Polychromatic Immunophenotyping Reveals the Complexity of Regulated Cell Death Processes

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Regulated Cell Death - RCD

- Classic apoptosis, RIP1-dependent apoptosis, necroptosis, parthanatos, DNA damage, pyroptosis, autophagy

- Accidental cell death - Oncosis

- Programmed cell death – developmental & homeostatic apoptosis

- Senescence & ER Stress?
  - Simultaneous flow cytometric immunophenotyping of necroptosis, apoptosis and RIP1-dependent apoptosis.
  - Lee HL¹, Pike R², Chong MHA¹, Vossenkamper A¹, Warnes G³.
  - Flow cytometry work was performed on a ACEA Bioscience Novocyte 3000

Nomenclature Committee on Cell Death - NCCD 2015
MINIREVIEW Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells Susan L. Fink1 and Brad T. Cookson2*

Mechanisms of some forms of cell death

**Diagram:**
- Normal Cell
- Death stimulus
- Apoptosis
  - Initiator caspase
  - Effector caspase
  - Substrate cleavage
  - DNA damage
  - Apoptotic bodies
- Autophagy
  - Lysosomal degradation
  - Autophagic vacuoles
- Oncosis
  - +/- DNA damage
  - Organelle swelling
  - Inflammatory cytokines
- Pyroptosis
  - Caspase-1
  - DNA damage
- Loss of Membrane Integrity
- Necrosis
  - Inflammatory contents
  - Apoptotic necrosis
  - Inflammatory cytokines

**Keywords:**
- PS Flipping
- Mitochondrial Dysfunction
- Caspase 3 activation
- LC3B – marker of Autophagy
- Mitochondrial Dysfunction
- Caspase-1 activity

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MINIREVIEW Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells Susan L. Fink1 and Brad T. Cookson2*
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Cell Death Phenotypes of TNF Induced Apoptosis, RIP1 Dependent Apoptosis & Necroptosis

RIP1 Dependent Apoptosis

Necroptosis

Apoptosis

Regulated necrosis: disease relevance and therapeutic opportunities

Marcus Conrad *, José Pedro Friedmann Angell *, Patrick Vandendaelme ** and Brent R. Stockwell
Programmed Necrosis – Necroptosis and Parthanatos

RIP3 up-regulation

Parthanatos: mitochondrial-linked mechanisms and therapeutic opportunities
Amos A Fatokun, Valina L Dawson and Ted M Dawson

The Endoplasmic Reticulum Stress & Autophagy

**ER Stress**

- ER stress
- Starvation
- TOR
- Atg1
- Autophagy
- Autophagosome
- Misfolded protein
- ER stress sensor

(Yorimitsu and Klionsky, 2007)

**Autophagy**

- Endosome
- Amphisome
- Lysosome
- Autophagosome
- Autolysosome
- Plasma membrane
- Initiation
- Elongation
- Closure
- Maturation
- Degradation

(Yang and Klionsky, 2010)
Biological Markers of Cell Death

- Apoptosis – Active Caspase-3 also RIP3<sup>-ve</sup>
- RIP1-dependent apoptosis – Upregulated RIP3/Caspase-3<sup>+ve</sup>
- Necroptosis - Upregulated RIP3/Caspase-3<sup>-ve</sup>
- Parthanatos – PARP
- DNA damage – H2AX
- Pyrotosis – Active Caspase-1
- Autophagy – Upregulated LC3B

- Accidental cell death – Oncosis – Marker?

- ER Stress - PERK, misfolded proteins, reticulophagy

- Usually analysed by Western Blot or Fluorescent microscopy
Drugs

- Apoptosis – Etoposide (1 µM), Staurosporine, UV irradiation
- Necroptosis – TNFα, Shikonin (0.5 µM),
- RIP1-dependent apoptosis - TNFα, Shikonin?
- Parthanatos – ?
- DNA Damage – UV irradiation
- Pyroptosis - infection
- Autophagy – Rapamycin & Chloroquine CQ (100 µM),
- ER Stress - Thapsigargin (7 µM Tg)
- Accidental Death – Oncosis - 0.25% Sodium Azide
Methodology

- After (24 h) induction of cell death label with Zombie NIR
- Fix with CalTag Fix Soln A for 15 min at RT
- Perm with 0.25% Triton X-100 for 15 min at RT
- Label with 1:400 Dilution of anti-LC3B
- Label with anti-rabbit AF-647
- Label with: PERK-AF488 (ER Stress); RIP3-PE (Necroptosis);
  PARP-PE-CF-595 (Parthanatos); H2AX-PeCy7 (DNA Damage);
  Active Caspase-3-BV650 (Apoptosis); LC3B-AF647 (Autophagy)

Flow Cytometry collected 100,000 events on a ACEA Bioscience Novocyte 3000. Analysis and single colour compensation performed on NovoExpress software version 1.2

Imaging Flow Cytometry, Xcyto10, Chemometec – 5 LED, 36 parameters, x20 magnification
Flow Cytometric Gating of RCD I

Untreated cells

Dead cells

Live cells

Autophagy-ER stress

DNA Damage-Apoptosis

RIP1 Dep-APO-Apoptosis

Parthanatos-DNA Damage

Apoptosis-Parthanatos

Parthanatos-DNA Damage-Apoptosis

Caspase-3-BV560
Zombie NIR
PARP-PeCF595
H2AX-PeCy7
LC3B-AF647

Live cells

Dead cells

Untreated cells
Flow Cytometric Gating of RCD II

Dead cells

- Dead necroptosis: 3.28% MFI 48,909±5,934
- Dead-RIP1 Dep-AP: 5.54% MFI 64,809±4,934
- Autophagy: 54.82% MFI 271±5.5%
- Apoptosis: 1811.3% MFI 5,5298±5,948
- RIP1 Dep Apoptosis: 15.312.6% MFI 46,809±4,934
- Necroptosis: 42.06% MFI 271±5.5%
- Double Negative: 42.06% MFI 271±5.5%

Live cells

- Necroptosis: 2.82% MFI 48,909±5,934
- RIP1 Dep-AP: 2.68% MFI 64,809±4,934
- Autophagy: 54.82% MFI 271±5.5%
- Apoptosis: 1811.3% MFI 5,5298±5,948
- RIP1 Dep Apoptosis: 15.312.6% MFI 46,809±4,934
- Autophagy: 2.82% MFI 48,909±5,934
- Necroptosis: 2.82% MFI 48,909±5,934
- Double Negative: 2.82% MFI 48,909±5,934

MFI values:
- Live cells: 30,296±5,948
- Dead cells: 46,809±4,934
Flow Cytometric Gating of RCD III

Dead Autophagic Cells

Parthanatos-
DNA Damage

Apoptosis-
DNA Damage

Apoptosis-Parthanatos

Necroptosis-
Apoptosis

Live Autophagic Cells

Parthanatos-
DNA Damage

Apoptosis-
DNA Damage

Apoptosis-Parthanatos

Necroptosis-
Apoptosis
Polychromatic Analysis of Apoptosis I

More cell death±Caspase-3
More Parthanatos±Caspase-3
More DNA Damage with Caspase-3
More DNA Damage with Parthanatos

Caspase-3-BV650
Zombie NIR
RIP3-PE
Caspase-3-BV650
Zombie NIR
PARP-PE-CF595
Caspase-3-BV650

Apoptotic Caspase-3\(^{\text{ve}}\) are RIP3\(^{\text{ve}}\)
All Caspase-3\(^{\text{ve}}\) are PARP\(^{\text{ve}}\)
Half of Caspase-3\(^{\text{ve}}\) have DNA Damage
More Parthanatos±DNA Damage

Parthanatos-DNA Damage
36±5%
35±2.5%
7±1.8%
***

DNA Damage-Apoptosis
36±5%
31±4.4%
9±1.9%
***

Apoptosis-Parthanatos
38±7.4%
5±1.7%
43±6.4%
25±5.3%
*

RIP1 Dep-APO-Apoptosis
22±2.8%
53±4.4%
28±5%
9±1.9%

DNA Damage-Apoptosis
22±2.8%
53±4.4%
28±5%
9±1.9%

Autophagy-ER stress
20.91% 6.25%
10.9%

DNA Damage-Apoptosis
26±2.1% 8.05%
31±11.6%

RIP1 Dep-APO-Apoptosis
26±2.1% 8.05%
31±11.6%

Apoptosis-Parthanatos
38±7.4%
5±1.7%
43±6.4%
25±5.3%
*

** P<0.01, *** P<0.001

More cell death±Caspase-3
More Parthanatos±Caspase-3
More DNA Damage with Caspase-3
More DNA Damage with Parthanatos

Caspase-3-BV650
Zombie NIR
RIP3-PE
Caspase-3-BV650
Zombie NIR
PARP-PE-CF595
Caspase-3-BV650

Apoptotic Caspase-3\(^{\text{ve}}\) are RIP3\(^{\text{ve}}\)
All Caspase-3\(^{\text{ve}}\) are PARP\(^{\text{ve}}\)
Half of Caspase-3\(^{\text{ve}}\) have DNA Damage
More Parthanatos±DNA Damage

Parthanatos-DNA Damage
13±1.9%
46±2.6%
35±2.5%
7±1.8%
**

DNA Damage-Apoptosis
13±4%
12±3.1%
***

Apoptosis-Parthanatos
38±7.4%
5±1.7%
43±6.4%
25±5.3%
*

RIP1 Dep-APO-Apoptosis
3±1%
54±6.1%
41±3.1%
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
7±1.8%
***

MFI
36,394±3,762
MFI
29,899±8,389
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
7±1.8%
***

MFI
36,394±3,762
MFI
29,899±8,389
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
7±1.8%
***

MFI
36,394±3,762
MFI
29,899±8,389
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
7±1.8%
***

MFI
36,394±3,762
MFI
29,899±8,389
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
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MFI
36,394±3,762
MFI
29,899±8,389
NS

DNA Damage-Apoptosis
32±1.5%
11±1.8%
36±5%
35±2.5%
7±1.8%
***

MFI
36,394±3,762
MFI
29,899±8,389
NS
Polychromatic Analysis of Apoptosis II

Dead Apoptotic cells

RIP1 Dep-Apoptosis

Apoptosis

Necroptosis

Double Negative

Live Apoptotic cells

RIP1 Dep-Apoptosis

Apoptosis

Necroptosis

Double Negative

Live Apoptotic cells

Dead Apoptotic cells

All phenotypes had more Parthanatos with DNA Damage
Necroptosis, RIP1 Dependent Apoptosis, DN had more Parthanatos
Necroptosis had more DNA Damage

* P < 0.05, ** P < 0.01, *** P < 0.001

P > 0.05 NS, P < 0.05 *, P < 0.01 **, P < 0.001 ***
Polychromatic Analysis of Necroptosis I

Necroptosis

Dead cells

Live cells

Autophagy-ER stress

DNA Damage-Apoptosis

RIP1 Dep-APO-Apoptosis

Parthanatos-DNA Damage

Apoptosis-Parthanatos

More cell death±Caspase-3
More Parthanatos
More Parthanatos with DNA Damage & Caspase-3
More DNA Damage with Caspase-3

RIP3 is 30% up-regulated – defines necroptosis
Apoptotic Caspase-3±ve are RIP3±ve
More apoptosis & RIP1-dependent apoptosis
All Caspase-3±ve are PARP±ve
More DNA Damage with PARP & Caspe-3
Less DNA Damage

P>0.05 NS, P<0.05*, P<0.01**, P<0.001***

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RIP1-dependent apoptotic cells showed more Parthanatos±DNA Damage & less DNA Damage
DN showed more Parthanatos

Necroptotic cells showed more Parthanatos ±DNA Damage
RIP1-dependent apoptotic cells showed more Parthanatos with DNA Damage
Apoptotic cells showed more Parthanatos ±DNA Damage
Polychromatic Analysis of Autophagy I

More autophagy
Less DNA Damage
More DNA Damage±Caspase-3,
More Parthanatos without Caspase-3
Less Parthanatos with Caspase-3

More autophagy
Less DNA Damage
More autophagy
Less DNA Damage

P>0.05 NS, P<0.05*, P<0.01**, P<0.001***
Polychromatic Analysis of Autophagy II

**Dead cells**
- Autophagy: 51±7.2% 48±1.2% 22±2.5% 22±1.8% 51±0.7%
- Necroptosis: 31±6.3% 33±4.2% 68±5.7% 7±0.7% 13±5.4%
- RIP1 Dep-Apoptosis: 35±10% 40±1.9% 8±0.7%
- Apoptosis: 12±4.6% 14±6.1%
- Double Negative: 35±12% 48±9.2%

**Live cells**
- Autophagy: 811±8% 75±13% 32±0.5% 3±0.9% 86±3%
- Necroptosis: 38±1.9% 3±0.15% 0.4±0.15% 0.4±0.1% 0.4±0.01%
- RIP1 Dep-Apoptosis: 21±2.8% 28±5.1% 48±9.2%
- Apoptosis: 14±16% 34±10%
- Double Negative: 37±2.8% 8±3.2%

**Live cells**
- Autophagy & Necroptotic phenotype had less DNA Damage
- RIP1-dependent apoptotic phenotype had less Parthanatos with DNA Damage
- Apoptotic phenotype had more Parthanatos with DNA Damage less without DN had less Parthanatos

**Dead cells**
- Autophagy, Apoptosis & DN phenotypes reduced Parthanatos but increased DNA Damage
- Necroptotic, RIP1-Dep-APO showed more DNA damage

- Dead cells: 51±7.2% 48±1.2% 22±2.5% 22±1.8% 51±0.7%
- Live cells: 811±8% 75±13% 32±0.5% 3±0.9% 86±3%

**P-values**
- P>0.05 NS, P<0.05*, P<0.01**, P<0.001***

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Polychromatic Analysis of Autophagy III

**Live Autophagic Cells**

- Autophagy reduced DNA Damage
- Also reduced Apoptosis and RIP1-Dependent Apoptosis phenotypes

**Dead Autophagic Cells**

- Autophagy increased DNA Damage
- with or without Caspase-3,
- Also increased Necroptosis

*P* >0.05 NS, *P* <0.05*, *P* <0.01**, *P* <0.001***
Conclusions

- Live & dead cells in apoptotic cultures showed that Caspase-3$^{\text{+ve}}$ are mostly RIP3$^{-\text{ve}}$ and PARP$^{\text{+ve}}$
- Live & dead Apoptotic, Necroptotic, DN & RIP1-dependent apoptotic phenotypes had more parthanatos with DNA damage after induction of apoptosis
- Shikonin induced necroptosis in live cell population defined by an up-regulation of RIP3 (30%); and RIP-1 dependent apoptosis both of which had more parthanatos with DNA damage
- All live caspase-3 cells were PARP$^{\text{+ve}}$ with more DNA Damage than controls
- All dead necroptotic & apoptotic cell phenotypes had more parthanatos ±DNA Damage, while RIP1-dependent apoptosis had more parthanatos with DNA Damage
- Confirmation that autophagy maintains cell health by reducing DNA Damage in live cells. Dead autophagic cells showed less parthanatos but had more DNA Damage
Further Work

- Have tested for ACD and ER stress
- Used apoptosis & necroptosis blockers zVAD and necrostatin-1
- Does prior induction of autophagy protect against other forms of RCD like it does with ER Stress
- Measure Pyrotosis – active caspase-1
- Thank you to Chemometec for the Image flow cytometer Xcyto demo
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