ARTICLE

Intestinal epithelial Caspase-8 signaling is essential to prevent necroptosis during *Salmonella* Typhimurium induced enteritis

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Although induction of host cell death is a pivotal step during bacteria-induced gastroenteritis, the molecular regulation remains to be fully characterized. To expand our knowledge, we investigated the role of the central cell death regulator Caspase-8 in response to *Salmonella* Typhimurium. Here, we uncovered that intestinal *salmonellosis* was associated with strong upregulation of members of the host cell death machinery in intestinal epithelial cells (IECs) as an early event, suggesting that elimination of infected IECs represents a host defense strategy. Indeed, *Casp8*^{ΔIEC} mice displayed severe tissue damage and high lethality after infection. Additional deletion of *Ripk3* or *Mlkl* rescued epithelial cell death and lethality of *Casp8*^{ΔIEC} mice, demonstrating the crucial role of Caspase-8 as a negative regulator of necroptosis. While *Casp8*^{ΔIEC}*Tnfr1*^{-/-} mice showed improved survival after infection, tissue destruction was similar to *Casp8*^{ΔIEC} mice, indicating that necroptosis partially depends on TNF-α signaling. Although there was no impairment in antimicrobial peptide secretion during the early phase of infection, functional Caspase-8 seems to be required to control pathogen colonization. Collectively, these results demonstrate that Caspase-8 is essential to prevent *Salmonella* Typhimurium induced enteritis and to ensure host survival by two different mechanisms: maintenance of intestinal barrier function and restriction of pathogen colonization.

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INTRODUCTION

The enteroinvasive bacterial pathogen *Salmonella enterica* serovar Typhi is the most common gastrointestinal pathogen that causes substantial human diseases ranging from gastroenteritis to systemic typhoid fever.¹ Infection with *Salmonella* Typhimurium leads to acute, self-limiting intestinal inflammation associated with diarrhea, vomiting, and abdominal pain.² The ability of pathogens, such as *Salmonella* to colonize and invade the gut is controlled by several factors, including the intestinal microbiota, the gut associated immune system and the intestinal epithelium.^{3,4}

In order to gain access into the host system and to initiate systemic infection, *Salmonella* initially has to cross the intestinal epithelium through enterocytes and M cells located above the Peyer's patches.^{5–7} The infectious cycle of *Salmonella* Typhimurium includes three important steps: invasion and colonization of the host cell, the establishment of the *Salmonella*-containing vacuole, a specific niche for replication,⁸ and the escape from the host cell to infect neighboring cells and tissues.^{9,10} All three phases are closely connected to the host cell death machinery, since during the initial step, epithelial cell death needs to be prevented, whereas in the last step *Salmonella* requires strategies to leave the host cells.^{11,12} Accordingly, previous publications have demonstrated that *Salmonella* infection in the gut is characterized by increased epithelial cell death.^{7,13} While epithelial cell death is an important pathological process and the major cause of tissue damage during infection, clearance of infected cells by active cell death has been characterized as a host defense strategy to limit pathogen replication and survival.¹⁴

Whether *Salmonella*-induced epithelial cell death represents a host response or rather a bacterial strategy to promote infection still needs to be elucidated. Until recently, apoptosis and pyroptosis mediated by inflammasome activation were considered as principal mechanisms of programmed host cell death in response to infection.^{15,16} Caspase-8 is well known for its function as an initiator caspase during receptor mediated apoptosis. Earlier studies have further implicated non-apoptotic roles for Caspase-8 in IL-1β maturation and inflammasome regulation in response to infection by microbial pathogens.^{17–19} A recent study further implicated a potential role for Caspase-8 in the expulsion of intestinal epithelial cells (IECs) by NLRC4 inflammasome activation.²⁰ While these studies suggest that Caspase-8 is a strong mediator of cell death and inflammation, research of the last decade has clearly demonstrated that Caspase-8 additionally has a rather prosurvival function by suppressing regulated necrosis (necroptosis). The role of regulated necrosis during bacterial infection is currently not well understood. Only a few murine studies reported evidence for necroptosis in infection related disease conditions and these data still remain controversial. While reduced levels of macrophage necroptosis have been demonstrated to increase susceptibility to systemic *Salmonella* Typhimurium infection in mice lacking interferon alpha receptor (*Ifnar*^{-/-}), the same study reported that *Ripk3*^{-/-} mice showed similar mortality after intravenous infection.²¹ Interestingly, while macrophage necroptosis seems to be only partially dependent on RIPK3, another study observed that RIPK1 kinase-dead mice (K45A mutation) are highly susceptible to

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Fig. 1 Infection of wildtype mice with *Salmonella Typhimurium* provokes caspase activation in the intestinal epithelium. **a–e** Wildtype (C57BL/6) animals were infected orally with 10^9 CFU of *Salmonella Typhimurium* (Δ aroA) and analyzed at indicated time points ($n = 3$ per time point). Experiments were repeated twice with similar results. **a** Quantification of colonic *Casp8* and *Casp3* mRNA expression relative to the expression of *Hprt*. Data are displayed as mean values + SD, * $p \leq 0.05$. **b** Western blot analysis of whole tissue lysates (colon) from mock treated and infected wildtype mice using antibodies against full length Caspase-8 (inactive, p55), activated Caspase-8 (p18), full length Caspase-3 (inactive, p35), activated Caspase-3 (p17/19), MLKL (54 kDa) and RIPK3 (57 kDa). β -Actin was used as a loading control. **c** Immunohistochemical staining using an antibody against activated CASP8 (red) in colon cross sections 3 days after infection (ti: tissue, lu: lumen; scale bar: 100 μ m). Hoechst 33342 (blue) was used for counterstaining. **d** Representative images of colon cross sections stained with H&E or immunohistochemically with an antibody against activated CASP3 (red) combined with TUNEL assay (green, scale bar: 100 μ m). Nuclei were counterstained with Hoechst 33342 (blue). **e** Gene transcription analysis of colonic *Ripk3* and *Mkl1*. Error bars indicate + SD, gene expression levels are shown relative to *Hprt*, * $p \leq 0.05$

Salmonella Typhimurium challenge, accompanied by an elevated bacterial burden and reduced macrophage death.²² Both studies reported that necroptosis is strongly dependent on interferon (IFN) signaling. Notably, we just recently identified that IFNs induce *Mkl1* gene transcription via activation of the transcription factor STAT1.²³ While previous studies indicate that macrophage necroptosis might contribute to systemic *Salmonella Typhimurium* infection, the role of regulated apoptotic and non-apoptotic epithelial cell death during enteric infection has not been elucidated.

Here, we demonstrate that the early epithelial response to *Salmonella Typhimurium* infection is characterized by a strong upregulation and activation of key components of the host cell death machinery. By using mice with an IEC specific deletion of the central cell death regulator Caspase-8 (*Casp8* ^{Δ IEC} mice) we identified that Caspase-8 is required to ensure a proper host response against *Salmonella Typhimurium* infection. Mechanistically, we uncovered that Caspase-8 primarily orchestrates the host cell death response by mediating apoptosis and suppression of necroptosis, but did not influence inflammatory activation. Thus, our data show that depending on the cellular context and the physiological situation Caspase-8 has fundamental functions that are essential to eliminate enteric infection.

RESULTS

Enteric pathogen infection triggers epithelial cell death

The early epithelial response to bacterial infection includes the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α that potentially orchestrate epithelial cell death.²⁴ In order to investigate the host cell death mechanism in response to invasive enteric pathogens, we initially evaluated the expression of factors known to be involved in regulated epithelial cell death in mice infected with *Salmonella Typhimurium*. While the abundance of gene transcripts involved in cell death regulation was not enhanced at early time points during infection, we observed elevated levels of central mediators of apoptosis such as Caspase-8 and Caspase-3, both on mRNA and protein levels 72 h post-infection (Fig. 1a, b). Similarly, we identified enhanced expression of factors that are well-known for their functions as cell death inducers, such as TNF- α (Figure S1A). Accordingly, Western blot analysis revealed enhanced activation of Caspase-8 and Caspase-3 already 24 h post-infection with increasing intensity during the course of infection (Fig. 1b). In line with these data, we observed enhanced epithelial cell shedding accompanied with strong signals for activated caspases in the colon and cecum of infected mice. Notably, caspase activation was most pronounced in cells at the upper part of the crypt during the early phase of infection (Fig. 1c, d).

These data suggest that caspase activation is linked to epithelial cell death and cell extrusion in response to *Salmonella* Typhimurium infection.

Previous studies of our own group and others have demonstrated that Caspase-8 is a central regulator of both apoptosis and

necroptosis.^{25,26} Interestingly, in addition to caspase activation, we observed elevated levels of central necroptosis mediators, including MLKL and RIPK3 during *Salmonella* Typhimurium infection (Fig. 1b, e). However, we could not observe phosphorylation of RIPK3 or MLKL, suggesting that Caspase-8 signaling is

Fig. 2 *Casp8^{ΔIEC}* mice are highly susceptible to *Salmonella* Typhimurium infection and develop severe enteritis. **a–i** Control (*Casp8^{fl}*) ($n = 4$) and *Casp8^{ΔIEC}* mice ($n = 4$) were mock treated or infected with 10^9 CFU of *Salmonella* Typhimurium (Δ aroA, attenuated). **d–i** Data derived at day 3 of infection. Experiments were repeated three times with similar results. **a** Body weight curve of animals. Data are displayed in percent relative to initial body weight $\pm$ SD. **b** Kaplan–Meier survival curve of control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice, $*p \leq 0.05$. **c** Representative endoscopic pictures of control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice 4 days after infection. **d** Representative pictures and statistical analysis of colon shortening ($n \geq 3$ per group; scale bar: 1 cm, $**p \leq 0.01$). **e** Representative H&E stained colon cross sections of mock treated and infected animals (scale bar: 100 μ m) and colon cross sections stained with an antibody against activated CASP3 (red) combined with TUNEL assay (green). Nuclei were counterstained with Hoechst 33342 (blue) (scale bar: 100 μ m). **f** Pathology score [maximum: 9] of uninfected and infected colon tissue of control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice. Data are displayed as mean value $\pm$ SD, $n \geq 3$ per group, $**p \leq 0.01$, $***p \leq 0.001$. **g** Representative images of colon cross sections stained with an antibody against β -Catenin (CTNNB1) (red) and counterstained with Hoechst 33342 (blue) (scale bar: 75 μ m). **h** Serum concentration of orally administered FITC-Dextran in control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice. Serum was separated from the collected blood and the concentration of FITC-dextran was determined by spectrophotofluorometry with an excitation of 485 nm and an emission of 528 nm using a serially diluted FITC-dextran standard. Data are displayed as individual values with indicated mean values $\pm$ SD ($n \geq 5$ per group, data are combined from two independent experiments). **i** Quantification of colonic *Villin* mRNA expression ($n \geq 3$ per group). Error bars indicate $\pm$ SD, gene expression levels are shown relative to *Hprt*, $*p \leq 0.05$

essential to prevent necroptosis during *Salmonella* Typhimurium induced enteritis (data not shown).

Until recently, the analysis of bacterial–epithelial interactions in the intestine has been hampered by the lack of reliable intestinal epithelium culture systems. In order to study the direct host–microbial interaction between IECs and *Salmonella* Typhimurium, we infected primary intestinal organoids in vitro. To visualize infection of organoids with *Salmonella* Typhimurium we used a green fluorescent protein (GFP) expressing strain of *Salmonella* Typhimurium (Figure S1B). Four hours post-infection we observed elevated mRNA expression of genes known to be involved in cell death regulation. Similar to the data obtained in vivo, members of both apoptosis and necroptosis were profoundly upregulated in infected organoids, indicating that both signaling pathways might influence epithelial cell death and barrier function in response to enteric infection (Figure S1C). Induction of epithelial cell death has been characterized as a host defensive mechanism to limit infection by enteric pathogens. Thus, increment of epithelial turnover is required to facilitate the repair of epithelial injuries and to decrease intestinal permeability induced by pathogens. Accordingly, we observed upregulation of the proliferation marker *Ki67* in infected organoids (Figure S1D). The innate immune system provides defense against invasive pathogens by inducing a variety of inflammatory responses, including expression of inflammatory cytokines and antimicrobial peptide (AMP) secretion.²⁷ To determine the immunological relevance of IECs we next evaluated the expression of intestinal epithelial derived cytokines in response to *Salmonella* Typhimurium infection. Infection of intestinal organoids resulted in a strong induction of pro-inflammatory cytokines, including *Ilfnb*, *Il1a/b*, and *Tnfa* (Figure S1E).

In summary these data implicate a potential role of epithelial Caspase-8 at the crossroad between IEC survival and regulated cell death including apoptosis and necroptosis in response to *Salmonella* Typhimurium.

Caspase-8 orchestrates epithelial apoptosis and prevents barrier dysfunction in response to *Salmonella* infection

In order to study a potential contribution of Caspase-8 to the pathogenesis of gastrointestinal infections, we subjected *Casp8^{ΔIEC}* mice and control littermates (*Casp8^{fl}* mice) to the streptomycin mouse model for *Salmonella* Typhimurium enteritis. *Casp8^{ΔIEC}* mice are maintained on a C57BL/6 background and previous studies have demonstrated that C57BL/6 are highly susceptible to infection with wild-type *Salmonella* Typhimurium due to an amino acid substitution in the natural resistance-associated macrophage protein-1 (*nramp^{D169}*) resulting in a non-functional protein.²⁸ Since we have previously shown that *Casp8^{ΔIEC}* mice are highly susceptible to lipopolysaccharide (LPS) administration and dextran sulfate sodium-induced colitis, we infected *Casp8^{ΔIEC}* mice and control littermates with the

attenuated *Salmonella* Typhimurium Δ aroA mutant.^{25,26} Although the Δ aroA mutant strain has impaired replication rates and thus reduced virulence, this strain sufficiently colonizes the colon and cecum and triggers pronounced inflammation and fibrosis in infected mice.^{28,29} While oral administration of the attenuated *Salmonella* strain had only a mild effect on wildtype mice, *Casp8^{ΔIEC}* mice displayed severe weight loss and high mortality already in the initial phases of infection (Fig. 2a, b). High-resolution endoscopy revealed severe signs of inflammation in the colon of *Casp8^{ΔIEC}* mice, including thickening of the bowel wall, reduced translucency, loss of regular vessel structure and fibrin extravasation (Fig. 2c). *Ex vivo* assessment of the infected colons further demonstrated a significantly reduced colon length compared to littermate controls (Fig. 2d). Accordingly, histological analysis revealed severe tissue destruction, accompanied by pronounced edema of the submucosa, massive epithelial cell death and inflammatory infiltrates in both the cecum and colon of *Casp8^{ΔIEC}* mice (Figs. 2e, f, 3a, b, S3A, B). While cell death in control mice was restricted to the upper part of the crypt and gut lumen, *Casp8^{ΔIEC}* mice showed massive cell death along the entire crypt axis both in the colon and cecum (Figs. 2e, 3c). β -Catenin immunostaining in these samples identified that massive cell death in *Casp8^{ΔIEC}* mice finally culminated in a general destruction of the intestinal epithelium (Fig. 2g, 3c). To investigate whether deficiency of Caspase-8 increases intestinal permeability, we next subjected *Salmonella* infected mice to fluorescein isothiocyanate-dextran (FITC-dextran) oral gavage. We noted highly elevated FITC serum levels in *Casp8^{ΔIEC}* mice as compared to control mice, suggesting a breakdown of intestinal barrier function (Fig. 2h). Loss of epithelial barrier was further confirmed by significantly reduced mRNA levels of the epithelial cell marker *Villin* (Fig. 2i). Notably, while we could not observe tissue destruction or inflammation in the small intestine of control mice, *Casp8^{ΔIEC}* mice also developed severe enteritis associated with massive epithelial erosions in the ileum (Fig. 3d).

To better characterize the kinetics behind *Salmonella* induced tissue destruction in *Casp8^{ΔIEC}* mice, we investigated the gut morphology at different time points following infection (24, 48, and 72 h). While 24 h post infection *Casp8^{ΔIEC}* mice showed highly increased expression of the pro-inflammatory marker *S100a9* (S2E), both control and *Casp8^{ΔIEC}* mice showed no remarkable changes in tissue morphology (Figure S2A, B). Epithelial cell death as visualized by TUNEL staining and barrier break down, demonstrated by β -Catenin staining and quantification of *Villin* mRNA levels, were detectable in *Casp8^{ΔIEC}* mice, but not in control littermates, starting from 48 h post infection in the colon and cecum (Figures S2C, D, S3C, D).

In summary these data demonstrate that Caspase-8 is essential to strengthen barrier function in response to *Salmonella* Typhimurium induced enteritis and that *Casp8^{ΔIEC}* mice provide

Fig. 3 Infection with *Salmonella* Typhimurium causes severe cecitis in *Casp8^{ΔIEC}* mice. **a–d** Control (*Casp8^{fl}*) ($n = 4$) and *Casp8^{ΔIEC}* mice ($n = 3$) were mock treated or infected with 10^9 CFU of *Salmonella* Typhimurium (Δ aroA, attenuated). Mice were sacrificed after 3 days. Tissue samples from cecum and terminal ileum were harvested and stored for further analysis. Experiments were repeated three times with similar results. **a** Representative images of H&E stained infected cecum cross sections (scale bar: 500 μ m, inset scale bar: 100 μ m). **b** Pathology score [maximum: 9] of infected ceca represented as mean values $\pm$ SD, $**p \leq 0.01$. **c** Representative images of cecum cross sections stained with antibodies against β -Catenin (CTNNB1) (red) or activated CASP3 (red) in combination with TUNEL (green). Hoechst 33342 (blue) was used for counterstaining of nuclei (scale bar: 100 μ m). **d** Representative images of H&E stained cross section of the infected terminal ileum (scale bar: 100 μ m)

a powerful tool to elucidate disease mechanism underlying inflammation during enteric infection.

Epithelial Caspase-8 expression is essential to restrict colonization during *Salmonella* Typhimurium infection. IECs constitute important host defense mechanisms to control access and survival of both commensal bacteria and pathogens. In order to study if intestinal epithelial Caspase-8 is essential to control colonization of the intestine, we infected mice with a wildtype *Salmonella* strain to exclude strain-dependent differences in replication efficiency. To better visualize bacterial loads, we used a bioluminescent wildtype *Salmonella* strain (UK1).²⁸ We observed inflammatory lesions and increased epithelial extrusion in the colon of wildtype animals (Figure S4A). Of note *Casp8^{ΔIEC}* mice showed massive tissue destruction and significantly decreased survival compared to control littermates, similar to the data obtained using the attenuated Δ aroA mutant strain (Figure S4). We observed an enhanced and equal colonization of the intestine at day 2 post infection in control and *Casp8^{ΔIEC}* mice (Fig. 4a). However, while control littermates successfully defended infection as demonstrated by *ex vivo* imaging of the gastrointestinal tract at day 4, *Casp8^{ΔIEC}* mice contained a high burden of *Salmonella* in the cecum and colon (Fig. 4b). These data implicate that epithelial

Caspase-8 signaling is an essential host defense mechanism to control intestinal colonization during *Salmonella* infection.

Previous studies have demonstrated that low expression of mucin glycoproteins was associated with a reduced thickness of the mucus layer which enables *Salmonella* to directly attach to the intestinal epithelium, to invade and finally to build microcolonies.^{30,31} Whereas *Salmonella* infection induced enhanced amounts of Mucin2 (MUC2) glycoproteins in wildtype animals, *Casp8^{ΔIEC}* mice failed to increase *Muc2* mRNA transcription and even showed a dramatic depletion of the mucus layer at later time points of infection (Fig. 4c, S5A). Hence, we hypothesized that depletion of the mucus layer facilitates commensal and non-commensal bacteria to directly attach to the intestinal epithelium and to translocate from the luminal side into subepithelial areas of *Casp8^{ΔIEC}* mice. Accordingly, fluorescence in situ hybridization with a eubacterial rRNA-specific probe revealed strong bacterial signals within the mucosa of *Casp8^{ΔIEC}* mice (Fig. 4d). In sharp contrast to *Casp8^{ΔIEC}* mice, bacteria remained most pronounced in the lumen of control mice (Fig. 4d). This was accompanied by massive infiltration of immune cells and an elevated expression of pro-inflammatory cytokines, like *Il6*, *S100a9*, and interferons, in *Casp8^{ΔIEC}* mice (Fig. 4e, S5B, C). We further observed a dramatic systemic spread of mucosal bacteria into other visceral organs

Fig. 4 Lack of epithelial Caspase-8 facilitates barrier break down and systemic spread of mucosal microbes. **a, b** Control (*Casp8^{fl/fl}*) ($n = 3$) and *Casp8^{ΔIEC}* ($n = 3$) mice were infected with 10^9 CFU of a bioluminescent strain of *Salmonella* Typhimurium (UK-1). Red areas represent regions with highest bacterial burden. Experiments were repeated three times with similar results. **a** Representative visualization and quantification of *Salmonella* Typhimurium burden in control and *Casp8^{ΔIEC}* mice at indicated time points. Data are displayed as mean values $\pm$ SD. **b** Representative *ex vivo* imaging and quantification of bioluminescent *Salmonella* Typhimurium in the gut of infected mice (ce cecum, col colon). Data are displayed as individual values with indicated mean values $\pm$ SD, $**p \leq 0.01$. **c–f** Control (*Casp8^{fl/fl}*) and *Casp8^{ΔIEC}* mice ($n \geq 3$ per group) were infected with *Salmonella* Typhimurium (Δ araA, attenuated) and analyzed at day 2 (f) or day 3 (c–e) of infection. Experiments were repeated three times with similar results. **c** Representative images of colon cross sections from unchallenged and infected animals stained with an antibody against MUC2 (red) combined with UEA-1 (green, scale bar: 75 μ m). Hoechst 33342 (blue) was used for counterstaining of nuclei. **d** Representative images of fluorescence *in situ* hybridization (FISH) with a universal eubacterial probe (red) in colon cross sections of infected mice (ti: tissue, lu: lumen; scale bar: 50 μ m). Hoechst 33342 (blue) was used for counterstaining. **e** Quantification of colonic *Il6* and *S100a9* gene expression relative to the expression of *Hprt*. Data are represented as mean values $\pm$ SD, $n \geq 3$, $*p \leq 0.05$. **f** Quantification of colonic *Reg3b* and *Reg3g* mRNA relative to the expression of *Hprt*, $n \geq 3$, $*p \leq 0.05$

such as mesenteric lymph nodes, liver, and spleen in *Casp8^{ΔIEC}* mice, suggesting that barrier defects and translocation of bacteria are responsible for the lethality of these mice (Figure S5D).

Invasion of IECs by *Salmonella* triggers an inflammatory response, leading to the release of AMPs. Previously, we have shown that *Casp8^{ΔIEC}* mice display defects in the antimicrobial defense due to a reduced expression of AMPs.²⁶ Therefore, we next evaluated if enhanced bacterial colonization of *Salmonella* Typhimurium in *Casp8^{ΔIEC}* mice is linked to defects in enteric AMP production. Analysis of AMP expression revealed a strong increase in *Reg3b* and *Reg3g* transcripts in response to *Salmonella* infection in both control and *Casp8^{ΔIEC}* mice 2 days post infection (Fig. 4f). Moreover, *Casp8^{ΔIEC}* mice displayed higher *Reg* gene expression, possibly as a host response adapted to the elevated bacterial burden. In order to investigate the direct interaction and host response of IECs to enteric pathogens, we infected intestinal organoids derived from Caspase-8 proficient and deficient animals with *Salmonella* Typhimurium *in vitro*. Similar to the results

obtained *in vivo*, both organoid cultures responded with strong induction of *Reg3g* mRNA expression, suggesting that Caspase-8 deficiency does not impair expression of AMPs (Figure S5E). These data indicate that at later time points during the infection, deficiency in AMP secretion caused by loss of IECs along with depletion of the mucus layer, allows *Salmonella* to colonize more efficiently in *Casp8^{ΔIEC}* mice.

In summary these data demonstrate that Caspase-8 is essential to promote host resistance to *Salmonella* Typhimurium by orchestrating the gut response to control bacterial burden and to prevent tissue colonization.

Distinct factors trigger necroptosis orchestrated by MLKL and RIPK3 during *Salmonella* infection

Apart from its function as a central regulator of apoptosis, recent studies identified novel roles of Caspase-8 downstream of the NLR4 inflammasome for modulating IL-1 β production and intestinal epithelial cell extrusion.^{20,32} Notably, infected Caspase-

Fig. 5 Blocking of IL-1 signaling does not improve barrier function or survival of *Casp8^{ΔIEC}* mice. **a–c** Control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice were mock treated or infected with 10^9 CFU of *Salmonella* Typhimurium (Δ aroA, attenuated). Mice were sacrificed at day 3 post-infection. **a** Quantification of *Il1b* mRNA in colonic tissue ($n \geq 3$ per group) and in vitro organoid cultures ($n = 3$ per group) relative to *Hprt*, + SD, * $p \leq 0.05$. **b** Control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice were treated intraperitoneally with a daily dose of PBS or Anakinra during infection with *Salmonella* Typhimurium (Δ aroA, attenuated) and analyzed at day 3 ($n = 3$ per group). Experiments were repeated two times with similar results. Representative images of H&E stained colon cross sections and immunohistochemical staining with an antibody against activated CASP3 (red) and TUNEL (green). Hoechst 33342 (blue) was used for counterstaining of nuclei (scale bar: 100 μ m). **c** Western blot analysis of colonic intestinal epithelial scrapings incubated with an antibody targeting the pro-form and the activated p10 fragment of Caspase-1. β -Actin was used as a loading control. **d** Representative images of mock treated and infected intestinal organoids derived from control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice 20 h after infection. For inhibition of Caspase-1, organoids were treated with 50 μ M Belnacasan (VX-765). Propidium Iodide (red) visualizes dead epithelial cells

8 deficient IECs exhibited a hyperactive production of *Il1b* mRNA in vivo and in vitro compared to control cells (Fig. 5a). Although IL-1 β is not directly required for cell death, IL-1 β production contributes to the inflammatory response elicited by cells undergoing Caspase-1 mediated pyroptosis. In order to investigate whether increased IL-1 β production is causally linked to excessive tissue destruction and inflammation in *Casp8^{ΔIEC}* mice during *Salmonella* Typhimurium infection, we treated *Casp8^{ΔIEC}* mice and control animals with a daily dose of the recombinant IL-1 receptor antagonist Anakinra, starting one day before the infection. While administration of Anakinra ameliorated pathogen induced inflammation and body weight loss in control animals, tissue destruction and barrier break down in *Casp8^{ΔIEC}* mice was not dependent on IL-1 signaling (Fig. 5b, S6A). These data demonstrate that IL-1 receptor signaling does not substantially contribute to barrier dysfunction in *Casp8^{ΔIEC}* mice. As mentioned above, Caspase-8 has also been shown to act downstream of the NLRC4 inflammasome. Therefore, we next investigated IEC inflammasome activation following *Salmonella* infection. In line with previous publications, we observed Caspase-1 processing in response to *Salmonella*

infection in epithelial scrapings of control and *Casp8^{ΔIEC}* mice (Fig. 5c). Notably, *Salmonella* infection potentially induced Caspase-1 cleavage to a similar extent in *Casp8^{ΔIEC}* mice and control littermates (Fig. 5b), suggesting that Caspase-1 mediated pyroptosis cannot explain excessive epithelial cell death as observed in *Casp8^{ΔIEC}* mice. To provide functional evidence, we infected organoids derived from control and *Casp8^{ΔIEC}* mice in vitro. Notably, we observed a more robust *Salmonella*-induced cytotoxicity accompanied with IEC death in Caspase-8 deficient organoids. Importantly, our data suggest that Caspase-1 is not involved in death of Caspase-8 deficient intestinal epithelial cells, since co-incubation of Caspase-8 deficient organoids with the potent and selective Caspase-1 inhibitor belnacasan (VX-765) could not restore viability of epithelial cells in response to *Salmonella* infection (Fig. 5d, S6B). These data suggest that Caspase-1 mediated pyroptosis does not substantially contribute to tissue injury observed in *Casp8^{ΔIEC}* mice. In order to address the contribution of Caspase-8 to intracellular replication of *Salmonella* Typhimurium, we infected Caspase-8 proficient and deficient HT-29 human colorectal cancer cells with *Salmonella* and evaluated

Fig. 6 Genetic ablation of necroptosis mediators restores barrier function and ensures survival of *Casp8^{ΔIEC}* mice. **a–h** Mice were mock treated or infected with 10^9 CFU of *Salmonella* Typhimurium (Δ aroA, attenuated). **a, d, g, h** Mice were analysed at day 3 of infection. Experiments were repeated two times with similar results. **a** Colonic expression of *Mkl1* mRNA in mock treated and infected control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice. Data are shown relative to the expression of *Hprt* + SD ($n \geq 3$ per group). **b, c** Control (*Casp8^{fl}*) ($n = 3$), *Casp8^{ΔIEC}* ($n = 6$), *Casp8^{ΔIEC} Mkl1^{-/-}* ($n = 4$) and *Mkl1^{-/-}* ($n = 3$) mice were mock treated or infected with *Salmonella* Typhimurium (Δ aroA). **b** Body weight of the animals is displayed relative to initial body weight $\pm$ SD, $**p \leq 0.01$. **c** Kaplan–Meier curve for survival of the animals after infection, $*p \leq 0.05$. **d** Colonic expression of *Ripk3* mRNA in mock treated and infected control (*Casp8^{fl}*) and *Casp8^{ΔIEC}* mice. Data are shown relative to the expression of *Hprt* + SD ($n \geq 3$ per group). **e, f** Control (*Casp8^{fl}*) ($n = 3$), *Casp8^{ΔIEC}* ($n = 3$), *Casp8^{ΔIEC} Ripk3^{-/-}* ($n = 3$) and *Ripk3^{-/-}* ($n = 4$) mice were mock treated or infected with *Salmonella* Typhimurium (Δ aroA). **e** Body weight of the animals is displayed relative to initial body weight $\pm$ SD, $**p \leq 0.01$. **f** Kaplan–Meier curve for survival of the animals after infection, $*p \leq 0.05$. **g** Representative H&E stained colon cross sections of mock treated or infected *Casp8^{ΔIEC}*, *Casp8^{ΔIEC} Mkl1^{-/-}*, *Casp8^{ΔIEC} Ripk3^{-/-}* animals (scale bar: 200 μm). **h** Pathology score of infected colon tissue collected from mock treated and infected *Casp8^{ΔIEC}*, *Casp8^{ΔIEC} Mkl1^{-/-}*, *Casp8^{ΔIEC} Ripk3^{-/-}* mice displayed as mean values $\pm$ SD, $n = 3$ per group, $**p \leq 0.01$, $***p \leq 0.001$

Fig. 7 Lethality of *Casp8^{ΔIEC}* mice partially depends on TNF-α. **a, b** Quantification of *Tnfa* mRNA in colonic tissue of mice infected for 4 days ($n \geq 3$) (**a**) and in organoids infected for 4 h ($n \geq 3$) (**b**) with *Salmonella* Typhimurium (Δ aroA, attenuated). Error bars indicate + SD, gene expression levels are shown relative to *Hprt*, * $p \leq 0.05$. Experiments were repeated three times with similar results. **c–e** Control (*Casp8^{fl}*) ($n = 4$), *Casp8^{ΔIEC}* ($n = 4$), and *Casp8^{ΔIEC} Tnfr1^{-/-}* ($n = 8$) mice were mock treated or infected with 10^9 CFU of *Salmonella* Typhimurium (Δ aroA, attenuated). Experiments were repeated two times with similar results. **c** Body weight of the animals is displayed relative to initial body weight $\pm$ SD, ** $p \leq 0.01$. **d** Kaplan–Meier curve for survival of the animals after infection, $n \geq 4$ per group, * $p \leq 0.05$. **e** Representative images of colon cross sections of infected mice stained with H&E, β-Catenin (CTNNB1) (red) or activated CASP3 (red) in combination with TUNEL (green). Hoechst 33342 (blue) was used for counterstaining of nuclei (scale bar: 100 μm)

the intracellular bacterial burden. Interestingly, while we could not observe differences in the viability of both cell lines, Caspase-8 deficient HT-29 cells showed a significantly higher burden of intracellular bacteria compared to wildtype cells (Figure S6C). Similar results could be obtained in murine colon adenocarcinoma cells (MC38 cells), which were treated with the Caspase-8 inhibitor Z-IETD-FMK (Figure S6D). To ascertain the influence of Caspase-1 to intracellular replication in Caspase-8 deficient epithelial cells, we infected HT-29 (Caspase-8 proficient and deficient) cells in the

presence or absence of a Caspase-1 inhibitor. We could not observe significant differences (Figure S6E). In summary these data suggest that during intestinal infection, Caspase-8 in IECs is essential to protect from pathogen invasion independent of Caspase-1.

Our previous publications have identified that Caspase-8 has an important function as a negative regulator of necroptosis in response to pro-inflammatory cytokines such as TNF-α, but also microbial molecules (LPS), suggesting that IECs in

Casp8^{ΔIEC} mice undergo necroptosis in response to bacterial infections.^{25,33}

While IEC death in infected control mice was associated with strong activation of Caspase-3, we observed massive TUNEL positive IECs that stained negative for activated caspases in *Casp8^{ΔIEC}* mice, suggesting that barrier dysfunction in these mice might be due to necroptosis of IECs (Fig. 2e, 3c, S3D). Accordingly, intestinal expression of central regulators of necroptosis, such as *MLK1* and *Ripk3* was highly increased in challenged mice (Fig. 6a, d). Of note infection of organoids with *Salmonella* Typhimurium revealed that *MLK1* and *Ripk3* mRNA was in particular upregulated in intestinal epithelial cells (Figure S7A). In line with these results, we also observed elevated protein levels of MLKL and RIPK3 most pronounced in isolated intestinal epithelial cells of infected *Casp8^{ΔIEC}* mice (Figure S7B). In order to provide direct functional evidence for a role of necroptosis in the lethal outcome of *Casp8^{ΔIEC}* mice, we subjected *Casp8^{ΔIEC}MLK1^{-/-}* and *Casp8^{ΔIEC}Ripk3^{-/-}* double deficient mice to *Salmonella* infection. The high lethality and severe body weight loss of *Casp8^{ΔIEC}* mice could be completely rescued by additional deletion of MLKL or RIPK3 (Fig. 6b, c, e, f). Histological analysis further revealed minor or no epithelial erosions in both double deficient strains, as demonstrated by H&E and β-Catenin staining (Fig. 6g, h, S8). In line with this, the number of dying IECs, indicated by TUNEL staining, was clearly reduced in animals lacking additionally MLKL or RIPK3 (Figure S8A + C). In summary these data demonstrate that epithelial Caspase-8 orchestrates epithelial apoptosis and prevents RIPK3 activation in response to *Salmonella* Typhimurium infection.

Among the large variety of pro-inflammatory cytokines which are expressed in the mucosa of *Salmonella* Typhimurium infected mice, several of these are candidates for triggering non-apoptotic cell death in the Caspase-8 deficient epithelium.² Previously, we have elucidated that LPS induced intestinal epithelial necroptosis is strictly dependent on TNF-α signaling, suggesting a potential contribution of TNF-α to barrier breakdown in *Casp8^{ΔIEC}* mice following infection.²⁵ Indeed, we observed high expression levels of *Tnfa* mRNA transcripts following infection with *Salmonella* Typhimurium in vivo (Fig. 7a). Notably, TNF-α was not only released by lamina propria cells, but also IECs as demonstrated by elevated *Tnfa* expression in infected organoids (Fig. 7b). To provide direct functional evidence for TNF-α, we infected *Casp8^{ΔIEC}Tnfr1^{-/-}* double deficient mice. Additional deletion of *Tnfr1* resulted in significantly less severe decrease in body weight and prolonged survival of *Casp8^{ΔIEC}Tnfr1^{-/-}* mice (Fig. 7c, d). However, histological analysis revealed a similar degree of tissue destruction with epithelial erosions in the colon irrespective of the presence or absence of TNF-receptor 1 (Fig. 7e). This was further confirmed by staining for the cell death marker TUNEL and β-Catenin to visualize barrier integrity (Fig. 7e). These data indicate that *Salmonella* Typhimurium induced colitis is partially dependent on this pathway and other cytokines might contribute, too.

DISCUSSION

Programmed cell death plays a crucial role for the regulation of inflammation and infection. Apoptosis and necroptosis are two pathologically relevant types of regulated cell death that strongly influence the pathogenesis of gastrointestinal disorders including inflammatory and infectious diseases.³⁴ The cysteine protease Caspase-8 is a central regulator of both apoptosis and necroptosis. Accordingly, we have previously demonstrated that excessive activation of Caspase-8 facilitates apoptotic cell death which can culminate in a general destruction of the intestinal barrier, whereas lack of Caspase-8 activity renders IECs susceptible to necroptosis.^{26,35} Here, we provide compelling evidence that the negative regulation of necroptosis by Caspase-8 plays a

fundamental role during enteric infection. We identified that epithelial Caspase-8 is essential to promote host resistance to *Salmonella* Typhimurium by orchestrating the gut response to control bacterial burden and prevent tissue colonization.

Programmed cell death in the context of host–pathogen interactions highlights the evolutionary conserved capacity of host cells to commit suicide during pathogen infection. This premature death of infected cells perhaps represents the most ancient defense strategy against lethal infection by limiting replication, colonization, and accompanied dissemination by alerting neighboring cells to impending infection.^{9,14,36} Accordingly, in this study we observed elevated levels of cell death mediators in IECs as a response to direct host–pathogen interaction. Importantly, we identified that key players of both the apoptosis and necroptosis pathways were significantly upregulated in the epithelium following *Salmonella* Typhimurium infection. These data also suggest that RIPK3-mediated necroptosis has been developed as an alternative, non-apoptotic form of epithelial cell death to ensure elimination of infected cells. Indeed, we observed that in case of Caspase-8 deficiency, IECs undergo RIPK3 and MLKL mediated necroptosis in response to *Salmonella* Typhimurium infection. While barrier function could be maintained under Caspase-8 proficient conditions, lack of Caspase-8 resulted in excessive tissue injury, barrier disruption, and death of mice. Additional deletion of MLKL or RIPK3 was sufficient to block excessive intestinal epithelial necrosis in *Casp8^{ΔIEC}* mice. These data suggest that expression of both necroptosis mediators particularly in the intestinal epithelium drives lethality of *Casp8^{ΔIEC}* mice. Importantly, while these data clearly indicate that necroptosis may represent a failsafe mode of innate immunity to preserve expulsion of infected cells and thus to control infection, we could not observe differences in the abundance of epithelial cell death in *MLK1^{-/-}* or *Ripk3^{-/-}* mice compared to control littermates. Thus in case of elimination of infected epithelial cells, Caspase-8 mediated apoptosis appears to be the preferred type of epithelial cell death, while necroptosis could represent a safeguard to ensure bacterial clearance. Indeed another study has demonstrated that *Ripk3^{-/-}Casp8^{-/-}* double deficient mice exhibited significantly elevated bacterial burdens and stool scores after oral infection with the mucosal pathogen *Citrobacter rodentium*.³⁷ Similar to our data it has been demonstrated that necroptosis is crucial to limit the pathological inflammation induced by *Staphylococcus aureus*. Accordingly, necroptosis has been described in macrophages during *Staphylococcus aureus* induced pneumonia³⁸ and in human keratinocytes infected with *S. aureus*.³⁹ Moreover, following challenge with *Serratia marcescens*, *S. aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and uropathogenic *Escherichia coli* macrophages, pretreated with inhibitors of RIPK1, RIPK3, or MLKL were protected against death.⁴⁰ Similarly, macrophages from *Casp8^{-/-}Ripk3^{-/-}* or *Ripk1^{-/-}* mice were remarkably resistant to cell death induced by *Yersinia pestis* and *Yersinia pseudotuberculosis*, but not *Ripk3* deficient macrophages.⁴¹ Notably, in sharp contrast to our data, macrophages deficient for Caspase-8 alone or in addition with RIPK3 were not resistant to cell death induced by *Salmonella* Typhimurium.^{32,41} It is currently believed that *Salmonella* Typhimurium predominantly triggers pyroptosis of infected macrophages.⁴¹

Interestingly, just recently it has been demonstrated that epithelial NLRC4 inflammasome is sufficient to significantly reduce *Salmonella* colonization in the cecum and translocation to mesenteric lymph nodes, liver and spleen. Of note it has been reported that Caspase-8 is recruited to the NLRC4 inflammasome upon *Salmonella* infection of macrophages. However, Caspase-8 deficiency in this setting did not affect cell death, possibly because of compensation of Caspase-1. Our results now suggest that in the intestinal epithelium Caspase-1 is insufficient to compensate for the loss of Caspase-8, since massive intestinal cell death in the

Caspase-8 deficient epithelium was not associated with increased activation of Caspase-1 and could be blocked by additional deletion of MLKL and RIPK3 but not pharmacological inhibition of Caspase-1. Notably, *Casp1*^{-/-}*Ripk3*^{-/-} double deficient mice showed similar disease activity (bacterial burden) in response to *Salmonella* infection compared to *Casp8*^{-/-}*Ripk3*^{-/-} mice.²⁰ Interestingly, mice lacking both caspases in addition to *Ripk3* (*Casp1*^{-/-}*Casp8*^{-/-}*Ripk3*^{-/-}) showed elevated tissue bacteria loads similar to our study. While these data are derived from full-knock out animals, they still support our main finding that epithelial Caspase-8 is essential to maintain intestinal barrier function by preventing necroptosis and controlling *Salmonella* colonization.

The release of intracellular contents from necrotic cells into the extracellular milieu not only initiates inflammatory reactions but also influences surrounding cells to activate the cell death machinery. By taking advantage of the organoid model we identified that the early epithelial inflammatory response to bacterial infection includes the expression of many factors, such as TNF- α , that are well known for their capability to activate apoptosis and necroptosis.^{23,25,42} These data suggest that cytokines may contribute to barrier breakdown in *Casp8* ^{Δ IEC} mice. Accordingly, we and others have previously shown that LPS-triggered epithelial apoptosis and necroptosis require the release of TNF- α by mucosal immune cells.^{26,43,44} Here, we uncovered that additional deletion of TNF receptor was sufficient to significantly improve survival but could not rescue lethality of *Casp8* ^{Δ IEC} mice following *Salmonella* Typhimurium infection. Similarly, depletion of TNF- α using a neutralizing antibody failed to prevent lethality of mice with an intestinal epithelial cell specific deletion of the Caspase-8 inhibitor cFLIP (*cFlip* ^{Δ IEC}).³⁵ Moreover, while *Casp8* ^{Δ IEC}*Tnfr*^{-/-} mice are protected from LPS-induced septic shock and mortality, inhibition of this signaling pathway is insufficient to block Paneth cell death or inflammation in these mice.^{25,35} This suggests that activation of epithelial apoptosis or necroptosis either occurs by a TNF- α independent pathway or that redundant signals are present during *Salmonella* Typhimurium infection. Further studies are required to address the contribution of other cytokines on intestinal homeostasis during enteric infection.

Collectively, our data uncover a crucial role for epithelial Caspase-8 in preventing *Salmonella* Typhimurium induced enteritis. Importantly, while Caspase-8 has been shown previously to predominantly influence inflammasome regulation in response to *Salmonella*, we observed that Caspase-8 is dispensable for this process in IECs. Of note, we provide solid evidence that epithelial Caspase-8 is an essential factor at the cross road between cell survival and death during *Salmonella* Typhimurium infection. We therefore propose that Caspase-8 functions at a convergent point of multiple host defense mechanisms that strongly depend on the cellular context.

MATERIALS AND METHODS

Mice

Ripk3^{-/-}⁴⁵ *Mkl1*^{-/-}²³ *Casp8* ^{Δ IEC}²⁶ *Casp8* ^{Δ IEC}*Ripk3*^{-/-}³⁵ and *Casp8* ^{Δ IEC}*Tnfr*^{-/-}³⁵ were described earlier. *Casp8* ^{Δ IEC}*Mkl1*^{-/-} were generated by crossing *Mkl1*^{-/-} mice to *Casp8* ^{Δ IEC} mice. Throughout the manuscript, we used littermate controls to exclude strain-dependent differences in susceptibility. Mice were routinely screened for pathogens according to FELASA guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Regierung von Unterfranken.

Salmonella Typhimurium infection

The *S. enterica* serovar *S. Typhimurium* strains UK1 (bioluminescent, provided by Dr. Christian Riedel, University of Ulm),⁴⁶ Δ aroA²⁸ and a GFP expressing wild-type strain⁴⁷ were cultured at 37 °C in Luria-Bertani broth, supplemented with erythromycin,

streptomycin or ampicillin respectively, under continuous shaking and aeration.

For in vivo infection mice were treated with a single dose of streptomycin by oral gavage 24 h before infection. Prior to infection, mice were starved for 8 h and subsequently orally gavaged with 10⁹ colony-forming units of *Salmonella*. For in vivo experiments mice were infected with either the *S. Typhimurium* strains UK1 (Fig. 4a, b and S4) or the attenuated Δ aroA strain (Figs. 1, 2, 3, 5, 6, 7, S1, S2, S3, S4, S5, S6, S7 and S8).

For in vitro infections organoids were rinsed from Matrigel, washed, and incubated with a GFP expressing wild-type strain, in crypt culture medium without antibiotics, for 30–45 min at 37 °C. After washing, the organoids were seeded in Matrigel and cultured in medium containing penicillin, streptomycin, and gentamycin. In some experiments organoids were co-incubated with Belnacasan (VX-765) a potent and selective inhibitor of Caspase-1 (50 μ M, Selleckchem). Organoid viability was monitored by light microscopy and propidium iodide (PI, BD Bioscience) staining (Life technologies).

Bioluminescent in vivo imaging

For in vivo imaging, mice infected with the bioluminescent UK1 strain of *Salmonella* Typhimurium, were anaesthetized with 2% isoflurane in oxygen and imaged using the IVIS Lumina II. Bioluminescent counts were recorded for 150 s from the abdomen as region of interest. Images were obtained and analyzed using LIVING IMAGE software (Xenogen).

Immunoblotting

Proteins were isolated from either whole colon tissue or epithelial scrapings using cell lysis buffer supplemented with PMSF (both Cell Signaling). Proteins were separated using MiniProtein-TGX gels (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked and probed with primary antibodies (for detailed information see Supplementary Table 2) overnight. HRP-linked anti-rabbit (Cell Signaling, #7074) was used as a secondary antibody. Blots were developed by chemiluminescence using an ECL substrate (Perkin Elmer or Merck Millipore).

Histology and immunohistochemistry

Histopathological analyses were performed on formalin-fixed paraffin-embedded tissue cross sections (terminal ileum, colon, and cecum) after Mayer's haematoxylin and eosin (H&E) staining. Immunofluorescence of tissue sections (colon and cecum) was performed using the TSA Cy3/Fluorescein system as recommended by the manufacturer (Perkin Elmer). Primary antibodies (for detailed information see Supplementary Table 2) were incubated overnight. Nuclei were counterstained with Hoechst 3342 (Invitrogen). Cell death (TUNEL) was analyzed using the In Situ Cell Death Detection Kit (Roche). Staining for immune cells was performed on cryosections. For immunohistochemical staining of the mucus layer, samples were fixed in Methanol-Carnoy's fixative.⁴⁸ Images were obtained using the microscope LEICA DMI 4000B together with the LEICA DFC360 FX or LEICA DFC420 C camera and the imaging software "LAS AF" (Leica, Wetzlar, Germany).

Gene expression analysis

Total RNA was extracted from intestinal tissue using the peqGOLD Total RNA Kit and from organoids using the peqGOLD Microspin Total RNA Kit (PqLab, Erlangen, Germany). cDNA was synthesized by reverse transcription using the SCRIPT cDNA Synthesis Kit (Jena Bioscience) and analyzed by real-time qPCR using SYBRGreen reagent (Roche), the LightCycler 480 (Roche) and specific QuantiTect Primer Assays (Qiagen) (for detailed information see Supplementary Table 1). Experimental values were normalized to levels of the housekeeping gene *hypoxanthine guanine phosphoribosyl transferase* (*Hprt*).

Statistical analyses

Differences were compared by Student *t*-test (2 groups) using GraphPad Prism 5, GraphPad Software Inc, (San Diego, CA). Quantitative results are presented as mean values with standard deviation (SD). *P* < 0.05 was considered significant (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

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AUTHOR CONTRIBUTIONS

C.G. and M.H. designed the research. M.H., I.S., C.G., and B.R. performed the experiments. M.M., S.W., G.W.H., M.F.N. provided material that made this study possible. M.H., M.F.N. and C.G. analysed the data and wrote the paper.

ADDITIONAL INFORMATION

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