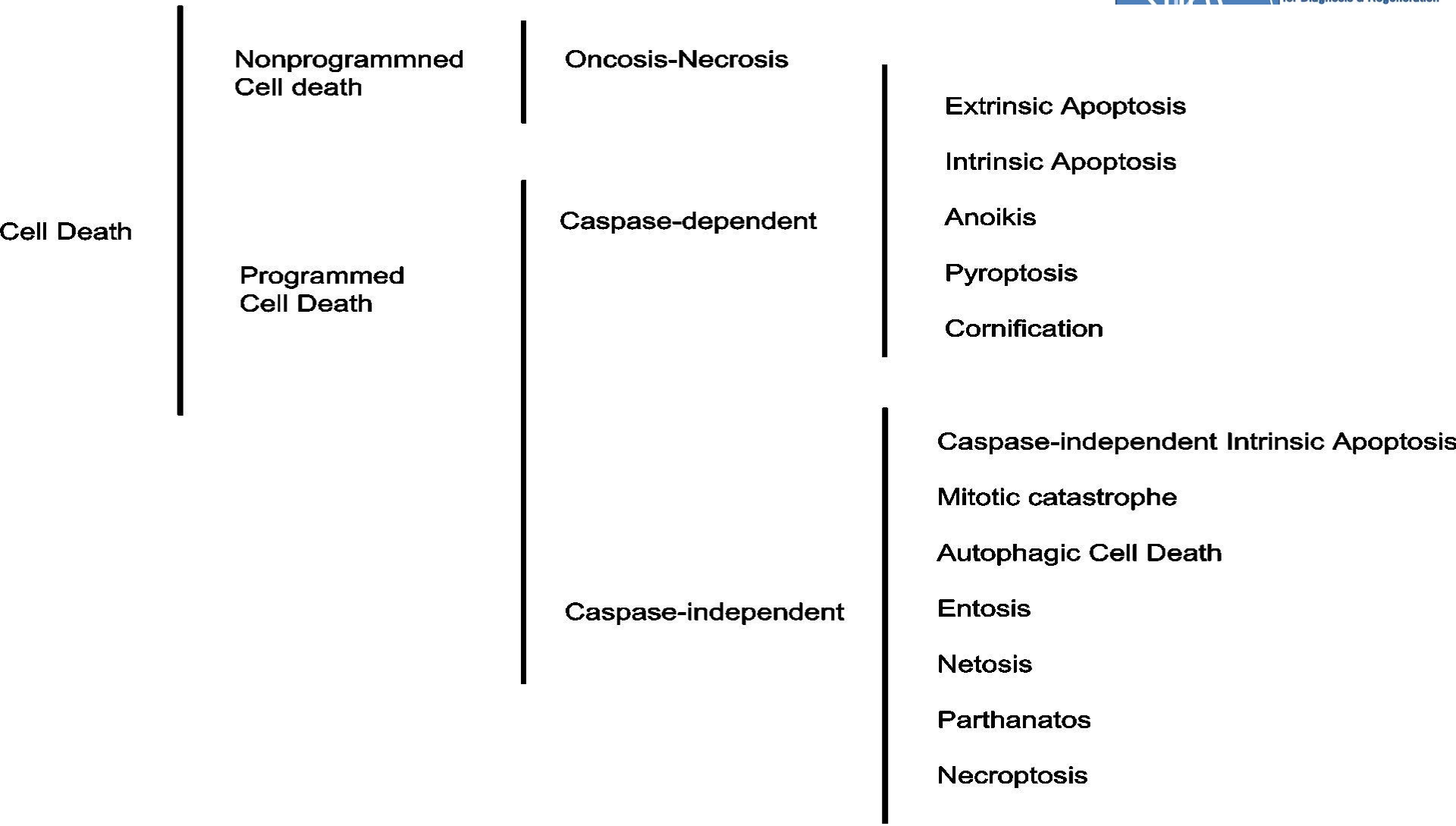
Ankersmit Laboratory
for Diagnosis & Regeneration in Thoracic Diseases & Applied Immunology

Role for RIP1 in mediating necroptosis in experimental intracerebral hemorrhage model both in vivo and in vitro

Haitao Shen, Chenglin Liu et al.

Cell death



Biochemistry, Genetics and Molecular Biology » "Apoptosis and Medicine", book edited by Tobias M. Ntuli, ISBN 978-953-51-0701-9, Published: August 16, 2012 under CC BY 3.0 license. © The Author(s). Cell Death and Cancer, Novel Therapeutic Strategies By Silvina Grasso, M. Piedad Menéndez-Gutiérrez, et al DOI: 10.5772/51285

Anoikis

- cytoskeleton is composed of microtubules, microfilaments and intermediate filaments
- Adherent cells become detached from the extracellular matrix or neighbouring cells
- caspase-dependent cell death called anoikis
- cytoskeleton components can modulate mitochondria

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Parthanatos

- Poly-ADP-Ribose Polymerase PARP synthesis is activated when DNA is fragmented in the presence of nuclear poly-ADP ribosylated proteins
- regulated necrosis in which PARP activation plays an important role
- PARP proteolysis facilitates nuclear disorganization and ensures irreversibility of the apoptotic process
- chromatin condensation and DNA fragmentation

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Cell death

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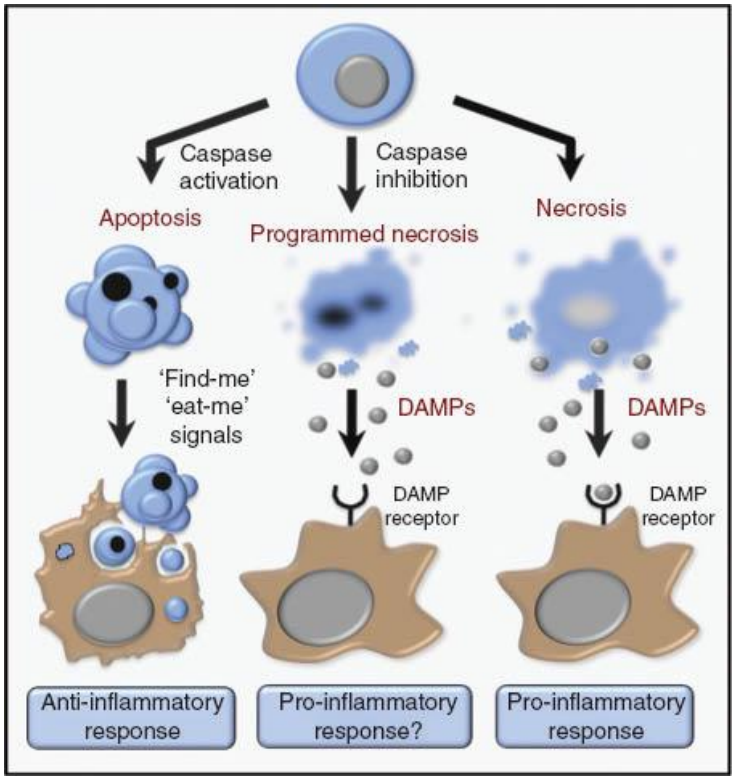
Table1 | **Characteristics of different types of cell death**

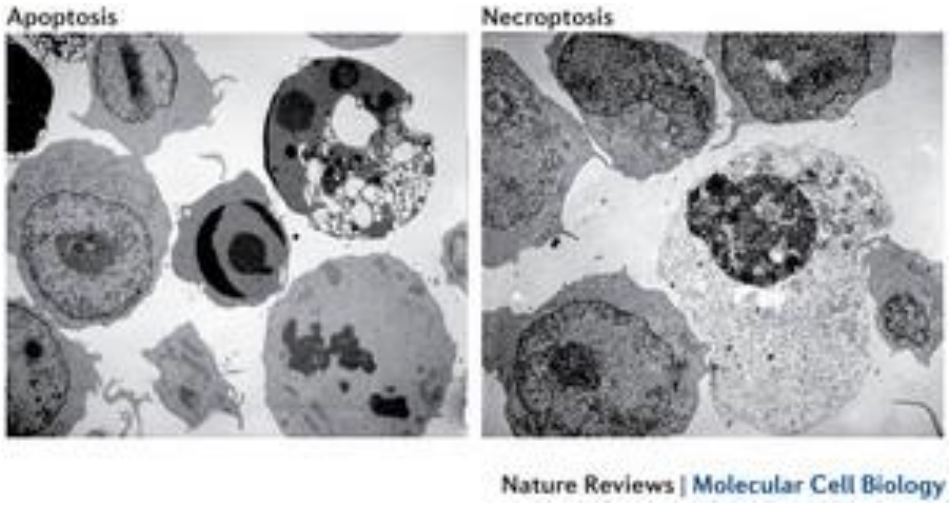
Type of cell death	Morphological changes			Biochemical features	Common detection methods
	Nucleus	*Cell membrane*	*Cytoplasm*		
Apoptosis	Chromatin condensation; nuclear fragmentation; DNA laddering	Blebbing	Fragmentation (formation of apoptotic bodies)	Caspase-dependent	Electron microscopy; TUNEL staining; annexin staining; caspase-activity assays; DNA-fragmentation assays; detection of increased number of cells in subG1/G0; detection of changes in mitochondrial membrane potential
Autophagy	Partial chromatin condensation; no DNA laddering	Blebbing	Increased number of autophagic vesicles	Caspase-independent; increased lysosomal activity	Electron microscopy; protein-degradation assays; assays for marker-protein translocation to autophagic membranes; MDC staining
Mitotic catastrophe	Multiple micronuclei; nuclear fragmentation	–	–	Caspase-independent (at early stage) abnormal CDK1/cyclin B activation	Electron microscopy; assays for mitotic markers (MPM2); TUNEL staining
Necrosis	Clumping and random degradation of nuclear DNA	Swelling; rupture	Increased vacuolation; organelle degeneration; mitochondrial swelling	–	Electron microscopy; nuclear staining (usually negative); detection of inflammation and damage in surrounding tissues
Senescence	Distinct heterochromatic structure (senescence-associated heterochromatic foci)	–	Flattening and increased granularity	SA-β-gal activity	Electron microscopy; SA-β-gal staining; growth-arrest assays; assays for increased p53, INK4A and ARF levels (usually increased); assays for RB phosphorylation (usually hypophosphorylated); assays for metalloproteinase activity (usually upregulated)

CDK1, cycline-dependent kinase 1; MDC, monodansylcadaverine; MPM2, mitotic phosphoprotein 2; SA-β-gal, senescence-associated β-galactosidase; RB, retinoblastoma protein.

Pathways of apoptotic and non-apoptotic death in tumour cells, Hitoshi Okada & Tak W. Mak
Nature Reviews Cancer 4, 592-603 (August 2004) doi:10.1038/nrc1412

Necroptosis





https://www.degruyter.com/view/j/bchm.2014.395.issue-10/hsz-2014-0164/hsz-2014-0164.xml last date of access 09.10.16

Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Dimitry Ofengeim & Junying Yuan Nature Reviews Molecular Cell Biology 14, 727–736 (2013) doi:10.1038/nrm3683

Necroptosis

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Apoptosis

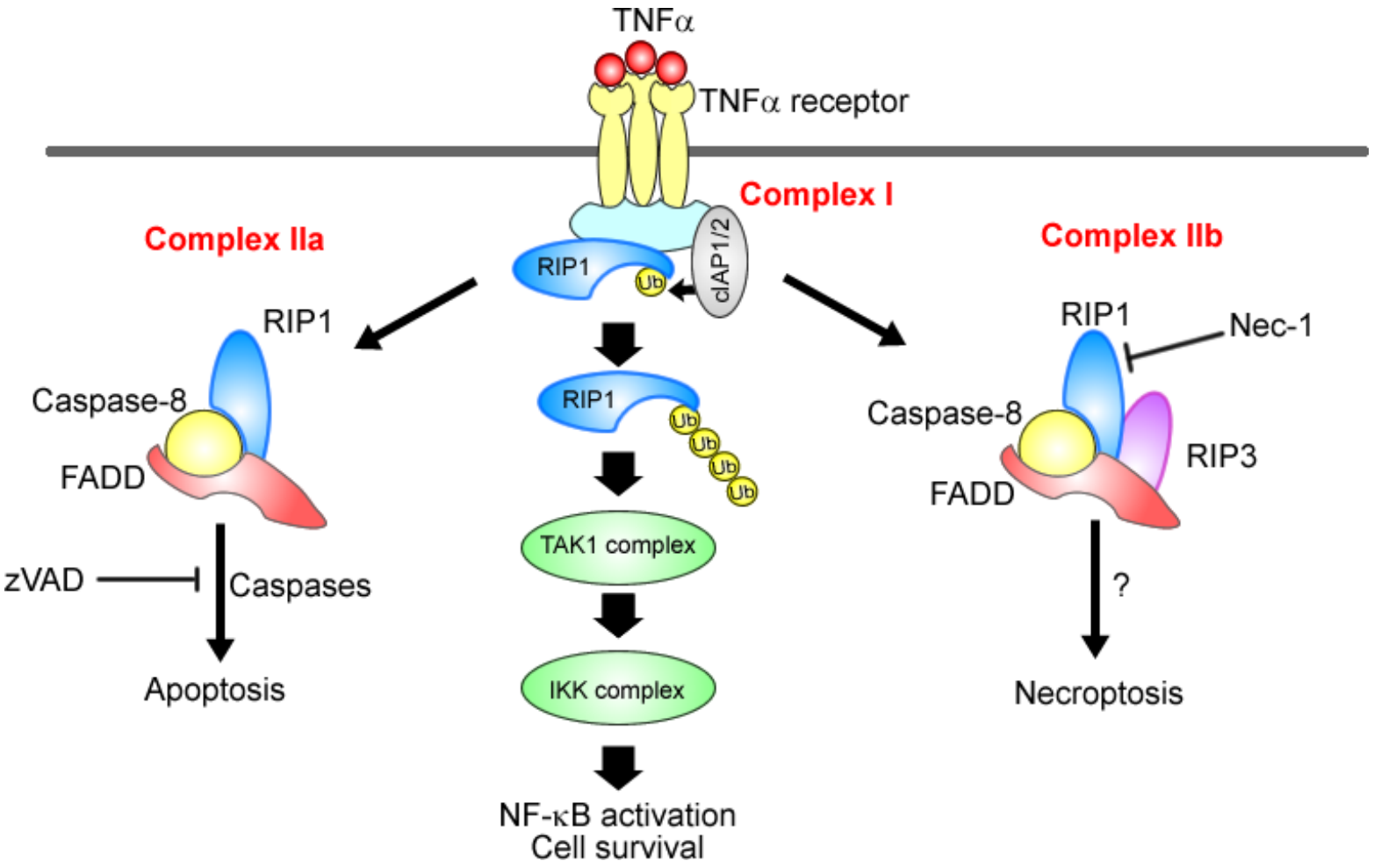
- Pyknosis
- Karyorrhexis
- Membrane blebbing
- Apoptotic bodies

Necroptosis

- Translucent cytoplasm
- Organelle swelling
- Membrane permeabilization
- Oncosis

Necroptosis and its role in inflammation. Pasparakis M, Vandenabeele P. ; Nature. 2015 Jan 15;517(7534):311-20. doi: 10.1038/nature14191.

Necroptosis



https://yuan.med.harvard.edu/discoveries
Last date of access 16.10.16

Intracerebral Hemorrhage (ICH)

- Second largest type of bleeding (15%)
- Mortality rate of ca. 40% in 1 month
- Symptoms:
  - Paralysis
  - Aphasia
  - Nerve function damage

Role for RIP1 in mediating necroptosis in experimental intracerebral hemorrhage model both in vivo and in vitro. Shen H, Liu C et al. Cell Death Dis. 2017 Mar 2;8(3):e2641. doi: 10.1038/cddis.2017.58.

Intracerebral Hemorrhage (ICH)

- Primary brain injury
  - Hematoma mass effect
  - Mechanical damage to adjacent brain tissues

- Secondary brain injury
  - Nerve function damage
  - Cell death
  - Cerebral edema
  - Blood-brain barrier damage
  - Inflammatory response
  - Proteolytic enzyme and toxic effect

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Necroptosis activation

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- TNF-α forms trimer with receptors
- RIP-1 binds on the death domain of TNF-α
- Activation via ubiquitination
- Activation of RIP 1 leads to recruitement of RIP3, MLKL and caspase-8
- Formation of the necrosome
- Overproduction of Reactive oxygen species (ROS)

  -> DNA damage, mitochondrial membrane permeability, lysosome damage, cell death

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Materials and Methods

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6-8 weeks old Sprague Dawley rats

- Experiment 1:
  - 9 groups with 6 rats
- Experiment 2:
  - 10 groups with 6 rats
- Experiment 3:
  - Six groups with 6 rats

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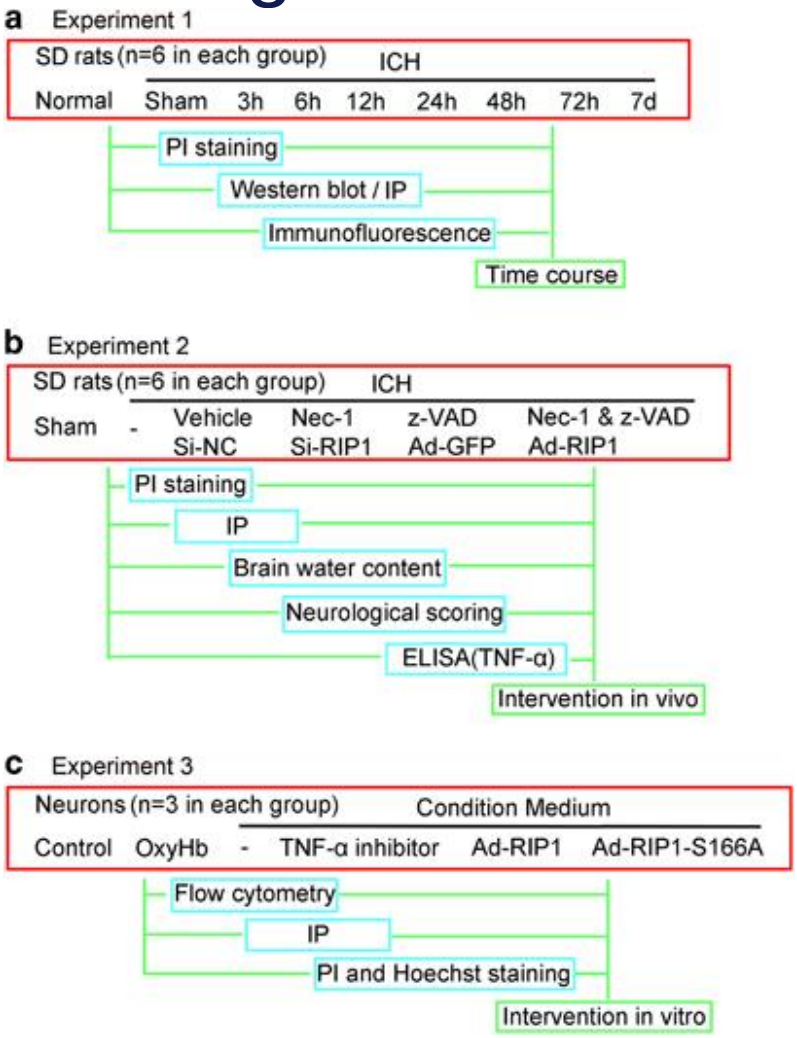
Experimental design



ICH model

- 100µl autologous blood collected from heart puncture
- Fixation in stereotaxic frame
- Drilling of a hole to right basal ganglia
  - 0.2mm anterior to the intersection between the coronal suture and sagittal midline and 3.5mm to the right sagittal suture)

- Microsyringe 5.5mm depth
- 100µl blood or NaCl injection
- Keeping the needle in the hole for 5 min
- Sealing of the hole with bone wax

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Methods

- Primary neuron- and microglial cultures
  - Neuron-enriched cultures from brains of fetal rats
  - Microglial-enriched cultures from brains of 1 day old rats

- Transfection of siRNA and adenoviruses (overexpression)

- Nec-1 and zVAD application in 3µl DMSO injected in lateral cerebral ventricle (1-2h before ICH)

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Methods

- Westernblot
- Immunoprecipitation
- Immunofluorescent staining
- PI and TUNEL staining
  - PI was injected intraperitoneally (1µg/g)
- Brain edema
- Blood brain barrier injury

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ICH model

Category	Behavior	Score
Appetite	Finished meal	0
	Left meal unfinished	1
	Scarcely ate	2
Activity	Walk and reach three corners	0
	Walk with some stimulations	1
	Almost always lying down	2
Deficits	No deficits	0
	Unstable walk	1
	Impossible to walk	2

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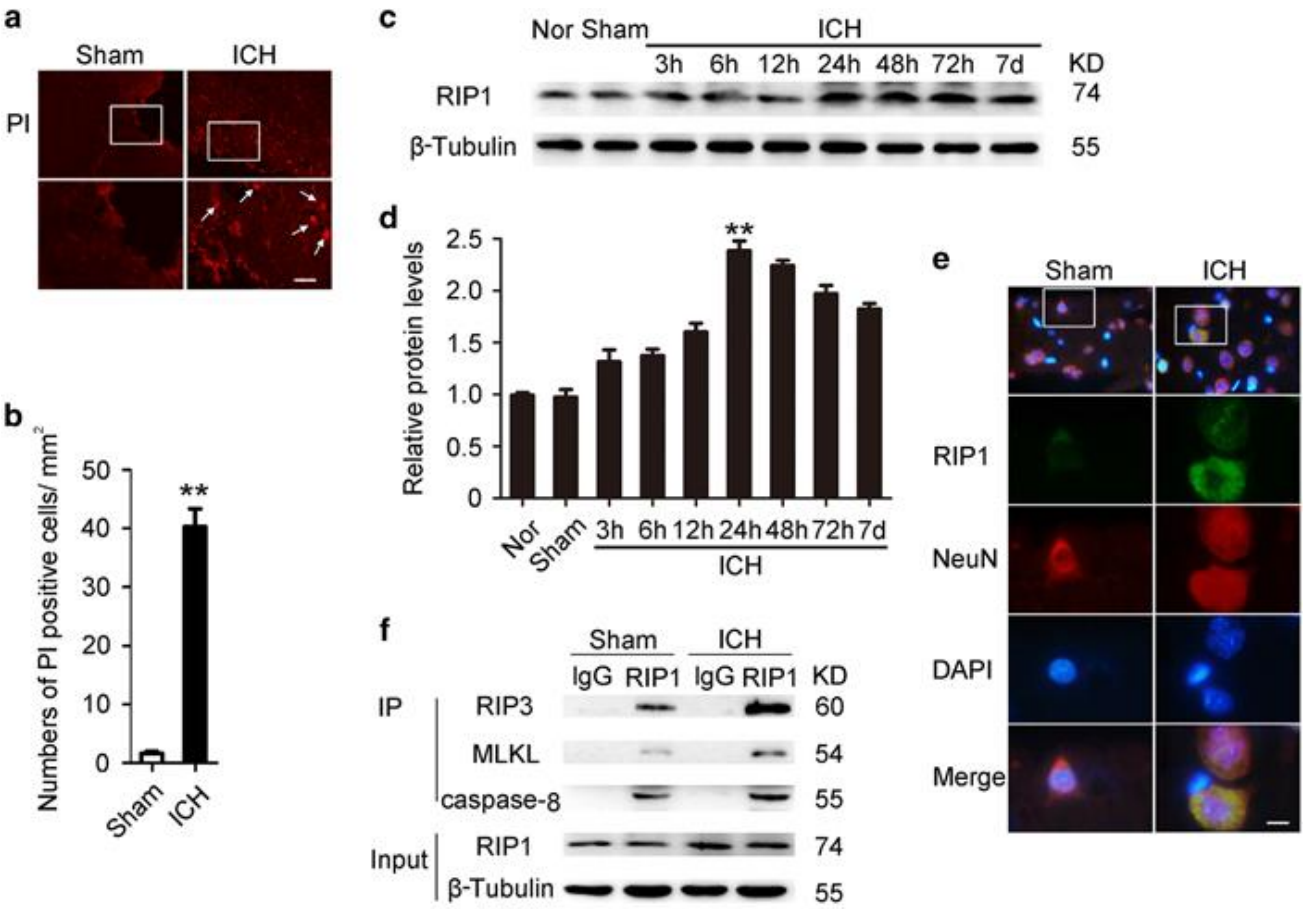
Results



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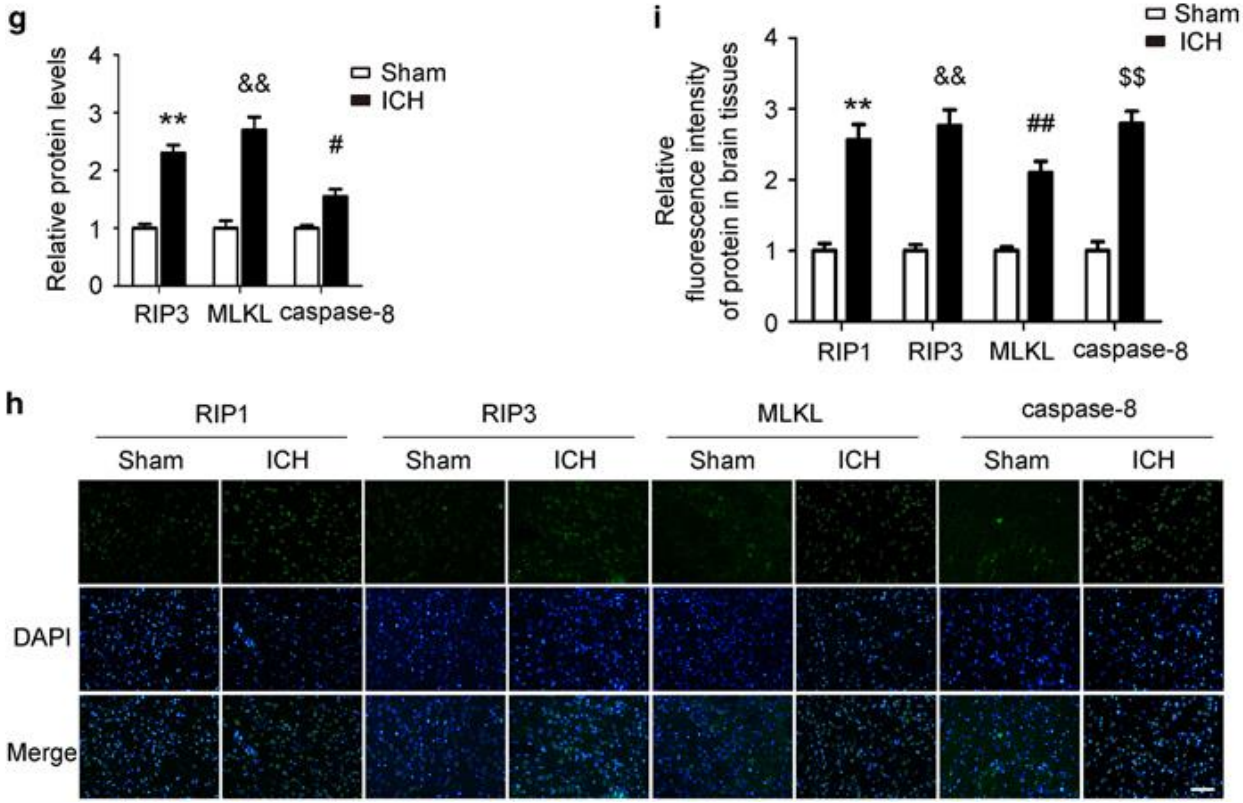
g
Relative protein levels
Sham
ICH
**
&&
#
RIP3
MLKL
caspase-8
i
Relative fluorescence intensity of protein in brain tissues
Sham
ICH
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&&
##
$$
RIP1
RIP3
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caspase-8
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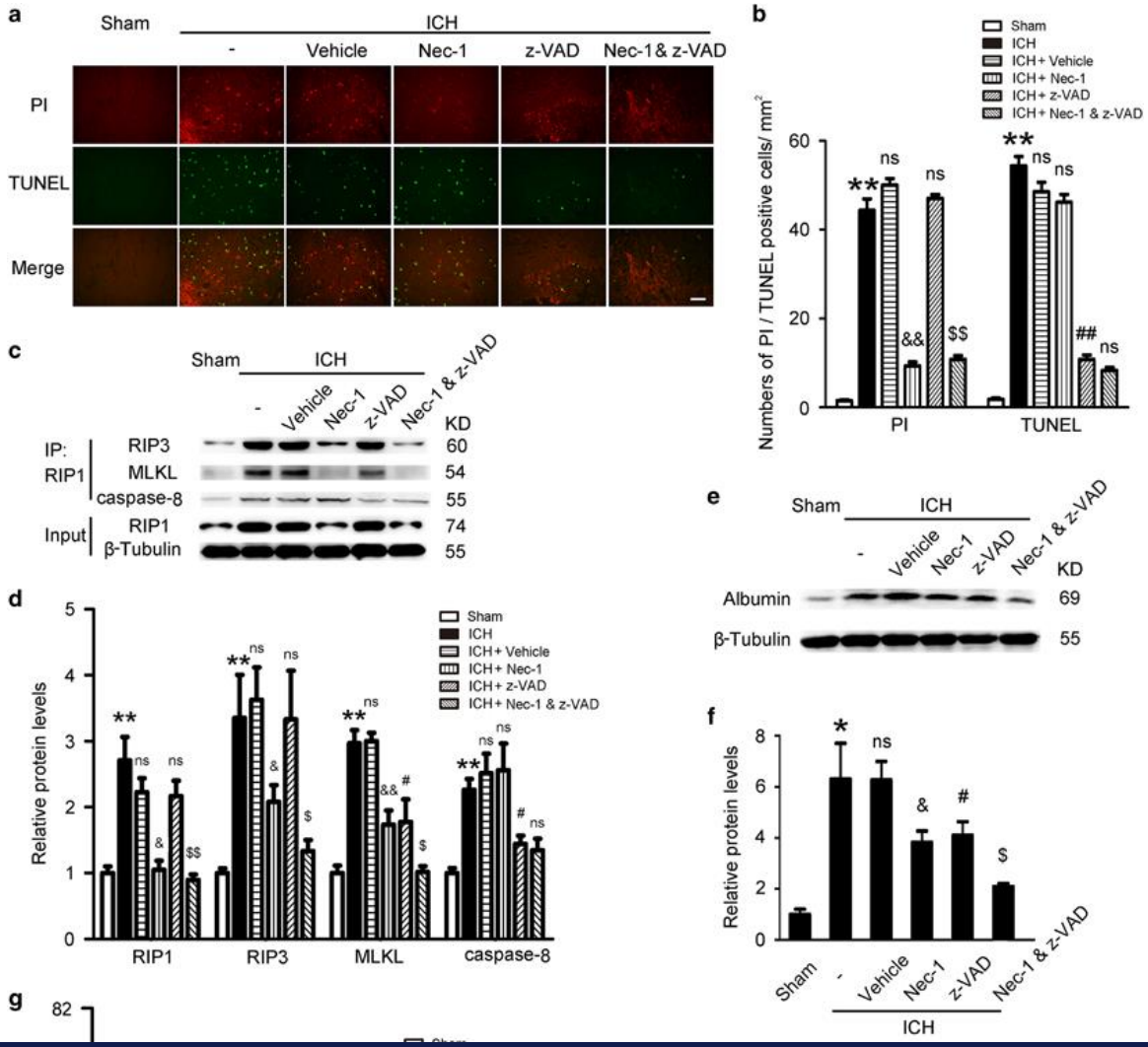
Results



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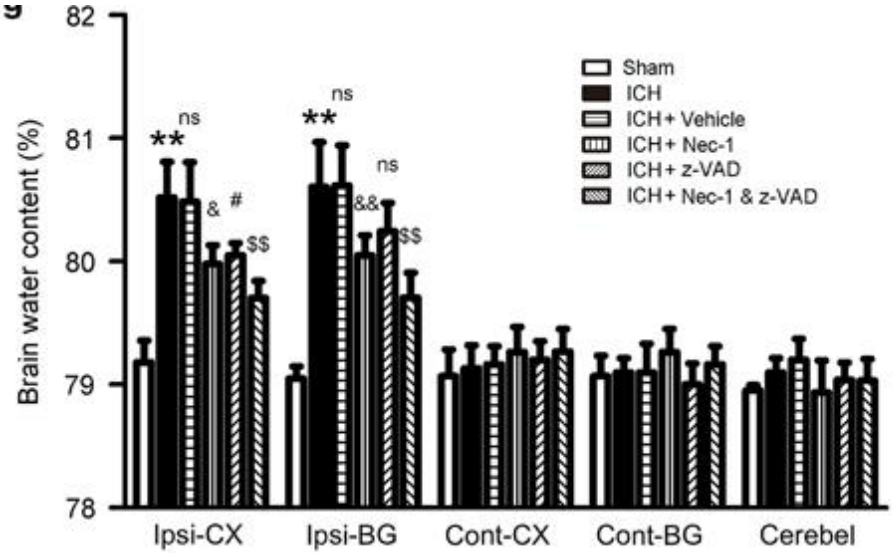

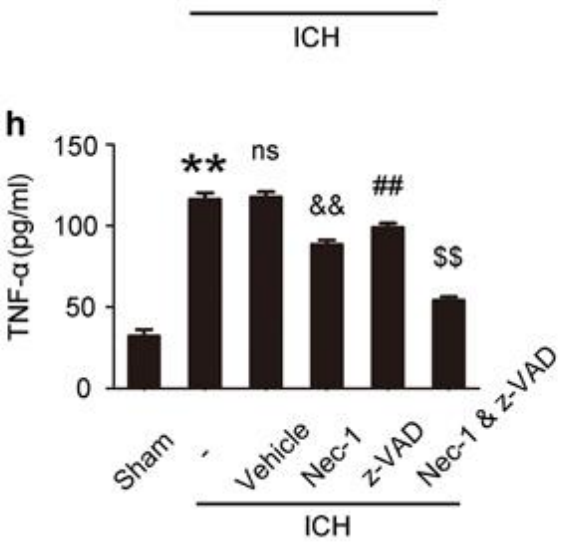

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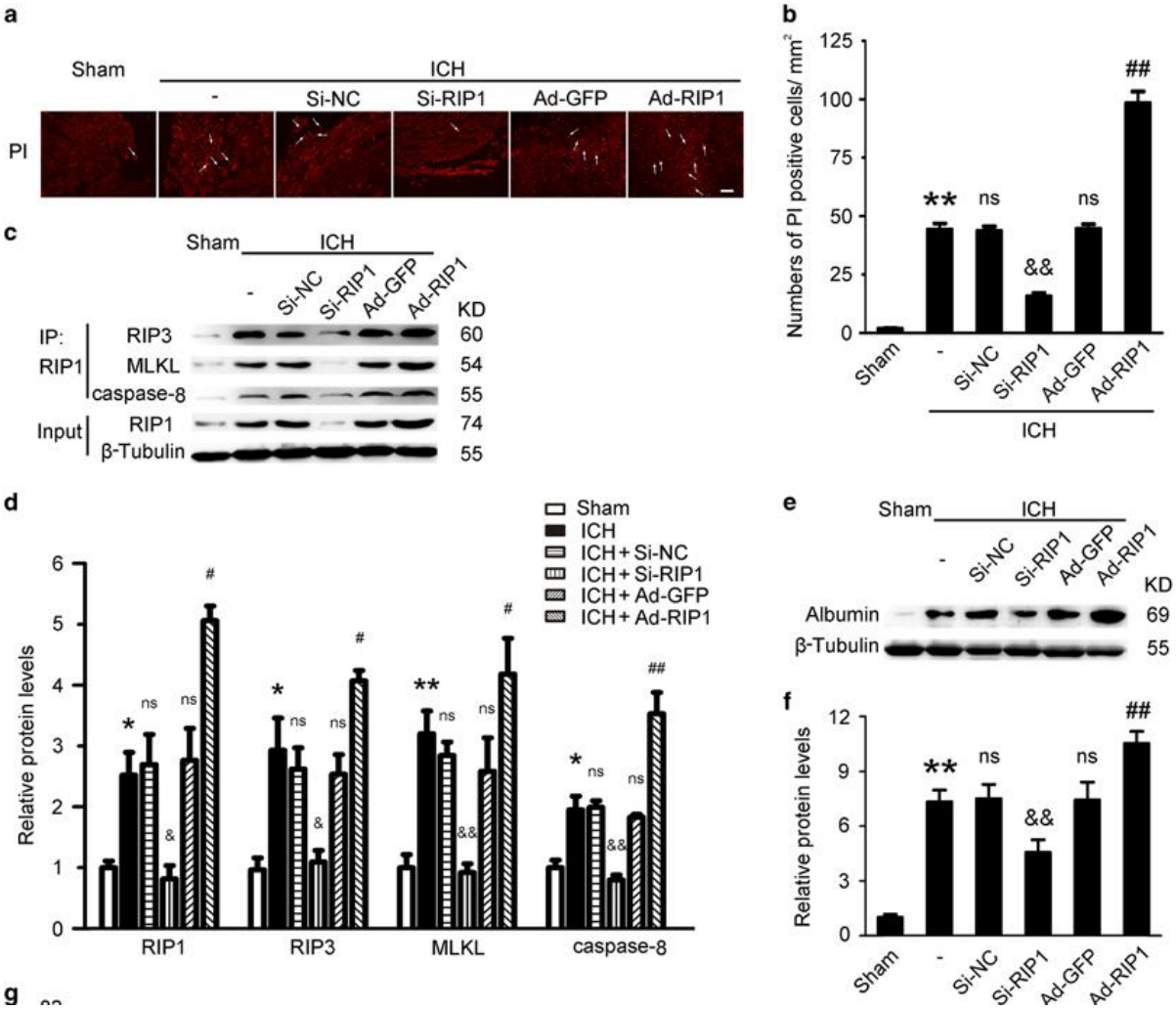
a
Sham
ICH
-
Si-NC
Si-RIP1
Ad-GFP
Ad-RIP1
PI
b
Numbers of PI positive cells/ mm2
125
100
75
50
25
0
**
ns
ns
&&
##
Sham
-
Si-NC
Si-RIP1
Ad-GFP
Ad-RIP1
ICH
c
Sham
ICH
-
Si-NC
Si-RIP1
Ad-GFP
Ad-RIP1
KD
IP:
RIP1
RIP3
MLKL
caspase-8
60
54
55
Input
RIP1
β-Tubulin
74
55
d
Sham
ICH
ICH + Si-NC
ICH + Si-RIP1
ICH + Ad-GFP
ICH + Ad-RIP1
Relative protein levels
RIP1
RIP3
MLKL
caspase-8
e
Sham
ICH
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Si-NC
Si-RIP1
Ad-GFP
Ad-RIP1
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Albumin
β-Tubulin
69
55
f
Relative protein levels
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9
6
3
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Ad-RIP1
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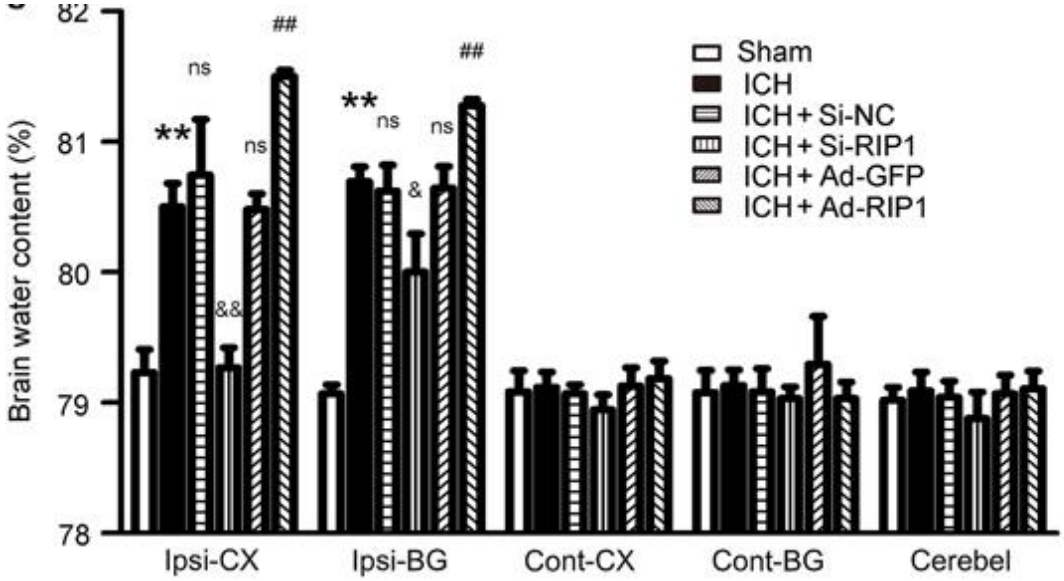
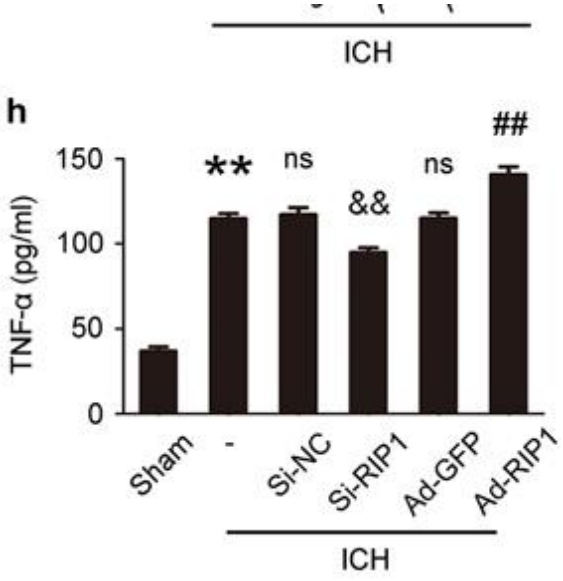
Results





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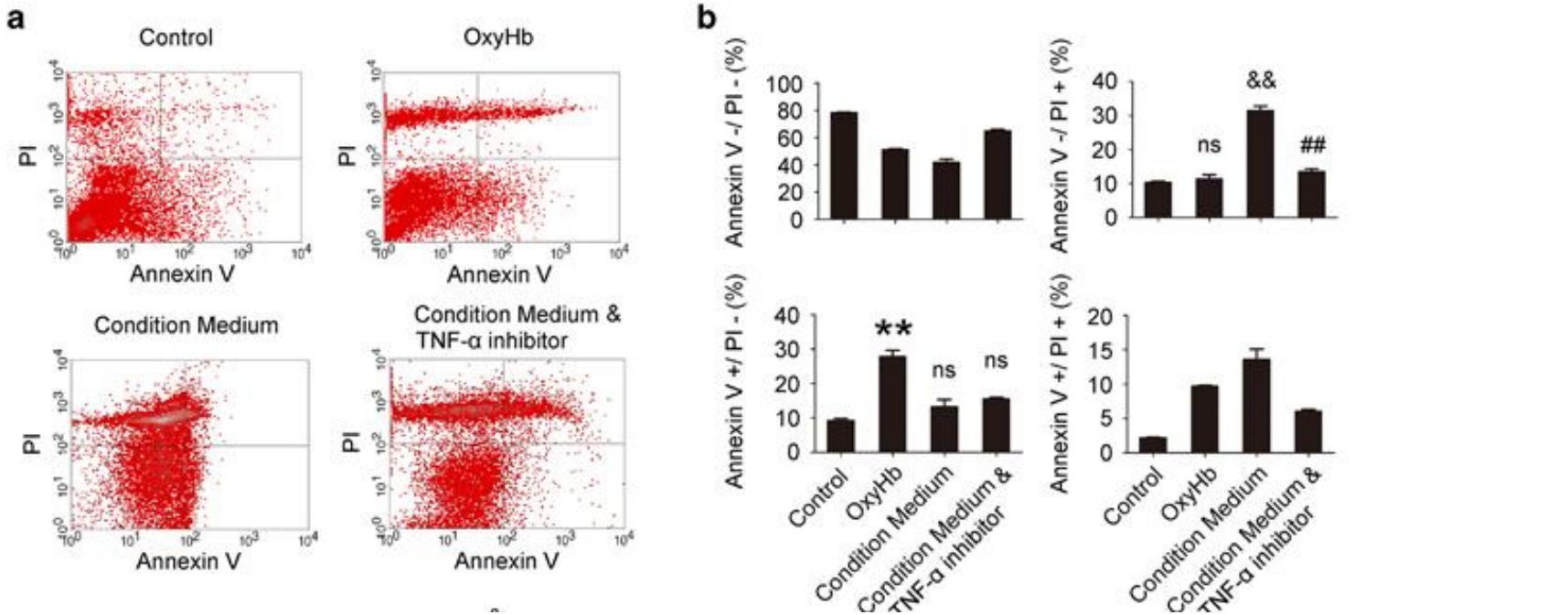

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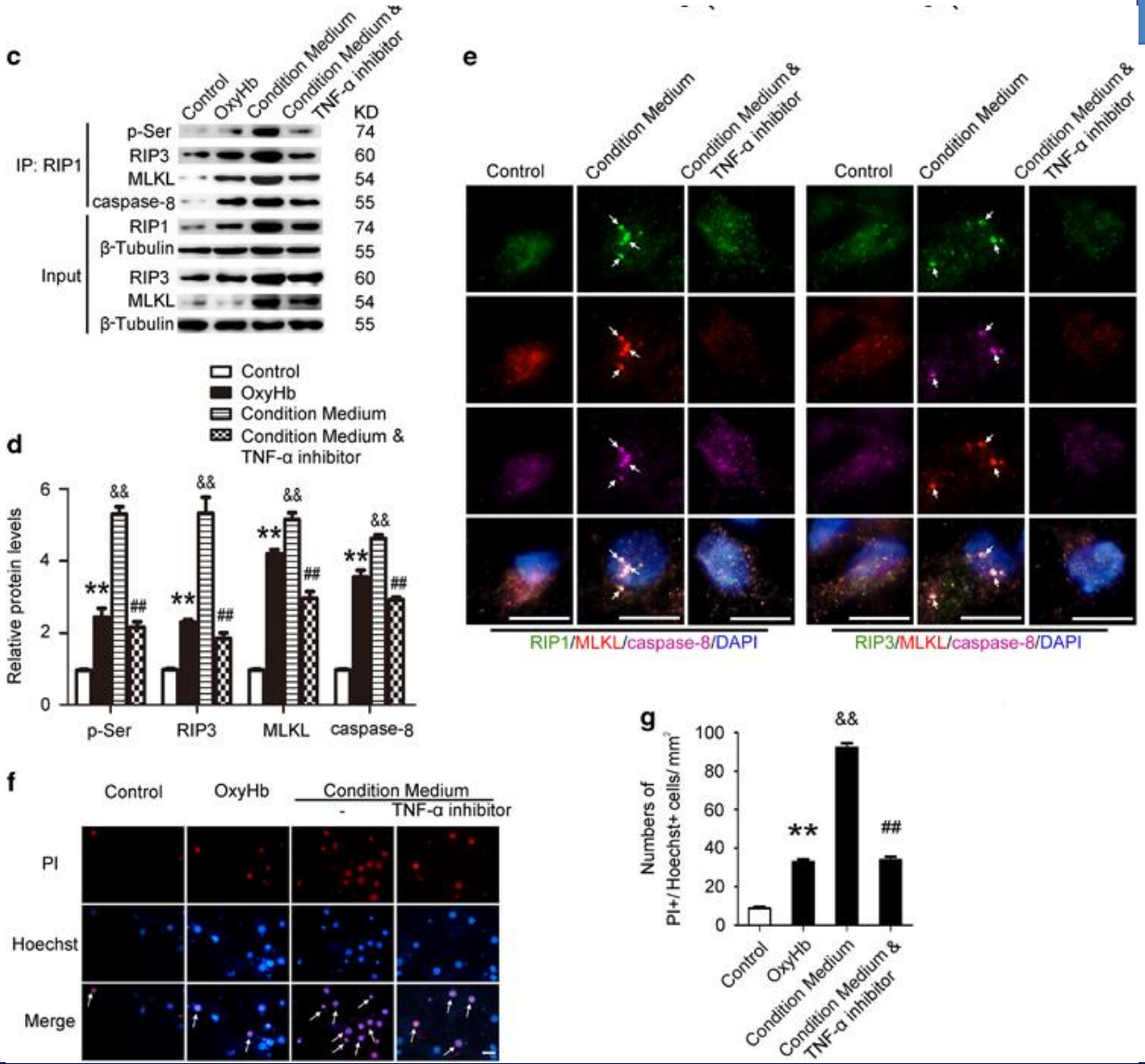

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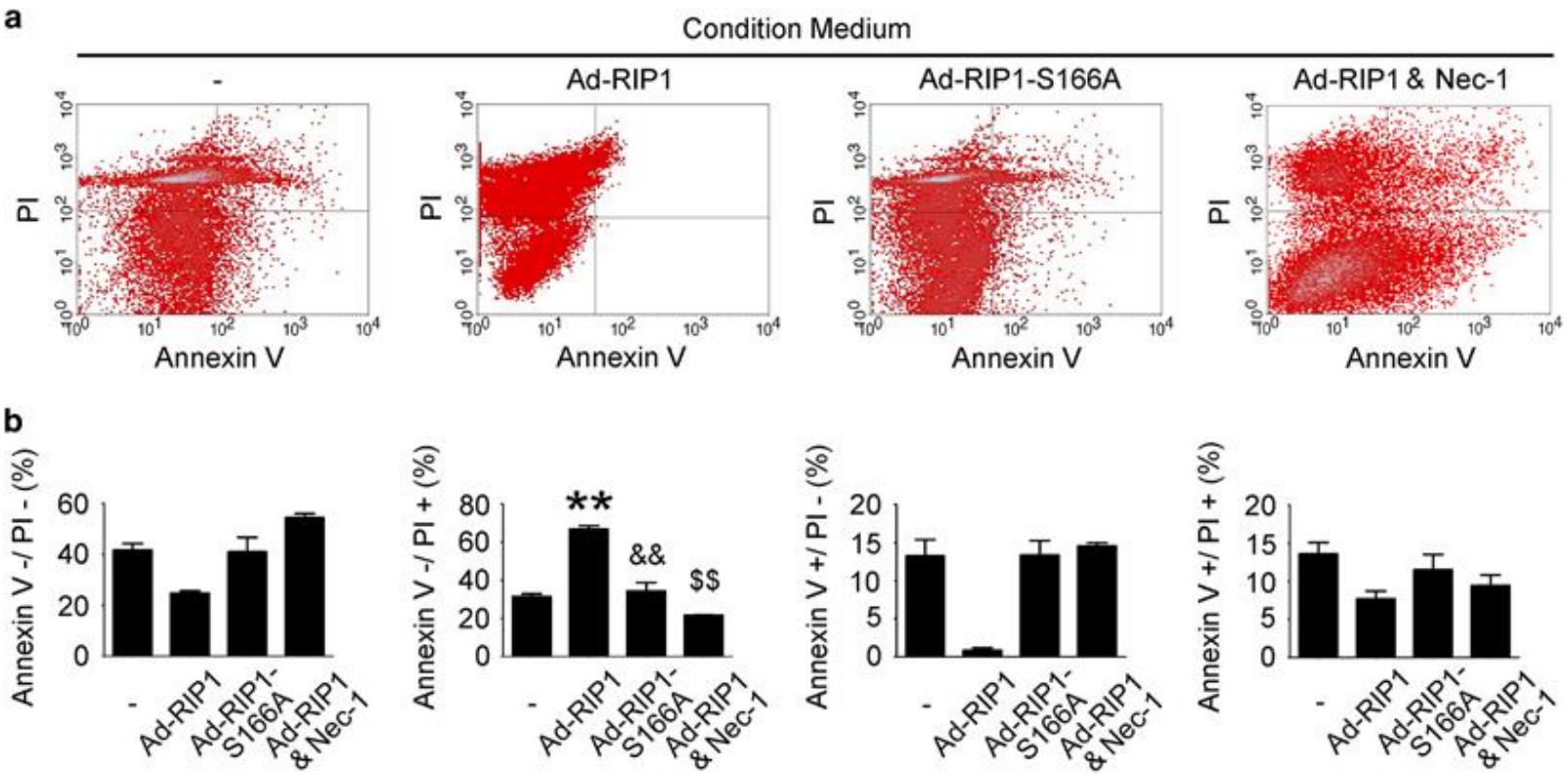

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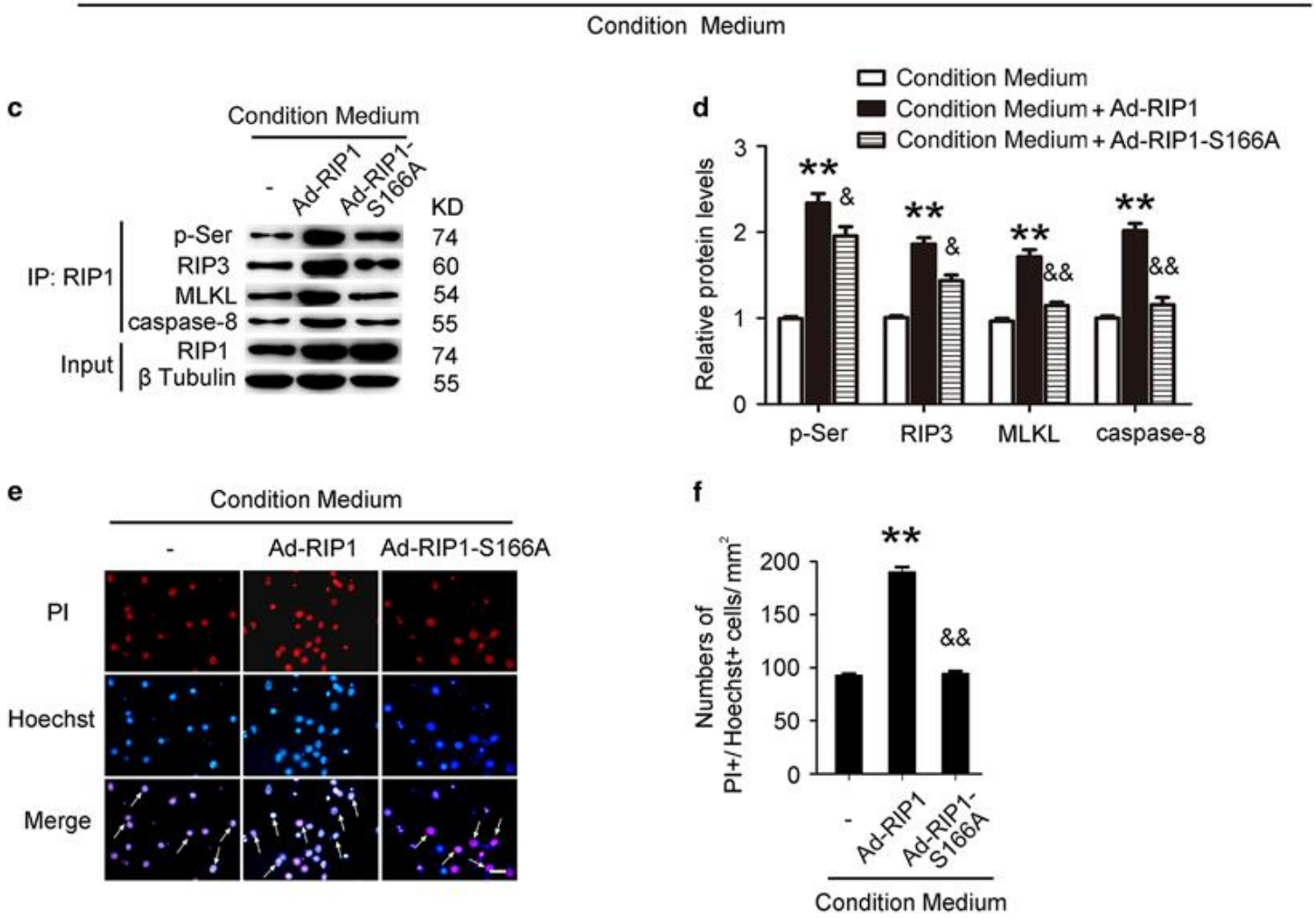

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Discussion

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- Dead necroptotic cells release danger associated molecular patterns (DAMPs) -> aggravation of secondary brain injury

- Necroptosis has an important role in neuronal dysfunction, brain edema and BBB permeability

- Necrostatin-1 can inhibit neuronal damage and BBB permeability

- Inflammatory factors secreted by microglia lead to necroptosis after ICH

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Thank you for your attention!

Necrostatin-1 protects against oleic acid-induced acute respiratory distress syndrome in rats. Pan L, Yao DC et al; Biochem Biophys Res Commun. 2016 Sep 30;478(4):1602-8. doi: 10.1016/j.bbrc.2016.08.163. Epub 2016 Aug 30