave substrates comprised of a 3 or 4 amino acid sequence containing Asp in the P1 position

4 groups:
1. Apoptosis: Initiator caspases (2, 8, 9, 10)
   - Caspase 8 coordinates response to TNF in the induction of inflammation, apoptosis, and necroptosis
   - Caspase 2 & 10 are alternates
2. Apoptosis: Effector caspases (3, 6, and 7)
   - Essential executioners to dismantle the cell via proteolytic cleavage
   - Are activated by initiator caspases, usually 8 & 9
3. Pyroptosis: Inflammatory caspases (1, 4, 5, 11, 12)
   - Caspase 1 initiates pyroptosis
   - Caspase 1, 4, 5, 11 cleave gasdermins leading to pyroptosis
   - Caspase 12 may have a role in ER stress RCD; is an anti-inflammatory regulator
4. Keratinization-relevant caspase 14
TNFR1 death receptor

Death Receptor
- TNFR1
- CD95 (Fas, APO-1)
- DR3
- TRAIL-R1 (DR4)
- TRAIL-R2 (DR4)

Death Ligand
- TNF
- CD95-ligand (CD95-L, Fas-L)
- TLIA
- TRAIL (Apo2-L)
- TRAIL (Apo2-L)

Tabel 1 and Figure 4 from D'Arcy, M. Cell death: a review of the major forms of apoptosis, necrosis, and autophagy. Cell Biol Int 43 (2019) 582–592.
### Key regulatory molecules

<table>
<thead>
<tr>
<th>Cell death mode</th>
<th>Genetic regulation</th>
<th>Morphological/biochemical features</th>
<th>Key regulatory molecules&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Y</td>
<td>Membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation, mitochondrial dysfunction, cytochrome c release, altered Bcl-2 family protein expression and activation</td>
<td>(+) Caspase-8, -9, -3, and -7; Bax; Bad; Bid; SMAC/DIABLO. (-) Bcl-2, Bcl-X&lt;sub&gt;L&lt;/sub&gt;, IAP1/2, XIAP.</td>
</tr>
<tr>
<td>Necrosis</td>
<td>N</td>
<td>Plasma membrane rupture, cell swelling and lysis, energy decline, DAMP release</td>
<td>None</td>
</tr>
<tr>
<td>Necroptosis</td>
<td>Y</td>
<td>Plasma membrane rupture, cell swelling and lysis, energy decline, DAMPs release</td>
<td>(+) RIPK1, RIPK3, MLKL. (-) Caspase-8.</td>
</tr>
<tr>
<td>Ferroptosis</td>
<td>Y</td>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;-dependent membrane lipid oxidation and membrane damage</td>
<td>(+) Fe&lt;sup&gt;2+&lt;/sup&gt;. (-) GPX4.</td>
</tr>
<tr>
<td>Pyroptosis</td>
<td>Y</td>
<td>Inflamasome activation membrane rupture, cell swelling and lysis, pore-induced intracellular traps, DNA fragmentation, nuclear condensation, DAMP release, proinflammatory cytokines</td>
<td>(+) Caspase-1 and -7, GSDMD.</td>
</tr>
<tr>
<td>MPT necrosis</td>
<td>Y</td>
<td>Plasma membrane rupture, cell swelling and lysis, mitochondrial permeability transition pore opening, mitochondrial swelling, energy decline, DAMP release</td>
<td>(+) Cyclophilin D (peptidyl-prolyl isomerase) ANT, voltage-dependent anion channel.</td>
</tr>
<tr>
<td>Parthanatos</td>
<td>Y</td>
<td>Cell swelling and lysis, DNA fragmentation, nuclear condensation, production of poly(ADP-ribose), release of mitochondrial apoptosis-inducing factor</td>
<td>(+) Poly(ADP-ribose) polymerase 1. (-) Poly(ADP-ribose) glycohydrolase.</td>
</tr>
<tr>
<td>Cellular senescence</td>
<td>N</td>
<td>Irreversible inhibition of the cell cycle</td>
<td>(+) p53, cyclin-dependent kinase inhibitor p16.</td>
</tr>
</tbody>
</table>

<sup>a</sup>(+) indicates molecules that activate the indicated pathway; (-) indicates molecules that negatively regulate the indicated pathway. ANT, adenine nucleotide translocator; IAP, inhibitor of apoptosis protein; SMAC/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor apoptosis-binding protein with low pl.

Oncosis to Necrosis

Normal cell

Cell suffers sudden shock (e.g., hypoxia, heat, chemical insult)

Cell damage is sudden and the cell is therefore unable to initiate programmed cell death

Oncosis

The cell becomes leaky and swells

Organelles

Necrosis

The cell eventually ruptures, spilling its contents into the surrounding environment

Figure 5 from D'Arcy, M. Cell death: a review of the major forms of apoptosis, necrosis, and autophagy. Cell Biol Int 43 (2019) 582–592.
Necrosis

- Uncontrolled
- Non-programmed or Accidental cell death (ACD)
- Loss of energy charge
- Triggers local inflammation
- Lysis: cell breaks open and spills content into the surrounding tissues
- Damage to surrounding tissues
- Damage Associated molecular Patterns (DAMP) release
- Sum of changes after the cell dies regardless of the pathway
- May trigger necroptosis and other forms of regulated cell death (RCD)

Necroptosis

- Regulated cell death (RCD) by a genetic program
- RIPKs activated by various cell-surface receptors: Death receptors (DRs), Toll-like receptors (TLRs), and the T-cell receptor (TCR)
- Receptor-interacting protein kinase RIPK1 and RIPK3 are key components of the necosome
- RIPK3-dependent phosphorylation of MLKL
- MLKL oligomerization
- RIPK3 & MLKL key effectors of injury propagation
- Ion channels
- Osmotic swelling and explosion
- Lysis: cell breaks open and spills content into the surrounding tissues
- Damage Associated Molecular Patterns (DAMP) release
- Propagate secondary inflammatory response
- Kidney and lung disease
- Beneficial to remove infected cells and activate anti-tumor response

Adapted from Figure 1 from Choi, M. E., et al. JCI Insight. 2019;4(15):e128834. https://doi.org/10.1172/jci.insight.128834.
Apoptosis

- Programmed RCD to detect damage
  - Intrinsic (intracellular)
  - Extrinsic (external)

- Cytosolic cysteine protease enzymes (caspases) are the primary mediators of apoptosis (the wrecking ball)
  - Initiator (caspase 8 & 9)
  - Executioner (caspase 3, 6, & 7)

- Once activated, initiate an irreversible cascade of events resulting in rapid cell death

- Cytoplasmic and nuclear condensation
- DNA fragmentation
- Expression of ligands for phagocytosis
- Apoptotic bodies
- Minimal damage to surrounding tissues
- Easily detect with
  - FLICA in vitro
  - FLIVO in vivo
  - Magic Red in real time

Pyroptosis

- Highly inflammatory programmed RCD; Infection response, pores, swelling
- A: Canonical inflammasome: ASC to activate Caspase-1, cleaves gasdermin D GSDMD
- B: Noncanonical inflammasome: bacterial LPS activates caspase-4/5/11 to cleave GSDMD
- C: New pathway: Caspase-3 to cleave gasdermin E GSDME, and C&N Terminal, Caspase-1

Necrosis vs. Apoptosis Kit

- Early-stage apoptotic cells exhibit a cell-retained green fluorescence (FLICA).
- Cell membrane compromised necrotic and very late-stage apoptotic cells exhibit red (7-AAD-only) fluorescence.
- Green and red fluorescing cells represent the population of Jurkat cells in mid to late-stage apoptosis.

Jurkat cells treated with 1 µM Staurosporine for 4 hours
Detection of apoptosis and necrosis...

Combine ICT’s polycaspase FLICA with the vital dye 7-aminoactinomycin D (7-AAD) to allow for simultaneous staining of live, early apoptotic, late apoptotic, and necrotic cells.
in vitro Apoptosis with FLICA®

- Inhibitor
- Detect active caspases; many different kits for different caspases
- Whole living cells & some tissues
- FLICA: Fluorescent-Labeled Inhibitor of CAspases
- Add directly to the media, incubate 15 minutes – 4 hours, wash, counterstain, freeze, fix, trypsinize, etc.
- Cell permeant probes that contain 3 components:
  1. Label (3 options)
     1. FAM (green)
     2. SR (red)
     3. 660
  2. Various caspase peptide sequences, such as YVAD, etc.
  3. FMK
- Image
  1. Fluorescence Microscope
  2. Flow cytometer
  3. Plate reader
- FLICA covalently binds to active caspases in vitro
Primary Rat Hippocampal Neurons

- Sprague-Dawley albino postnatal day 0 male pups
- 300,000 cells, 25-mm poly-l-lysine coverslips
- Blue Hoechst, cat #639, DNA label (A, 4 blue cells)
- Green FLICA \textit{in vitro} #94 Caspase-3/7 FAM-DEVD-FMK (B: 3 green cells)
- Red PI, cat. #638, membrane permeabilized (C, 2 red cells)
- 3 populations (D, composite image)

Data courtesy of Dr. Akozer, University of Maryland
Counterstaining

Live/Dead Stains
• Propidium Iodide
• 7-AAD

Nuclear Stains
• Hoechst 33342
• DAPI
Pyroptosis/Caspase-1 Assay, in Green and Far Red

• Green Catalog #9145 & 9146
• Far Red Catalog #9158
• Fluorescent microscope, fluorescent plate reader, flow cytometer
• Nigericin catalog #6698
  • Positive control for pyroptosis experiments
  • Included with kits
Pyroptosis Microscopy Data

*Salmonella* Infected epithelial cells labeled with FAM-FLICA Caspase-1 Assay (image courtesy of Knodler, et al. 2010. PNAS 107 (41) 17733-8)
Flow Data

No Nigericin Control

Nigericin, 1 hour

Nigericin, 3 hour

Nigericin, 6 hour

Nigericin, 12 hour

Nigericin, 24 hour

V1-L 94.8%
V1-R 5.2%

V1-L 78.9%
V1-R 21.1%

V1-L 55.6%
V1-R 44.4%

V1-L 34.0%
V1-R 66.0%

V1-L 9.6%
V1-R 90.4%
Plate Reader Data

- **Negative control (non-induced):** 11.5 RFU
- **Pyroptotic (induced):** 21.4 RFU
**Autophagy**

- Kit contains Autophagy Probe, Red, a cell-permeant dye that fluoresces brightly when inserted in the membranes of autophagosomes and autolysosomes.
- Easily monitor autophagy using a flow cytometer equipped with a green/yellow laser and appropriate filter.
- Optimal excitation/emission is 590 nm/620 nm.
Mitochondrial Membrane Potential with MitoPT™

1. TMRE (Cat # 9103)
2. TMRM (Cat # 9105)
3. JC-1 (Cat # 911)

• Cells experiencing oxidative stress may lose polarization along their inner and outer mitochondrial membranes.

• These potentiometric mitochondrial dyes can be used to assess the status of mitochondrial polarization.
Cell Death & Viability Assays

- Apoptosis
- Caspases
- Cathepsins
- Serine Proteases
- Necrosis
- Pyroptosis
- Autophagy
- Cytotoxicity
- Mitochondria
- Lysosomes
- Oxidative Stress
Thank You!

Technical Support
Fast Shipping
Conferences
ISO 9001:2015
Woman owned
Founded 1994

ImmunoChemistry Technologies
9401 James Ave S. #155, Bloomington, MN 55431 USA
952-888-8788 | 1-800-829-3194 | Fax: 952-888-8988
www.immunochemistry.com help@immunochemistry.com