Gasdermin D is the only Gasdermin that provides protection against acute Salmonella gut infection in mice

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Gasdermins (GSDMs) share a common functional domain structure and are best known for their capacity to form membrane pores. These pores are hallmarks of a specific form of cell death called pyroptosis and mediate the secretion of pro-inflammatory cytokines such as interleukin 1β (IL1β) and interleukin 18 (IL18). Thereby, Gasdermins have been implicated in various immune responses against cancer and infectious diseases such as acute Salmonella Typhimurium (S. Tm) gut infection. However, to date, we lack a comprehensive functional assessment of the different Gasdermins (GSDMA-E) during S. Tm infection in vivo. Here, we used epithelium-specific ablation, bone marrow chimeras, and mouse lines lacking individual Gasdermins, combinations of Gasdermins or even all Gasdermins (GSDMA1-3C1-4DE) at once and performed littermate-controlled oral S. Tm infections in streptomycin-pretreated mice to investigate the impact of all murine Gasdermins. While GSDMA, C, and E appear dispensable, we show that GSDMD i) restricts S. Tm loads in the gut tissue and systemic organs, ii) controls gut inflammation kinetics, and iii) prevents epithelium disruption by 72 h of the infection. Full protection requires GSDMD expression by both bone-marrow-derived lamina propria cells and intestinal epithelial cells (IECs). In vivo experiments as well as 3D-2D-, and chimeric enteroid infections further show that infected IEC extrusion proceeds also without GSDMD, but that GSDMD controls the permeabilization and morphology of the extruding IECs, affects extrusion kinetics, and promotes overall mucosal barrier capacity. As such, this work identifies a unique multipronged role of GSDMD among the Gasdermins for mucosal tissue defense against a common enteric pathogen.

immunology | microbiology | pathogen | pyroptosis

Gasdermins make up a protein family including Gasdermin A, B, C, D, and E (GSDMA, GSDMB, GSDMC, GSDMD, and GSDME, respectively) in humans and GSDMA1-3, GSDMC1-4, GSDMD, and GSDME in mice (1). All members share a common functional domain structure, in which an inhibitory C-terminal domain is linked to a membrane pore-forming N-terminal domain (1, 2). Upon cleavage at the linker region, the N-terminal domain is released to form membrane pores (3-6). These pores mediate lytic cell death and release inflammatory mediators, such as interleukin 1β (IL1β), interleukin 18 (IL18), and lipids into the extracellular milieu to alert neighboring cells (7-9). Gasdermins can be activated with different efficiency by the cysteine proteases Caspase-1, -3, -4, -8, and -11 and by secrete propeptes to execute cellular responses, thus mediating immunity against pathogens and cancer (7, 9-24). However, their individual roles, cell type specificities, and possible redundancies during oral bacterial infections, such as those caused by Salmonella enterica Serovar Typhimurium (S. Tm), have not been comprehensively explored.

S. Tm is a major foodborne pathogen, a prevalent cause of diarrheal disease worldwide (25), and a risk factor for inflammatory bowel diseases (26). As shown in streptomycin-pretreated mice—a commonly used mouse model for human Salmonella diarrhea—S. Tm frequently invades intestinal epithelial cells (IECs) during the acute gut infection, transgresses into the underlying lamina propria compartment, and spreads to systemic organs (27, 28). Innate host immune responses against S. Tm include the activation of the NAIPLNLR4 inflammasome, which senses invading S. Tm to induce cell death and interleukin release through a mechanism involving Caspase-1 (29). During S. Tm infection of streptomycin-pretreated mice, which develop pronounced Salmonella enterocolitis, the NAIPLNLR4 response, particularly in IECs, provides a first line of defense. This reduces S. Tm loads locally in the gut tissue as well as restricts pathogen accumulation at systemic sites like the mesenteric lymph nodes (mLN), spleen, and liver (8, 30–38). The IECs NAIPLNLR4 response limits pathogen spread predominantly by swiftly expelling infected IECs into the gut lumen (8, 31, 37, 39).

Significance

The host immune response against infection relies on programmed cell death that has recently been shown to involve Gasdermins—a family of membrane-pore-forming proteins. Despite abundant expression of multiple Gasdermins in mammalian gut tissue, we here find using a mouse line lacking all mouse Gasdermins at once that only Gasdermin D provides protection against oral Salmonella infection. To accomplish this protection, both gut epithelial cells and classical immune cells employ Gasdermin D to limit bacterial loads in the mucosa, to control inflammation, to prevent epithelial disruption, and to reduce systemic spread of the pathogen. Hence, this study sheds light on the differential impact of Gasdermins in infectious diseases.


The authors declare no competing interest.

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Importantly, epithelial NAIP/NLRC4 not only triggers cell death but also coordinates the detachment of the infected IEC from the epithelium (a process referred to as IEC extrusion), with a concomitant release of the aforementioned inflammatory mediators (8, 31, 37). In studies using bacteria and/or pure NAIP/NLRC4 ligands, it was shown that GSDMD affects the qualitative features of the IEC extrusion process (8, 21, 40). However, how and to which extent epithelial GSDMD contributes to the overall defense against S.Tm infection in vivo remains far from clear. Moreover, we have recently shown that a fraction of the extruding IECs feature activated forms of Caspase-3 and -8 (31), which raises the question if other Gasdermins, which can be activated by those caspases, could additionally be involved in IEC extrusion, and thereby contribute to the defense against S.Tm. Indeed, recent studies in mice suggested a role for epithelial GSDME in 2,4,6-trinitrobenzenesulfonic acid-induced colitis (41) and for epithelial GSDMC in worm-infected mice (23, 42). Finally, Gasdermins are originally known for their function in phagocytic immune cells. It has been shown that immune cells employ Gasdermins to promote gut inflammation and defense against several gut pathogens in vivo (11, 15, 43–46). These observations suggest that not only epithelial Gasdermins but also Gasdermins expressed by dedicated immune cells may contribute to pathogen restriction during S.Tm infection. However, the respective contribution(s) of particular Gasdermins in IECs and immune cells during acute Salmonella diarrhea has not yet been systematically addressed.

Here, we have performed a comprehensive assessment of the impact of Gasdermins during oral S.Tm infection. Surprisingly, out of all analyzed Gasdermins, only GSDMD appears to significantly protect against the acute S.Tm infection, lowering pathogen loads both in the gut mucosal tissue and at systemic organs. We show that epithelial GSDMD impacts how IECs are extruded into the lumen and that GSDMD in IECs and in bone-marrow-derived phagocytes of the lamina propria work together to prevent S.Tm spread beyond the intestinal barrier.

Results

Mice Deficient in All Gasdermins, or only Gasdermin D, Feature Elevated S.Tm CFUs in the Gut Tissue and in Systemic Organs.

Gasdermins are activated downstream of cell death pathways and play an important role against pathogens and cancer development (7, 9–24). However, little is known of how they combine to mediate protection against the prototypic gut pathogen S.Tm. Therefore, we addressed to what extent and how Gasdermins are involved in the immune response against S.Tm infection in mice. Streptomycin-pretreated mice were infected perorally with S.Tm (SL1344) for 48 h and RT-qPCR analysis of the cecum tissue—mainly via Gasdermin-deficient mice revealed that at least one homologue of each Gasdermin is expressed above detection limit (Fig. 1A). Using CRISPR/Cas9 genome editing, we generated a knockout mouse line globally lacking all mouse Gasdermins including the multiple homologues for GSDMA and GSDMC (hereafter referred to as GsdmA1−/−, GsdmC1−/−, and GsdmE−/−). In combination with single knockout mice, this multi-Gasdermin-deficient mouse line allows us to address a long-lasting question in the field, namely if redundancies among Gasdermins do exist. To limit microbiota-driven artifacts, these GsdmA1−/− mice were co-housed with wild-type (WT) mice for at least two weeks prior to infection. Interestingly, while Gasdermins had no impact on luminal colonization (SI Appendix, Fig. S1A), GsdmA1−/− mice had up to 10-fold elevated S.Tm loads in mLN at 48 h post infection (p.l.) suggesting that Gasdermin(s) do restrict S.Tm gut infection (Fig. 1B). To investigate whether one or several Gasdermins mediate this protection, we performed littermate-controlled infections with mice deficient in individual Gasdermins (GSDMA1−/−, GSDMC1−/−, GSDMD−/−, or GSDME-deficient mice). In line with the GsdmA1−/− mice, luminal S.Tm density was similar across genotypes (SI Appendix, Fig. S1 B–E). However, while we did not detect any CFU differences for GsdmA1−/− (hereafter referred to as GsdmA−/−), GsdmC1−/− (hereafter referred to as GsdmC−/−), and GsdmE−/− mice, we did enumerate up to 10-fold more CFUs in the mLN of GsdmD−/− mice, suggesting that GSDMD is the critical Gasdermin limiting S.Tm loads (Fig. 1C). Next, we co-housed WT, GsdmA1−/−, and GsdmD−/− mice and infected them together to verify that the phenotype in GsdmA1−/− mice is attributable to GSDMD. In addition, we expanded our analysis and plated cecum tissue as well as other systemic organs such as the spleen and liver. In support of the observations above, we found again higher CFUs for GsdmA1−/− and GsdmD−/− mice in the mLN and also in the cecum tissue and spleen and liver (Fig. 1 D–G and SI Appendix, Fig. S1F for luminal colonization). Furthermore, CFU counts appeared similar between GsdmA1−/− and GsdmD−/− animals, indicating that these mice feature a similar phenotype (Fig. 1 D–G). Of note, none of the other Gasdermin-deficiencies led to a detectable CFU difference in any of the organs (SI Appendix, Fig. S1 G–O). Overall, these data suggest that in contrast to GSDMA1−/−, GSDMC1−/−, and GSDME, GSDMD reduces S.Tm loads in the gut tissue as well as in systemic organs. Thus, GSDMD-deficiency phenocopies GsdmA1−/−, which highlights a unique role for GSDMD during acute S.Tm gut infection.

GSDMD Reduces Lamina Propria S.Tm Loads and Protects the Gut Tissue Integrity by 72 h of Infection.

Since the phenotype of GsdmA1−/− mice was fully attributable to GSDMD (Fig. 1), we decided to focus our analysis on GSDMD. To exclude any differences at steady-state between GsdmD−/− and GsdmD−/− mice in terms of gut inflammation, we analyzed non-infected littermates. In line with previous work (47), the gut mucosa appeared normal in GSDMD-deficient mice, and baseline expression levels of inflammatory mediators were indistinguishable from those from matched littermate controls (SI Appendix, Fig. S2 A–C). GSDMD is activated by Caspase-1 downstream of inflammasomes such as the NAIP/NLRC4 inflammasome (7, 9). Mouse deficient in the NAIP/NLRC4 inflammasome accumulate higher S.Tm loads in the lamina propria which results in a TNF-driven collapse of the epithelial barrier by days 2–3 after orogastric infection (31). In remiscence to this NAIP/NLRC4 phenotype, microscopy-based analysis of the cecum tissue at 48 h.p.i. revealed elevated S.Tm loads in the lamina propria of GSDMD-deficient mice (Fig. 2A and B) and cecum TNF levels were significantly increased (Fig. 2C). Of note, at this time point of infection, in both GsdmD−/− and GsdmD−/− littermates, we measured high levels of the inflammatory marker lipocalin-2 (LCN2) in the feces (SI Appendix, Fig. S3A).

One day later at 72 h.p.i., S.Tm CFU loads in the gut tissue as well as at systemic sites were still higher in GSDMD-deficient mice than in the heterozygous littermate controls (Fig. 2 D–F and SI Appendix, Fig. S3 B and C). Interestingly, S.Tm loads remained high in the lamina propria (Fig. 2G) and the epithelium became severely disrupted by 72 h.p.i. in GsdmD−/− mice, but not in the GSDMD-proficient controls (Fig. 2 H and I). Accordingly, we observed significantly reduced numbers of IECs and more epithelial gaps than in the corresponding control animals at this point of the infection (Fig. 2 H and I and SI Appendix, Fig. S3D). This appeared remarkably similar to the day 3 infection phenotypes previously seen in NAIP/NLRC4-deficient mice (31). These observations were confirmed by independent experiments using...
an alternative GSDMD-deficient mouse line (GsdmD_6X−/−), in which the deficiency is caused by a genetic frameshift instead of a deletion (SI Appendix, Fig. S3 E–P). Overall, these data confirm that GSDMD is protective against S.Tm infection at ~48 to 72 h p.i., in partial analogy to NAIP/NLRC4 (8, 31, 37). Both GSDMD and NAIP/NLRC4 limit S.Tm loads in the deeper gut mucosal tissue, as well as in systemic organs, and prevent the loss of epithelial barrier integrity by 48 to 72 h of infection.

Bone-Marrow-Derived Cells Employ GSDMD to Restrict S.Tm Tissue Loads. Since GSDMD is known for the induction of pyroptosis in bone marrow (BM)-derived macrophages and we observed elevated S.Tm loads in the lamina propria of GSDMD-deficient mice, we addressed whether GSDMD in BM-derived cells of the lamina propria restricts S.Tm in vivo. WT mice were gamma-irradiated and reconstituted with BM from either WT (CD45.1+) or GSDMD-deficient donors, which resulted in >92% transfer efficiency (SI Appendix, Fig. S4A). When infected, both groups exhibited similar luminal S.Tm colonization (SI Appendix, Fig. S4B). However, GsdmD−/− BM recipients harbored significantly elevated S.Tm loads in the cecum tissue, mLN, and spleen at 72 h p.i. (Fig. 3 A–C). Moreover, fluorescence microscopy revealed elevated S.Tm loads specifically in the lamina propria compartment (Fig. 3 D and E). This demonstrates that lack of GSDMD exclusively in BM-derived cells is sufficient to observe higher lamina propria S.Tm loads after 72 h of infection. Similar observations were made in 48-h infections, or in BM chimeric mice derived from GSDMD-deficient donors, which resulted in >92% BM–derived S.Tm loads in the mesenteric lymph nodes of littermate-controlled 48-h infections with GSDMA(A1–A3)-deficient, GSDMC(C1–C4)-deficient, GSDMD-deficient, and GSDME-deficient mice. (D–G) At 48 h p.i., GSDMD-deficient mice (GsdmD−/−) phenocopy mice deficient in all Gasdermins (GsdmACDE−/−) in terms of S.Tm pathogen loads. S.Tm pathogen loads in (D) cecum tissue, (E) mesenteric lymph nodes, (F) spleen, and (G) liver. In A, 5 mice were analyzed. Means with SD are indicated. In B–G, each data point represents one mouse. ≥5 mice per group from ≥2 independent experiments for each comparison. Line at median. The dotted line represents the detection limit. Mann-Whitney U test (ns—not significant, *P < 0.05, **P < 0.01, ***P < 0.001).
explored whether GSDMD deficiency leads to increased Tm, Fig. S6 SI Appendix.

We depleted neutrophils with anti-Ly6G or macrophages with anti-CSF1R antibodies and investigated whether GsdmD−/− mice still featured elevated Tm organ loads compared to heterozygous littermates. Surprisingly, neither the depletion of neutrophils, nor of macrophages, attenuated the GSDMD-dependent S.Tm restriction (SI Appendix, Fig. S6 A–G). Guided by this observation, we explored whether GSDMD deficiency leads to increased S.Tm loads in any specific lamina propria cell type. Flow cytometry analysis of lamina propria cells from infected GsdmD−/− and GsdmD+/− mice was performed to determine the predominant cell type(s) that harbor S.Tm (SI Appendix, Fig. S6H).

Interestingly, while cellular composition was only marginally different between genotypes, multiple cell populations including neutrophils, monocytes, and macrophages (which are most frequent and harbor the highest S.Tm loads), but also eosinophils (which are less frequent and harbored lower S.Tm loads) all featured elevated fractions of S.Tm infected cells when comparing GSDMD-deficient mice to their littermate controls (Fig. 3 I and J). Taken together, GSDMD deficiency increases S.Tm loads across different types of BM-derived lamina propria cells, particularly in neutrophils, monocytes, macrophages, and eosinophils.

Epithelial GSDMD Is Dispensable for IEC Extrusion but Affects the Qualitative Features of the Extrusion Process and Promotes Gut Inflammation. The results above demonstrate a restrictive role of GSDMD in lamina propria BM–derived cells. However, it remained incompletely resolved if epithelial GSDMD also contributes significantly to the defense against S.Tm, e.g., by cytokine release or by controlling the extrusion of infected IECs. Work in NLR4-deficient mice had established that epithelial-dependent phenotypes are particularly prominent during the first day of the infection of streptomycin-pretreated mice, or the first few hours in enteroid infection models (8, 31, 37, 40). To tackle this question, we established enteroids from WT and GSDMD-deficient mice, which were infected in-bulk ex vivo with a S.Tm reporter strain that turns GFP positive upon host cell invasion (S. Tm–G′, ref. 49). Notably, in these infected enteroids, we detected cleaved GSDMD to a similar extent as when the enteroids were bulk ex vivo with a S.Tm–G′ reporter strain that turns GFP positive upon host cell invasion (S. Tm–G′, ref. 49). Notably, in these infected enteroids, we detected cleaved GSDMD to a similar extent as when the enteroids were pretreated mice, or the first few hours in enteroid infection models (8, 31, 37, 40). To tackle this question, we established enteroids from WT and GSDMD-deficient mice, which were infected in-bulk ex vivo with a S.Tm reporter strain that turns GFP positive upon host cell invasion (S. Tm–G′, ref. 49). Notably, in these infected enteroids, we detected cleaved GSDMD to a similar extent as when the enteroids were exposed to the NAIP/NLRC4 agonist RodT ox (SI Appendix, Fig. S7A). Furthermore, using Nlr4−/− enteroids as a positive control (31, 40), we quantified S.Tm–G′ infection foci in more than 70 enteroids per replicate (which represents a sufficient sampling size to obtain statistically valid results; SI Appendix, Fig. S7B), which revealed that GsdmD−/− enteroids harbored significantly more intracellular S.Tm than the WT controls.
The S. Tm counts in the GsdmD+/− enteroids were, however, still much lower in comparison to NLRC4-deficient enteroids (Fig. 4 A and B).

Given that NLRC4 counteracts S. Tm infection by expelling infected IECs into the gut lumen (8, 31, 37, 40), we next sought to address whether and how epithelial GSDMD might contribute to this defense mechanism. To this end, we established enteroid-derived monolayers atop loose hydrogels (50), infected these with S. Tm, and followed the IEC extrusion process by differential interference contrast (DIC) and fluorescence live cell microscopy. As expected, NLRC4-deficient monolayers failed to extrude infected IECs (Fig. 4 C and D). By sharp contrast, in WT and GsdmD+/− monolayers, we could detect many extruding IECs within 120 min of S. Tm infection (Fig. 4 C and D). Importantly, this process was morphologically distinct between WT and GSDMD-deficient monolayers. IECs extruding from the GsdmD+/− monolayers appeared round and bright in DIC and remained impermeable for the dye Draq7, while extruding WT IECs were as a rule translucent and eventually all became Draq7 positive (Fig. 4 C and D). These observations held true even for chimeric monolayers in which we focused on GSDMD-proficient (RFP-labeled) and deficient IECs that were located next to each other, suggesting that this phenomenon is mediated by a cell-intrinsic mechanism (Fig. 4 E and F). Hence, GSDMD is not required for extrusion per se, but it cell-intrinsically affects the qualitative features of extruding IECs and, in line with previous work (8, 21, 40), the time point of membrane permeabilization.

To investigate the contribution of epithelial GSDMD in vivo, we infected streptomycin-pretreated mice for 18 h, which led to cleaved GSDMD in the cecum tissue (SI Appendix, Fig. S7D). Of note, one infected mouse out of five did not show a band for cleaved GSDMD, indicating that GSDMD cleavage kinetics differ between mice, that GSDMD deficiency leads to a general increase of infected lamina propria cells. Fluorescence microscopy-based quantification of S.Tm-LPS+ and GsdmD−/− lamina propria cells at 72 h p.i. (G) Microscopy-based quantification of S.Tm-LPS+ cells in the lamina propria. (H) Relative percentage of the quantification in G. (I and J) Lamina propria cells more frequently harbor S.Tm in GSDMD-deficient mice. Flow cytometry analysis of lamina propria cells from 72 h infected GsdmD+/- and GsdmD−/− littersates. (I) Total cell population sizes in the lamina propria. (J) Relative percentage of the quantification in I. (L) Total S. Tm-LPS+ cell numbers in the lamina propria. In A–C and E–I, each data point represents one mouse. Data are combined from ≥2 independent experiments for each comparison except for F, where only one representative experiment is shown out of 2. Line at median. The dotted line represents the detection limit. Mann-Whitney U test (ns—not significant, *p < 0.05, **p < 0.01).
increased loads of S.Tm-G⁺ in the mucosal tissue of GsdmD⁻/⁻ mice, which was mostly attributable to infected IECs (Fig. 4 G and H). In contrast to the profoundly reduced numbers of expelling infected IECs that we had observed in NLRC4-deficient mice (31, 37), we still observed considerable numbers of dislodged IECs in the GSDMD-deficient mice (Fig. 4I). However, GSDMD-deficient mice did feature a trend toward reduced numbers of dislodged IECs, had fewer intraluminal neutrophils, and were significantly less inflamed, as judged by lipocalin-2 levels (Fig. 4 I and J and SI Appendix, Fig. S7 E and F). In line with previous observations (8), ASC speck formation appeared to be more prevalent in dislodged IECs of GsdmD⁻/⁻ mice, even though our analysis has been underpowered to obtain statistically significant evidence for such ASC phenotype (SI Appendix, Fig. S7 G and H). Of note, despite abundant active Caspase-3 in IECs captured in the extrusion process (SI Appendix, Fig. S8A), a point mutation rendering GSDMD insensitive to inactivation by Caspase-3, did not alter S.Tm tissue loads (SI Appendix, Fig. S8 B–F). Moreover, although GSDEME can be cleaved by apoptotic Caspases (11, 15–18), we could not observe a similar gut mucosal phenotype in GSDME-deficient mice at 18 h p.i. (SI Appendix, Fig. S8 G–K), not even in a GSDMD-deficient background (SI Appendix, Fig. S8 L and P).

Overall, from these data, we can conclude that although GSDMD is dispensable for IEC extrusion, it can to an extent restrict S.Tm in the epithelium (but not by the same magnitude as NAIP/NLRC4). Moreover, GSDMD is essential for prompt cell membrane lysis upon IEC extrusion, which also appears linked to neutrophil recruitment and the initiation of inflammation in the gut mucosa.

Epithelial GSDMD Contributes to Anti-S.Tm Defense in the Mucosa. To directly address whether this epithelial GSDMD phenotype at 18 h p.i. contributes to elevated S.Tm loads in cecum tissue, and at systemic sites later during infection, we again generated BM chimeras in which we replaced the BM of GsdmD⁺/⁺ and GsdmD⁻/⁻ littermates with WT (CD45.1) BM. Strikingly, despite high transfer efficiency of GSDMD-proficient cells (SI Appendix, Fig. S9A), we could still detect a tendency toward increased S.Tm loads in cecum tissue, as well as significantly elevated loads in mLN, spleen, and liver of GSDMD-deficient recipient mice (Fig. 5 A–C and SI Appendix, Fig. S9 B and C). Also, in these mice, we enumerated more S.Tm-G⁺ in the lamina propria (Fig. 5 D and E).

To exclude that this difference is attributable to remaining recipient BM-derived cells and to verify the S.Tm restriction capacity of epithelial GSDMD, we generated epithelial-specific GsdmD⁻/⁻ mice by crossing floxed-GsdmD mice with the intestinal-epithelial-specific expressing Cre-recombinase mouse line (VilCre-mice). Indeed, at 48 h p.i., while luminal colonization was similar across genotypes (SI Appendix, Fig. S9D), we observed elevated S.Tm loads in the cecum tissue, mLN, spleen and liver from GsdmDΔIEC compared to GsdmΔIBR mice (Fig. 5 F–H and SI Appendix, Fig. S9E). Again, we stained for S.Tm-LPS and counted significant more S.Tm-LPS' cells in the lamina propria of epithelial-specific GSDMD-deficient animals (Fig. 5 I and J). This formally demonstrates that epithelial GSDMD contributes to the defense against S.Tm in vivo. However, this protection is less pronounced than in the mice with full-body ablation of GSDMD, which is in line with an additional protective function of GSDMD in non-epithelial cells.

Taken together, our combined infection data from BM chimera and mice with cell-type-specific ablation suggest that both, epithelial and lamina propria cell GSDMD, contribute to restricting S.Tm tissue loads upon oral S.Tm infection.

**Discussion**

Gasdermins are key executors of multiple pathogen restriction mechanisms. A limited number of in vivo studies have shown that Gasdermins C, D, and E can reduce organ loads of diverse pathogens, or are involved in immunopathology (11, 22, 23, 41–43, 52, 53). Nevertheless, to date, we still lack a systematic assessment of their potential restrictive role(s) during oral S.Tm infection. Our experiments in streptomycin-pretreated mice establish how Gasdermins contribute to S.Tm restriction and demonstrate a central function of GSDMD. Moreover, IECs and lamina propria cells both employ GSDMD to restrict S.Tm tissue infection and to switch the gut mucosa as a whole toward an anti-S.Tm state.

GSDMD is the best-studied Gasdermin to date. GSDMD-deficient mice feature elevated organ pathogen loads during infections with the lung pathogen *Burkholderia cenocepacia* (53). Moreover, GSDMD also plays an important role in protection against gut pathogens such as *Yersinia pseudotuberculosis* and *Citrobacter rodentium* (11, 22, 52). With regard to S.Tm infections, it was reported that GSDMD mediates NETosis in neutrophils upon non-canonical Caspase-11 activation by the attenuated S.Tm strain ΔsifA (48, 54). SifA-deficiency is known to promote egress of S.Tm from the *Salmonella* containing vacuole (55), which should enhance pathogen detection in the host cell's cytosol by Caspase-11. Interestingly, *GsdmD⁻/⁻* mice intraperitoneally infected with this mutant strain exhibited elevated pathogen loads in the spleen. This appeared to be dependent on the formation of neutrophil extracellular traps (NETs), since DNase I treatment increased spleen pathogen loads in WT mice, but not in GSDMD-deficient animals. Notably, due to the use of a mutant S.Tm strain and the intraperitoneal administration (which bypasses the gut tissue invasion steps of the normal infection process), it remained unclear whether this holds true in oral infection with WT S.Tm. Here, we demonstrate that GSDMD indeed restricts orally administered WT S.Tm not only at the level of the IEC but also in BM-derived lamina propria cells, as well as in systemic organs. However, GSDMD deficiency in a fraction of BM-derived cells appears enough to increase overall tissue S.Tm loads and frequency within neutrophils, but also several other immune cells showed elevated S.Tm numbers in *GsdmD⁻/⁻* mice. This suggests that GSDMD acts globally to restrict S.Tm in the mucosal tissue. Since neither the separate depletion of neutrophils, macrophages, or the inflammasome-dependent cytokines IL18 or IL1β significantly impacted the GSDMD phenotype, we speculate that multiple mechanisms may explain this restrictive effect of GSDMD. It is likely that GSDMD in macrophages and neutrophils i) promotes cell death, ii) accelerates mucosal inflammation, and iii) traps S.Tm in pore-induced intracellular traps (PITs) and NETs, respectively (54, 56), thereby preventing subsequent re-infections into adjacent host cells. These mechanisms are supported by other previous studies in vivo (43, 48). Nevertheless, it is plausible that additional mechanism(s) may also contribute to the GSDMD-dependent restriction in the lamina propria.

The data from the infection of 3D enteroids, enteroid-derived 2D monolayers (Fig. 4), the early (first 18 h p.i.) infections in GsdmD-deficient mice (Fig. 4), the later time point (48 h p.i.) infections with the BM chimeras (Fig. 5) and the epithelial-specific *GsdmDΔIEC* experiments (Fig. 5) prove that epithelial GSDMD also contributes to restricting S.Tm, most likely during the initial phase of the infection. Importantly, this restriction is much weaker compared to that conferred by epithelial NAIP/NLRC4. This can be explained by the prominent role for NAIP/NLRC4 in driving extrusion of infected IECs, a process which can still be executed in the absence of GSDMD. However, in GSDMD-deficient enteroid–monolayers, we found that the qualitative features of extruding...
cells appear remarkably different. In line with a recent publication, we show that epithelial GSDMD impacts the time point of membrane lysis during the extrusion process, which also influences how efficiently, and with what kinetics, infected IECs can be removed from the epithelium (21). Furthermore, a delay of cell membrane permeabilization toward much later time points (that is at or after the end of the IEC extrusion process) should also impact the levels of reduced exposure of the lamina propria to IEC marker genes in the cecum tissue of GsdmD-deficient mice (22, 57, 58). However, in the unperturbed gut, we did show that epithelial GSDMD impacts the time point of membrane lysis during the extrusion process, which also influences how efficiently, and with what kinetics, infected IECs can be removed from the epithelium. Chimeric enteroid–monolayers with GSDMD-proficient (VIIIFP) and deficient (Gsdmd−/−) IECs were infected with S.Tm in vivo and promotes induction of inflammation. (E) Representative micrographs of time-lapse microscopy. Filled arrowheads indicate lytic cell death and empty arrowheads indicate non-lytic cell death. (F) Quantitative analysis of the Dra7 signal from time-lapse microscopy. (G-J) 18-h infections with S.Tm harboring a pssag-GFP reporter indicate that epithelial GSDMD restricts S.Tm in vivo and promotes induction of inflammation. (G) Representative micrographs of time-lapse microscopy. (H) Microscopy-based quantification of S.Tm-GFP+ IECs. Arrowheads indicate S.Tm-GFP+ in epithelial cell line. Lu.–lumen. (I) Microscopy-based quantification of dislodged IECs. (J) Quantification of inflammation by Lipocalin-2 levels of cecum content. In A–F, combined results, or representative results from ≥2 replica. In D and F, mean ± range are plotted. In H–J, each data point represents one mouse. ≥5 mice per group from ≥2 independent experiments for each comparison. Line at median. The dotted line represents the detection limit. Mann-Whitney U test (**P < 0.01, ****P < 0.0001).

Fig. 4. Epithelial GSDMD is dispensable for IEC extrusion but affects the qualitative features of the extrusion process and promotes gut inflammation. (A and B) Epithelial GSDMD restricts S.Tm loads in epithelium but not to the same extent as epithelial NAIP/NLRC4. 3D enteroids were infected in bulk with S.Tm harboring a pssag-GFP reporter (renders the bacterium GFP-positive upon host cell entry, S.Tm-GFP) for 4 h. (A) Representative micrographs of infected 3D enteroids. Arrowheads indicate S.Tm-GFP+; (B) Microscopy-based quantification of S.Tm-GFP+ in epithelium of enteroids. (C and D) Epithelial GSDMD impacts qualitative features of extruding infected IECs. Enteroid–monolayers were infected with S.Tm in the presence of the membrane-impermeable dye Dra7 to track membrane lysis. (C) Representative micrographs of time-lapse microscopy. Filled arrowheads indicate lytic cell death and empty arrowheads indicate non-lytic cell death. (D) Quantitative analysis of the Dra7 signal from time-lapse microscopy. (E and F) GSDMD acts cell-intrinsically on the qualitative features of extruding IECs. Chimeric enteroid–monolayers with GSDMD-proficient (VIIIFP) and deficient (Gsdmd−/−) IECs were infected with S.Tm in the presence of the membrane-impermeable dye Dra7. (E) Representative micrographs of time-lapse microscopy. Filled arrowheads indicate lytic cell death and empty arrowheads indicate non-lytic cell death. (F) Quantitative analysis of the Dra7 signal from time-lapse microscopy.

Recent work in mice has demonstrated that other Gasdermins, e.g., GSDMC and GSDME, also take part in the defense against pathogens and in inflammation (11, 41, 42). In particular, GSDME was shown to induce Caspase-8-driven pyroptosis in neutrophils, which helps to control systemic Yersinia infections (11). Additionally, GSDME in epithelial cells promotes inflammation during chemically induced colitis (41). Therefore, it is somewhat surprising that we do not detect any protective effect for GSDME during S.Tm infection. GSDME seems to neither influence IEC extrusion efficiency nor to restrict S.Tm pathogen loads in the gut tissue, or at systemic sites, not even in a GSDMD-deficient background. Furthermore, GSDMC2 and GSDMC3, which were shown to be highly expressed in epithelial cells of worm-infected mice (42), along with GSDMC1 and GSDMC4 also fail to impact S.Tm infection, at least in the streptomycin-pretreated mouse model. The same holds true for GSDMA1-3. Why only GSDMD plays an important protective role during S.Tm infection is not fully clear. A reason could be that S.Tm is an intracellular pathogen in contrast to worms, and innate immunity against S.Tm is consequently dominated by inflammasome signaling. Also, we cannot rule out that S.Tm may
express yet unidentified virulence factors blocking the action of some Gasdermins. Notably, based on the results from mice lacking all Gasdermins, we were able to answer a long-lasting question in the field and can exclude any redundant protective role among Gasdermins during acute S.Tm infection. This is specific for the mouse model used in our experiments and not otherwise specified. Where indicated, S.Tm reporter strain harboring the plasmid p975 (pssAG-GFPmut2) was used (49). S.Tm was cultured overnight in LB/0.3M NaCl (Sigma Aldrich) with appropriate antibiotics (ca. 12 h) before sub-culturing in 1:20 dilution for 4 h in the same media without antibiotics. For mouse infections, S.Tm were washed once and reconstituted with PBS (BioConcepts) before oral gavage. For 2D enteroid infections, S.Tm were washed with PBS and reconstituted in DMEM/F12 (STEMCELL) supplemented with 3% FCS (Thermo Fisher). For 2D enteroid-derived monolayer infections, S.Tm were reconstituted in DMEM/F12 (Gibco) and diluted in complete mouse IntestiCult (STEMCELL) without antibiotics to the desired concentration.

Mouse Infections. All mice used were specific pathogen free (SPF) and were housed in individually ventilated cages of the ETH Zürich mouse facility (EPIC Mouse Infections.). WT mice were C57BL/6 (congenic marker CD45.2+) originally from Charles River (Sulzfeld, Germany). For generation of bone marrow chimeras, B6.SJL-Ptpcr^Pepc mice (congenic marker CD45.1^) were used as WT where indicated. All genetic modified mice were of C57BL/6 background. Specifically, the following mouse lines were used: Nlrc4^−/− (B6.C2-Nlrc4tm1Vmd, ref. 59), GsdmA^−/− (C57BL/6J-Gsdma^1-^em1Broz^x^Gsdmd^1-^em1Broz^x^Gsdmc1^−/−, this study), Gsdmd^−/− (9), Gsdmd^−/− (C57BL/6J-Gsdmd^1-^em1Broz^x^Gsdmc1^−/−, ref. 60), Gsdmc1^−/− (C57BL/6J-Gsdmc1^−/−, ref. 60), Gsdmc1^−/− (C57BL/6J-Gsdmc1^−/−, ref. 11), Gsdmc1^−/− (C57BL/6J-Gsdmc1^−/−, this study), ActRFP (B6.Cg-Tg[CAG-GFP, ref. 61], Il18^−/− (B6.129P2-Ily1tm1Aki, ref. 62), Gsdmdem1Broz (B6.SJL-Tg[VilCre]997GumJ^1/2)^x^C57BL/6J-Gsdmd^1-^em1Broz^x^Gsdmc1^−/−, this study). Genotyping of mice was done by PCR or sequencing. Heterozygous littermates were used as control animals except for Gsdmc1^−/− mice. Gsdmc1^−/− mice were co-housed in (A–D) Replacement of the BM of Gsdmd^−/− and Gsdmc1^−/− littermates with WT BM cells (CD45.1^) does not restore the S.Tm pathogen load phenotype of Gsdmd^−/− mice locally and systemically at 48 h p.i. S.Tm pathogen loads in (A) cecum tissue, (B) mesenteric lymph nodes, and (C) spleen. (D) Representative micrographs of cecum tissue sections, stained for S.Tm. Arrowheads indicate S.Tm in the lamina propria. Lu.–lumen. (E) Microscopy-based quantification of S.Tm^LPS^ cells in the lamina propria. (F) IEC-specific Gsdmd deficiency results in elevated S.Tm pathogen loads locally and systemically at 48 h p.i. S.Tm pathogen loads in (F) cecum tissue, (G) mesenteric lymph nodes, and (H) spleen. (I) Representative micrographs of cecum tissue sections, stained for S.Tm-LPS. Arrowheads indicate S.Tm in the lamina propria. Lu.–lumen. (J) Microscopy-based quantification of S.Tm-LPS^ cells in the lamina propria. In A–C, E–H, and I, each data point represents one mouse. ≥5 mice per group from ≥2 independent experiments for each comparison. Line at median. The dotted line represents the detection limit. Mann–Whitney U test (ns–not significant, **P < 0.01, ***P < 0.001).

Methods

Bacterial Strains, Plasmids, and Culture Conditions. All infection experiments were done with Salmonella Typhimurium (S.Tm) SL1344 (SB300, SmR) if not otherwise specified. Where indicated, S.Tm reporter strain harboring the

Fig. 5. Epithelial GSDMD contributes to anti-S.Tm defense in the mucosa. Epithelial GSDMD contributes to restricting S.Tm tissue loads upon oral S.Tm infection. (A–D) Replacement of the BM of Gsdmd^−/− and Gsdmc1^−/− littermates with WT BM cells (CD45.1^) does not restore the S.Tm pathogen load phenotype of Gsdmd^−/− mice locally and systemically at 48 h p.i. S.Tm pathogen loads in (A) cecum tissue, (B) mesenteric lymph nodes, and (C) spleen. (D) Representative micrographs of cecum tissue sections, stained for S.Tm. Arrowheads indicate S.Tm in the lamina propria. Lu.–lumen. (E) Microscopy-based quantification of S.Tm^LPS^ cells in the lamina propria. (F) IEC-specific Gsdmd deficiency results in elevated S.Tm pathogen loads locally and systemically at 48 h p.i. S.Tm pathogen loads in (F) cecum tissue, (G) mesenteric lymph nodes, and (H) spleen. (I) Representative micrographs of cecum tissue sections, stained for S.Tm-LPS. Arrowheads indicate S.Tm in the lamina propria. Lu.–lumen. (J) Microscopy-based quantification of S.Tm-LPS^ cells in the lamina propria. In A–C, E–H, and I, each data point represents one mouse. ≥5 mice per group from ≥2 independent experiments for each comparison. Line at median. The dotted line represents the detection limit. Mann–Whitney U test (ns–not significant, **P < 0.01, ***P < 0.001).
with WT or GsdmD<sup>−/−</sup> mice for at least 2 wk prior to infection. In general, 8- to 15-wk-old mice were infected according to the streptomycin mouse model (64). Briefly, mice were orally pretreated with 25 mg streptomycin sulfate (5m, AppliChem) 1 d before infection with ~5 × 10<sup>7</sup> CFU S.typhimurium per gavage. Mice were monitored daily, and organs were harvested at the indicated time points. Organs were homogenized in PBS containing 0.5% tertigol and 0.5% BSA using a tissue lyser (Qiagen) and plated on MacConkey agar (Oxoid) with 5m. Cecum tissue was first washed in PBS, incubated for 30 to 60 min in PBS/400 µg/ml gentamycin and washed extensively (6 ×) in PBS before plating. To generate bone marrow chimeras, mice were gamma-irradiated (1,000 Rad) and 5 × 10<sup>6</sup> bone marrow cells from the respective mouse line were transferred via the tail vein. Mice received Boral (Verenirania AG) in the drinking water for 3 wk and kept at least for 6 wk before infection. Transfer efficiency was measured by flow cytometry. Briefly, the spleen was pressed through a 40 µm cell strainer, and cells were collected in ice-cold PBS. Cells were pelleted (600 g, 5 min, 4 °C) and incubated in BD Cytofix/Cytoperm (BD Biosciences) for 15 min. Fixation was stopped by adding PBS, and cells were washed once with PBS before staining. For staining, cells were incubated in FACS buffer containing CD45.1 and 1G7, and cells were washed once in FACS buffer and filtered before acquisition. For intravenous infections, 10<sup>5</sup> S.typhimurium in 100 µL PBS from an overnight or 4 h subculture were injected in the tail vein. For IL18 or IL1β depletion experiments, 200 µg/mouse anti-IL18 (BioXCell, YIGIF74-1G7) or anti-IL1β (BioXCell, B1222), respectively, was injected intraperitoneal on the day of pretreatment and infection. For neutrophil depletion experiments, 500 µg/mouse anti-ly6G (BioXCell, 1A8) was injected intraperitoneal daily starting at pretreatment. For macrophage depletion, 1,000 µg/mouse anti-CSF1R (BioXcell, AFS98) was injected intraperitoneal 4 d prior infection and 300 µg/mouse every following day until harvest. All animal experiments were approved by the Kantonale Veterinärämter Zürich (licences 193/2016, 158/2019, and 108/2022).

**Generation of Conditional GsdmD, GsdmA<sup>−/−</sup>, GsdmC<sup>−/−</sup>,** and GsdmACDE<sup>−/−</sup> Knockout Mice. To generate conditional GsdmD knockout mice (C57BL/6-J-GsdmD<sup>−/−</sup>, Iox<sup>−</sup>) sites were inserted up and downstream of exon 2 of GsdmD using the following gRNAs (1: TACGCTGCTTATAACGGTGGT and 2: TCATCAATTCCACTTCTTTGG-GGG). To generate GsdmD<sup>−/−</sup> knockout mice (C57BL/6-J-GsdmD<sup>−/−</sup>-g3<sub>−/−</sub>), the complete GsdmAT1-3 locus was deleted by using the following 2 gRNAs (3: TACGGGTGATACGCTCTGTG and 4: ACAAGGGCCGCGCAGGAGGAA). To generate GsdmC<sup>−/−</sup> knockout mice (C57BL/6-J-GsdmC<sup>−/−</sup>-g4<sub>−/−</sub>), the complete GsdmC1-4 locus was deleted by using the following 2 gRNAs (5: GTGCGTAGTAAACGAGCTGGG and 6: GATGGTGGAATATGACGCTGGG). To generate GsdmA<sup>−/−</sup> knockout mice (C57BL/6-J-GsdmA<sup>−/−</sup>-g5<sub>−/−</sub>), the entire GsdmA<sup>−/−</sup> genomic locus was deleted between the binding site of gRNA3 and PCR<br>

**Establishment and Infection of 2D Murine Enteroid-Derived Monolayers.** 2D murine enteroid-derived monolayers were established as previously described (40). In brief, enteroids were split as described above and cultured in freshly prepared CV medium, i.e., complete mouse IntestiCult supplemented with 3 µM CHIR99021 (Cayman Chemicals) and 1 mM valproic acid (Cayman Chemicals) for 1 wk. The medium was exchanged for fresh CV medium every 2 to 3 d. Glass-bottom 8-well chamber slides (Cellvis) were pre-coated with 75 µg/mL Poly-L-Lysine (Sigma Aldrich) at RT overnight and washed three times with PBS (Gibco). Chamber slides were then dried for 2 h before a 1 mg/mL collagen (Coling) solution in collagen neutralization buffer (200 µM HEPES/5 mM sodium bicarbonate/nitrogen dioxide to equilize acid from the collagen stock) was added to the wells, and the hydrogels were left to solidify for 1 h at 37 °C as previously described (66). CV-pre-treated enteroids were dissociated by mechanical shearing and incubation in Gentle cell dissociation reagent (STEMCELL) for 10 min, and then washed with PBS, washed with PBS, and then left to solidify for 10 min. The culture medium was exchanged every 2 d. Every 5 to 7 d, the cultures were split by mechanical shearing in RT Gentle dissociation reagent, and the enteroids were re-seeded in 50 µL Matrigel domes (splitting ratio 1.4 to 1.6). Stable enteroid cultures were cryopreserved and thawed for experimentation. S<sub>4b</sub> tml infections were performed after at least 2 wk of culture maintenance. To this end, domes containing ~100 enteroids were dissolved in ice-cold DMEM/MEM/F12/10% FCS by pipetting carefully up and down. The enteroids were pelleted by centrifugation (300g, 5 min, 4 °C), and re-suspended in pre-warmed DMEM/F12/10% FCS without antibiotics. For western blotting, enteroids were infected with S<sub>4b</sub> at an estimated MOI of 100 for the indicated durations (37 °C, 5% CO<sub>2</sub>), and harvested at 6 wk. The enteroids were pelleted by centrifugation (300g, 5 min, 4 °C), and the pellets were snap frozen in liquid nitrogen, and kept at −80 °C until western blotting. For microscopy analysis, 5.1m harboring a pssA-GFP reporter was used to infect the enteroids at an estimated MOI of 100 for 40 min (37 °C, 5% CO<sub>2</sub>). After infection, RT DMEM/F12/10% FCS containing 100 µg/mL gentamycin (AppliChem) was added for 15 min (37 °C, 5% CO<sub>2</sub>) to kill extracellular bacteria. The enteroids were pelleted (300g, 5 min, 4 °C), resuspended in complete IntestiCult supplemented with 25 µg/mL gentamycin and seeded in 25 µL Matrigel domes in prewarmed 8-well glass chamber slides (Thermo Scientific). Domes were solidified for 10 min (37 °C, 5% CO<sub>2</sub>), RT complete IntestiCult containing 25 µg/mL was added, and the enteroids were incubated (37 °C, 5% CO<sub>2</sub>) until fixation with 4% paraformaldehyde (PFA; Sigma Aldrich) at 4 h. After fixation, samples were washed three times with PBS, permeabilized with PBS/0.5% TX-100 (Sigma Aldrich) for ≥10 min, blocked with PBS/10% Normal Goat Serum (Gibco) for ≥30 min, and incubated for ≥40 min with TRITC-conjugated Phalloidin (Fluoprobes) and DAPI (Sigma Aldrich). Stained enteroids were extensively washed with PBS and ddH<sub>2</sub>O, the chambers were carefully removed from the glass slides, and the samples were covered with a glass slip using one drop of Mowiol (WWR International AG) per dome.
pre-warmed DMEM/F12, and the medium was exchanged for complete mouse IntestiCult without Y-27632. Thereafter, the medium was exchanged for fresh complete mouse IntestiCult every 1 to 2 d. Monolayer infections were performed 72 to 96 h post establishment. Prior to infection, the monolayers were washed once with pre-warmed DMEM/F12 and complete IntestiCult without antibiotics containing 1.5 μM Drag7 was added to each well. After placing the chamber slide in the pre-warmed microscope chamber (37 °C, 5% CO₂), the prepared 0.1 μm inçoculum was added at an MOI of 0.5 to 2 and imaging was started immediately.

**Time-Lapse Imaging of 2D Murine Enteroid-Derived Monolayers.** Time-lapse imaging of 2D murine enteroid-derived monolayers was performed on a custom-built microscope based on an Eclipse Ti2 body (Nikon) with 60×, 0.7 numerical aperture Plan Apo Lambda air and 40×/0.6 Plan Apo Lambda air objectives (Nikon) and a back-lit sCMOS camera (pixel size 11 μm, Prime 95B; Photometrics). Samples were maintained at 37 °C, 5% CO₂, in a moisturized chamber during imaging. Bright-field imaging was performed using differential interference contrast (DIC), and fluorescence was acquired with an excitement light engine Spectra-X (Lumencor) and emission collection through a quadruple bandpass filter (89402; Chroma).Infected monolayers were imaged in 2D intervals for a total of 120 min. To quantify IEC permeabilization in response to infection, images were thresholded in Fiji (a version of ImageJ ref; 67) using the same threshold value for all time-lapse movies from the same experiment, and the area above threshold was enumerated.

**Immunofluorescence Staining and Wide-Field and Confocal Microscopy.** Upon harvesting, mouse cecum tissue was fixed in 4% PFA, saturated in 20% sucrose, and submerged in Optimal Cutting Temperature compound (OCT, Tissue-Tek) before flash freezing in liquid nitrogen. Samples were kept at −80 °C until further analysis. Ceca were cut in 10- to 20-μm thick cross-sections and mounted on glass slides (SuperFrost+ +, Thermo Scientific). Air-dried sections were rehydrated with PBS, permeabilized with PBS/0.5% Tx-100 (Sigma Aldrich), and incubated with PBS/10% Normal Goat Serum (NGS; Reactolab SA) before fluorescence staining. For fluorescence staining the following primary/secondary antibodies and dyes diluted in PBS/10% NGS were used: α-EpCam/CD326 (clone G8.8, Biolegend), α-cleaved Caspase 3 (clone #661, Cell Signaling Technology), α-S1m LPS (O-antigen group B factor 4-5, Difo), α-ICAM-1/CD54 (clone 3E2, BD Biosciences), α-ASC (N-15, Santa Cruz Biotechnology), α-Ly46.2 (clone 7/4, Bio-Rad), α-rabbit-AlexaFluor488 (Abcam Biochemicals), α-rabbit-Cy3 (Bethyl Laboratories), α-rat-FITC (Jackson), α-rat-Cy3 (Jackson), α-rat-Cy5 (Jackson), α-hamster-Cy5 (Jackson), CruzFluor488-conjugated Phalloidin (Santa Cruz Biotechnology), TRITC-conjugated Phalloidin (Fluorobeta), AlexaFluor647-conjugated Phalloidin (Molecular Probes), and DAPI (Sigma Aldrich). Mowiol (VWR International AG) was used to cover the stained sections with a coverslip. Microscopy was performed using a Zeiss Axiovert 200 m microscope with 10 to 200× objectives, a spinning disc confocal lased unit (Visitron), and an Evolve 512 EMCCD camera (Photometrics). Images were processed or analyzed with Visview (Visitron) and/or ImageJ. Microscopy quantification was done manually and blindly on at least 2 sections per mouse as previously described (31).

**Flow Cytometric Analysis of Lamina Propria Cells.** Cecum lamina propria cells were isolated and stained as previously described (33). For cell surface staining, cells were incubated in 1 µg/sample Mouse BD Fc Block (BD Biosciences) for 10 min, washed in PBS containing 1.5 µg/sample Mouse BD Fc Block (BD Biosciences) to block Fc recepts before staining. Cells were incubated in 1× FACS buffer (eBioscience, 00310, Dharmacon) for 30 min. After washing in PBS containing 1× FACS buffer (eBioscience, 00310, Dharmacon), the following antibodies were used: α-S1m LPS (O-antigen group B factor 4-5, Difo), α-EpCam/CD326 (clone G8.8, Biolegend), α-cleaved Caspase 3 (clone #661, Cell Signaling Technology), α-S1m LPS (O-antigen group B factor 4-5, Difo), α-ICAM-1/CD54 (clone 3E2, BD Biosciences), α-ASC (N-15, Santa Cruz Biotechnology), α-Ly46.2 (clone 7/4, Bio-Rad), α-rabbit-AlexaFluor488 (Abcam Biochemicals), α-rabbit-Cy3 (Bethyl Laboratories), α-rat-FITC (Jackson), α-rat-Cy3 (Jackson), α-rat-Cy5 (Jackson), α-hamster-Cy5 (Jackson), CruzFluor488-conjugated Phalloidin (Santa Cruz Biotechnology), TRITC-conjugated Phalloidin (Fluorobeta), AlexaFluor647-conjugated Phalloidin (Molecular Probes), and DAPI (Sigma Aldrich). Mowiol (VWR International AG) was used to cover the stained sections with a coverslip. Microscopy was performed using a Zeiss Axiovert 200 m microscope with 10 to 200× objectives, a spinning disc confocal lased unit (Visitron), and an Evolve 512 EMCCD camera (Photometrics). Images were processed or analyzed with Visview (Visitron) and/or ImageJ. Microscopy quantification was done manually and blindly on at least 2 sections per mouse as previously described (31).

**Flower of the whole mount tissues was directly fixed in 4% paraformaldehyde for 1 h, washed with PBS, infiltrated with 30% sucrose in PBS for 1 h, and embedded in OCT. The OCT was then cut into 10-μm-thick sections and stained with a cocktail of antibodies against α-S1m LPS, α-ICAM-1, and DAPI.**

**Histology.** For histology analysis, cecum tissue embedded in OCT was snap frozen in liquid nitrogen, cut into 5-μm sections, air-dried, and stained with hematoxylin and eosin. The histology score was determined blindly as described previously (64).

**Lipocalin-2 and TNF ELISA.** Feces or cecal content was used for Lipocalin–2 ELISA and ca. a 5-mm piece of extensively washed cecal tissue for TNF ELISA. The cecal tissue sample was homogenized in PBS/0.5% Tergitol/0.5% BSA (Sigma Aldrich, Chemie Brunschwig AG) supplemented with protease inhibitor cocktail (Roche). Lipocalin-2 (R&D Systems) and high-sensitivity TNF (Invitrogen) ELISA was performed according to the manufacturer’s protocols.

**Western Blotting.** Cecum tissue samples were homogenized in ice-cold PBS (ThermoFisher Scientific) supplemented with protease inhibitor cocktail (Merck) for 20 s using an Omni International TH tissue homogenizer. Cells were then lysed in LDS sample buffer (ThermoFisher Scientific) + 10 mM DTT and boiled at 98 °C for 10 min before western blotting. The pelleted enteroids (~500 enteroids/sample) were resuspended in 250 μL TrisCl pH 7.4 + 4% SDS supplemented with protease inhibitor cocktail and passed 10 times through a 25-μm sieve. Samples were then diluted in LDS sample buffer + 10 mM DTT before boiling at 98 °C for 10 min. Protein extracts were run on SDS-PAGE gels and then blotted onto nitrocellulose membranes (Amersham). Membranes were blocked in 5% milk in TBS + 0.1% Tween20 (TBS-T) and incubated with primary antibodies overnight at 4 °C. Membranes were subsequently washed three times in TBS-T and incubated with secondary antibody for 1 h at RT. Following three further washes in TBS-T, membranes were developed with ECL using an iBright imaging device.

**Statistical Analysis.** The Mann–Whitney U test was used to assess statistical significance where applicable as indicated in the figure legends.

**Data, Materials, and Software Availability.** All study data are included in the article and/or SI Appendix.

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