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Title:

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

Date:

2021-12

Citation:

Engel, S., Doerflinger, M., Lee, A. R., Strasser, A., Herold, M. J., Bedoui, S. & Bachem, A. (2021). Caspase-2 does not play a critical role in cell death induction and bacterial clearance during Salmonella infection. *Cell Death and Differentiation*, 28 (12), pp.3371-3373. <https://doi.org/10.1038/s41418-021-00893-x>.

Persistent Link:

<https://hdl.handle.net/11343/340375>

1 **Functional flexibility and plasticity in immune control of systemic *Salmonella* infection**

2

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13

14 **Abstract**

15 Immunity to systemic *Salmonella* infection depends on multiple effector mechanisms.  
16 Lymphocyte-derived interferon gamma (IFN- $\gamma$ ) enhances cell-intrinsic bactericidal capabilities  
17 to antagonize the hijacking of phagocytes as replicative niches for *Salmonella*. Programmed  
18 cell death (PCD) provides another means through which phagocytes fight against intracellular  
19 *Salmonella*. We describe remarkable levels of flexibility with which the host coordinates and  
20 adapts these responses. This involves interchangeable cellular sources of IFN- $\gamma$  regulated by  
21 innate and adaptive cues, and the rewiring of PCD pathways in previously unknown ways. We  
22 discuss that such plasticity is likely the consequence of host-pathogen coevolution and raise  
23 the possibility of further functional overlap between these seemingly distinct processes.

## 24 **Introduction**

25 Oral infection with *Salmonella enterica* causes two types of pathologies with very different  
26 consequences [1]. Gastroenteritis, a self-limiting infection, ensues when the bacteria are  
27 controlled at the epithelial level [2]. In contrast, spread of *Salmonella* into deeper tissues can  
28 result in systemic dissemination and enteric fever [3], a life-threatening disease that affects  
29 10-20 million people worldwide and kills >100,000 individuals per annum [4]. Observations  
30 from *Salmonella* infections in inherited or infection-induced immunodeficiency indicate a  
31 major role for the immune system during both types of infections [5]. However, many aspects  
32 of precisely how immune mechanisms enable infected cells to control, purge and eliminate  
33 *Salmonella* remain unclear [6]. This opinion article focuses on systemic *Salmonella* infections  
34 in mice and discusses recent developments about the role of phagocytes, cell-intrinsic and cell-  
35 extrinsic immune effector mechanisms, and their regulation.

36

## 37 ***Salmonella* replicates in cells specialized for the destruction of intracellular bacteria**

38 *Salmonella* replicates primarily within phagocytes in the spleen and liver during systemic  
39 infection [7]. Key to this important virulence trait are two needle-like type III secretion systems  
40 (T3SS) encoded by *Salmonella* Pathogenicity Island (SPI)-1 and SPI-2 that enables *Salmonella*  
41 to inject dozens of effector proteins into the cytosol of phagocytes [8]. These proteins prompt  
42 the repurposing of a host cell-derived membrane compartment into *Salmonella*-containing  
43 vacuoles (SCVs), which promote bacterial replication and shield the bacteria from cytosolic  
44 effector mechanisms, such as production of reactive oxygen and nitrogen species (ROS, NO)  
45 [9]. Not all bacteria remain confined to these sanctuaries [10]. Some are shed into the cytosol  
46 from where they regain access to the extracellular space and spread further, enabling the  
47 infection of more host cells, and restarting the cycle of establishing SCVs. This transitioning  
48 in and out of the extracellular space provides the host with additional opportunities to mount

49 immune responses against the bacteria. Free bacteria can be engulfed by monocytes,  
50 macrophages and neutrophils. These phagocytes are equipped with Toll-like receptors (TLRs)  
51 capable of detecting vital components of *Salmonella* [11], such as flagellin or  
52 lipopolysaccharides (LPS). Stimulation of these TLRs and other pathogen recognition  
53 receptors induces the secretion of pro-inflammatory cytokines, such as tumour necrosis factor  
54 alpha (TNF- $\alpha$ ), interleukin (IL)-6 and IL-12, which provide important cues for the  
55 orchestration of the initial immune response against this infection.

56

### 57 **Do CD4<sup>+</sup> T cells enhance resistance of phagocytes against infection through IFN- $\gamma$ ?**

58 Dendritic cells (DCs) residing at sites of infection can also capture *Salmonella* [12]. These  
59 specialized phagocytes process proteins from engulfed bacteria and upon migration to the  
60 lymph node present peptide antigens to naïve T cells in complex with major histocompatibility  
61 complex (MHC) molecules [13]. Naïve CD4<sup>+</sup> T cells recognizing MHC class II-restricted  
62 *Salmonella*-derived antigens (pMHC) on activated, IL-12 secreting DCs differentiate into T  
63 helper type 1 (Th1) cells and migrate to sites of infection. Here, they scan cells for matching  
64 pMHC, and upon T cell receptor (TCR) stimulation synthesize and release IFN- $\gamma$ . This cytokine  
65 promotes phagolysosome fusion, acidification of the lysosomal content and ROS/NO  
66 production [14]. These bactericidal activities combine to inactivate microbial proteins, oxidize  
67 lipids and destroy microbial DNA, thus leaving the bacteria irreversibly damaged and  
68 preventing their replication. Mice lacking CD4<sup>+</sup> T cells fail to clear *Salmonella* infections [15,  
69 16] and patients with reductions in CD4<sup>+</sup> T cells caused by acquired immunodeficiency  
70 syndrome (AIDS) are particularly susceptible to severe *Salmonella* infection, especially by  
71 serovars that normally only cause self-limiting gastroenteritis [17, 18]. Together with IFN- $\gamma$ -  
72 deficient mice failing to control *Salmonella* infections [16, 19] and humans with mutations in

73 the receptor for IFN- $\gamma$  being particularly susceptible to *Salmonella* infections [20], these  
74 observations suggest that *Salmonella*-specific Th1 cells are the critical source of IFN- $\gamma$  [5].

75

#### 76 **Antigen-independent provision of IFN- $\gamma$ from interchangeable cellular sources**

77 We have previously examined the relative roles of CD4<sup>+</sup> T cells and IFN- $\gamma$  during murine  
78 systemic infection using a growth-attenuated *Salmonella* strain that mimics key modalities of  
79 human enteric fever. Mice lacking CD4<sup>+</sup> T cells controlled this infection over the first three  
80 weeks, but ultimately failed to clear the bacteria. Notably, this outcome differed substantially  
81 from the lethal salmonellosis that developed in IFN- $\gamma$ -deficient mice, where even the initial  
82 control of bacterial replication failed [16]. Although these observations are generally consistent  
83 with important roles for CD4<sup>+</sup> T cells and IFN- $\gamma$  during *Salmonella* infection, they also imply  
84 that CD4<sup>+</sup> T cells are at least partially redundant for the provision of IFN- $\gamma$ . In exploring this  
85 redundancy further, we found that adoptive transfer of either CD8<sup>+</sup> T cells or NK cells could  
86 restore some control of bacterial growth in IFN- $\gamma$ -deficient mice [21, 22]. We did, however,  
87 also find that *Salmonella* control was intact in mice lacking CD8<sup>+</sup> T cells or NKT cells,  
88 suggesting that as long as IFN- $\gamma$  could be provided by any one type of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T  
89 cells or non-conventional T cells, such as NKT cells, the infection could be controlled [16].  
90 Intriguingly, this provision of protective IFN- $\gamma$  did not depend on *Salmonella*-specific  
91 responses by lymphocytes. In fact, memory CD8<sup>+</sup> T cells specific for an unrelated viral  
92 infection conferred significant control to *Salmonella* infected *Ifng*<sup>-/-</sup> mice [21]. This was not  
93 due to TCR cross-reactivity but resulted from an alternative, antigen-independent cellular  
94 response, in which IL-18 stimulated protective IFN- $\gamma$  secretion from CD8<sup>+</sup> T cells, CD4<sup>+</sup> T  
95 cells and NK cells [21-23]. Antigen-independent IFN- $\gamma$  responses therefore compensated for  
96 the absence of *Salmonella*-specific Th1 cells as crucial providers of IFN- $\gamma$ . Interestingly, we

97 also found that control of the infection was possible without IL-18 (unpublished data), and only  
98 broke down to levels comparable to IFN- $\gamma$ -deficient mice in *Rag*  $\times$  *Il2rg*<sup>-/-</sup> mice lacking all  
99 lymphocytes (i.e. T cells, B cells and NK cells) [21, 22]. It therefore appears that innate triggers  
100 can compensate for a lack of TCR-driven IFN- $\gamma$  provision by *Salmonella*-specific Th1 cells,  
101 and conversely, that TCR-driven provision of IFN- $\gamma$  is also sufficient without innate  
102 stimulation.

103

#### 104 **Control of *Salmonella* infection through inflammasomes and pyroptosis**

105 The implication of IL-18 in the response to *Salmonella* suggested a role for the nucleotide-  
106 binding oligomerization domain-like receptors C4 (NLRC4) and its upstream adaptor proteins  
107 of the NLR apoptosis inhibitory protein (NAIP) family that together form the so-called NLRC4  
108 inflammasomes [24]. These large protein structures serve as platforms for the activation of  
109 caspase-1, which proteolytically processes pro-IL-1 $\beta$  and pro-IL-18 into their bioactive forms.  
110 Active caspase-1 also cleaves N-terminal fragments from gasdermin D (GSDMD). The  
111 fragments then oligomerize and introduce pores into the host cell plasma membrane, allowing  
112 the release of IL-1 $\beta$  and IL-18 into the extracellular space [25]. Notably, the resulting influx of  
113 fluids and molecules initiates a form of PCD termed pyroptosis [26], which can also be  
114 triggered by the activation of caspase-11 [27]. With *Salmonella*-derived flagellin, components  
115 of its SPI-1 encoded T3SS, and LPS capable of triggering pyroptosis [28], it seemed possible  
116 that infected phagocytes would deploy this complex process to purge *Salmonella* from these  
117 cells. Indeed, NLRC4 and caspase-1 were then found to be required for the prevention of  
118 *Salmonella* replication through inflammasome-induced pyroptosis *in vitro* [29, 30]. However,  
119 subsequent *in vivo* studies painted a more complex picture, with *Casp1/11*<sup>-/-</sup> mice only having  
120 surprisingly minor, if any defects in controlling systemic infections with the growth-attenuated  
121 *Salmonella* strain SL1344  $\Delta$ *aroA* [31].

122

123 **Caspase-8-mediated apoptosis can compensate for the lack of pyroptosis**

124 More recently it became clear that the initiator caspase-8 can compensate for the absence of  
125 caspases-1 and -11 by inducing GSDMD cleavage and pyroptosis instead [32]. This was  
126 intriguing, as caspase-8 was thought to play an exclusive role in the initiation of the extrinsic  
127 apoptosis pathway. In this alternative form of PCD, caspase-8 is activated by extracellular  
128 ligands of the TNF superfamily, including TNF- $\alpha$  and FAS ligand (FASL). Activated caspase-  
129 8 then proteolytically activates the so-called executioner caspases-3 and -7 that set-in motion  
130 cell death by cleaving hundreds of substrate proteins to orchestrate the ordered demolition of  
131 the dying cells. Caspase-8 can also activate the executioner caspases indirectly by engaging the  
132 intrinsic apoptotic pathway through proteolytic activation of the BH3-interacting-domain death  
133 agonist (BID) leading to Bcl-2-associated X and K protein (BAX/BAK) mediated  
134 mitochondrial outer membrane permeabilization (MOMP) and consequent activation of  
135 caspase-9 [33, 34]. To test if caspase-8 can compensate for the lack of caspases-1 and -11 by  
136 providing an alternative path to GSDMD-dependent pyroptosis *in vivo*, we infected  
137 *GsdmD/Bid*<sup>-/-</sup> mice (unpublished data) and *Casp8/Ripk3*<sup>-/-</sup> mice with growth-attenuated  
138 *Salmonella*. However, both mouse strains had no defect in controlling *Salmonella* infection  
139 that was greater than the defect caused by the absence of caspases-1 and -11. In striking  
140 contrast, control of bacterial replication failed when caspases-1, -11 and -8 were absent in  
141 combination [31], demonstrating that a caspase-8-dependent mechanism not only compensated  
142 for the lack of caspases-1 and -11 but also for pyroptosis itself. Intrinsic apoptosis, necroptosis  
143 and the CARD-containing caspases, caspase-12 [31] and caspase-2 [35], seemed to play only  
144 minor if any roles in the control of systemic growth-attenuated *Salmonella* infection in mice.  
145 Mechanistically, the compensation between caspase-1/caspase-11 and caspase-8 involved the  
146 rewiring of the known apoptosis and pyroptosis signaling pathways in previously unknown

147 ways. For example, caspase-1 could functionally replace caspase-8 by stimulating MOMP  
148 formation and triggering the executioner caspases-3 and -7. The reverse was also the case,  
149 where caspase-8 could take over the functions of caspase-1 in driving pyroptosis. In fact, we  
150 made the surprising discovery that as long as caspase-1 and caspase-8 were present, cells  
151 infected with wild-type *Salmonella* SL1344 could undergo cell death independently of all  
152 known executioner caspases and pore forming molecules [31]. Phagocytes therefore seem to  
153 have at their disposal a system of cell death-inducing pathways that they can deploy with  
154 remarkable plasticity to control *Salmonella* infections. This crosstalk between apoptosis,  
155 necroptosis and pyroptosis with caspases-1 and 8 as core mediators is reminiscent of recent  
156 reports demonstrating important overlapping roles during embryogenesis and development [36,  
157 37].

158

159 **Are IFN- $\gamma$ , lymphocytes and programmed cell death linked in the control of *Salmonella***  
160 **infection?**

161 The functional interchangeability of caspase-1 and caspase-8 was reminiscent of the  
162 redundancies we have identified in the cellular sources that can provide IFN- $\gamma$  to control  
163 *Salmonella* infections. Moreover, the lethal type of salmonellosis that ensued in the absence of  
164 pyroptosis and caspase-8-mediated apoptosis had comparable kinetics to that occurring in mice  
165 lacking all lymphocytes or IFN- $\gamma$ . Recent studies point to a possible explanation for these  
166 similarities. IFN- $\gamma$  can prime macrophages to become susceptible to TLR or TNF- $\alpha$ -induced  
167 cell death [38, 39] and this type of cell death was characterized by the activation of caspase-8  
168 and the executioner caspases-3 and -7. In the case of TLR-induced cell death, this also appeared  
169 to involve NO and imbalances between inhibitors and inducers of the intrinsic apoptosis  
170 pathway. Precisely how IFN- $\gamma$  priming facilitates the activation of caspase-8 was not examined,  
171 but TNF- $\alpha$  is a known trigger of the extrinsic apoptosis pathway and has previously been linked

172 to the innate control of *Salmonella* and *Mycobacterium tuberculosis* infections [40, 41]. In this  
173 context it is also interesting to consider that IFN- $\gamma$  increases the expression of MHC class II  
174 molecules on phagocytes and that effector CD4<sup>+</sup> T cells do not only secrete IFN- $\gamma$  but also  
175 increase the expression of FASL. It is therefore possible that a so far unexplored relationship  
176 between cytokines and PCD exists during *Salmonella* infection. IFN- $\gamma$  or TNF- $\alpha$  could prime  
177 phagocytes for extrinsic apoptosis, and the eventual triggering of caspase-8 could be regulated  
178 in an antigen-specific manner by CD4<sup>+</sup> T cells through FASL. It will be interesting to  
179 investigate this in detail and also to compare whether the use of attenuated *Salmonella* strains  
180 in *in vivo* studies influences the levels of redundancy discussed here.

181

182

### 183 **Conclusions and Perspectives**

184 Our exploration of how particular processes of the immune system contribute to *Salmonella*  
185 control has revealed a remarkable extent of flexibility and plasticity [31, 42]. Critical cytokines,  
186 such as IFN- $\gamma$ , can be released through innate immune pathways when adaptive immune  
187 responses are absent and *vice versa*. Moreover, different types of lymphocytes can act as  
188 interchangeable sources of IFN- $\gamma$ . We also found a remarkable degree of flexibility in the  
189 contribution of different types of PCD and their molecular regulation to the control of  
190 *Salmonella* infections. This propensity for functional compensation is likely related to the  
191 ongoing struggle between pathogens seeking to evade protective host responses [43, 44] and  
192 the host developing strategies to offset these attempts. For example, flagellin is rapidly  
193 downregulated once *Salmonella* infects a cell [30]. This likely serves to prevent NLRC4  
194 activation, as demonstrated by the rapid NLRC4 and caspase-1/caspase-11-dependent  
195 clearance of *Salmonella* strains engineered to continuously express flagellin [45]. Similar  
196 mechanisms occur with SPI-1 T3SS-derived agonists of NLRs [30, 46]. In this context it is also

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interesting to note that the *Salmonella* SPI-2 effector protein SteD can induce the ubiquitination and subsequent depletion of MHC class II molecules from the surface of infected cells [47]. This ability has been thought of as a means to prevent the priming of *Salmonella*-specific CD4<sup>+</sup> T cells. However, it seems equally possible that the bacteria utilize this mechanism to prevent FASL-mediated induction of extrinsic apoptosis by CD4<sup>+</sup> T cells recognizing MHC class II-restricted *Salmonella* antigens on infected phagocytes.

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204

## 205 **ACKNOWLEDGEMENTS**

206 We are indebted to all members past and present of the Bedoui, Strugnell, Strasser and Herold  
207 laboratories for their dedicated work and the many insightful discussions. This research was  
208 supported by the NHMRC (1159658 to M.J.H. and S.B.; and 1194482 to S.B. and 1156095 to  
209 M.J.H.), the Australian Research Council (1901102213) and the German Research Council  
210 (GRK2168 to S.E. and S.B.). Figures were created using BioRender.

211

## 212 **Legends**

### 213 **Fig. 1 Cell-intrinsic and cell-extrinsic immune effector mechanisms and their** 214 **regulation during systemic *Salmonella* infection**

215 (A) The detection of cytosolic *Salmonella* proteins, including flagellin and structural  
216 components of the *Salmonella* Pathogenicity Island (SPI)-1 encoded T3SS, initiates the  
217 assembly of inflammasomes. Following recruitment and activation within the NLRC4  
218 inflammasome, caspases-1 and -8 cleave GSDMD. The N-terminal GSDMD fragment  
219 translocates to the plasma membrane and oligomerises to form pores resulting in pyroptotic  
220 cell death. In contrast, caspase-11 directly senses cytosolic *Salmonella* and is activated by LPS.  
221 Activated caspase-11 can also cleave GSDMD, thus providing an alternative path towards cell

222 lysis. Pyroptotic cell death results in the release of pro-inflammatory cytokines, such as IL-1 $\beta$   
223 and IL-18, which are cleaved into their bioactive forms by caspase-1. IL-18 can induce the  
224 release of IFN- $\gamma$  by activated NK cells, CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells. By acting on  
225 phagocytes, IFN- $\gamma$  stimulates antimicrobial capacities, such as ROS production, antigen  
226 presentation and expression of death receptors. (B) *Salmonella* limits inflammasome activation  
227 and pyroptosis by downregulation of SPI-1 and flagellin, and interferes with MHC class II  
228 antigen presentation. While caspase-8-dependent apoptosis can compensate for the lack of  
229 pyroptotic cell death, it is not clear what triggers caspase-8 under these circumstances.  
230 Considering that the SPI-2 effector protein SteD depletes mature MHC class II molecules from  
231 the surface of infected cells, that effector CD4<sup>+</sup> T cells can express FASL and that IFN- $\gamma$   
232 enhances the expression of MHC class II molecules and the receptor for FASL, it is possible  
233 that the caspase-8-dependent switch from pyroptosis to apoptosis is orchestrated by  
234 *Salmonella*-specific CD4<sup>+</sup> T cells.

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