Final report:

K-125224

The role of caspase-9 in cross-regulation of immunogenic and tolerogenic cell death pathways

The overall goal of the proposal was to explore the molecular backround of driving cross-regulation in the course of inflammatory/immunogenic and tolerogenic cell death pathways. This project relies on the hypothesis that 1) Caspase-9 is essential for inducing necroptosis, while 2) Caspase-9 plays an essential role in the regulation of the immunological outcomes of the cell death pathway.

The project was based on two main aims:

Aim I. Investigate the role of caspase-9 in necroptosis

The points listed in this AIM are covered almost entirely by the publication:

Caspase-9 acts as a regulator of necroptotic cell death Molnár T, Pallagi P, Tél B, Király R, Csoma E, Jenei V, Varga Z, Gogolák P, Odile Hueber A, Máté Z, Erdélyi F, Szabó G, Pettkó-Szandtner A, Bácsi A, Virág L, Maléth J, **Koncz G.** *FEBS J.* 2021 Apr 25. doi: 10.1111/febs.15898.

In this manuscript:

- We dissected the molecular background of necroptosis and identified necropotic signaling steps that may be directly dependent on caspase-9. We have shown that caspase-9 regulates the upsteram steps of necrosis. Because RIPK1-RIPK3 association and RIPK1 / RIPK3 phosphorylation are blocked in the absence of capase-9 following necroptotic stimuli. These results were confirmed by RIPK1-RIPK3 overexpression, which restored the necroptotic sensitivity of caspase-9-deficient cell lines.
- We determined Caspase-9 binding partners during necroptosis. Two new interaction partners for caspase-9 were identified, Auroa kinase A and RIPK3, which molecules were associated with caspase-9 under necroptotic conditions.
- We demonstrated that inhibition of either AURKA or its known substrate, GSK3ß have restored necroptosis sensitivity of Caspase-9 deficient cells.
- We demonstrated the role of caspase-9 in death receptor-independent necroptosis, demonstrating that caspase-9 is required for LPS- and HSV-1-induced necroptosis.
- We demonstrated the role of caspase-9 in necroptosis also by *in vivo* studies. We investigated acute pancreatitis in the pancrease acinar cell-specifc deletion of Caspase-9 using the ptf1a-cre mice. In collaboration with the group of Dr József Maléth, we presented that cerulein-induced pancreatitis was significantly reduced in mice with acinar cell restricted Caspase-9 gene knockout.

These results highlighted a new role of Caspase-9 in the regulation of necroptosis. We demonstrated a new regulation pathway of necroptosis, a conceptually new role of Caspase-9 and we posed Caspase-9 as an attractive target for controlling the transition between intrinsic apoptosis and necroptosis.

Examination of some points in the project plan did not support the initial hypothesis. These results have not yet been published, although some of these results may lead to further publications:

- We crossed caspase-9 LoxP animals with (myeloid specific) lysozyme M-cre mice. We investigated the TNFα/zVad-induced lethal shock, we monitored the drop in body temperature (figure 1) and the level of 40 different cytokines were analyzed by cytokine array form the serums of wild type and Caspase-9 deficient animals, but we could not detect differences in these parameters comparing the Caspase-9 deficient mice to wild type counterparts.
- Since this (our primary) *in vivo* model did not support the hypothesis, we worked on two alternative models. The model based on acinar cell-specific deletion of caspase-9 has been published. We are currently investigating the effect of endothelial cell-specific deletion of caspase-9, with promising preliminary results. (figure 1) The role of caspase-9 in endothelial cell necroptosis is also supported by measurements of serum cytokine and chemokine levels. (data not shown)

  The unique *in vivo* mouse model of the project resulted in the formation of two new relevant international collaborations with David Wallach (Weizmann Institute of Science, Israel) to investigate skin and liver specific caspase-9 deletion, and with Andreas Linkermann (Medizinische Klinik und Poliklinik III Universitätsklinikum, Germany) to investigate kidney specific caspase-9 deletion.
- Examination of caspase-9 associated molecules by tandem mass spectrometry did not lead to clearly identified binding partners of caspase-9. (The kinetics and dose of activation, the immunoprecipitating antibodies used, can all cause these approaches to fail.)
- We transfected GFP-caspase-9 construct into caspase-9-deficient Jurkat cells, but no changes in caspase-9 localization could be detected under necroptotic conditions.
- We encountered a number of technical difficulties in the production of recombinant caspase-9 in the bacterial system. Because caspase-9 binding partners were identified by immunoprecipitation using Jurkat cell lines, this aspect of the project became less important.

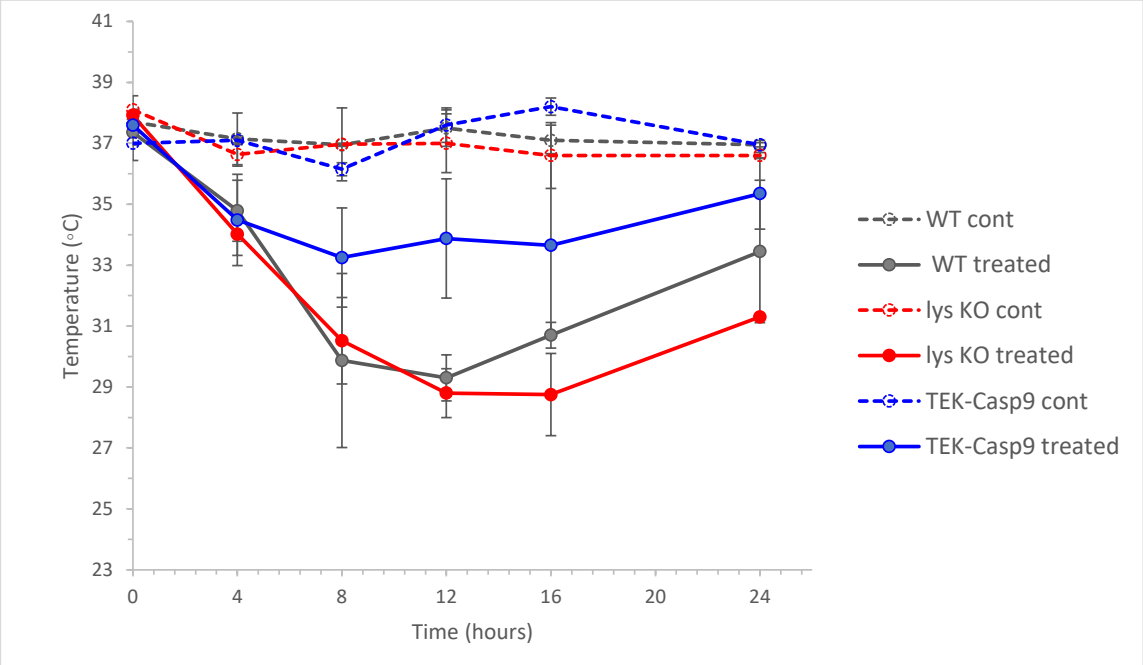


Figure 1: *Endotheial celll specific, but not myeloid specific knock down of caspase-9 protect mice from TNF-zVAD induced sepsis. Caspase-9 LoxP animals were crossed with TEK-Cre (TIE2) mice and lysoysme M-Cre mice. Sepsis was induced with 100 µg zVAD and. 12 µg TNF and mice were monitored for a drop in body temperature.*

AIM II. The interplay of apoptosis and programmed necrosis in the context of caspase-9 functional activities

The points listed in this AIM are partly covered by these publications:

- *Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis.* Varga Z, Molnár T, Mázló A, Kovács R, Jenei V, Kerekes K, Bácsi A, **Koncz G.** *Cancer Immunol Immunother*. 2020 May 29.
- *Cytotoxic activity of human dendritic cells induces RIPK1-dependent cell death* Varga Z, Rácz E, Mázló A, Korodi M, Szabó A, Molnár T, Szöőr Á, Veréb Z, Bácsi A, **Koncz G.** *Immunobiology. 2021 Jan;226(1):152032. doi: 10.1016/j.imbio.2020.152032.*

In these manuscripts we highlighted that:

- Macrophages are relatively resistant to most apoptotic stimuli, but are highly sensitive to necroptosis.

- M2 macrophages were highly sensitive, but M1 macrophages were unaffected by TAK1 inhibitor-induced necroptosis
- Inhibitors of caspase-9 binding partner AURKA rendered the two macrophage populations equally sensitive to TAK1 inhibitor-induced cell death
- At least two different necroptotic pathways operate in macrophages.
- Following PRR activation (LPS or CL075) the supernatants of human immature monocyte-derived DCs induce TNF-dependent apoptosis of Jurkat cells by allowing the immature DC to phagocytose and cross-present dead cell-derived antigens.
- Dendritic cell supernatant activates both RIPK1-dependent and -independent apoptosis
- Dexamethasone-induced tolerogenic conditions reduce the cytotoxic capacity of immature dendritic cells.

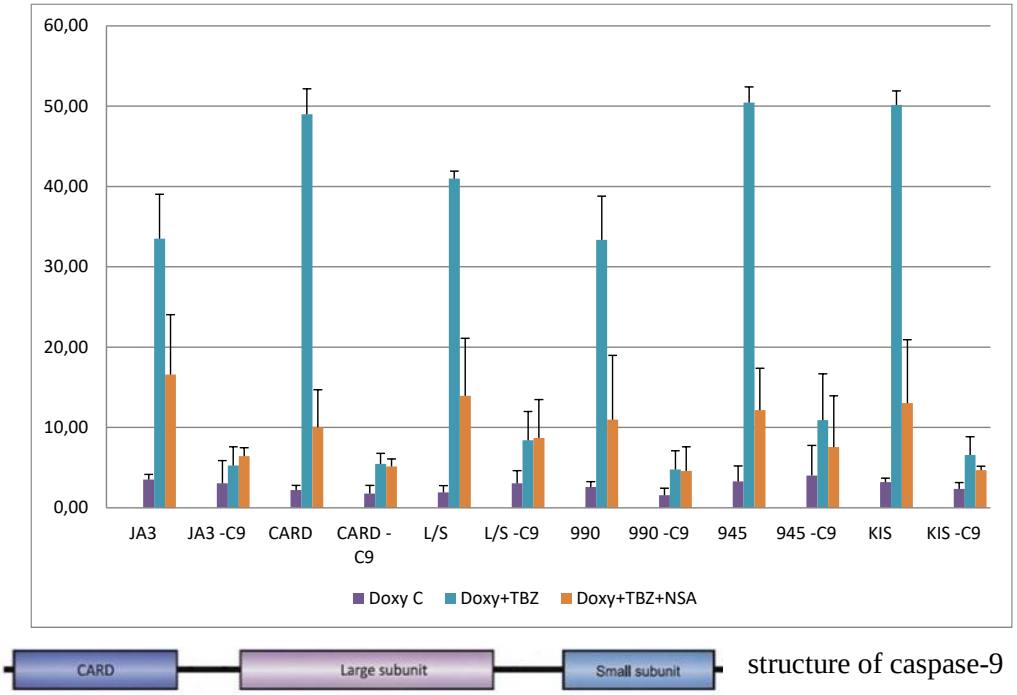
These results highlighted the translational potential of necroptosis in apoptosis resistant cells. Our results indicate a new concept to regulate the ratio of classically activated and alternatively activated macrophages by targeted elimination of each phenotypes. We present novel findings that DC-induced immunogenic cytotoxicity could be relevant for the initiation of cross-presentation and thus activating naive cytotoxic T cells.

Examination of some points in the project plan did not support the initial hypothesis. Although some of this result may lead to further publications, these results have not yet been published:

- The caspase-9 deficient and wild-type Jurkat cell line was transfected with different caspase-9 mutants (wild-type, catalytically inactive, non-cleavable, blocked in dimerization) or different fragments of caspase-9. We could not detect significant differences in the necroptosis susceptibility of caspase-9-deficient Jurkat cells compared to cells transfected with any particular domain of caspase-9. These results indicate that the unique domains of caspase-9 are unable to restore or to block the susceptibility of Jurkat cells to necroptosis. (Figure 2)
- We could not detect posttranslational modifications in caspase-9 under necroptotic conditions, either by tandem mass spectrometry or with phospho-specific antibodies.
- We could not detect differences between wild-type and caspase-9-deficient cells during different inflammasome activations, either by examining MEF cell lines or bone marrow-derived macrophage cell pairs.
- Caspase-9a or Caspase-9b isoforms were overexpressed into Caspase-9 deficient Jurkat cells. Both isoforms have been shown to restore the necroptotic capacity of caspase-9-deficient cells, indicating that caspase-9b is sufficient for necroptosis but does not mediate an apoptotic response. We could

not detect significant differences in caspase-9a / caspase-9b ratio between samples from normal skin or melanoma.

Figure 2.



structure of caspase-9

Figure 2. *JA3 cellls and its caspase-9-deficient counterpart (JA3-C9) were transfected with CARD domain, large subunit (990), catalytically inactive large subutint (945), small subunit (KIS), large and small subunits (L/S) of caspase-9 with doxicyklin inducible system. Necroptosis was induced by TNF, BV6 and Z-Vad stimuli in the presence or absence of NSA (necroptosis inhibitor) After 24 h, the extent of cell death was determined by PI staining.*

Deliverables:

New international collaborations

The development of the project resulted in the formation of two new international collaborations, with David Wallach (Weizmann Institute of Science, Israel) and with Andreas Linkermann (Medizinische Klinik und Poliklinik III Universitätsklinikum, Germany) The created caspase-9 LoxP animals will be crossed with mice strains expressing Cre recombinase in different tissues to investigate the cell type specific role of caspase-9 in non-apoptotic cell death pathways. (in kidney, liver and skin models)

Publications (with grant number):

- *The Role of Indoleamine-2,3-Dioxygenase in Cancer Development, Diagnostics, and Therapy.* Hornyák L, Dobos N, **Koncz G**, Karányi Z, Páll D, Szabó Z, Halmos G, Székvölgyi L.*Front Immunol.* 2018 Jan 31;9:151.
  IF:4,716 Q1 citations 173 (google scholar)

- *Ultraviolet radiation-mediated development of cutaneous melanoma: An update*.Emri G, Paragh G, Tósaki Á, Janka E, Kollár S, Hegedűs C, Gellén E, Horkay I**, Koncz G**, Remenyik É. *J Photochem Photobiol* B. 2018 Aug;185:169-175.
  IF:4,067 Q1 citations 43 (google scholar)

- *Current translational potential and underlying molecular mechanisms of necroptosis.* Molnár T, Mázló A, Tslaf V, Szöllősi AG, Emri G, **Koncz G.** *Cell Death Dis*. 2019 Nov 12;10(11):860.
  IF:6,304 D1 citations: 36 (google scholar)

- *Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis.* Varga Z, Molnár T, Mázló A, Kovács R, Jenei V, Kerekes K, Bácsi A, **Koncz G.** *Cancer Immunol Immunother*. 2020 May 29.
  IF:6.968 D1, citations:3 (google scholar)

- *Cytotoxic activity of human dendritic cells induces RIPK1-dependent cell death* Varga Z, Rácz E, Mázló A, Korodi M, Szabó A, Molnár T, Szöőr Á, Veréb Z, Bácsi A, **Koncz G.**
  Immunobiology. 2021 Jan;226(1):152032. doi: 10.1016/j.imbio.2020.152032.
  IF:3,144 Q2 citations:(google scholar)

- *Caspase-9 acts as a regulator of necroptotic cell death* Molnár T, Pallagi P, Tél B, Király R, Csoma E, Jenei V, Varga Z, Gogolák P, Odile Hueber A, Máté Z, Erdélyi F, Szabó G, Pettkó-Szandtner A, Bácsi A, Virág L, Maléth J, **Koncz G.** *FEBS J*. 2021 Apr 25. doi: 10.1111/febs.15898.
  IF:5,542, Q1 citations:2 (google scholar)

- *Multiple levels of immunological memory and their association with vaccination*: Bugya Z, Prechl J, Szénási T, Nemes É, Bácsi A, **Koncz G.** *Vaccines* 2021 Feb 19;9(2):174. doi:0.3390/vaccines9020174.
  IF:4.422, Q1 citations:1 (google scholar)

Comprehensive analysis of different tumor cell-line produced soluble mediators on the differentiation and functional properties of monocyte-derived dendritic cells. Sára Burai, Tamás Molnár, Márta Tóth, Tímea Szendi-Szatmári, Viktória Jenei, Zsuzsanna Bíró-Debreceni, Shlomie Brisco, Margit Balázs, Attila Bácsi, **Gábor Koncz**[c], Anett Türk-Mázló[c]. ***Sent for publication*** (c correspondent Author)

PhD dissertations based on the topic of the OTKA grant:

- Varga Zsófia: A RIPK1-függő sejthalál szerepe az immunválasz szabályozásában" (defence: 2021 May)
- Molnár Tamás: A szabályozott nekrózis jelátvitelének tanulmányozása (defence: 2021 November)

Conferences:

Conference appearances related to the listed publications are not indicated.

In the current epidemiological situation, we could not attend any conferences in 2020 and 2021.

5th European Congress of Immunology 2018, Amsterdam:

Flagellin increases death receptor-mediated celldeath in a RIP1-dependent manner

Tamas Molnar, Dora Hancz, Aniko Szabo, Zsofia Varga, Aniko Hancz, Andrea Gregus, Anne-Odile Hueber, Eva Rajnavolgyi, **Gábor Koncz**

The supernatant of immature dendritic cells mediates RIP1-dependent apoptosis

Zsófia Varga , Evelin Jakab-Rácz , Anikó Szabó, Éva Rajnavölgyi , **Gábor Koncz**

Magyar Immunológiai Társaság 46. Vádorgyűlése, 2018, Velence

Flagellin increases death receptor-mediated celldeath in a RIP1-dependent manner

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Iap antagonists in the regulation of immune response

Varga Zsófia, Rácz Evelin, Szabó Anikó, Korodi Mónika, Salamon Pál, Albert Beáta, Rajnavölgyi Éva, **Koncz Gábor**