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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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To the Editor:

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 [4–8], 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 (Fig. 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 (Fig. 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 Fig. 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] (Fig. 1a and Supplementary Fig. 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 (Fig. 1a and Supplementary Fig. 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 (Fig. 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 (Fig. 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] (Fig. 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 (Fig. 1c). Consistent with this interpretation, bacterial titres were comparable between mice of these two genotypes when analysed at the time of death (Supplementary Fig. 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

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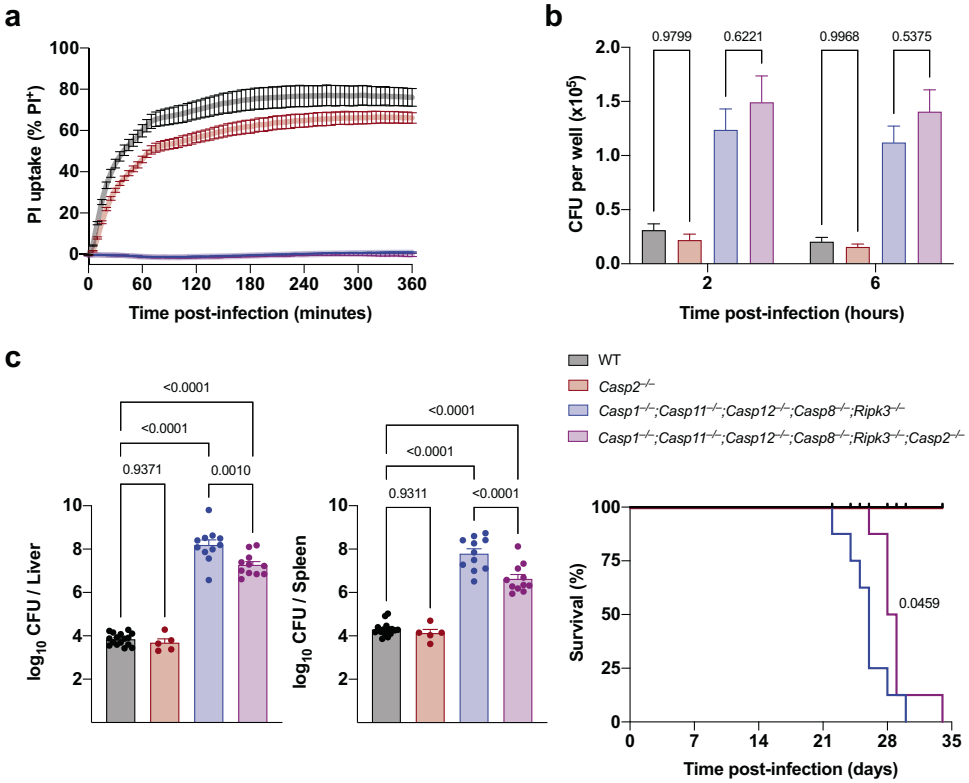

Fig. 1 Caspase-2 does not play a critical role in cell death induction and *Salmonella* control. a-b Wild-type (WT; depicted in black), *Casp2*$^{-/-}$ (red), *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$ (blue) and *Casp1*$^{-/-}$;*Casp11*$^{-/-}$;*Casp12*$^{-/-}$;*Casp8*$^{-/-}$;*Ripk3*$^{-/-}$;*Casp2*$^{-/-}$ (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 $n \geq 3$. 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.

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

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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.

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AUTHOR CONTRIBUTIONS

AS, SB, MJH and AB conceptualised and designed the study; SE and AB designed, performed and analysed experiments and generated the figures; MD and ARL provided technical and material support; SE and AB 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.

ETHICS STATEMENT

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

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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