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Caspase-2 does not play a critical role in cell death induction and bacterial clearance during *Salmonella* infection

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Members of the family of aspartate-specific cysteine proteases (caspases) aid in the removal of infected cells through their involvement in diverse programmed cell death (PCD) processes[1]. Despite substantial advances in understanding the individual roles of the different caspases in these processes, the function of caspase-2 remains relatively poorly understood[2, 3]. Caspase-2 has been linked to the host response against intracellular infections[4, 5, 6, 7, 8], DNA damage, endoplasmic reticulum stress and mitosis[9, 10]. PCD pathways are tightly interconnected and regulated by a remarkable level of redundancy, whereby caspases can operate in multiple pathways and thus substitute for the absence of other caspases[1, 11]. Therefore, and given previous reports of a role for caspase-2 in controlling PCD during *Salmonella* infection of macrophages[6], we hypothesised that so far unknown roles for caspase-2 could be uncovered under conditions where all key caspases required for the host response to *Salmonella* infection are absent[12].

To test this, we generated mice lacking caspases-1, -11, -12, -8, -2 and receptor-interacting serine/threonine-protein kinase 3 (RIPK3; the latter to prevent necroptosis caused by the loss of caspase-8) and compared their ability to control *Salmonella* infections to wild-type (WT) and caspase-2 deficient animals. This approach of deleting multiple effectors and regulators of PCD was chosen to mimic evasion strategies employed by bacteria, such as *Salmonella*, that can interfere with PCD pathways at many levels, often by targeting multiple components simultaneously[1]. We first examined how bone marrow-derived macrophages (BMDMs) responded to infection with *Salmonella enterica* serovar Typhimurium SL1344 (*S.* Typhimurium). Up to 70% of WT BMDMs were killed within 2 h of infection as determined by propidium iodide (PI) uptake (Figure 1a). *Casp2*$^{-/-}$ BMDMs showed a slightly reduced rate of cell death compared to WT BMDMs, which was only significant in the first hour of infection (Figure 1a). We performed a lactate dehydrogenase (LDH) release assay as a different

measurement of cell death. The observed differences in the PI assay between WT and *Casp2*$^{-/-}$ BMDMs within the first hour of infection were not evident in this assay (Supplementary Figure 1a), overall suggesting no critical role of caspase-2 in *Salmonella*-induced killing of macrophages *in vitro*. As previously reported, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ BMDMs were resistant to cell death upon SL1344 infection[12] (Figure 1a and Supplementary Figure 1a). Similar but not greater resistance was seen in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ BMDMs that additionally lacked caspase-2, indicating neither a potential pro- nor anti-apoptotic role for caspase-2 during *Salmonella* infection (Figure 1a and Supplementary Figure 1a). These findings were in line with bacterial growth as no differences in bacterial titres could be ascribed to the absence of caspase-2 alone or in combination with caspases-1, -11, -12, -8 and RIPK3 at 2 and 6 h post-infection (Figure 1b). These observations extend on previously published results which revealed that caspase-2 was required for early cell death induction by *Salmonella*[6]. However, overall findings obtained from diverse *in vitro* assays indicate that caspase-2 does not play a substantial primary or compensatory role in *Salmonella*-induced killing of BMDMs and hence the associated control of *Salmonella* replication.

The full redundancy of the cell death processes that ensure host protection during infections becomes obvious under *in vivo* conditions. Given that the role of caspase-2 in bacterial clearance has not yet been determined *in vivo*, we infected mice with 200 colony forming units (CFU) of the growth-attenuated *S.* Typhimurium strain BRD509, which results in a systemic infection that can be controlled in WT mice[13]. Focusing our analysis on the peak of infection, we found that bacterial titres in the liver and spleen 3 weeks post-infection were comparable in *Casp2*$^{-/-}$ and WT mice (Figure 1c), suggesting no critical role for caspase-2 in *S.* Typhimurium control. As shown previously, such control was compromised in *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice, resulting in severe disease[12] (Figure 1c). The

additional absence of caspase-2 did not cause a marked difference with only a minor drop in bacterial titres and a slight delay in the survival evident in the *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ mice compared to *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ mice (Figure 1c). Consistent with this interpretation, bacterial titres were comparable between mice of these two genotypes when analysed at the time of death (Supplementary Figure 1b). These results indicate that caspase-2 does not play a substantial role in cell death induction and *Salmonella* control *in vivo*, even under conditions that obviate potential compensatory roles by other caspases.

Collectively, these findings reveal that the absence of caspase-2 causes no major impairment of *Salmonella* control *in vitro* and *in vivo* and therefore argue against a significant role for caspase-2 operating as a fail-safe mechanism in the complex PCD network[1]. The mechanism by which the absence of caspase-2 reduces (albeit to a minor extent) the increase in bacterial burden caused by the loss of caspases-1, -11, -12, -8 and RIPK3 is not known. It may relate to its proposed roles in cell survival and cell division. In the complex situation of an *in vivo Salmonella* infection, caspase-2 could act as a pro-survival factor for activated macrophages in the absence of other caspases. Its absence would thus lead to a decrease in the number of macrophages that can be infected, which would reduce the replicative niche for the bacteria.

The lack of a clear phenotype of the caspase-2 knockout mice following *Salmonella* infection together with other studies indicating a limited role of caspase-2 in pathogen-induced cell death, raises the question whether caspase-2 plays any role in this context. There are some reports demonstrating that caspase-2 is of importance in infections with *Brucella abortus* and *Brucella suis* of macrophages[4, 5, 8]. Rough *Brucella* variants appear to induce a so-called hybrid

93 form of cell death that combines features of both apoptosis and pyroptosis[8] and is accompanied
94 by endoplasmic reticulum stress leading to mitochondrial damage, inflammasome activation
95 and pro-inflammatory cytokine release[14]. However, these *Brucella* species naturally occur as
96 smooth strains that prevent macrophage death to establish replication and chronic infection[4, 5]
97 and other studies found no evidence for a role of caspase-2 following *Brucella* infection[15]. The
98 described hybrid cell death of macrophages induced by attenuated rough *Brucella* variants
99 suggests that there might be specific conditions under which caspase-2 can contribute to
100 bacteria-induced cell death. However, our findings argue against a major role for caspase-2 in
101 the host response to intracellular bacterial pathogens.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICS STATEMENT

All animal experiments were approved by The University of Melbourne Animal Ethics Committee under project number 1714194.

AUTHOR CONTRIBUTIONS

A.S., S.B., M.J.H. and A.B conceptualized and designed the study; S.E. and A.B. designed, performed and analysed experiments and generated the figures; M.D. and A.R.L. provided technical and material support; S.E. and A.B. wrote the original draft of the manuscript; all authors contributed to writing, editing and revision of the manuscript; all authors read and approved the final manuscript.

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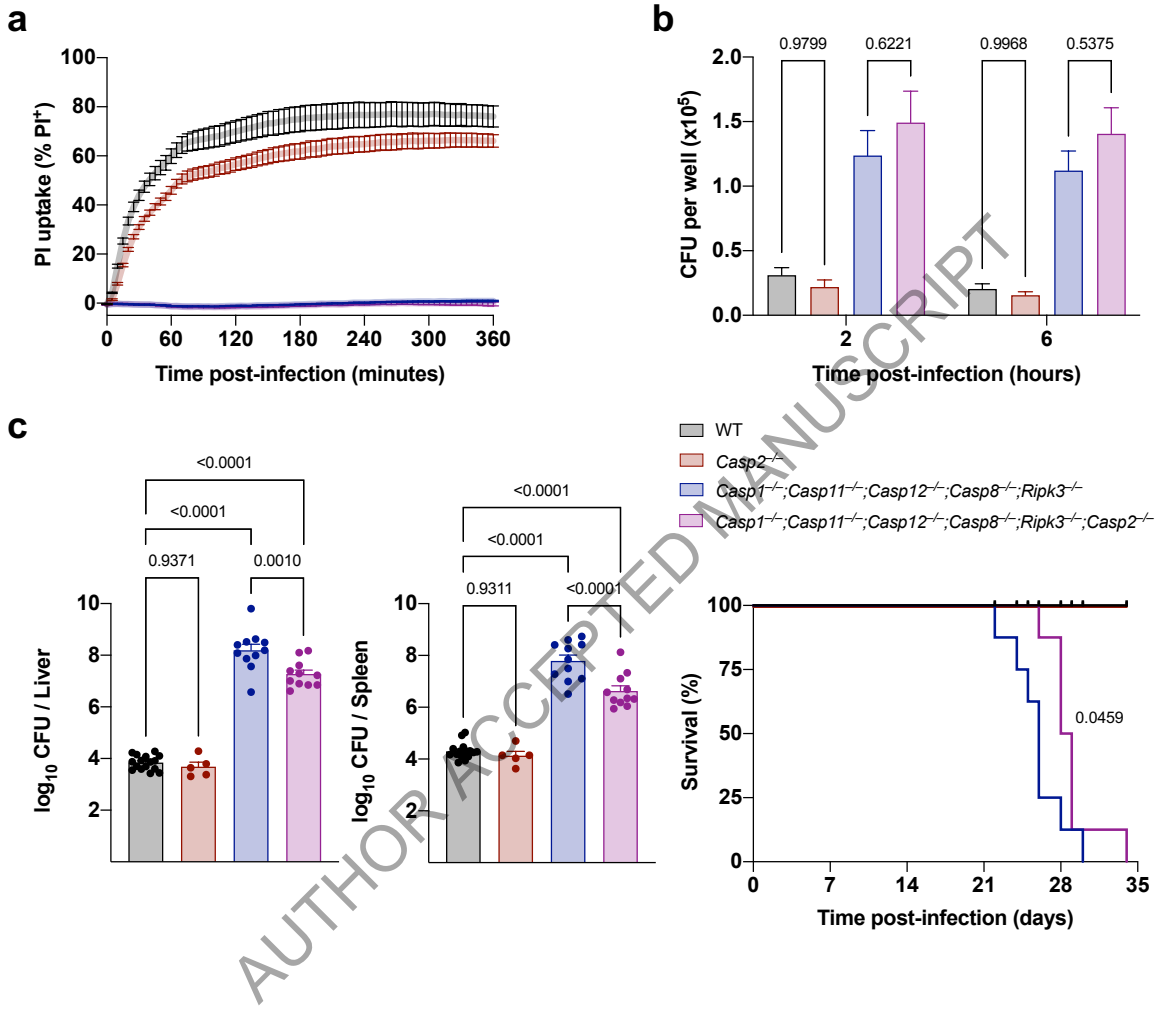
DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files.

FIGURE LEGEND

Figure 1 Caspase-2 does not play a critical role in cell death induction and *Salmonella* control

a-b Wild-type (WT; depicted in black), *Casp2*$^{-/-}$ (red), *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ (blue) and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ (purple) bone marrow-derived macrophages (BMDMs) were infected *in vitro* with *Salmonella* Typhimurium SL1344 (1 h; MOI 25-50) followed by gentamicin treatment to remove extra-cellular bacteria. **a** The uptake of propidium iodide (PI; a marker of cell death) of BMDMs was measured over a time period of 6 h post-infection. **b** Intracellular bacterial colony forming units (CFU) of surviving BMDMs per well were determined at the indicated time points post-infection. **c** WT, *Casp2*$^{-/-}$, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ mice were infected intravenously with 200 CFU of the growth-attenuated *Salmonella* Typhimurium strain BRD509. Bacterial titres in the liver and spleen 3 weeks post-infection and survival of infected mice were determined. All experiments were performed two to three times. *In vitro* assays were performed with ≥ 3 technical repeats. *In vivo* experiments were performed with each experimental group including n ≥ 3. Data are pooled and are expressed as mean ± SEM. Statistically significant differences were determined by either multiple unpaired t tests (a), two-way ANOVA (b) or one-way ANOVA (c). Mouse survival data were analysed using log rank (Mantel Cox) test; calculated *p*-values are depicted.



SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1 Caspase-2 is not essential for cell death induction and *Salmonella* control

a Wild-type (WT; depicted in black), *Casp2*$^{-/-}$ (red), *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ (blue) and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$;*Casp2*$^{-/-}$ (purple) bone marrow-derived macrophages were infected *in vitro* with *Salmonella* Typhimurium SL1344 (1 h; MOI 50) followed by gentamicin treatment to remove extra-cellular bacteria, and lactate dehydrogenase (LDH) release was analysed as an indicator for cell death. **b** WT, *Casp2*$^{-/-}$, *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$;*Casp2*$^{-/-}$ mice were infected intravenously with 200 colony-forming units (CFU) of the growth-attenuated *Salmonella* Typhimurium strain BRD509. Bacterial titres in the liver and spleen were determined at the time of sacrifice. All experiments were performed twice. *In vitro* assays were performed with ≥ 3 technical repeats. *In vivo* experiments were performed with each experimental group including n ≥ 3. Data are pooled and are expressed as mean ± SEM. Statistically significant differences were determined by either two-way ANOVA (a) or one-way ANOVA (b); calculated *p*-values are shown.

MATERIALS AND METHODS

Mice

C57BL/6 (WT), *Casp2*$^{-/-}$, *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$ and *Casp1*$^{-/-}$*;Casp11*$^{-/-}$*;Casp12*$^{-/-}$*;Casp8*$^{-/-}$*;Ripk3*$^{-/-}$*;Casp2*$^{-/-}$ mice were bred and maintained on a C57BL/6 background at The Walter and Eliza Hall Institute of Medical Research Animal Facility and The Doherty Bioresources Facility. Age- and sex-matched animals between 8 and 14 weeks of age were used for *in vivo* and *in vitro* studies. All animal experiments were approved by The University of Melbourne Animal Ethics Committee. Mice were euthanised at experimental end point or based on disease severity (including body condition, appearance and behaviour), described as 'mouse survival'.

Bone Marrow-Derived Macrophages

For the generation of murine bone marrow-derived macrophages (BMDMs), bone marrow was flushed from femurs and tibiae. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM 1g/L D-glucose, L-glutamine and 110 mg/L sodium pyruvate; Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 15% L929-conditioned medium (as a source of M-CSF), 100 U/mL penicillin (Clifford Hallam Healthcare) and 200 µg/mL streptomycin (Sigma-Aldrich) for 6 to 7 days in non-tissue culture treated dishes at 37°C and 5.6% CO_2. Differentiated BMDMs were seeded in antibiotic-free medium into 24- or 96-well plates at a density of 2 x 10^5 or 1 x 10^5 cells/well, respectively. BMDMs were allowed to rest for at least 12 h before infection and downstream analysis as described below.

In vivo and *in vitro* Infections

For *in vivo* infections, streptomycin-resistant *Salmonella* Typhimurium BRD509 were grown shaking at 37°C in Luria-Bertani (LB) broth supplemented with 50 µg/mL streptomycin and used in the exponential growth phase. Bacteria were washed and diluted in phosphate-buffered saline (PBS) and 200 colony-forming units (CFU) BRD509 were injected into the tail vein in a volume of 200 µL PBS. The numbers of replicating bacteria were determined by homogenising organs from infected mice in 5 mL of sterile PBS. The homogenates were serially diluted and plated onto LB agar plates supplemented with 50 µg/mL streptomycin and incubated at 37°C for 24 h. The bacterial burden of mice infected with BRD509 was comparable in female and male mice. For *in vitro* infections of primary BMDMs, streptomycin-resistant *Salmonella* Typhimurium SL1344 were grown shaking at 37°C overnight in LB broth supplemented with 50 µg/mL streptomycin and OD_{600} was determined using a spectrophotometer to calculate multiplicity of infection (MOI).

Propidium Iodide Uptake Assay

BMDMs were infected with SL1344 at a MOI of 50 in antibiotic-free medium and 50 µg/mL gentamicin (Sigma-Aldrich) was added 1 h after infection to remove extra-cellular bacteria. Cell death kinetics of *Salmonella*-infected BMDMs were measured by the uptake of propidium iodide (PI; Sigma-Aldrich) using the CLARIOstar Plus microplate reader (BMG LABTECH). The percentage of cell death at each time point was calculated by normalising the PI uptake of infected cells to that of paraformaldehyde (ProSciTech) treated cells, used as a control for maximum cell lysis.

Lactate Dehydrogenase Release Assay

BMDMs were infected with SL1344 at a MOI of 50 in antibiotic-free medium and 50 µg/mL gentamicin was added 1 h after infection to remove extra-cellular bacteria. The lactate

dehydrogenase (LDH) release of BMDMs at the indicated time points was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). The percentage of cytotoxicity at each time point was calculated by normalising the LDH release of *Salmonella*-infected cells to the maximum LDH release control.

Intracellular Bacterial Counting

BMDMs were infected with SL1344 at a MOI of 25 in antibiotic-free medium. After 1 h, cells were washed twice with PBS and the medium was replaced for 1 h with DMEM (+10% FBS) containing 100 µg/mL gentamicin, followed by DMEM (+10% FBS) with 10 µg/mL gentamicin for the remaining time to prevent growth of extracellular bacteria. *Salmonella*-infected BMDMs were washed twice with PBS and lysed with 1% TritonX-100 (Sigma-Aldrich) in distilled water at indicated time points. The numbers of replicating intracellular bacteria were determined by serially diluting and plating the cell lysates onto LB agar plates supplemented with 50 µg/mL streptomycin. Plates were incubated at 37°C for 24 h.

Quantification and Statistical Analysis

Prism v9.0.1 (GraphPad Software) was used to perform statistical tests. Groups were compared by either multiple t tests, one-way ANOVA or two-way ANOVA. Mouse survival data were analysed using log rank (Mantel Cox) test with the Bonferroni-corrected threshold of 0.0083; data of the *Casp2*$^{-/-}$ group were incremented by -0.5 data units on the Y axis for the purpose of visualisation. Please refer to the legend of the figures for description of the sample sizes (n).