Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP3 inflammasome assembly

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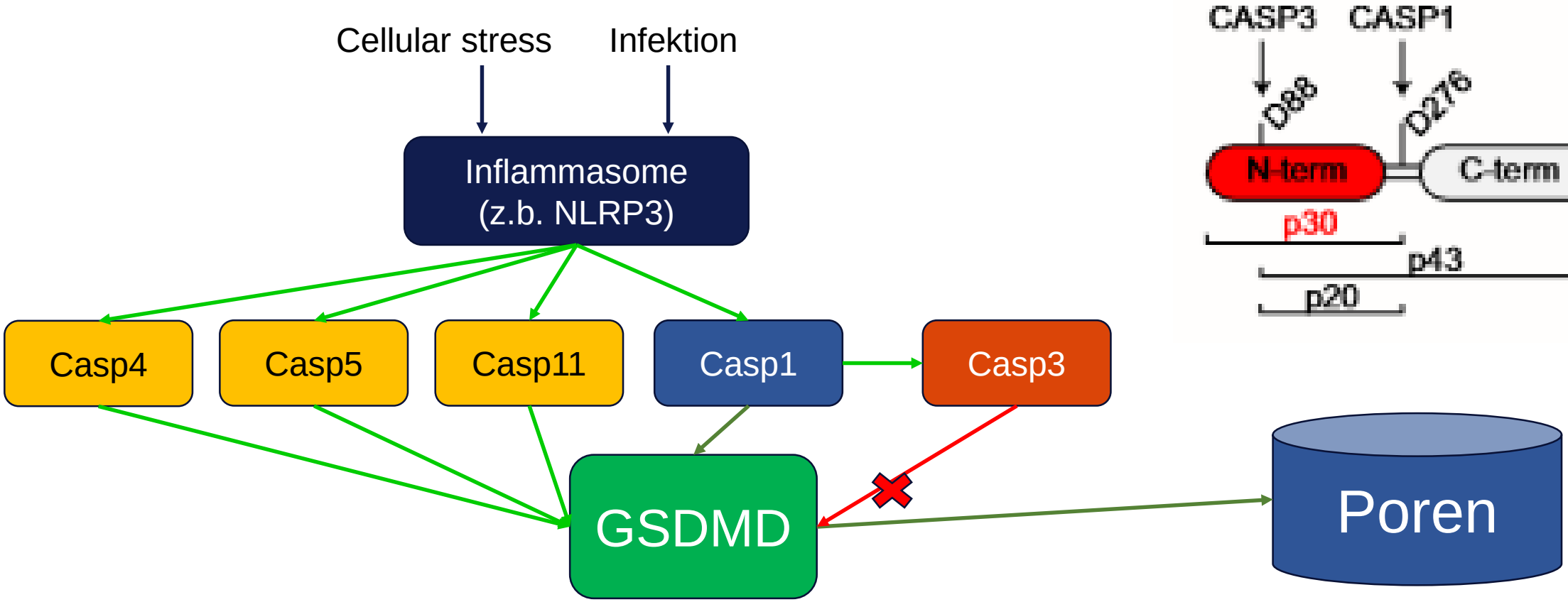
Background

Begriffdefinitionen

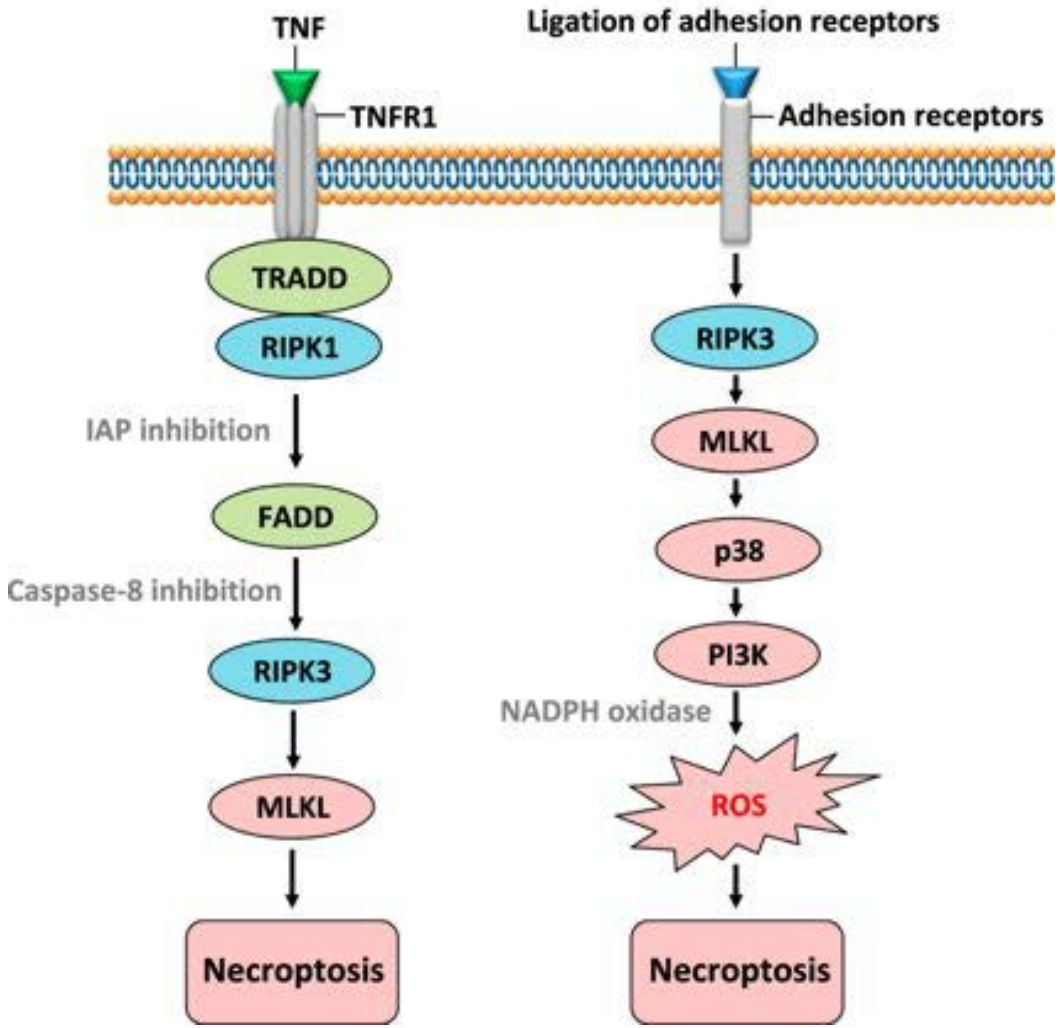
- Nekrose: akzidenteller Tod von außen durch Toxine, Hypoxie, Hypothermie, Infektionen,..
- Apoptose: immunologisch ruhiger Zelltod (ohne Entzündung, Teil des Stoffwechsels)
- Sekundäre Necrose: Zerfall von Apoptosekörpern bei zu langsamer Phagozytose
- Pyroptose: lytischer, stark entzündlicher Zelltod (angeboren, gegen intrazelluläre Pathogene)
- Necroptose: RIPK3-abhängige **regulierte** Nekrose, Aktivierung von Todesrezeptoren wie bei Apoptose

Pyroptose

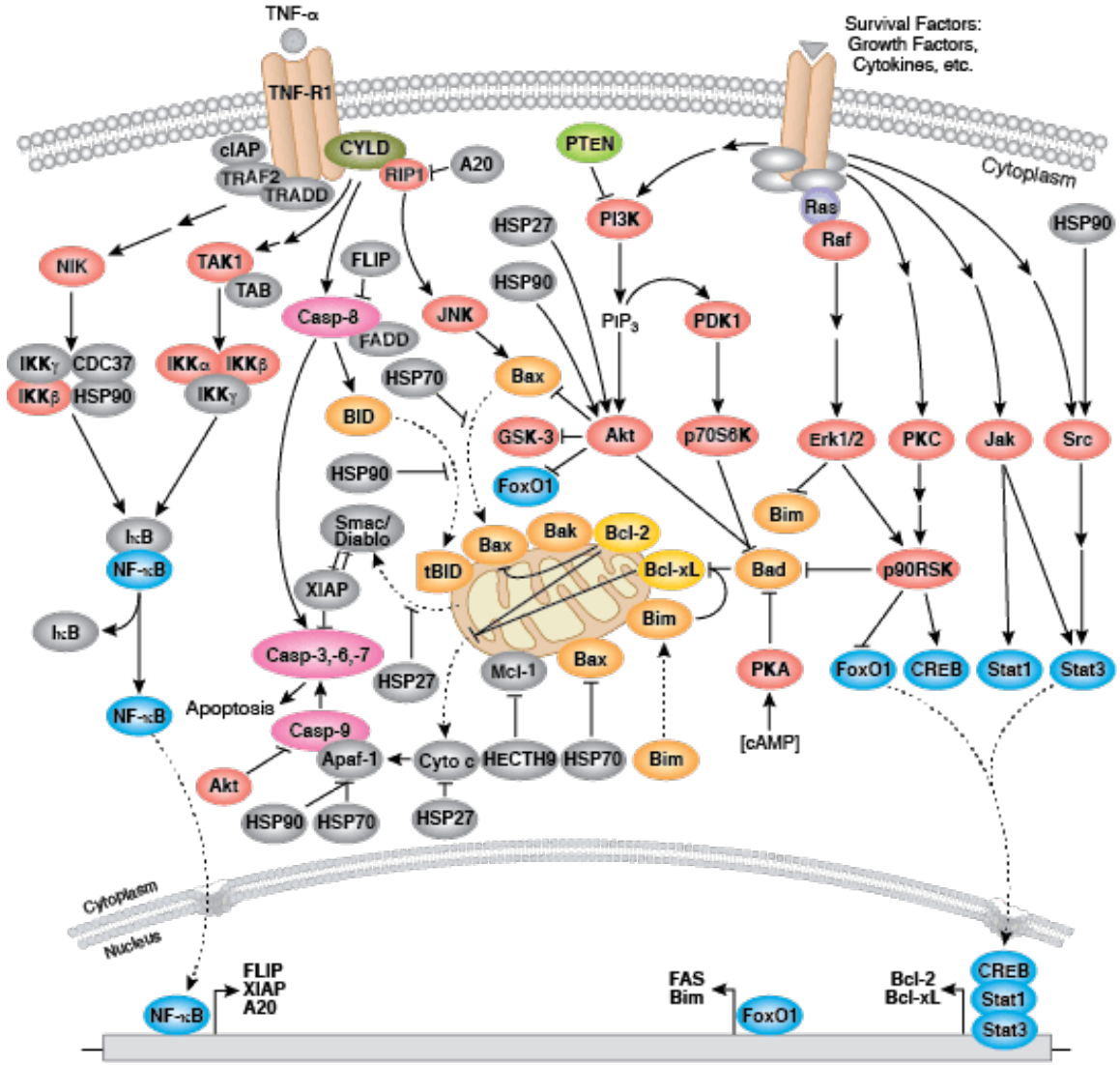

Cellular stress
Infektion
Inflammasome
(z.b. NLRP3)
Casp4
Casp5
Casp11
Casp1
Casp3
GSDMD
Poren

CASP3
CASP1
D88
D276
N-term
C-term
p30
p43
p20

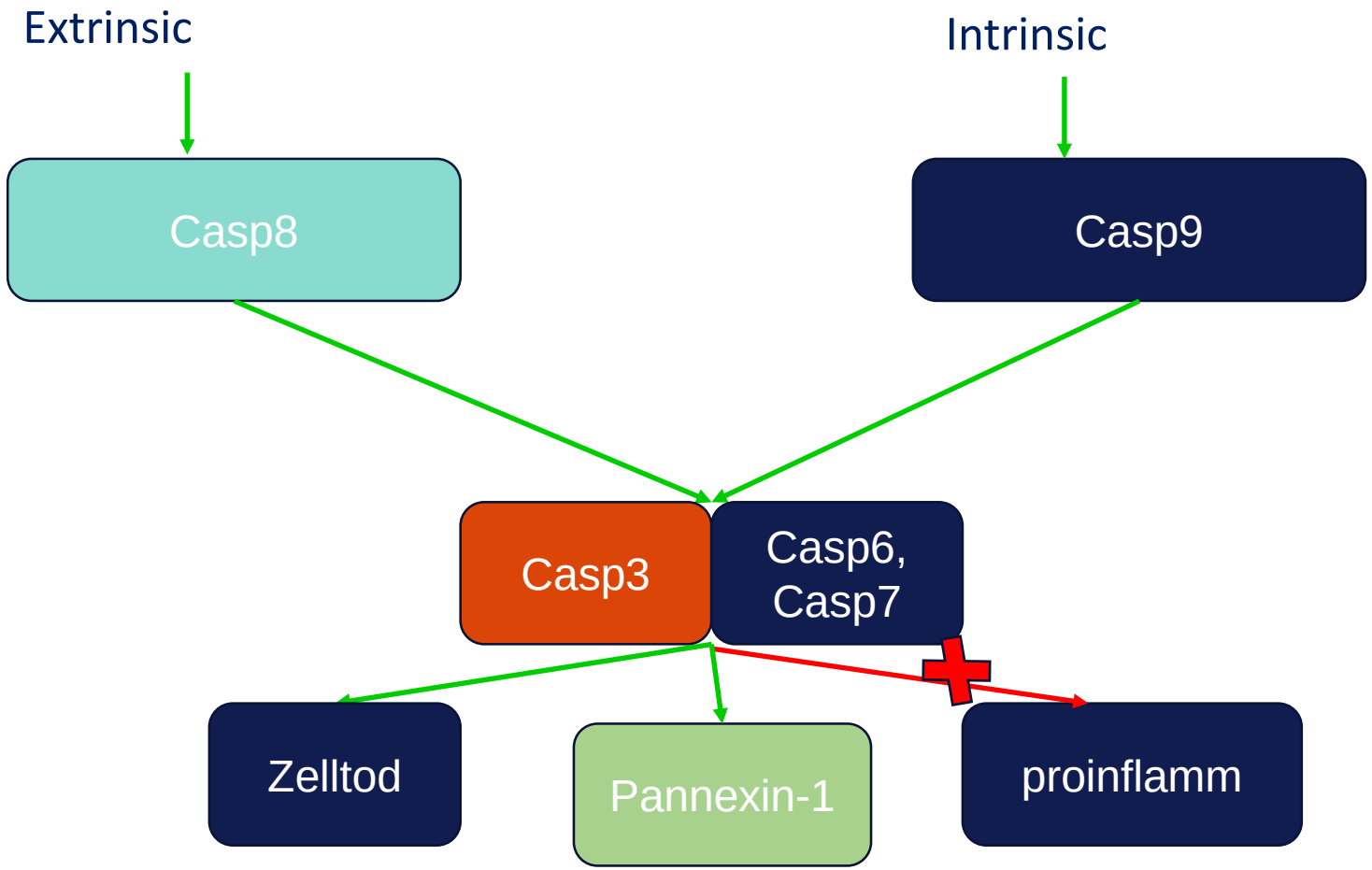
Necroptose


TNF
TNFR1
TRADD
RIPK1
IAP inhibition
FADD
Caspase-8 inhibition
RIPK3
MLKL
Necroptosis
Ligation of adhesion receptors
Adhesion receptors
RIPK3
MLKL
p38
PI3K
NADPH oxidase
ROS
Necroptosis

Apoptose


TNF-α
TNF-R1
cIAP
TRAF2
TRADD
CYLD
RIP1
A20
NIK
TAK1
TAB
FLIP
Casp-8
FADD
JNK
IKKγ
CDC37
IKKβ
HSP90
IKKα
IKKβ
IKKγ
HSP70
Bax
BID
GSK-3
Akt
FoxO1
HSP90
Smac/
Diablo
IκB
NF-κB
XIAP
tBID
Bax
Bak
Bcl-2
Bcl-xL
Bad
Bim
p90RSK
IκB
Casp-3,-6,-7
HSP27
Mcl-1
Bax
PKA
[cAMP]
FoxO1
CREB
Stat1
Stat3
NF-κB
Apoptosis
Casp-9
Apaf-1
Cyto c
HECTH9
HSP70
Bim
Akt
HSP90
HSP70
HSP27
Cytoplasm
Nucleus
NF-κB
FLIP
XIAP
A20
FAS
Bim
FoxO1
Bcl-2
Bcl-xL
CREB
Stat1
Stat3
Survival Factors:
Growth Factors,
Cytokines, etc.
PTEN
PI3K
HSP27
HSP90
PIP3
PDK1
Ras
Raf
Erk1/2
PKC
Jak
Src
HSP90
p70S6K
Cytoplasm

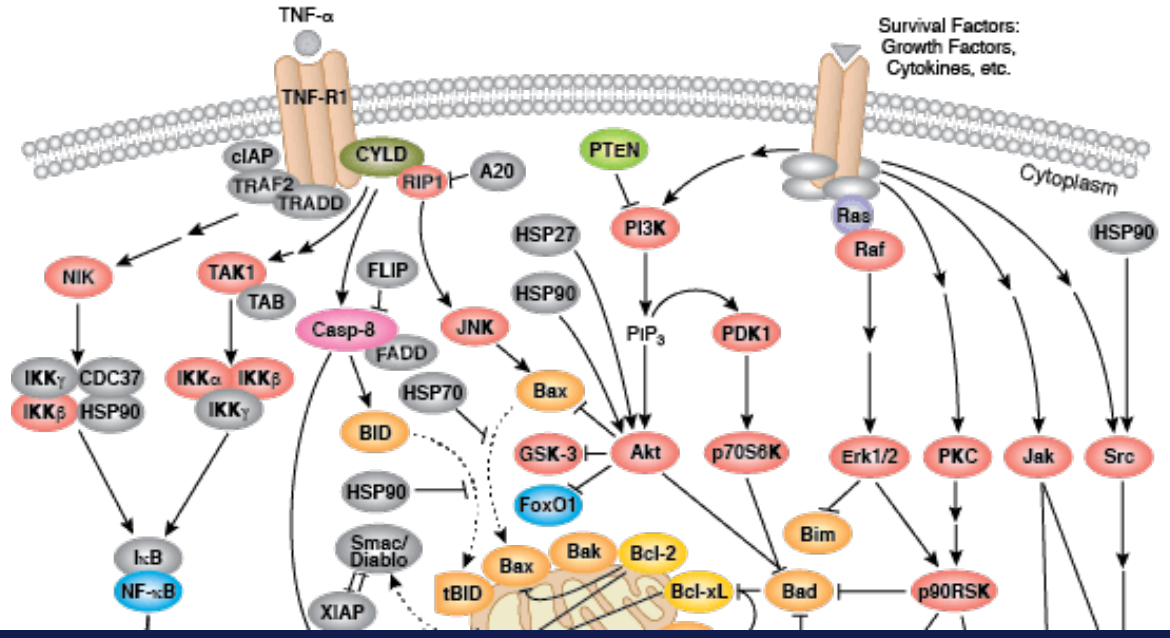
Apoptose



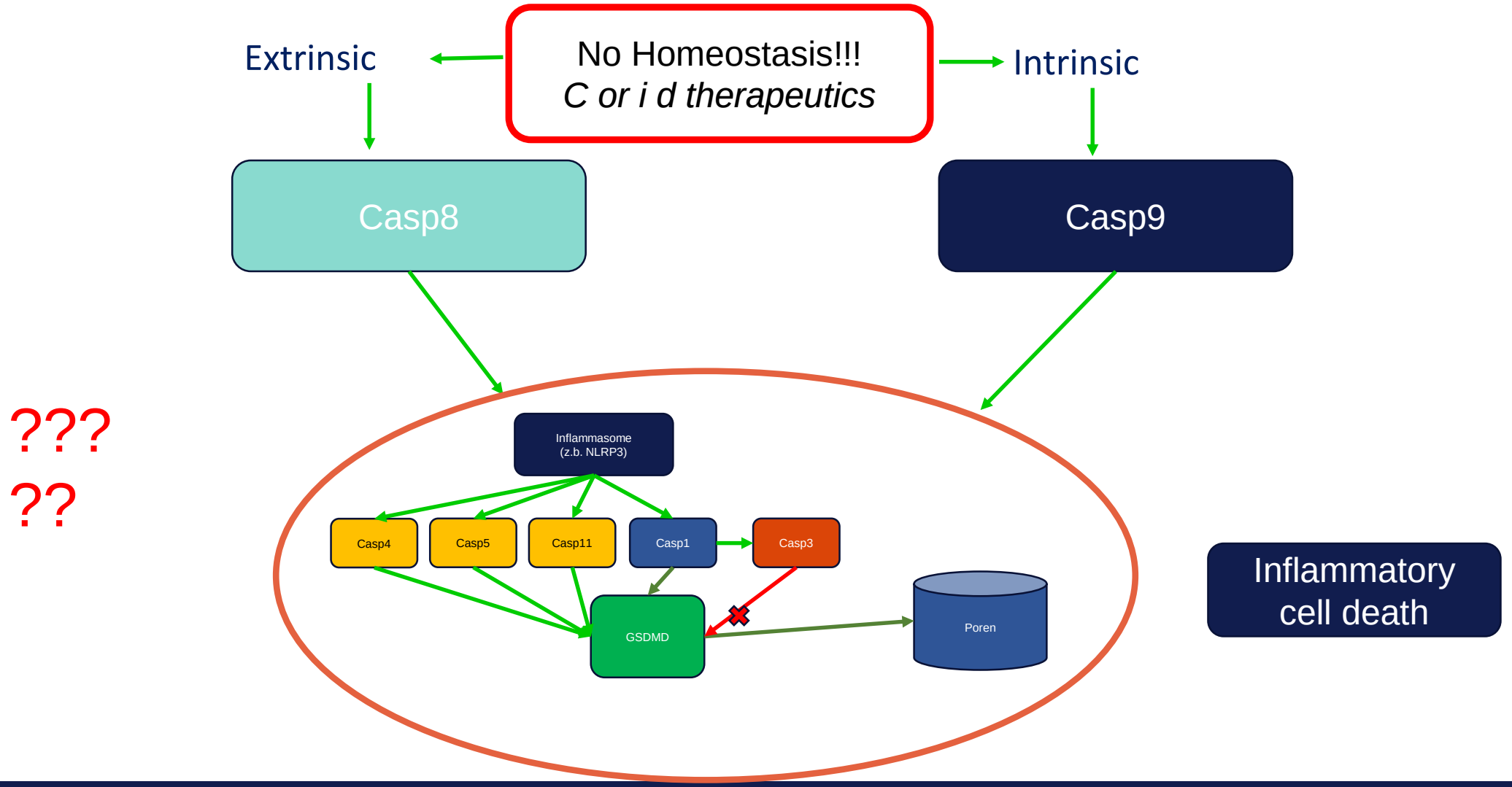
Homeostasis!!!

Chemotherapie or inflammatory disorder therapeutics

- SMAC mimetics (XIAP, cIAP1, cIAP2 antagonist) ***[extrinsic Apoptosis]***
- TAK1 inhibitor ***[extrinsic Apoptosis]***
- BH3 mimetics (binding and inhibiting select atiapoptotic BCL-2 member) ***[intrinsic Apoptosis]***



Chemotherapie or inflammatory disorder therapeutics



Methoden

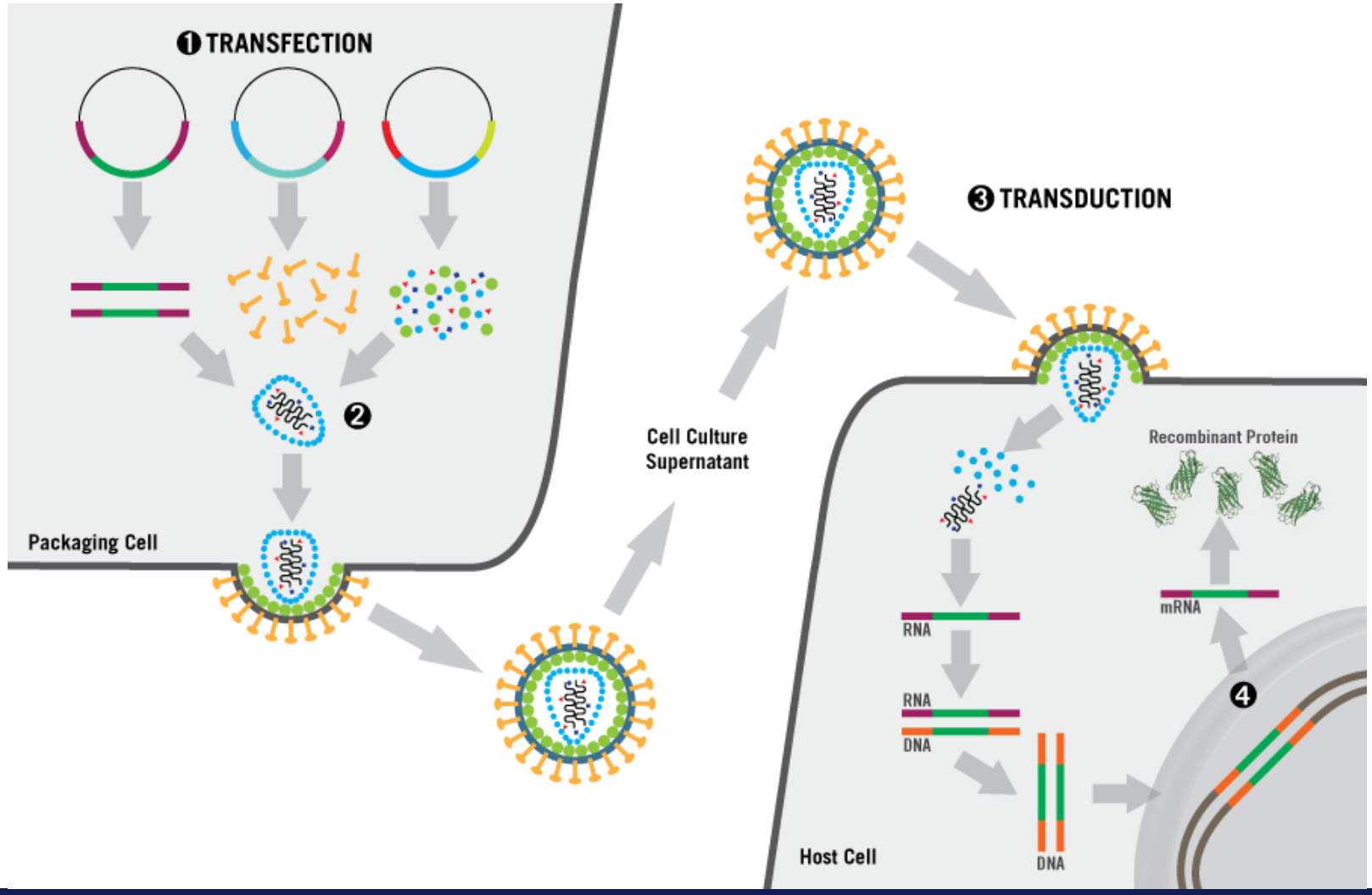
Methoden

- **Zellkultur:** Bone marrow-derived macrophages (BMDMs), Immortalized BMDMs, HEK293T cells, HeLa cells
- **Lentivirale Transduction**
- **Generation of CRISPR knockouts in immortalized BMDMs**
- **Mice:** C572BL/6J, CRISPR/Cas9 genome targeting, KAPA Hotstart mouse Genotyping Kit, PCR
- **HEK293T DmrB-caspase-8 dimerization system:** Transfected with Plasmid + Polyethylenimine, later Doxycicline to induce DmrB-cas8 expression, later B/B homodimerizer to induce DmrB-casp-8 homodimerization

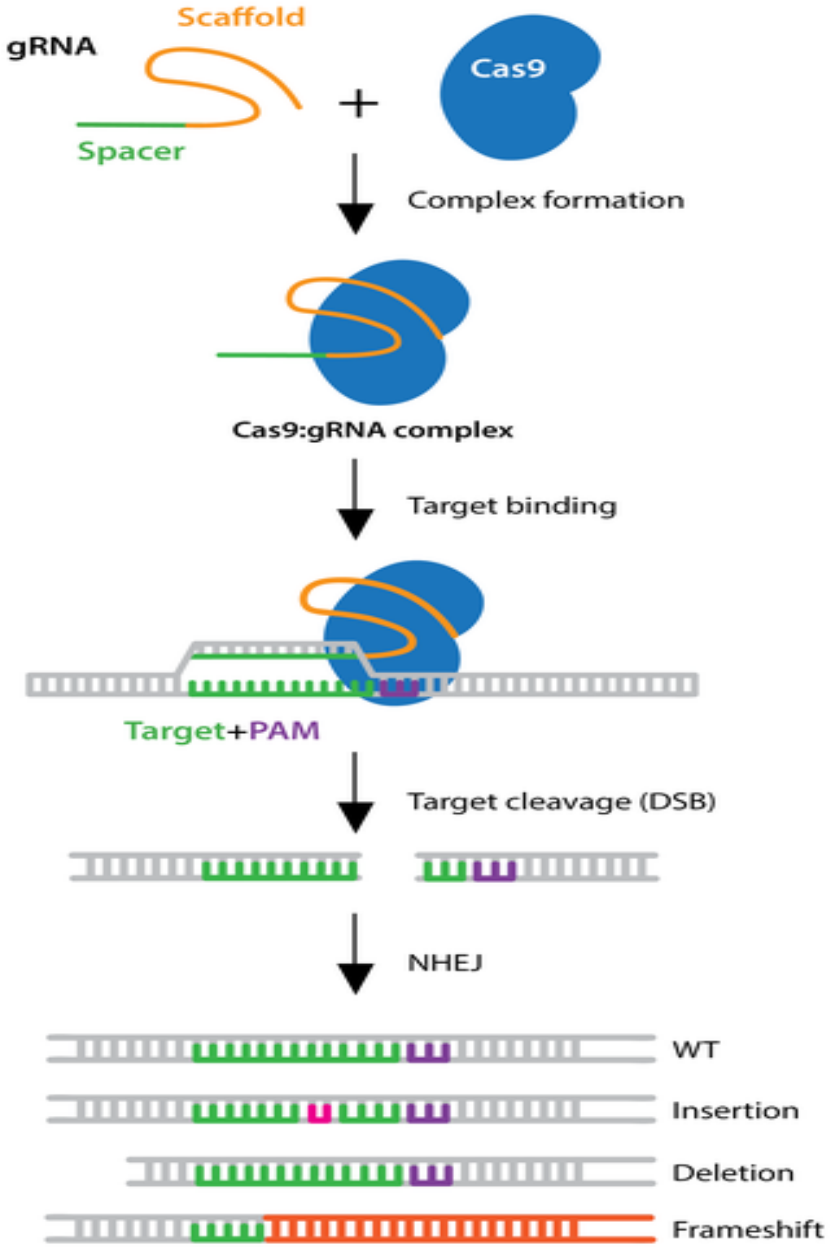
Methoden

- **Apoptosis and necroptosis assay:**
  - Intrinsic: ABT-737, S63845
  - Extrinsic: recombinant murine TNF, SMAC-mimetic AZD 5582, TAK inhibitor 5z 7-oxozeanol
  - Necroptosis: LPS, Q-VD-Oph
  - TLR4-apoptosis: E. coli K12 LPS, AZD 5582
- **Inflammasome assay:** LPS, nigericin, probenecid and trovafloxacin
- **LDH release assay:** CytoTox 96 non-radioactive cytotoxicity assay
- **Westernblotting:** immunoblotting with defined Antibodies, supernatant + lysed cell extract
- **Live cell imaging:** stained with propidium iodide

Lentivirale Transduction

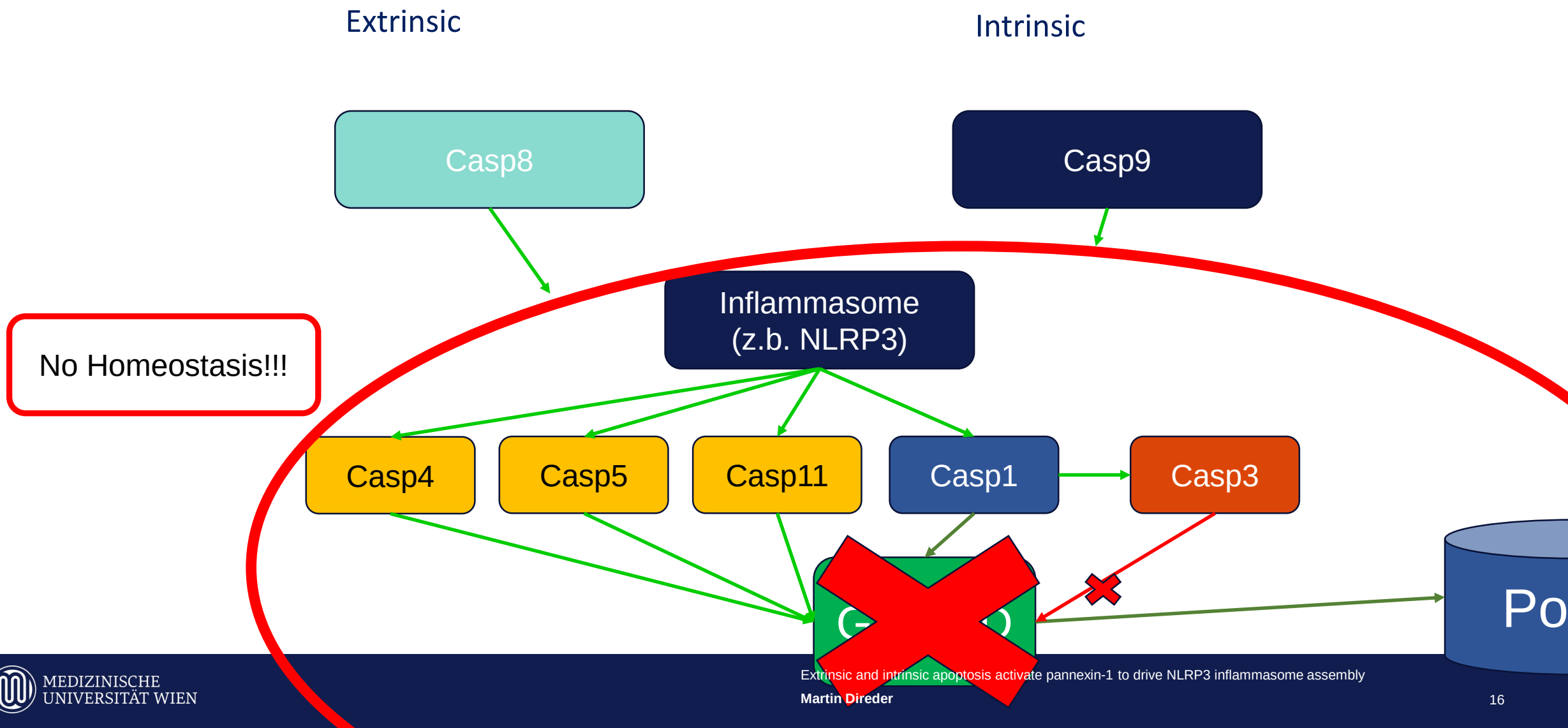


CRISPS/Cas9


gRNA
Scaffold
Spacer
Cas9
Complex formation
Cas9:gRNA complex
Target binding
Target+PAM
Target cleavage (DSB)
NHEJ
WT
Insertion
Deletion
Frameshift

Results

Chemotherapie or inflammatory disorder therapeutics



Extrinsic apoptosis trigger GSDMD-dependent and caspase-3/7-dependent necrosis

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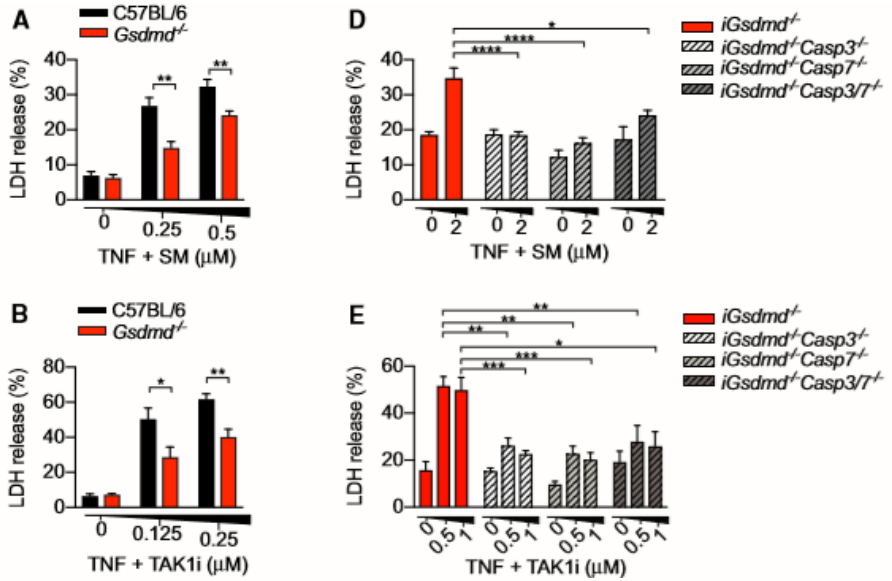
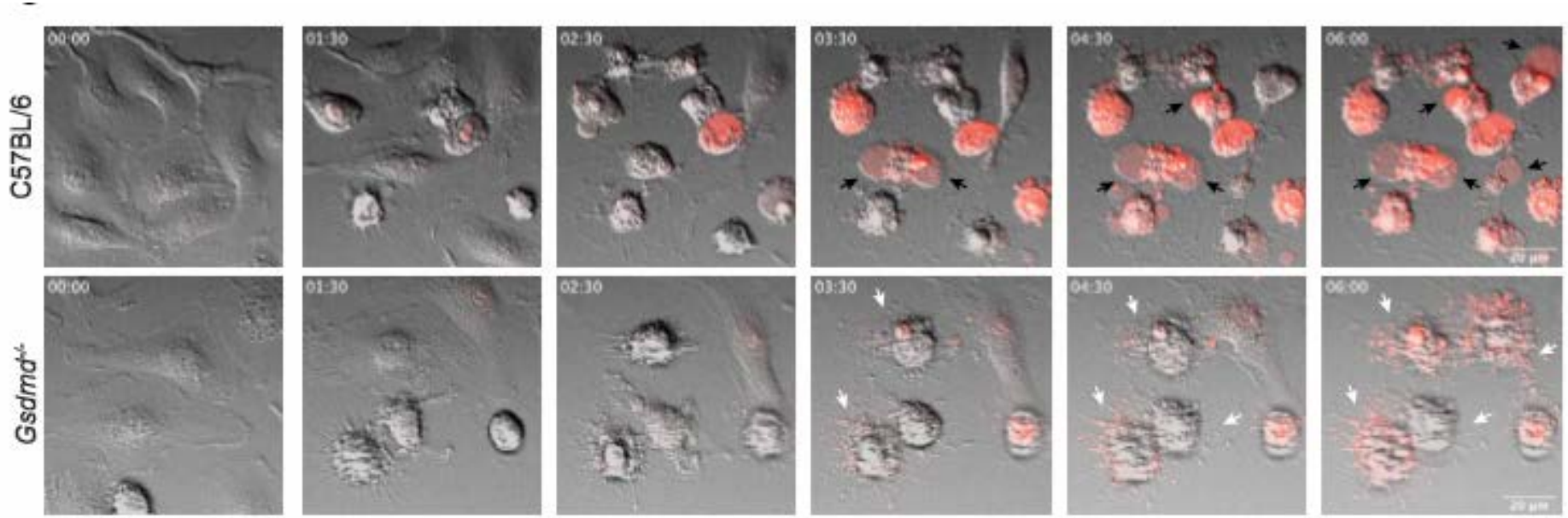


Figure 1. Extrinsic apoptosis trigger GSDMD-dependent and caspase-3/7-dependent necrosis.

A–E Primary (A, B) or immortalized BMDMs (D, E) were stimulated with recombinant murine TNF (100 ng/ml) in combination with (A, D) SM or (B, E) TAK1i for 6 or 4 h, respectively. (C) Time-lapse confocal images (hour:min) of BMDMs stimulated with recombinant murine TNF (100 ng/ml) and SM (250 nM) stained with propidium iodide (red) for 6 h. Black arrowheads indicate membrane ballooning, while white arrowheads indicate apoptotic bodies.

Data information: Data are means ± SEM of pooled data from (A-B) five or (D-E) eight independent experiments. Statistical analyses for normally distributed data sets were analysed using the parametric *t*-test, whereas non-normally distributed data sets were analysed using non-parametric Mann–Whitney *t*-tests. Data were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ or $^{****}P < 0.0001$. (C) Data are representative of three independent experiments.

Extrinsic apoptosis trigger GSDMD-dependent and caspase-3/7-dependent necrosis



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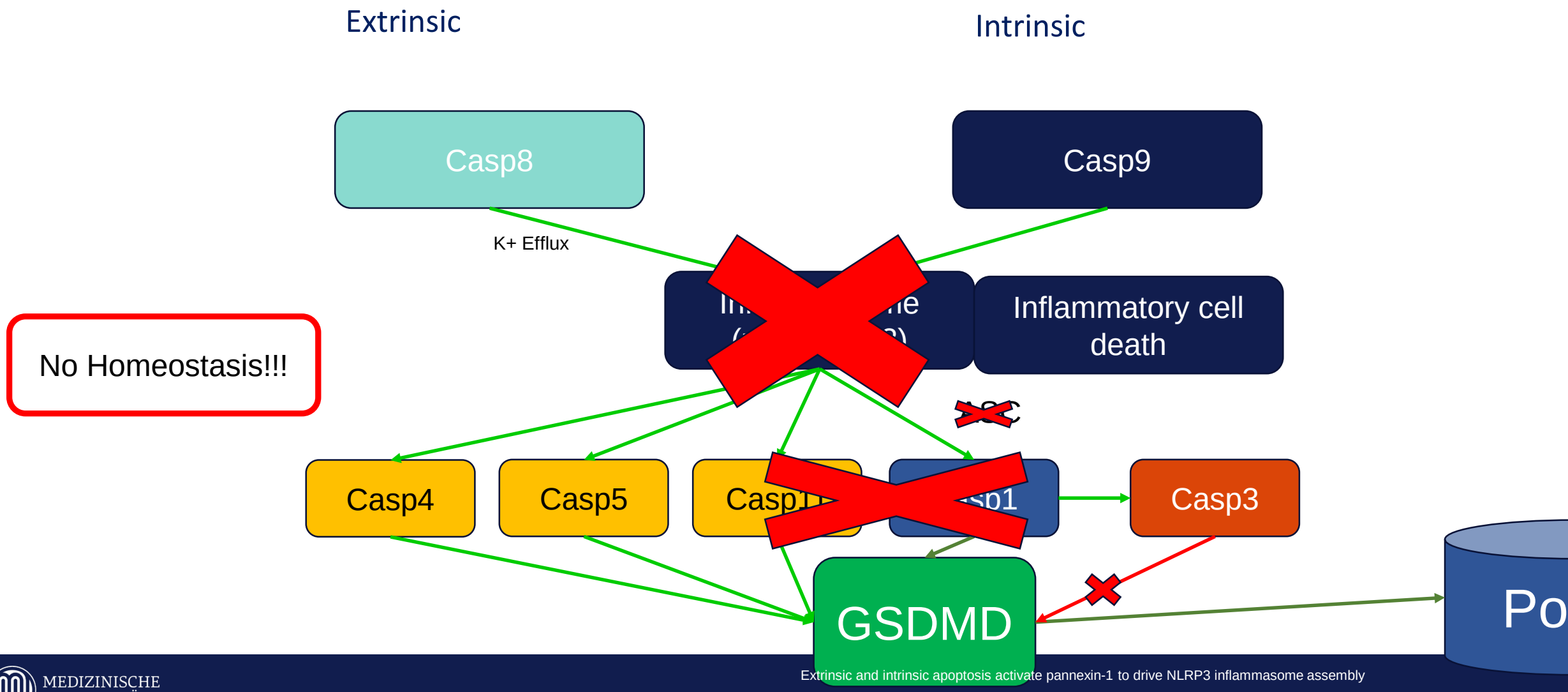
Chemotherapie or inflammatory disorder therapeutics



Extrinsic apoptosis triggers caspase-1/11- independent GSDMD processing and GSDMD/E-independent NLRP3 activation

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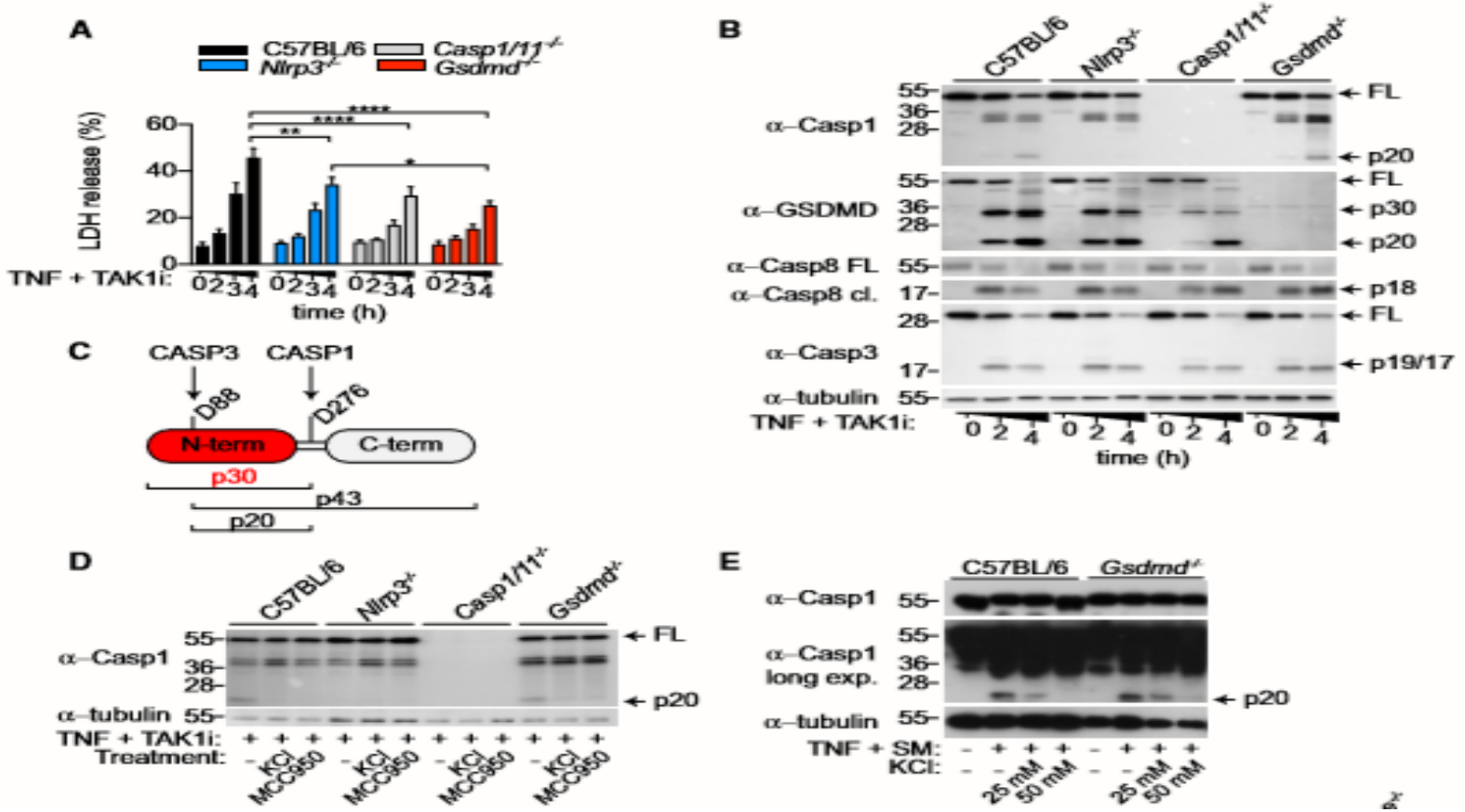
Panx1 promotes NLRP3 during apoptosis *Kaiwen W Chen et al*



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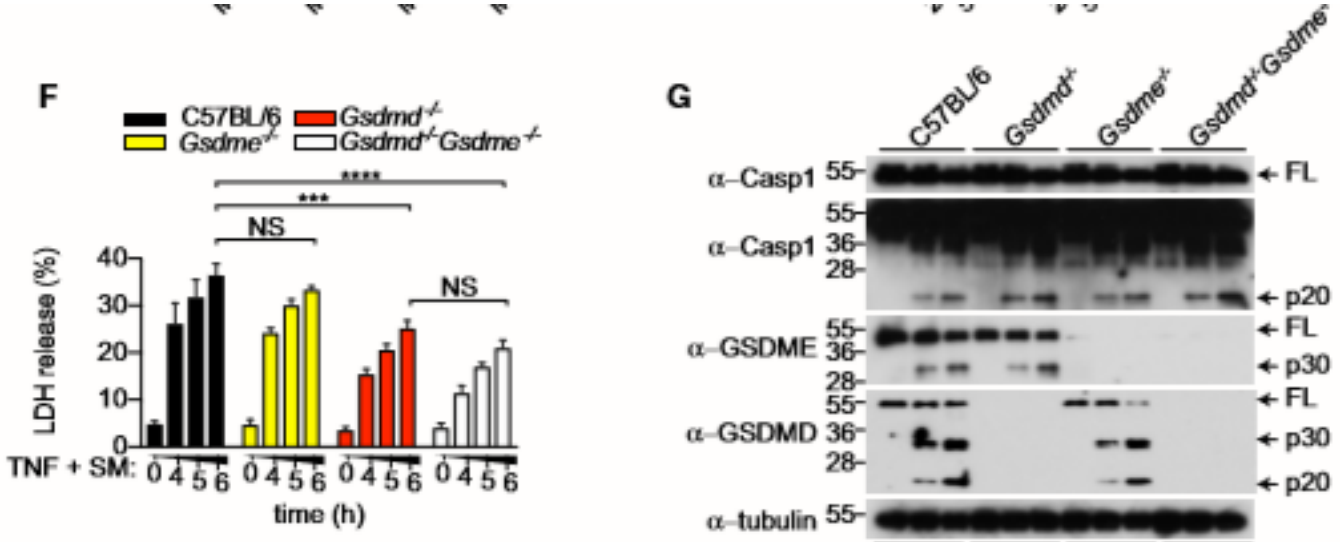


Figure 2. Extrinsic apoptosis triggers caspase-1/11-independent GSDMD processing and GSDMD/E-independent NLRP3 activation.

A, B BMDMs were stimulated with TNF (100 ng/ml) in combination with TAK1i (125 nM) for the indicated time points. (A) LDH release and (B) mixed supernatant and cell extracts were analysed.

C Representation of known caspase cleavage site and molecular weight of corresponding cleavage fragment in mouse GSDMD.

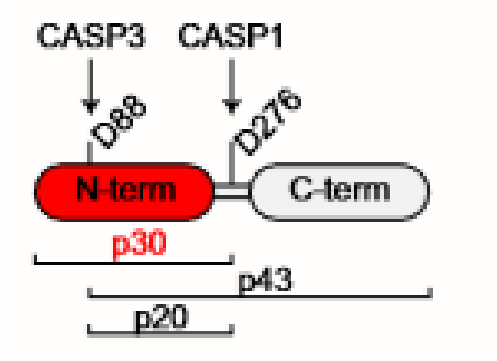
D BMDMs were costimulated with TNF (100 ng/ml) and TAK1i (125 nM) for 4 h in the presence or absence of KCl (50 mM). Where indicated, cells were pre-incubated with MCC950 (10 μM) 20–30 min prior to TNF/TAK1i stimulation.

E–G BMDMs were costimulated with TNF (100 ng/ml) and SM (E) (250 nM; 6 h), mixed supernatant and cell extracts were analysed by immunoblot, or (F) LDH release in the cell culture supernatant was quantified at the indicated time points.

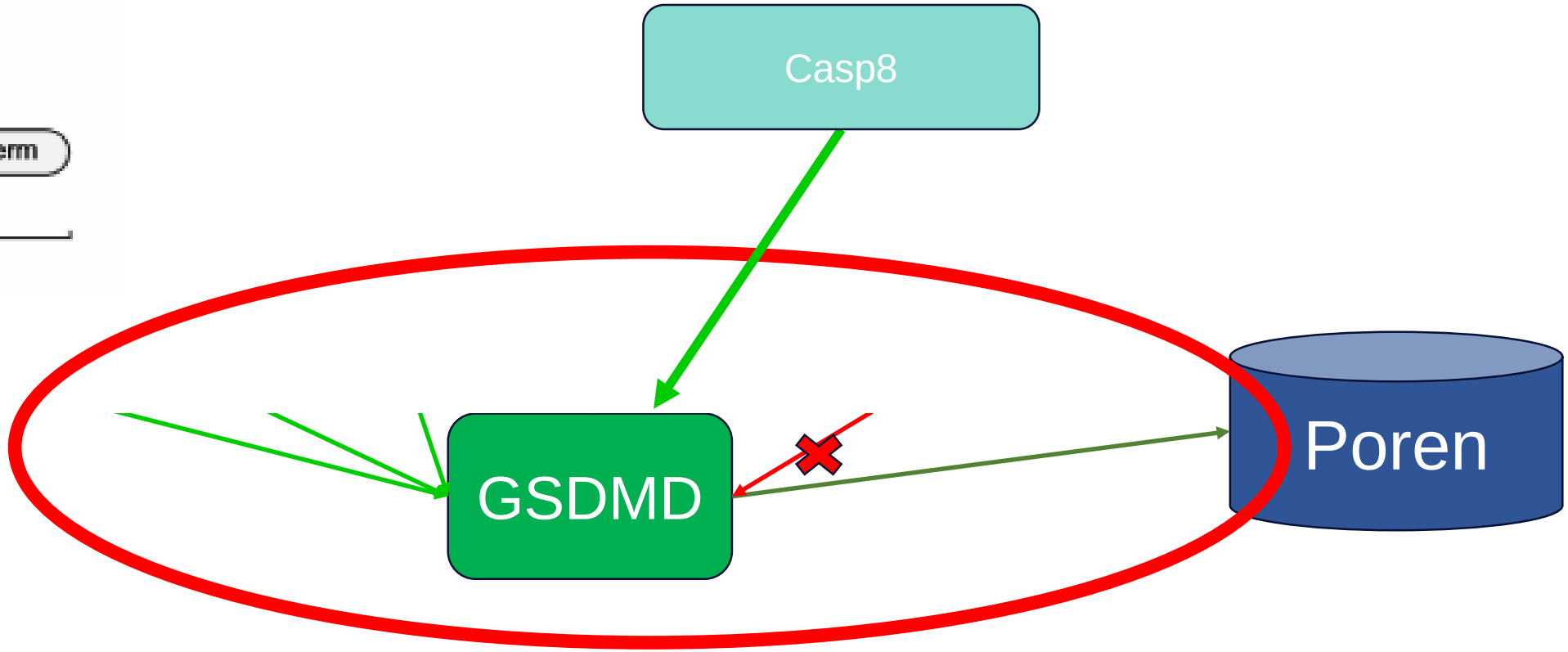
Data information: Data are means ± SEM of pooled data from (A) four or (F) five independent experiments. Statistical analyses were performed using a two-way ANOVA. Data were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ or $^{****}P < 0.0001$. All immunoblots are representative of three independent experiments. Source data are available online for this figure.

Chemotherapie or inflammatory disorder therapeutics

No Homeostasis!!!



Extrinsic



Caspase-3 suppresses caspase-8-dependent GSDMD activation and cell lysis during extrinsic apoptosis

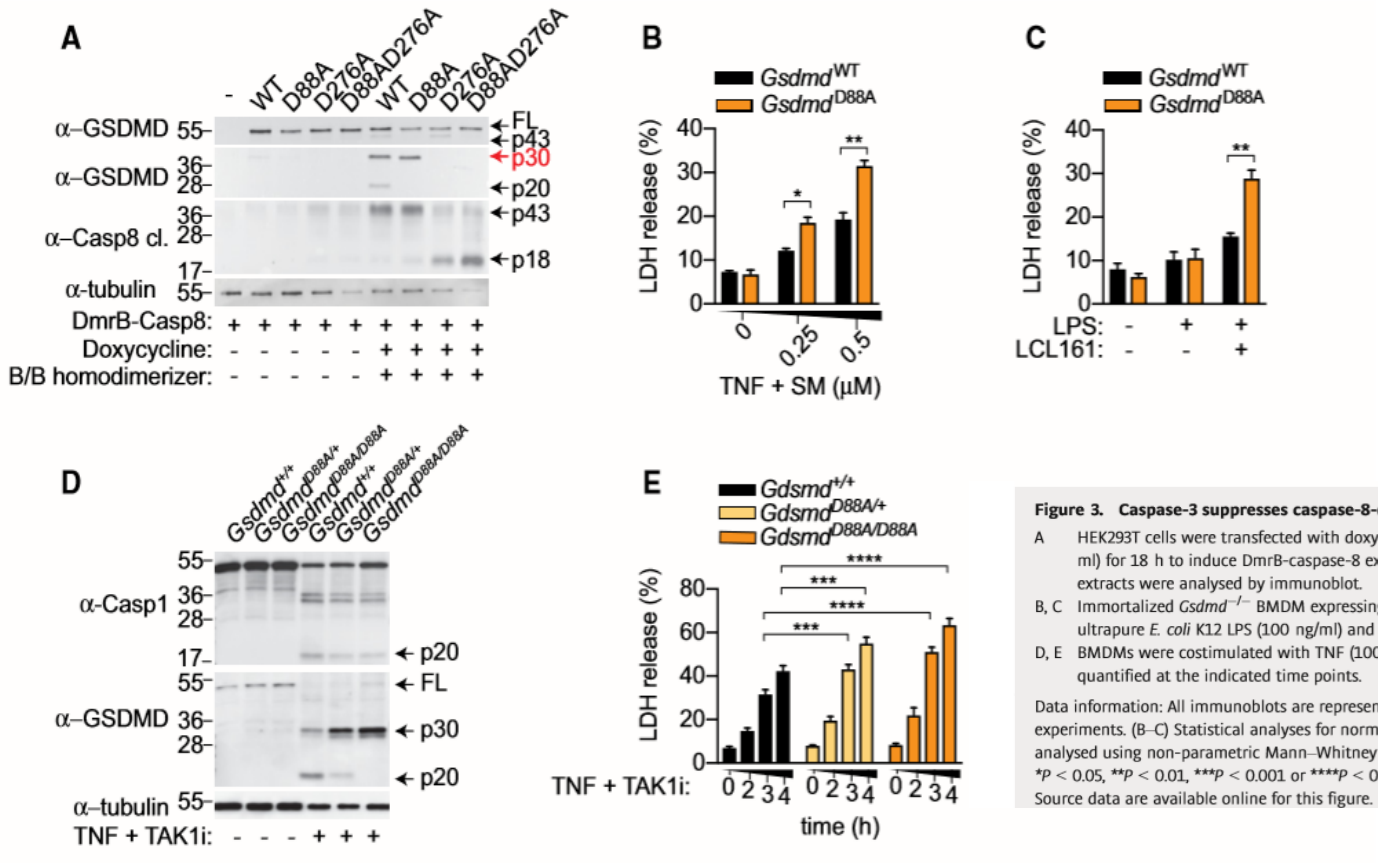


Figure 3. Caspase-3 suppresses caspase-8-dependent GSDMD activation and cell lysis during extrinsic apoptosis.

A HEK293T cells were transfected with doxycycline-inducible DmrB-caspase-8 and the indicated GSDMD constructs. Cells were stimulated with doxycycline (10 μg/ml) for 18 h to induce DmrB-caspase-8 expression and exposed to B/B homodimerizer (12.5 nM) for another 2 h to activate caspase-8. Mixed supernatant and extracts were analysed by immunoblot.

B, C Immortalized *Gsdmd*$^{-/-}$ BMDM expressing GSDMD^{WT} and GSDMD^{D88A} were (B) costimulated with TNF (100 ng/ml) and SM for 6 h or (C) primed for 3 h with ultrapure *E. coli* K12 LPS (100 ng/ml) and stimulated with LCL161 (1 μM) for 24 h, and LDH release was quantified.

D, E BMDMs were costimulated with TNF (100 ng/ml) and TAK1i for 4 h, (D) mixed supernatant and extracts were analysed by immunoblot, or (E) LDH release was quantified at the indicated time points.

Data information: All immunoblots are representative of three independent experiments. Data are means ± SEM of pooled data from (B–C) three or (E) seven individual experiments. (B–C) Statistical analyses for normally distributed data sets were analysed using the parametric *t*-test, whereas non-normally distributed data sets were analysed using non-parametric Mann–Whitney *t*-tests. (E) Statistical analyses were performed using a two-way ANOVA. Data were considered significant when $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ or $****P < 0.0001$.

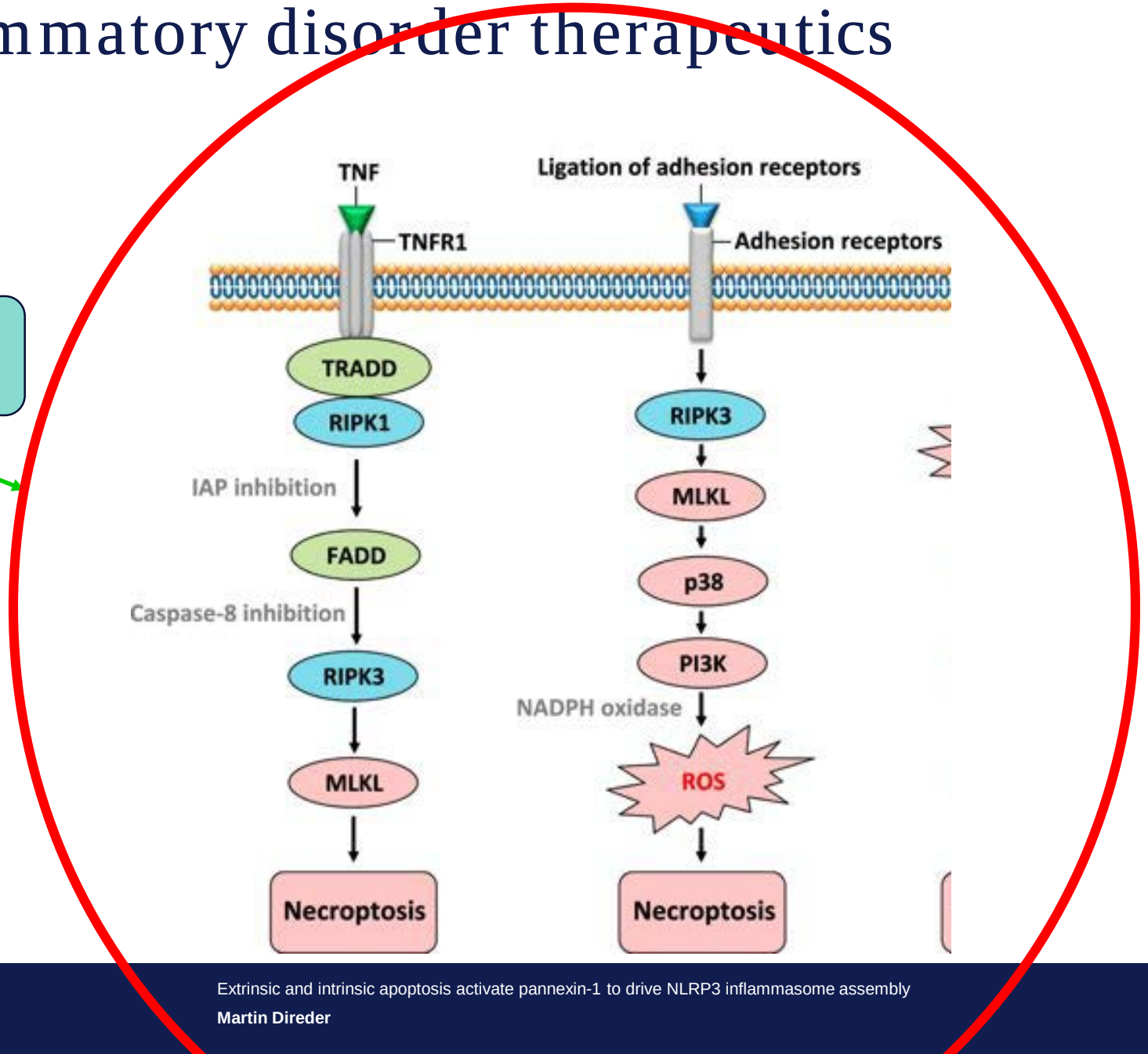
Source data are available online for this figure.

Chemotherapie or inflammatory disorder therapeutics

Extrinsic

Casp8

No Homeostasis!!!



RIPK3 promotes caspase-3 activation and pannexin-1 activity to drive NLRP3 assembly during extrinsic apoptosis

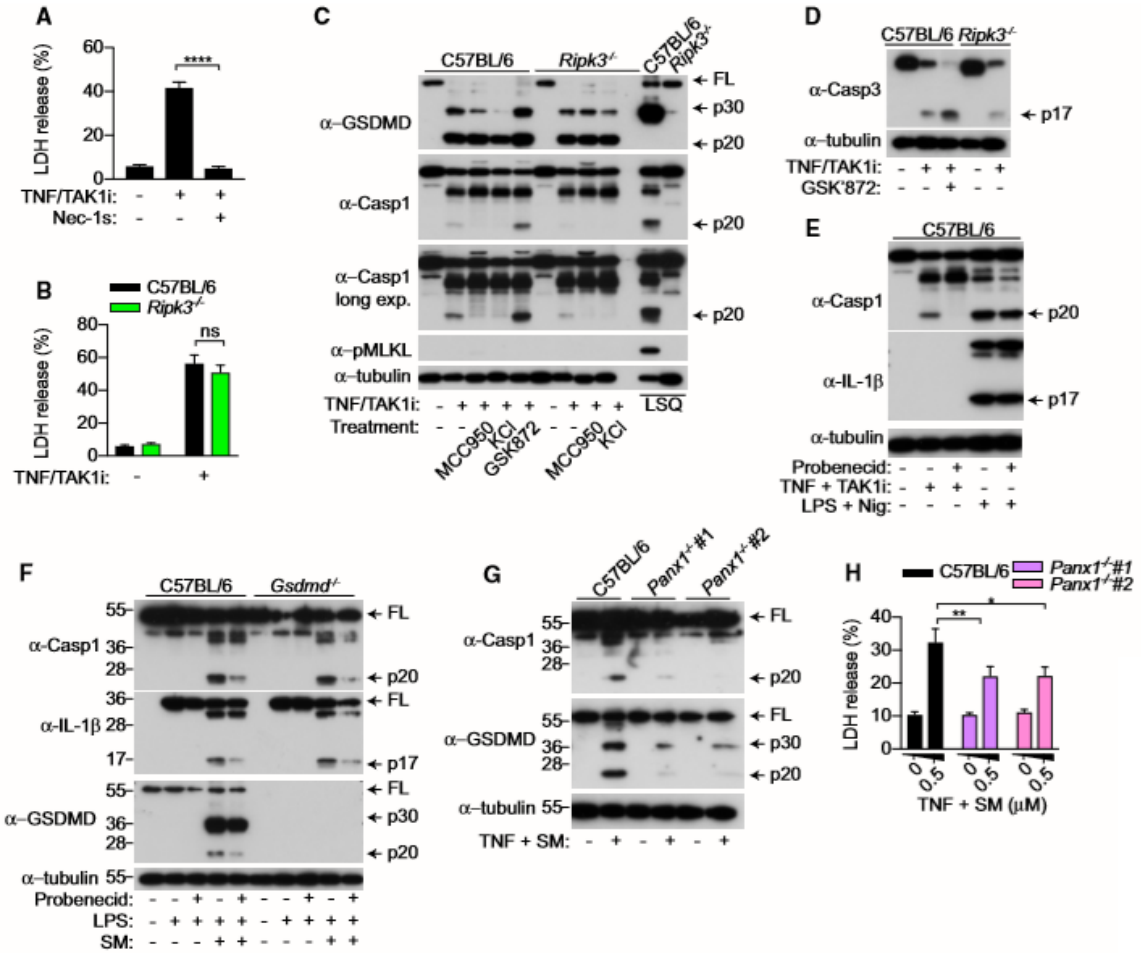


Figure 4. RIPK3 promotes caspase-3 activation and pannexin-1 activity to drive NLRP3 assembly during extrinsic apoptosis.

A–E BMDMs were costimulated with TNF (100 ng/ml) and TAK1i (125 nM) for 4 h, (A, B) LDH release was quantified, or (C, D, E) mixed supernatant and extracts were analysed by immunoblot. Where indicated, cells were treated with the inhibitors Nec-1s (50 μM), MCC950 (10 μM), GSK'872 (1 μM), probenecid (1 mM) 20–30 min prior to cell stimulation. KCl (50 mM) was added together with TNF and TAK1i. (C) To induce necroptosis, BMDMs were primed for 3 h with ultrapure *E. coli* K12 LPS (100 ng/ml) and Q-VD-OPh (10 μM) was added at the last 20–30 min of priming and stimulated with SM (500 nM) for 4 h. (E) To activate the NLRP3 inflammasome, BMDMs were primed with ultrapure *E. coli* K12 LPS (100 ng/ml) for 4 h and stimulated with nigericin (10 μM) for 1 h.

F BMDMs were primed for 3 h with ultrapure *E. coli* K12 LPS (100 ng/ml) and stimulated with SM (0.5 μM) for a further 4 h. Probenecid (1 mM) was added 20–30 min prior to cell stimulation, and mixed supernatant and extracts were analysed by immunoblot.

G, H BMDMs were stimulated with TNF (100 ng/ml) and SM (0.5 μM) for 6 h, (G) mixed supernatant and extracts were analysed by immunoblot, or (H) LDH release was quantified.

Data information: All immunoblots are representative of three independent experiments. (A, B, H) Data are means ± SEM of pooled data from (A, H) four or (B) three independent experiments. Statistical analyses for normally distributed data sets were analysed using the parametric *t*-test, whereas non-normally distributed data sets were analysed using non-parametric Mann–Whitney *t*-tests. Data were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{****}P < 0.0001$.
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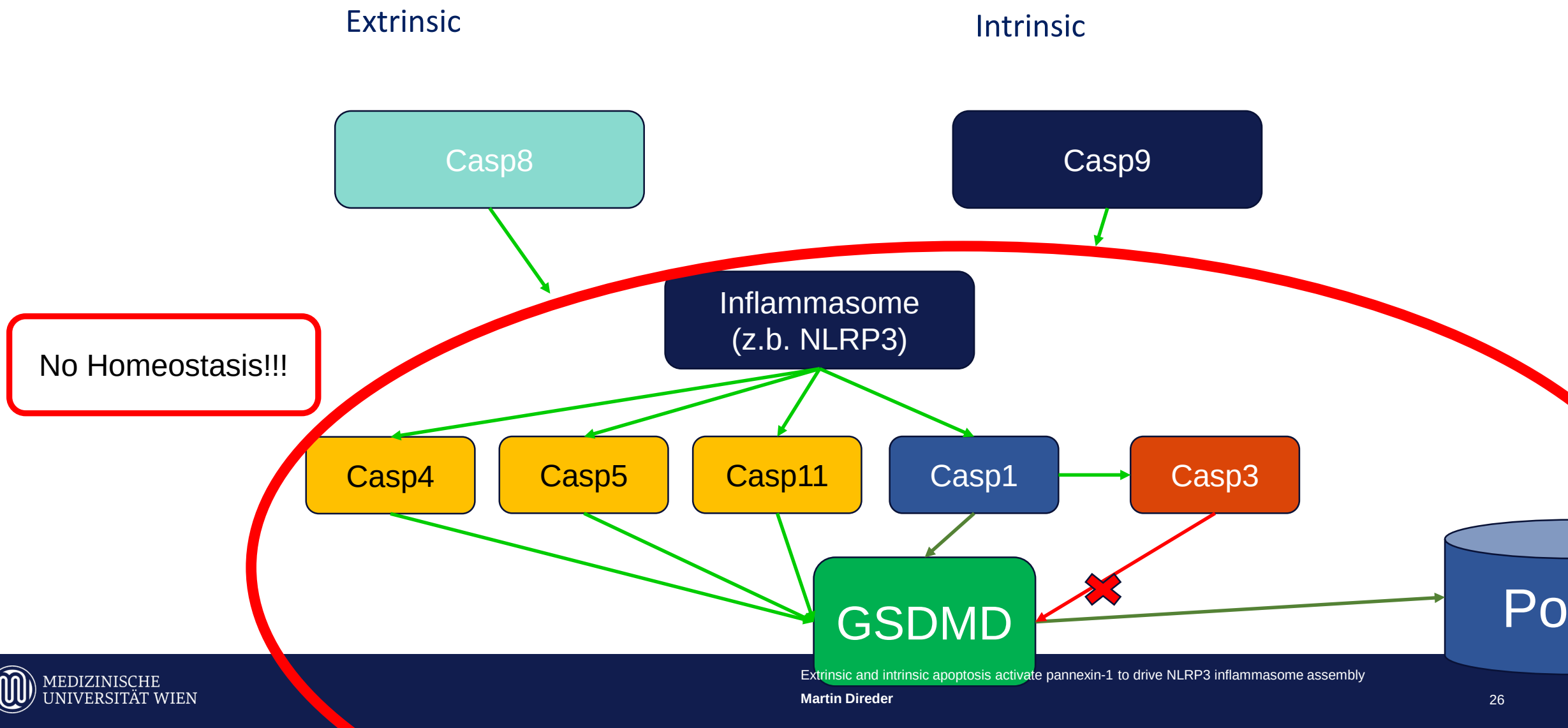
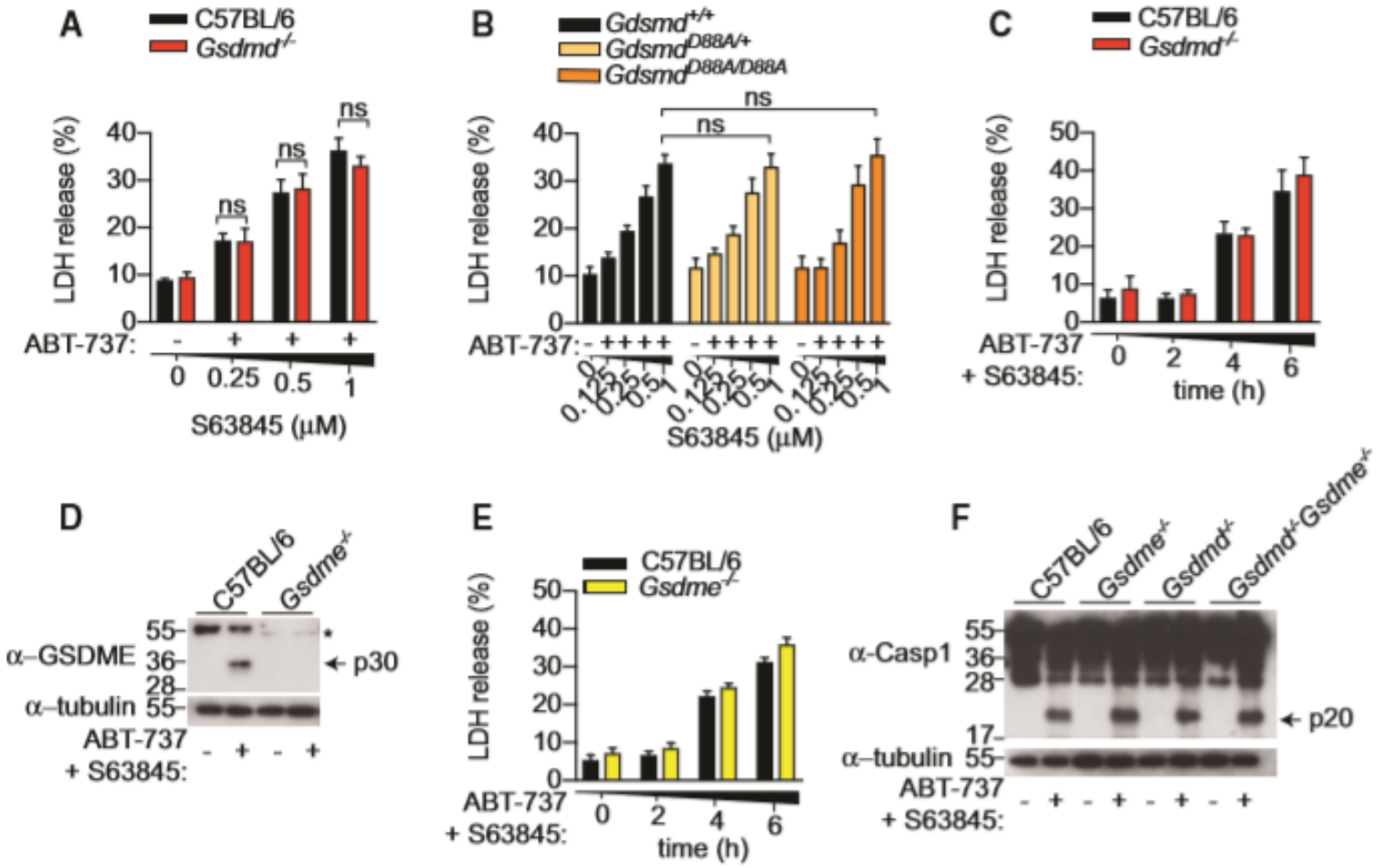
Chemotherapie or inflammatory disorder therapeutics



Intrinsic apoptosis drives gasdermin-independent cell lysis but promotes NLPR3 assembly through pannexin-1 activity



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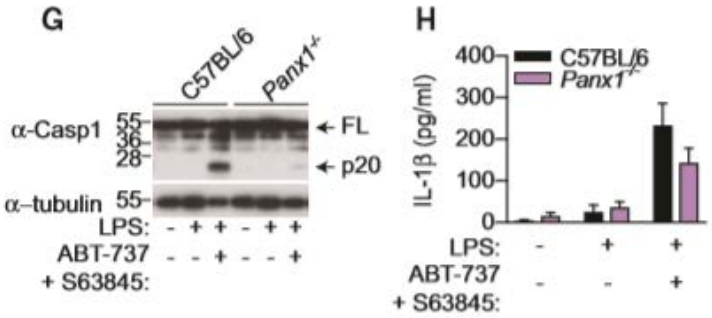

Figure 5. Intrinsic apoptosis drives gasdermin-independent cell lysis but promotes NLPR3 assembly through pannexin-1 activity.

A, B BMDMs were stimulated with an increasing dose of S63845 in the presence of ABT-737 (0.5 μM), and LDH release was quantified at 6 h.

C–F BMDMs were stimulated with ABT-737 (0.5 μM) and S63845 (0.5 μM), LDH release was quantified (C, E), or mixed supernatant and extracts were analysed by immunoblot at 6 h (D, F).

G, H BMDMs were primed with ultrapure *E. coli* K12 LPS (100 ng/ml) for 3 h and further stimulated with ABT-737 (1 μM) and S63845 (1 μM) for 24 h, mixed supernatant and extracts were analysed by immunoblot (G), and IL-1β in cell-free supernatant was quantified by ELISA (H).

Data information: All immunoblots are representative of three independent experiments. Data are means ± SEM of pooled data from (A) four, (B) five or (C, E, H) three independent experiments.
Source data are available online for this figure.

Discussion

Diskussion

- Theorie casp8-> GSDMD pores-> Kefflux->NLRP3 activation wiederlegt
- Genetic evidence dass Casp8 akkumulation von p30 keinen effekt auf Casp1 spaltung hat
- RIPK3 Gerüstfunktion ausschlaggebend für Pannexinporen….
- Gasdermin wird durch intrinsic Apoptosis aktiviert, aber kein Effekt auf Zelllyse
- Pannexin-1 aktiviert NLRP3 bei intrinsischer und extrinsischer Apoptose durch K efflux
- Cancer Chemotherapeutics verursachen Entzündungsreaktionen über Pyroptose pathway…. Gespaltene Theorien über bedeutung für Tumorentwicklung

Danke für Ihre Aufmerksamkeit

Literatur