Uncoupling of invasive bacterial mucosal immunogenicity from pathogenicity

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There is the notion that infection with a virulent intestinal pathogen induces generally stronger mucosal adaptive immunity than the exposure to an avirulent strain. Whether the associated mucosal inflammation is important or redundant for effective induction of immunity is, however, still unclear. Here we use a model of auxotrophic *Salmonella* infection in germ-free mice to show that live bacterial virulence factor-driven immunogenicity can be uncoupled from inflammatory pathogenicity. Although live auxotrophic *Salmonella* no longer causes inflammation, its mucosal virulence factors remain the main drivers of protective mucosal immunity; virulence factor-deficient, like killed, bacteria show reduced efficacy. Assessing the involvement of innate pathogen sensing mechanisms, we show MYD88/TRIF, Caspase-1/Caspase-11 inflammasome, and NOD1/NOD2 nodosome signaling to be individually redundant. In colonized animals we show that microbiota metabolite cross-feeding may recover intestinal luminal colonization but not pathogenicity. Consequent immunoglobulin A immunity and microbial niche competition synergistically protect against *Salmonella* wild-type infection.
Mounting a functional anti-microbial adaptive immune response depends on concomitant induction of an innate immunogenic response through pattern recognition receptor (PRR) activation. PRRs sense conserved microbial molecular structures, such as bacterial lipopolysaccharide (LPS), peptidoglycan, and flagellin, that are conserved across pathogenic and non-pathogenic microorganisms. Pathogen-specific virulence factors such as type 3 secretion system (T3SS) components and intracellular toxin action have also been shown to be specifically sensed by PRRs. The integration of diverse PRR signals is believed to regulate immune responses according to the nature of the microbial threat. Natural and artificial PRR signaling agonists are consequently exploited pharmacologically as pro-immunogenic additives or adjuvant components of vaccines. Besides the immunogenic response, PRR activation by pathogens may also drive inflammation and innate anti-microbial defense. This arm of the innate immune system is important for the control of primary pathogen infection, but is also responsible for the adverse effects of inflammation and defense that damage host tissue and symbiotic microbiota, which may be exploited by some mucosal pathogens.

The intestinal mucosal membranes are colonized continuously with a diverse symbiotic microbiota and are guarded by a complex mucosal immune system. The mucosa is well adapted to stable symbiosis with non-pathogenic microbes. Multiple physical and chemical barriers as well as active immune tolerance avoid the unnecessary activation of immune defense mechanisms by harmless symbiotic microbes or food antigens. Only virulent mucosal pathogens normally induce inflammatory responses. Avirulent, fully attenuated pathogens are inefficient and deficient intestinal bacteria also in the absence of PRR signals by the mucosal barrier it then combines virulence factor-dependent immunogenicity and avirulence with the added benefit of pathogen niche competition.

Results

Proliferation-incompetent STmAux induces functional immunogenicity. Mucosal tissue invasion and virulence of STm are mediated by two type 3 secretion systems (T3SS) encoded on Salmonella pathogenicity islands, SPI1 and SPI2 (refs. 5,17–19). Activity of the SPI1-encoded T3SS induces early mucosal inflammation. As the invading and tissue-overgrowing virulent bacteria responsible for this are not to pronounced population bottle necks, we hypothesized that a strain of live STm encoding functional virulence factors would retain its invasiveness with associated adaptive immunogenicity, despite being unable to replicate and overall avirulent.

To test this hypothesis we generated an auxotrophic STm strain (STmAux) that strictly requires supplementation with the essential peptidoglycan constituents D-alanine (D-Ala) and meso-diaminopimelic acid (m-Dap) to grow and survive cell division (Supplementary Fig. 1A, B). Like the homologous model in commensal Escherichia coli developed previously, STmAux colonized the gastrointestinal tract of germ-free mice only transiently, allowing rapid and full recovery to germ-free status, as neither host metabolism nor diet could substitute the auxotrophic requirement for these metabolites (Fig. 1a, b). Salmonella T3SS competence or deficiency had no effect on STmAux colonization kinetics. Bacterial quantitation in small intestinal (Supplementary Fig. 2A) and cecal (Supplementary Fig. 2B) contents at early time points revealed small intestinal transit of STmAux in quantities similar to wild-type STm until 2.5 h following inoculation. At 4.5 h, STmAux had transited from the small intestine into cecum without evidence for replication (Supplementary Fig. 2A, B; compare STmAux numbers between small intestine at 2.5 h and cecum at 4.5 h), whereas wild-type STm populations had begun to expand in the cecum. By 34 h after inoculation wild-type STm stably colonized all intestinal segments, whereas STm Aux densities had sharply declined. No spontaneous D-Ala/m-Dap-independent revertants have been isolated ex vivo during these experiments.

D-Ala/m-Dap auxotrophic bacteria depleted of D-Ala or m-Dap, analogous to wild-type bacteria exposed to beta-lactam antibiotics, remain active until self-destruction by programmed autolytic cell death occurring at the onset of cell division. Accordingly, D-Ala- and m-Dap-depleted STmAux displayed normal cell invasiveness, as demonstrated by immunofluorescence microscopy and gentamicin protection assay (Fig. 1g, h). In germ-free mice, following enteral administration of 10¹⁰ colony-forming units (CFU) of STmAux by gavage, the invasive auxotroph was found to be completely avirulent. In contrast to wild-type STm, STmAux was rarely recoverable from mesenteric lymph nodes (mLNs), liver, or spleen (Fig. 1f). It no longer induced detectable levels of typhococilis (inflammation of the cecum, the main enteric histopathology in the non-typhoidal invasive salmonellosis mouse model) as determined either by quantification of cecal luminal inflammation marker lipocalin-2 (Fig. 1c) or by histopathologic scoring (Fig. 1d, e). Quantification of early mRNA markers of chemokine and other innate activation...
signals in total cecum tissue supported the conclusion that STmAux is avirulent (Supplementary Fig. 2C).

Transitory intestinal mucosal conditioning by live STmAux bacteria (Fig. 2a) induced an adaptive immune response highly protective against the re-challenge of the germ-free animals with non-auxotrophic wild-type STm. While immunity had no effect on the large intestinal luminal load of the challenge strain (Fig. 2b), it protected against its intestinal pathogenesis (Fig. 2c, d) and limited penetration to the mLNs, liver, and spleen (Fig. 2e). Protective immunity was associated with high STm-specific titers of intestinal secretory IgA measured by live bacterial flow cytometry (Fig. 2f, g), and was abolished in B cell- and antibody-deficient 129−/− mice (Fig. 2h–j). B and T cell-deficient RAG-deficient mice phenocopied JH-deficient mice (Supplementary Fig. 3A–D). Hence, B cell immunity is functionally required for STmAux-induced intestinal protective immunity. The live STmAux dose–response relationship was examined by comparing the mucosal conditioning with doses of 10¹⁰, 10⁸, and 10⁶ live STmAux, which revealed that induction of functional immunity required doses greater than 10⁸ live STmAux (see extended dataset.
**Fig. 1** Transient colonization of GF mice with STmAux. a Mice were inoculated at day 0 with 10^{10} CFU of either auxotrophic (Aux; red symbols) or non-auxotrophic control (black symbols) STm strains that were either type 3 secretion competent (STm/STmAux, filled symbols) or isogenic type 3 secretion-deficient mutants (STmΔT3SS−/STmAux ΔT3SS−, open symbols). b Time course of viable bacteria of each strain recoverable from feces (STmAux n = 32, STmΔT3SS− n = 15, STmAux ΔT3SS− n = 12, STm n = 11, animals examined over nine independent experiments). c Lipocalin-2 concentration in cecal contents at 9 h after inoculation (STmAux n = 5, STmΔT3SS− n = 5, STm n = 5 animals). d Cecal histopathology score at 9 h after inoculation. Each symbol represents one individual (STmAux n = 5, STmΔT3SS− n = 5, STm n = 5 animals). e Cecal histology at 9 h after inoculation with indicated STm strains. H&E staining. Scale bar: 100 μm (STmAux n = 5, STmΔT3SS− n = 5, STm n = 5 animals). f Organ loads of T3SS-proficient STmAux and STm in mLN, liver, and spleen on day 1 (mLN: STmAux n = 18, STm n = 3; liver: STmAux n = 6, STm n = 2; spleen: STmAux n = 9, STm n = 3 animals examined over four independent experiments) and 3 (mLN: STmAux n = 2, STm n = 3; liver: STmAux n = 2, STm n = 3; spleen: STmAux n = 2, STm n = 3 animals examined over two independent experiments) post inoculation. g Immunofluorescence of HeLa cells infected for 2 h with wild type (STm), SPII T3SS-deficient (STmΔinvC), auxotrophic SPII T3SS-proficient (STmAux), and auxotrophic SPII T3SS-deficient (STmAux ΔinvC) STm. Cells were stained with DAPI (DNA/nuclei, blue), and with anti-STm group B antisera and labeled secondary antibodies consecutively before, and after membrane permeabilization to differentiate extracellular (red + green) and intracellular (green only) STm. Scale bar: 10 μm (six samples were examined over two independent experiments for each condition). h Quantification of gentamicin-protected intracellular STmAux ΔinvC (black open circles, n = 6 wells examined over two independent experiments), STmΔinvC (black filled circles, n = 6 wells examined over two independent experiments), STmAux (red open triangles, n = 6 wells examined over two independent experiments), and STmΔinvC (red filled triangles, n = 6 wells examined over two independent experiments) in HeLa cells 2 h after infection. Statistics: bars indicate mean (c, f, h) or median (d) values. Horizontal dotted lines indicate the lower limit of detection (b, c, f, h). Panel c was analyzed with ordinary one-way ANOVA and Tukey’s test for multiple comparison. Panel d was analyzed with a two-sided multicomparison Kruskal-Wallis test and Dunn’s post hoc test. Panel f was analyzed with unpaired two-tailed t-test for each day. Panel h was analyzed with two-way ANOVA (vivulence and auxotrophy as the two factors) and Sidak multiple comparison correction. Source data are provided as a Source Data file. Detailed statistical metrics are available in the Supplementary Statistical Analysis file.

Optimal protective efficacy of STmAux is viability dependent. We next addressed how relevant STmAux viability is for the induction of functional intestinal immunity. The replication incompetency of live STmAux in germ-free mice allowed us to quantitatively compare the functional effects of mucosal exposure to live versus killed STm: both live and killed STmAux cells are sterile entities in germ-free mice. Parallel groups of germ-free mice were intestinally conditioned by gavage with STmAux inocula administered either live or following inactivation by peracetic acid (PAA) treatment. PAA killing is highly effective and has been shown to preserve mucosally protective STm surface B cell epitopes26,27. Naïve germ-free animals served as negative controls. Four weeks after the first treatment, the germ-free animals of all three groups were challenged orally with virulent wild-type STm and studied at days 1 and 4 after the challenge, respectively (Fig. 3a). Compared to live STmAux-conditioned mice, PAA-killed STmAux-induced STm-specific IgA titers were reduced at day 1 of challenge (Fig. 3b, Supplementary Fig. 5). Yet, by day 4 this difference was no longer apparent. However, while pretreatment with either PAA killed or live STmAux were similarly protective against early wild-type STm-induced mucosal inflammation at day 1 after challenge, only live STmAux preconditioning provided effective protection from intestinal pathology and organ infection until day 4 (Fig. 3c–g). Notably, live STmAux-induced immunity not merely delayed the onset of disease, but protected the germ-free mice from lethal STm infection. Live STmAux-conditioned germ-free mice that were followed up for 3 weeks following challenge remained free of macroscopic evidence of severe infection and were recovering at the time of sacrifice (Supplementary Fig. 6). None of these effects were explained by differences in fecal or cecal luminal colonization levels of the challenge strain, which were similar across all experimental groups (Fig. 3h, i, Supplementary Fig. 6b).

**Salmonella** type 3 secretion signifies robust immunogenicity. We next asked whether or not the viability-dependency of functional mucosal immunogenicity of STmAux is virulence factor related. We hypothesized that host interaction through *Salmonella* T3SSs (whose function is energy and viability dependent) signifies the functional immunogenicity of live STmAux. If this was true, T3SS deficiency would diminish the mucosal efficacy of live STmAux.

We tested this hypothesis by comparing the protective effect of the enteral conditioning of germ-free mice with matching doses of live T3SS-competent and isogenic T3SS-deficient mutant strains of STmAux (STmAux T3SS−). Two different isogenic STmAux T3SS− mutants were tested: a complete SPII and SPI2 genomic island deletion mutant (ΔSPII ΔSPIII) devoid of T3SS genes entirely28, and a ΔinvC ΔssaV mutant expressing defective T3SSs29,30. Mice treated with equivalent doses of PAA-killed STmAux or naïve mice served as controls. Four weeks after the first treatment, the germ-free animals were enterally challenged with wild-type STm, and studied at days 1 and 3 after challenge (Fig. 4a). Analysis of the severity of challenge infection and mucosal pathology at day 3 revealed that live, T3SS-deficient STmAux strains induced less robust functional protective immunity than T3SS-competent STmAux, and their efficacy against intestinal mucosal pathology (Fig. 4b, c) and bacterial penetration to mLN (Fig. 4d) was no longer significantly better than that of PAA-killed STmAux. Genetic deletion of the three most important SPI1 effector protein genes (sopE, sopE2, and sipA) required for early SPI1 T3SS-mediated intestinal STm pathogenesis31 also resulted in reduced efficacy (Supplementary Fig. 7). This suggests that not merely immune recognition of a functional T3SS apparatus but rather mucosal pathogenesis-related type 3 effector protein functions are driving the superior immunogenicity of the T3SS-competent STmAux strain.

As in the previous experiment (Fig. 3b) killed STmAux− as well as STmAux T3SS−–preconditioned mice displayed reduced STm-specific IgA titers at day 1 of challenge (Fig. 4e, Supplementary Fig. 8). Yet, by day 3 mice of all three treatment groups had similar intestinal IgA titers. Immunoglobulin repertoire sequencing analysis of small intestine and mLN revealed overlapping IgA repertoires following mucosal conditioning with live T3SS-competent STmAux− versus T3SS-incompetent STmAux that clustered separately from those of naïve germ-free control mice.

in Supplementary Fig. 4A–G). Thus, STmAux allowed us to probe mucosal immunity in a strictly dose-dependent manner. This data showed the threshold effects of STmAux conditioning in germ-free mice, which would not be achievable with conventional non-auxotrophic bacteria that would exponentially expand rapidly to reach high intestinal densities independently of inoculum size.

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Supplementary Fig. 9). Repertoire overlap was measured by calculating the geometric mean of relative overlap frequencies between CDR3 amino acid sequence usage (see Methods section). Preprocessed clonotype amino acid sequences and metadata description are available as supplementary data files (Supplementary Data 1–21).

O-serotype specific IgA has been shown previously to be a necessary component of any intestinal immune protection induced by killed or live STm12,26,27. O-antigen is a dominant polysaccharide antigen and in binding assays tends to mask other surface epitopes from antibody recognition, which is the basis of O-serotyping. To specifically study O-serotype-independent Salmonella surface binding IgA, germ-free mice were pre-conditioned with STmAux but challenged with the different Salmonella serotype Enteritidis (SEn) (Supplementary Fig. 10A). The resulting intestinal IgA had reduced surface reactivity towards O-antigen-deficient (rough) STm compared to wild-type (smooth) STm, as expected (Supplementary Fig. 10B, C). However, the non-O-antigen-specific IgA cross-reacted between rough STm and rough SEn (Supplementary Fig. 10B, C). It also cross-reacted with smooth wild-type SEn, suggesting that it contributes to serotype-independent Salmonella surface reactivity (Supplementary Fig. 10B, C). Although the O-serotype-independent IgA component alone is insufficient12,26,27, it may complement...
O-antigen-specific IgA in protective mucosal immunity. Supporting this idea, we found that, although both killed and T3SS-deficient STmAux preconditioning at day 3 of challenge resulted in robust IgA titers towards smooth STm (Fig. 4e, panel Day 3), IgA binding to rough STm was significantly reduced (Fig. 4f).

These data show that Salmonella T3SS-dependent virulence functions signify the mucosal immunogenic efficacy of live STmAux in absence of inflammation. The underlying T3SS-dependent IgA B cell response is characterized by a less O-antigen-restricted bacterial surface reactivity.
PRR signaling redundancy in induction of immunity. In innate pathogen recognition through PRRs is critical in the defense against primary STm infection. MYD88 knockout mice lacking TLR and IL1R family downstream signaling are consequently severely impaired in innate immunity against mucosal STm infection. Canonical Caspase-1-dependent and non-canonical Caspase-11 (Caspase-4 in humans)-dependent inflammasome activation have also been implicated in innate immune control of STm infection. The NLRC4 inflammasome is activated by the SPI1 T3SS needle complex proteins and therefore may mediate innate recognition of T3SS-competent intestinal STm specifically. Moreover, the NOD1/NOD2 nodosome has been reported to respond to bacterial pathogenicity by sensing the cytoplasmic activities of Salmonella SPI1 T3SS-1 effector proteins.

Are these also factors individually important for the induction of functional adaptive immunity in the absence of an inflammatory response? To address this we tested the hypothesis that deficiencies for innate recognition pathways critical in innate immune defense also affect induction of functional adaptive immunity by live STmAux. Mice deficient in (i) TLR/IL1R family adaptor proteins MYD88 and TRIF, (ii) Caspase-1 and Caspase-11, (iii) NLRC4, and (iv) NOD1 and NOD2 were derived germ free and compared with innate immunocompetent control mice for their adaptive immune responses towards live STmAux.

Using STmAux avoids bacterial overgrowth of severely innate immunodeficient hosts that lack control and containment of intestinal microbes, leading to increased mucosal penetration also of attenuated, avirulent, and commensal bacteria. In MYD88/TRIF double-deficient mice this has been shown to result in abnormally high systemic exposure to gut commensals and consequently compensatory B cell immunity. Involvement of redundant innate signaling pathways triggered by massively increased microbial loads has been postulated to be responsible but has not been characterized further. The STmAux model in germ-free mice, however, uniquely fixes the bacterial load per animal and consequently avoids bacterial overgrowth to skew immune activation.

First, germ-free MYD88/TRIF double-deficient and wild-type control mice were enterally conditioned with live STmAux. Four weeks after the first treatment all mice were challenged orally with wild-type STm harboring an intracellular inducible GFP reporter plasmid (pM973) and studied at day 3 after the challenge (Fig. 5a). Control groups of both genotypes were challenged but without STmAux preconditioning. Quantification of cecal mucosal (Fig. 5b, Supplementary Fig. 11A), mLNs, liver, and spleen (Fig. 5c) burdens of STm[pM973] revealed that the induction of functional immunity by live STmAux was robust even in the highly susceptible MYD88/TRIF double-deficient mice. At day 3 post challenge, bacterial loads in mLNs, livers, and spleens of STmAux-treated MYD88/TRIF-deficient mice were similar to, and in cecal mucosal tissues even lower than, those in the STmAux-treated wild-type animals. In accordance with this relatively greater effect of STmAux treatment in mutant than wild-type mice, a two-way ANOVA revealed a significant interaction between the effects of genotype and STmAux treatment ($p < 0.0001$ for all panels; for detailed statistical metrics see Statistical Analysis file available as Supplementary Information). Quantitation of cecal luminal lipocalin-2 and histopathology scores both confirmed the protective effects of STmAux treatment within each mouse genotype (Supplementary Fig. 11B, C), although these two readouts themselves are MYD88/TRIF-dependent and should therefore be compared between both mouse genotypes with caution.

We next tested the ability of live STmAux to induce adaptive immunity in NOD1/NOD2-double-knockout mice (Fig. 6a–c), NLRC4-deficient mice, and Caspase-1/11 double-deficient mice (Fig. 6d–f), all of which at day 3 of challenge were found to have no deficiency in mounting functional mucosal immunity towards live STmAux conditioning (two-way ANOVA, for detailed statistical metrics see Statistical Analysis file available in the Supplementary Information).

These results show that MYD88/TRIF, Caspase-1/Caspase-11 inflammasome, and NOD1/NOD2 nodosome signaling were individually redundant for the induction of adaptive immunity by live STmAux in the absence of inflammation. Their role in complementing adaptive immunity in pathogen clearance at later stages of secondary infection is likely functionally important, although not apparent at day 3 of challenge.

Microbiota-dependent colonization and niche competition. So far, the fully reversible germ-free mouse model uniquely had allowed the quantitative study of the immunogeneity of different phenotypes of STmAux in a very clean system. However, in real-life situations STmAux would interact also with the indigenous gut microbiota, which we hypothesized to provide crossfeeding of the required cell wall metabolites in vivo. This may delay STmAux intestinal luminal clearance in colonized mice. We tested this hypothesis using a well-established gnotobiotic mouse model that is stably colonized with 12 representative murine intestinal taxa [stable defined moderately diverse mouse microbiota (sDMDMm)] all of which are fully sequenced and openly available as pure cultures from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ)36,37. The sDMDMm model has proven merit for the study of intestinal STm infection and its interaction with the commensal microbiota without the need for harsh antibiotic treatments, and shows relevant phenotypic effects such as limiting the colonization of STm38.
Following a single inoculation with $10^7$ STmAux by gavage, sDMDMm mice showed efficient and stable colonization of STmAux, reaching luminal densities similar to those of isogenic non- auxotrophic strains, including partly attenuated SPI2 TTSS-deficient (AssaV) and avirulent SPI1/SPI2 double-deficient ($\Delta invC \Delta saaV$) STm (Fig. 7a). STmAux did not revert to lose its auxotrophic phenotype during these experiments (no recovery of STm growth from ex vivo intestinal samples in non-supplemented control medium). Even STmAux re-isolated from an sDMDMm mouse after 8 months colonized germ-free mice fully reversibly. Following gavage of $10^{10}$ CFU of either the 8-month re-isolate or the original lab strain, all mice ($n = 5$...
per group) had recovered to germ-free status at day 2 post inoculation. Despite efficient luminal colonization and evidence for epithelial invasion of STmAux (Fig. 7e), neither deep mucosal penetration to mLN and systemic organs nor mucosal pathology were evident in either wild type (Fig. 7b–d) or MYD88/TRIF-deficient (Fig. 7f) sDMDMm mice. Thus, while crossfeeding by sDMDMm organisms can rescue gut luminal colonization, it was insufficient to recover pathogenicity of STmAux, which is consistent with the local intestinal luminal confinement of the crossfeeding microbiota and the activity of d-amino acid degrading enzymes in host tissues and intestinal mucus.

Nevertheless, induction of STm-specific IgA was seen after 4 weeks of colonization, which, in contrast to colonization efficiency, was dependent on SPI1 T3SS competence (Fig. 7g, Supplementary Fig. 12). As an added host benefit, stably colonizing STmAux further provided robust niche competition to a subsequent oral challenge by wild-type STm (Fig. 7h blue symbols, and Supplementary Fig. 13B). Notably, pre-colonization with SPI1 T3SS-incompetent STmAuxDmC provided only partial niche competition (Fig. 7h, black symbols, Supplementary Fig. 13B). SPI1/SP12 double-deficient STmAuxΔmC showed the exact same phenotype (Fig. 7h, green symbols, Supplementary Fig. 13B), supporting the conclusion that SPI1 T3SS-dependent virulence factors are mainly responsible. In RAG knockout mice also T3SS-competent STmAux showed inefficient intestinal niche competition (Supplementary Fig. 14A–D). These findings are consistent with the interpretation that STmAux-induced host

**Fig. 5** Mucosal induction of adaptive immunity by live STmAux is robust in MYD88/TRIF double-deficient mice. **a** Germ-free MYD88+/−TRIF+/+/ps mice (open symbols) and wild-type control mice (filled symbols) were enterally conditioned with three doses of 10¹⁰ CFU of live STmAux (red triangles, n = 7 MYD88/TRIF KO animals and n = 9 wild-type animals examined over two independent experiments) or left untreated as controls (gray circles, n = 6 MYD88/TRIF KO animals and n = 10 wild-type animals examined over two independent experiments). Twenty-seven days after the first treatment (day 0) mice were challenged with wild-type STm (10³ CFU) harboring ssag::eGFP reporter plasmid m973. The mice were studied at day 3 after challenge. Each symbol represents one individual. **b** Quantification of intracellular wild-type STm expressing eGFP in the cecal mucosa. **c** Bacterial burden of wild-type STm recoverable from mLN, spleen, and liver at day 3 after challenge. Statistics: bars indicate mean (b, c). Horizontal, dotted lines indicate the lower detection limit (b, c). Panels **b** and **c** were analyzed with two-way ANOVA (host genotype and treatment as factors) and Sidak multiple comparison correction. The data were pooled from two independent experiments. Source data are provided as a Source Data file. Detailed statistical metrics are available in the Supplementary Statistical Analysis file.
immunity synergizes with niche competition by STmAux in protection against wild-type STm challenge. Measurements of cecal luminal lipocalin-2 and challenge bacterial burden in mLN, liver, and spleen at day 4 of wild-type STm challenge support this conclusion (Fig. 7i, j, Supplementary Fig. 14D). In a second context, streptomycin pre-treated conventional mice, a widely used mouse model for nontyphoid invasive salmonellosis9,24,40, were also permissive for extended gut luminal colonization of STmAux (Supplementary Fig. 13C).

These data show that in the colonized mouse model, microbiota-syntrophic STmAux more closely mimics the natural pathogen in terms of intestinal luminal colonization and virulence factor-driven translocation of wild type STm to organs.
induction of IgA immunity. Thus, independently of germ-free conditions, also stable intestinal STmAux colonization allows uncoupling of intestinal immunogenicity from pathogenicity, with the added benefit of luminal pathogen niche competition.

Discussion

Fully attenuated or inactivated pathogens have long been noted to be poorly protective mucosal immunogens compared to more virulent strains. This has been attributed mainly to the capacity of virulent pathogens to induce more vigorous innate immune responses and to penetrate into and overwhelm inductive sites of the mucosal immune system. Here we show specifically that invasive Salmonella cells expressing live type 3 secretion systems are recognized by the immunogenic response, independently of their propensity to deeply penetrate and replicate as live organisms inside host tissues or the induction of a marked mucosal inflammatory response. These data suggest that the mucosal immune system reacts not only to a damaging infection but can also recognize stereotypical activities of pathogens more directly, and thus potentially more sensitively and rapidly. Consequently, a small number of highly transient mucosal exposures with virulence factor-competent STmAux robustly induce highly effective immunity in germ-free mice, in the absence of an inflammatory response. The underlying B cell response induced by live, virulence factor-competent STmAux is characterized by the production of intestinal IgA with increased O-serotype-independent Salmonella surface reactivity. Additional future work will be required to address which effector T cell activities may additionally contribute to STmAux-induced immunity.

It has been described previously that bacterial viability itself is an important determinant of bacterial immunogenicity, independent of pathogenicity and replication competence. Live pathogenic bacteria are more immunogenic live than killed when administered parenterally. This difference was revealed to be mediated by innate immune recognition of bacterial messenger RNA (mRNA), highly unstable, hence normally viability-associated, molecules. The underlying sensing pathway for bacterial mRNA was shown to be dependent on TLR8 and TRIF in humans, and on TRIF, Caspase-1 and Caspase-11 in mice. Here we observed only a minor difference in intestinal mucosal immunogenicity between avirulent live and killed STmAux (see Fig. 4), which may be mediated by the same mechanism. Virulence factor-competent invasive STmAux, however, was much more efficacious. Its epithelial invasiveness may increase subepithelial live antigen delivery and consequently prime immunity more efficiently by delivering live bacteria into the tissues. However, its immunogenicity was robust even in MYD88/TRIF−/− deficient mice, and thus may not be fully explained by the same live bacterial sensing pathway. Our data confirm and extend previous findings of functional redundancy between innate and adaptive immune responses in the control of intestinal commensal bacteria and the efficacy of established model vaccines with adjuvant in MYD88/TRIF double-deficient mice. Here we show that this extends also to intestinal pathogenic bacteria. The remarkable robustness of this system may represent an evolutionary adaptation to pathogens that evade or alter the innate immune defense.

Long-established live STm vaccine strains like SPI2 T3SS-deficient and aromatic amino acid auxotrophic aro mutants of STm also are effective mucosal immunogens, but are not fully growth deficient in host tissues and consequently considered dangerous for HIV positive and other immunocompromised individuals (reviewed in ref. 14). This has so far ruled out approval for human application. On the other hand, peptidoglycan metabolite auxotropic STm strains similar to the one we presented in this paper have been developed previously but in this form have been considered insufficiently immunogenic because of their poor mucosal penetration. This conclusion is however predicated on the preclinical study mainly in conventional rodent models that are (like humans) intestinally colonization resistant against Salmonella. In this context, when STmAux proliferation in the intestinal lumen is inhibited by the competing microbiota, its colonization dynamics would be expected to be more similar to the germ-free mouse model, and it may consequently require very high oral doses (as we saw in germ-free mice) to be efficacious. Instead, the field has moved into the direction of developing more sophisticated strains that display regulated delayed in vivo attenuation/lethality phenotypes, allowing for transient survival, replication, and tissue invasion in vivo. These highly innovative approaches are inherently more difficult to combine with safety parameters matching those of the constitutively d-Ala/Dap auxotrophic strain. The presented experiments in non-colonization-resistant mouse models highlight yet another possible strategy. The remarkably efficient gut luminal microbiota-syntrophy permitted extended mucosal stimulation with live virulence factor-competent STmAux, without compromising the strain’s deficiency in causing pathology and systemic infection. This phenotype could potentially be exploited further by metabolic engineering of STmAux strains to gain intestinal colonization efficiency, or by temporal reduction of colonization resistance in the host at the time of treatment (preferably other than by antibiotic treatment). However, given that our conclusions so far are based onmouse models that have laboratory levels of microbiota complexity, additional work in more relevant preclinical models will be necessary to assess potential translatable of these findings for veterinary or human medical applications.
Methods

**Bacterial strains, plasmids, and culture media.** The bacterial strains and plasmids used in this study are listed in Table 1. Auxotrophic strain HA135 (STmΔmet, UK-1 background) \(\Delta\)metC::TetRA \(\Delta\)alr \(\Delta\)dadX \(\Delta\)asd was generated from strain \(\chi\)9052 \(\Delta\)alr3 \(\Delta\)dadB4 \(\Delta\)asdA33 by replacing the coding region of metC with a TetRA resistance cassette by Lambda Red recombineering using recombineering plasmid pSIM5 (ref.50) as described in ref.51. Isogenic mutant alleles \(\Delta\)invC::aphT, \(\Delta\)invC::aphT \(\Delta\)ssaV::cat, and \(\Delta\)invC::aphT \(\Delta\)invH::cat \(\Delta\)ssaG-ssaU::aphT were transferred into the STm and STmΔmet backgrounds by phage P22-mediated transduction using the donor strains M736, M73831 and \(\chi\)9650 (ref. 28), respectively, as described52. Auxotrophic strain HA623 (SL1344 background) \(\Delta\)metC \(\Delta\)alrN \(\Delta\)alrP \(\Delta\)asd was generated from strain SL1344 (SB300) by in-frame deletion of each gene. This was achieved by generation of four single deletion mutants in SL1344 using the plasmid pSIM6 encoded Lambda Red recombinase system50 for allelic exchange of the coding sequence (leaving the stop codon) with a Tet selectible tetA-sacB cassette, followed by four sequential rounds of P22

![Image](https://example.com/image1.png)

**Fecal densities of indicated strain**

- **a** Fecal densities of indicated strain
- **b** Lipocalin-2 in cecal content
- **c** STm<sup>+</sup>, day 4
- **d** Translocation of indicated strain to organs
- **e** Translocation of indicated strain to organs
- **f** Translocation of indicated strain to organs
- **g** IgA level against wild type STm
- **h** Fecal densities of precolonizer and challenge strain
- **i** Lipocalin-2 in cecal contents
- **j** Translocation of wild type STm

Transgenic mice were injected via tail vein with 5 × 10<sup>8</sup> overnight grown bacteria and sacrificed by CO<sub>2</sub> asphyxiation at 1 and 3 days post-injection. Feces were collected to determine bacterial density (CFU/g) and cecal contents were analyzed by ELISA for lipocalin-2 levels (ng/g). Translocation to various organs was followed by CFU determination (CFU/g). IgA levels were determined by ELISA against wild type STm. Data is presented as mean ± SEM. Plots show statistical significance relative to control by two-tailed Student’s t-test.

**Transgenic mice** were injected via tail vein with 5 × 10<sup>8</sup> overnight grown bacteria and sacrificed by CO<sub>2</sub> asphyxiation at 1 and 3 days post-injection. Feces were collected to determine bacterial density (CFU/g) and cecal contents were analyzed by ELISA for lipocalin-2 levels (ng/g). Translocation to various organs was followed by CFU determination (CFU/g). IgA levels were determined by ELISA against wild type STm. Data is presented as mean ± SEM. Plots show statistical significance relative to control by two-tailed Student’s t-test.
Fig. 7 Efficient colonization and immune induction by auxotrophic STm in microbiota-associated mice. a sDMDMm mice were gavaged with a single dose of 10^7 CFU of either STm^aux^ (blue open circles, n = 21), STm^T3SS^- (green squares, n = 9), STm^asav^ (black triangles, n = 5), or wild-type STm (red triangles, n = 6). Time course of viable bacteria of each strain recoverable from feces. b Lipocalin-2 concentration in cecal contents at day 2, 4, and 28 after inoculation with the indicated STm strains. Pictures show representative H&E stainings of ceca at day 4 post inoculation with either STm^WT^ or STm^aux^. c Representative cecal histology on day 4 after colonization with STm^aux^, H&E staining, scale bar 100 µm. d Bacterial burden of indicated STm strains recoverable from mLn, spleen, and liver at day 2, 4, and 28 after initial colonization. e Confocal immunofluorescence microscopy of cecum tissue showing an epithelial cell invaded by STm^aux^, Green, STm^asav^ harboring ssag::eGFP reporter plasmid pM973; blue, DNA (DAPI); gray, F-actin (phalloidin). Dotted yellow lines outline the border of the epithelium facing the intestinal lumen (Lu) and lamina propria (Lp), respectively. Scale bar = 10 µm (n = 6 animals). f Bacterial burden of indicated STm strains recoverable from mLn, liver, and spleen at day 3 post inoculation in MYDB8/TRIF double KO mice (n = 3 animals per treatment group). g Wild-type STm-specific titer of intestinal IgA at day 24 post inoculation of sDMDMm mice with either STm^aux^ (blue open circles, n = 3 animals) or STm^aux^ T3SS^- (green open squares, n = 5 animals). h sDMDMm mice were inoculated by gavage with a single dose of 10^7 CFU of either STm^aux^ (blue open circles, n = 5 animals), STm^aux^ T3SS^- (green open squares, n = 3 animals), STm^asav^ (black open triangles, n = 4 animals), or left untreated (n = 5 animals), and at day 28 challenged with 10^7 CFU wild-type STm (red open triangles). Mice were studied 4 days after challenge (day 32). I Lipocalin-2 concentration in cecal contents quantified at day 32 (4 days after challenge). J Bacterial burden of wild-type STm recoverable from mLn, liver, and spleen at day 32. Each symbol represents on individual. Statistics: connecting lines connect means (a, f), bars indicate mean (b–e, g, h).

Table 1 Bacterial strains and plasmids.

| Strain (acronym)/plasmid | Genetic background | Relevant genotype | Known resistances | Comments | Origin or reference |
|--------------------------|--------------------|-------------------|-------------------|----------|-------------------|
| x4138 (STm) x4650 (STm^aspi^ ΔSPI2) | UK-1 UK-1 | Wild-type strain ΔavrA-invH::cat Δ(ssoG-ssoU::aphT) | Nal | Virulent wild-type control | 28 |
| 9052 M736 | ATCC 14028s derivative | ΔavrA ΔoabBΔ ΔasidA33 ΔinvC::aphT | Nal, Kan | 68 |
| HA135 (STm^aux^) | UK-1, 9052 | ΔmetC::tetRA ΔavrA ΔoabBΔ ΔasidA33 ΔinvC::aphT | Tet | This study |
| HA618 (STm^aux^ ΔinvC::aphT) | UK-1, STm^aux^ | ΔmetC::tetRA ΔavrA ΔoabBΔ ΔasidA33 ΔinvC::aphT | Tet, Kan | This study |
| HA218 (STm^aux^ ΔinvC ΔssoV::cat) | UK-1, STm^aux^ | ΔmetC::tetRA ΔavrA ΔoabBΔ ΔasidA33 ΔssoV::cat ΔssoG-ssoU::aphT | Tet, Kan, Cam | This study |
| HA208 (STm^aux^ ΔSPI2) | UK-1 | ΔmetC::tetRA ΔavrA ΔoabBΔ ΔasidA33 ΔssoV::cat ΔssoG-ssoU::aphT | Tet, Kan, Cam | This study |
| pH7937 | pWKS30 (ref. 70) | | | | |
| SB300 (STm) | SL1344 | Wild-type strain ΔmetC ΔoabN ΔoabP Δasd | Amp | eGFP under control of the ssoG promoter | 17 |
| HA623 | SL1344 | ΔmetC ΔoabN ΔoabP Δasd | Str | | |
| HA630 (STm^aux^) | SL1344, HA300 | ΔmetC ΔoabN ΔoabP Δasd::tetRA | Str, Tet | | |
| HA705 (STm^T3SS^-) | SL1344, SB300 | ΔinvC::aphT ΔssoV::cat | Str, Kan, Cam | Used in Fig. 7 and corresponding Supplementary figures | |
| HA706 (STm^asav^) | SL1344, SB300 | ΔinvC::aphT ΔssoV::cat | Str, Cam | Used in Fig. 7 | |
| HA702 (STm^aux^ T3SS^-) | SL1344, SB300 | ΔinvC::aphT ΔssoV::cat | Str, tet | Used in Fig. 7 | |
| HA700 (STm^aux^ ΔinvC) | SL1344, SB300 | ΔmetC ΔoabN ΔoabP Δasd::tetRA | Kan, Cam | Used in Fig. 7 | |
| HA727 (STm^aux^ ΔTriple-Eff) | SL1344, SB300 | ΔinvC::aphT ΔssoV::cat ΔssoG-ssoU::aphT ΔspaP::aphT ΔspaE::pM218 | Str, Tet, Kan, Cam | Used in Fig. S7 | Protrophic derivative of HA727 | This study |
| HA733 (STm^ΔTriple-Eff) | SL1344, SB300 | ΔmetC ΔoabN ΔoabP Δasd::tetRA ΔinvC::aphT ΔssoG-ssoU::aphT ΔssoP::pM218 ΔspaP::aphT | Str, Tet, Kan, Cam | | This study |
| SK112 (rough STm) | SL1344, SB300 | ΔmetC ΔoabN ΔoabP Δasd::tetRA ΔinvC::aphT ΔssoG-ssoU::aphT ΔssoP::pM218 ΔspaP::aphT | Str, Tet, Kan, Cam | | This study |
| M1525 (Sen) | S. Enteritidis 125109 | Wild-type strain Δf55 | | | |
| M1627 (rough Sen) | S. Enteritidis 125109 | | | | |

transduction followed by Lambda Red recombination mediated removal of the tetRA-sacB cassette by counterselection as described, leading to quadruple deletion mutant HA623. HA630 was generated by Lambda Red recombination of a tetRA resistance cassette into the asid deletion site of HA623. The mutagenesis primers used are listed in Table 2. Auxotrophic SPI1 effector gene sopE sopE2 sipA triple mutant H727 was constructed in parent strain SL1344 as described previously. The reported avirulence phenotype of the sopE sopE2 sipA6 mutation was confirmed by gentamicin protection assay (see Fig. S7D) and by P22 transduction of the wild-type alleles of asid and oabN to recover prototrophy in resultant strain HA733, which was then confirmed to be of strongly reduced intestinal virulence in germ-free mice (histopathological score at day 2 of infection = 2.5 ± 0.5 [mean ± range; n = 2]; wild-type control = 11).

Luria-Bertani (LB) medium (Sigma-Aldrich) was used as standard bacterial culture medium. Ampicillin (Sigma; 100 µg/mL), tetracycline (Sigma; 12.5 µg/mL), kanamycin (Sigma; 50 µg/mL), chloramphenicol (Sigma; 6 µg/mL), nalidixic acid (Sigma; 30 µg/mL), meso-diaminopimelic acid (m-Dap; Sigma, 50 µg/mL), and/or D-alanine (D-Ala; Sigma; 200 µg/mL) were added to the medium as appropriate.

Cellular invasion assays. HeLa (Kyoto) cells were seeded into 24-well dishes and were grown for 1 day until 80% confluence was obtained. HeLa cells were cultured
Table 2 Primers used for bacterial mutagenesis.

| Primer name | 5’—3’ sequence | Additional information | Applicable background |
|-------------|-----------------|-----------------------|----------------------|
| STM-metC-TetRA-F | TTGGCAAAATTTCATGTCATACGAGTCGCAGCGGAGGGTCCA | Allelic exchange of metC with TetRA | UK-1 |
| STM-metC-tatRA-R | GAACAGCTGAGCGGTTATCAGGTTATAGGTCAGGGGCCGA | Allelic exchange of metC with TetRA | UK-1 |
| STM-metC-cntr-F | AACCCACACTACACAGTGCCTGACCCTCCATC | metC control primer | UK-1 |
| STM-metC-cntr-R | CTGGTATTCTCTGTTATCGAGGTTATAGGTCAGGGGCCGA | metC control primer | UK-1 |
| STM-asd-TetA-SacB-F | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Allelic exchange of asd with TetA-SacB | SL344 |
| STM-asd-TetA-SacB-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Allelic exchange of asd with TetA-SacB | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |
| STM-asd-rmlv-R | GCGGATACGCGGGAGGCAGGTTATCAGGTTATAGGTCAGGGGCCGA | Removal of TetA-SacB cassette | SL344 |

in Dulbecco modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated at 37 °C under an atmosphere containing 5% CO2. Shortly before Stm infection, the adherent cells were incubated in Hanks’ buffered salt (HBSS) medium. Stm strains were inoculated from a single colony in 10 mL of LB medium. Bacteria were grown overnight at 25 °C with shaking. The next day, the bacterial suspension was diluted 1:20 into 40 mL fresh medium and incubated at the same conditions for 5 h. Grown cultures were centrifuged at 3,000 g for 10 min and used to infect the HeLa cells.

Animal experiments. Animal experiments were performed in accordance to animal experiment licenses (BE94/11, BE91/14, BE36/15, BE85/17) approved by the Bernese Cantonal Ethical committee for animal experiments and carried out in accordance with Swiss Federal law for animal experimentation.

Mice were maintained under axenic barrier conditions at the Clean Mouse Facility of the University of Bern. The housing conditions in the whole facility are strictly controlled. The ambient temperature is 23–25 °C and the relative humidity is 52–60%. All mice received at all times standard diet (Kliba 3307) and water ad libitum. The genetic background of all mice used was C57BL/6. MYD88−/− TRIFlps/lps mice were provided by Bruce A. Beutler, The Scripps Research Institute, CA, USA and maintained germ free in the Clean Mouse Facility. Mice were purchased from MGI (B6N.129S2-Casp1tm1Flv Casp4del/J58) were purchased from Jackson Immunoresearch, diluted in blocking buffer. Subsequently, cells were permeabilized with 0.5% Triton-X 100 in PBS, incubated again in blocking buffer, and stained again with rabbit-anti Stm O-antigen group B antiserum (Becton Dickinson) diluted in blocking buffer, washed twice in blocking buffer, and incubated in goat anti-rabbit CY3 (Jackson Immunoresearch) diluted in blocking buffer. Subsequently, cells were permeabilized with 0.5% Triton-X 100 in PBS, incubated again in blocking buffer, and stained again with rabbit-anti Stm O-antigen group B antiserum (Becton Dickinson) diluted in blocking buffer. Subsequently, cells were permeabilized with 0.5% Triton-X 100 in PBS, incubated again in blocking buffer, and stained again with rabbit-anti Stm O-antigen group B antiserum (Becton Dickinson).
The Jackson Laboratory in the form of cryopreserved embryos and transferred into germ-free recipients in the Clean Mouse Facility, University of Bern. Germ-free and sDMDMm wild-type C57BL/6 mice for 4 weeks. Gnotobiotic sDMDMm mice have been generated at the Clean Mouse Facility, University of Bern. Mice were infected with 200 μl of the murine intestinal bacterial consortium Oligo-MM12 (ref.38) and challenged with cold PBS, and resuspended to the appropriate density. For peracetic acid treatment, bacterial loads were quantified by plating on LB agar. Where necessary, β-Ala (200 μg/mL) and m-Dap (50 μg/mL) were added.

Infection and colonization experiments were performed under strict aseptic conditions. Mice were derived and maintained germ free in flexible film isolators (including the duration of transient atoxic bacterial conditioning) or autoclaved Sealsafe-plus IV cages (Tecniplast, Italy; during STm challenge and short-term infections) at the Clean Mouse Facility (CMF) of the University of Bern. Animals were provided with autoclaved mouse chow (Kliba 3307) and water ad libitum. Germ-free status of all animals was routinely monitored using culture-based methods including the Clean Mouse Facility, DKF University of Bern. Mice were infected with 200 μl STm suspension.

Bacteria for enteral inoculation were grown under SPI1-inducing conditions. A taxonomic STm were inoculated into 10 ml β-Ala (200 μg/mL) and m-Dap (50 μg/mL) supplemented LB containing 0.3 M NaCl and incubated shaking at 150 rpm, at 37 °C for 16 h. The resulting bacterial cultures were diluted 108-fold in 500 mL fresh medium and incubated under the same conditions for 15 more hours. STm were harvested by centrifugation (15 min, 4816 × g, 4 °C), washed twice with cold PBS, and resuspended to the appropriate densities. For peracetic acid (PAA) inactivation, an aliquot of autotrophic STm was resuspended in 10 ml 1% peracetic acid for 1 h at room temperature. The inactivated STm suspension was washed with PBS and resuspended to the appropriate densities. Sterility of PAA-killed inocula was confirmed by standard culture methods. Wild-type STm cultures were inoculated from a single colony in 10 ml 0.3 M sodium-chloride/LB and incubated at 150 rpm, at 37 °C for 16 h. Wild-type STm cultures were diluted 1:20 into 40 ml fresh medium and incubated at the same conditions for 5 h.

Bacterial loads in organs and feces. Organs, feces, and cecum content were aseptically collected. Organs were homogenized in 0.5 ml 0.5% tergitol/PBS, feces and cecum content in 0.5 ml PBS using a tissue lyzer (TissueLyzer LT, Qiagen, 5 min, with a stainless steel bead). Bacterial loads were quantified by plating on LB agar. Where necessary, β-Ala (200 μg/mL) and m-Dap (50 μg/mL) were added.

Isolation of intestinal secretory IgA. Intestinal IgA lavages were collected by rinsing the small intestine with 5 ml of 1% soybean-trypsin inhibitor/0.05 M EDTA/PBS. The intestinal lavages were spun at 4816 × g, 20 min, 4 °C. The supernatant was sterile-filtered (0.22 μm cut-off size) to remove bacteria-sized particles and stored long-term in aliquots frozen at −20 °C.

*Immunoglobulin repertoire sequencing. Germ-free C57BL/6 mice were orally administrated with 3 × 1010 STmAux or STmAux ΔinvC ΔssaV (three times at 7-day intervals). Naive germ-free mice served as naive controls. Twenty-eight days post last administration, ileum and MLN were dissected and snap-frozen in Trizol reagent (Life Technologies) using liquid nitrogen. Thawed tissues were homogenized (Retsch bead-beater) in 1 mL of Trizol reagent. Two hundred microliters chloroform was added to samples and centrifuged (12,000 × g, 15 min, 4°C), was performed. The upper phase containing RNA was collected, and RNA was precipitated with ice-cold isopropanol. After washing once with 75% ethanol, RNA pellet was dried and resuspended in RNase-free water. Nanodrop2000 (Thermo Scientific) was used to quantify RNA concentrations and purity.

To prepare IgA amplicons, cDNA was synthesized by mixing 700 ng of RNA, 1 μl of 2 μM gene specific primer mix (as previously described63, 1 μl of 10 mM dNTP (containing dATP, dCTP, dGTP, and dTTP, and dTTP at a final concentration of 10 mM, Invitrogen) and topped with dH2O to 13 μl. Samples were heated to 65 °C for 5 min, and then cooled for 1 min on ice. Four microliters 5X first strand buffer (Invitrogen), 1 μl 782 0.1 M DTT (Invitrogen), 1 μl RNaseOUT (Invitrogen), and 1 μl Superscript III RT enzyme (Invitrogen) were added to each reaction, mixed, and incubated at 55 °C for 50 min. A heat inactivation step at 70 °C for 15 min was done to stop the reaction. Five microliters of synthesized cDNA library was used as a template DNA for amplicon PCR PlatinumTaq PCR buffer (Qiagen) following the manufacturer’s instructions. Primers used in the PCR reaction listed below were described previously65. PCR products were ethanol precipitated on 1.5% agarose gel and purified with the QIAquick Gel Extraction kit (Qiagen). The purified DNA was quantified using Qbit (Thermofisher). Sequencing adapters (Nextera® XT Index Kit, Illumina) were linked to each amplicon by doing a second PCR. After testing with a Fragment Analyzer® (Advanced Analytical), amplicons with sequencing adapters were pooled for sequencing on the MiSeq illumina sequencer using paired 250 bp mode.

For primer sequences used view Table 3.

**Antibody repertoire sequencing analysis.** B cell IgA receptor heavy chain libraries were prepared as previously described66 and sequenced on the Illumina MiSeq platform (2 × 250 cycles, paired-end). Output files were preprocessed (VDJ alignment, clonotyping) using MiXCR (v3.0.12). Clonotypes were defined by 100% amino acid sequence identity of CDR3 regions. Annotation of the different segment was defined by MiXCR according to the nomenclature of the Immunogenetics database (IMGT)64. MiXCR output files were further processed in the post-processing tool-suite: VDJtools64. Further filtering was applied in order to keep only productive sequences if: (i) they were composed of at least four amino acids and (ii) had a minimal read count of 2 (ref.63) and were in-frame.

**Table 3 Primers used for antibody repertoire sequencing.**

| IgH Forward Mix | 5’ – 3’ sequence Illumina Adapter sequence read 1 + Diversity region + VH 5’ specific region |
|-----------------|---------------------------------------------------------------------------------------------|
| lgH-UAd-fw1     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw2     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw3     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw4     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw5     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw6     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw7     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw8     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw9     | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw10    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw11    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw12    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw13    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw14    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw15    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw16    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw17    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw18    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |
| lgH-UAd-fw19    | TCCTGCCGACGCGTACAGTGTTGATAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |

| IgH Reverse Primer | 5’ – 3’ sequence Illumina Adapter sequence read 2 + Diversity region + IgA constant region specific |
|-------------------|---------------------------------------------------------------------------------------------|
| lgA-const-rev     | GTCTCGTGGGCTCGAGTGTGTAAGAGACACGNNNGKAGTTRMACGTTCCAGGAGTCT |

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Fluorescence microscopy of tissue invaded STM. Tissue invaded intracellular STM harboring sueG::GFP reporter plasmid PM973 were visualized and quantified in cecum cryosections prepared from paraformaldehyde-fixed and cryo-embedded cecum tissue as described previously25. Sections were stained with DAPI (Sigma, diluted 1:2000) and Phalloidin ATTO 647N (Sigma, diluted 1:500). Up to 12 non-consecutive sections per animal were quantified visually using a Zeiss AXIO Imager.M1 microscope equipped with an EC-Plan-NEOFOLTAR 40C/1.3 Oil objective and x10/23 oculars. One high-power field measures approx. 40,000 μm². Quantitation was carried out in a blinded manner. Images were recorded on a Zeiss LSM 710 laser scanning confocal microscope using the Zeiss ZEN 3.1 software. Images were analyzed with the Image J Fiji package.

mRNA quantification in cecal tissue by qPCR. Cecum tissue was collected 6 h after infection. Immediately, the tissue was washed in PBS and snap-frozen in RNAlater (Qiagen). The total RNA was extracted from approximately 15 mg tissue, using the RNeasy mini kit (Qiagen). The extraction quality was assessed with Agilent RNA Nano 600 Nano Kit (Qiagen) and reached minimally RIN 9. In total, 5 pg mRNA samples were reversed with RT² easy first strand kit (Qiagen). cDNA libraries were analyzed in a Viia7 Real-Time PCR System and the Viia7 Real-Time PCR System acquisition software (Thermo Scientific) using a the RT² profiler PCR array quantifying murine Crohn’s disease-related markers (PAMM-169Z, Qiagen) and SYBR green reagents (Qiagen). Five housekeeping genes (Acb, Bzm, Gapdh, Gusb, and Hsp90ab1) were averaged and used for calculating ΔCt (ΔCtsample−ΔCthoukkeeping). The upper CT limit was fixed to 35 cycles.

Enzyme-linked immunosorbent assays (ELISA). Total lipocalin-2 concentrations of cecal content and fecal pellets were determined by sandwich ELISA using a commercial mouse lipocalin-2/NALG ELISA DuoSet (R&D, DY1857), according to the manufacturer's instructions. Immunoglobulin A (IgA) concentrations were quantified from mouse intestinal lavages by sandwich ELISA. ELISA plates were coated with goat anti-mouse IgA (Southern BioTech, 1040-01) and IgA was detected with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (A4789, Sigma). A purified monoclonal IgA isotype antibody (Becton Dickinson, clone M18-254, 553476) served as standard. Absorbance was measured in a 96-well microplate reader (Variskan Flash, version 4.0.033) at 405 nm. Lipocalin-2 and IgA titers were analyzed in Prism 8 for Windows (GraphPad software Inc). β-CG of each sample/standard was calculated by a four-parameter curve fitting.

Live bacterial flow cytometry. Live bacterial flow cytometry quantification of bacterial-specific intestinal IgA titers (expressed as LogEgcU values) were determined as previously described in ref.25. Briefly, STm were cultured under SPI1-inducing conditions26 as described in the Cellular Invasion Assays section. Subsequently, 1 mL of the culture was pelleted at 4816 × g for 5 min, and washed twice in sterile-filtered 2% BSA/0.005% NaN3/PBS. Intestinal IgA lavages were collected as described above. Intestinal lavages were serially diluted in sterile-filtered 2% BSA/0.005% NaN3/PBS. Serially diluted Ig-solutions and bacterial suspension were mixed 1:1 and incubated at 37°C for 1 h. Bacteria were washed twice in sterile-filtered 2% BSA/0.005% NaN3/PBS before re-suspension in monoclonal FITC-anti-mouse IgA (clone 10;3; Becton Dickinson) or PE-anti-mouse IgG1 (clone A85-1; Becton Dickinson) and FITC-anti-mouse IgG2b (clone R12-3; Becton Dickinson). After a further hour of incubation, the bacteria were washed once with PBS/2% BSA/0.005% NaN3/PBS and then resuspended in 2% paraformaldehyde (PFA)/PBS for acquisition on a Becton Dickinson FACSAarray SORP or Beckman Coulter Cytoflex S using Fsc (forward scatter) and Ssc (side scatter) parameters in logarithmic mode. Flow cytometric gating strategy is shown in Fig. S15. Data were analyzed using FlowJo software (Tree Star), and titers were calculated by fitting four-parameter logistic curves27.
B. thurivirginiae-prokaryota-lytic activity (3446–3454).

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