ATP released by intestinal bacteria limits the generation of protective IgA against enteropathogens

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T cell dependent secretory IgA (SIgA) generated in the Peyer’s patches (PPs) of the small intestine shapes a broadly diverse microbiota that is crucial for host physiology. The mutualistic co-evolution of host and microbes led to the relative tolerance of host’s immune system towards commensal microorganisms. The ATP-gated ionotropic P2X7 receptor limits T follicular helper (Tfh) cells expansion and germinal center (GC) reaction in the PPs. Here we show that transient depletion of intestinal ATP can dramatically improve high-affinity IgA response against both live and inactivated oral vaccines. Ectopic expression of Shigella flexneri periplasmic ATP-diphosphohydrolase (apyrase) abolishes ATP release by bacteria and improves the specific IgA response against live oral vaccines. Antibody responses primed in the absence of intestinal extracellular ATP (eATP) also provide superior protection from enteropathogenic infection. Thus, modulation of eATP in the small intestine can affect high-affinity IgA response against gut colonizing bacteria.
E nteric pathogens such as enteropathogenic E. coli and non-typhoidal Salmonella are a major health burden in both humans and animals. The rapid spread of antibiotic resistance in these species highlights the need for better disease prophylaxis. Protection from infection is most effective when strong mucosal immune responses have been induced, either by prior infection or by oral vaccination. High-affinity secretory IgA (SlgA) promotes enteropathogen enchainment and aggregation to disable and clearly destroy invasive species from the intestinal lumen. However, balancing safety of the vaccination strain with sufficient immune stimulation has proved challenging.

T follicular helper (Tfh) cells express high levels of the ATP-gated P2X7 receptor, a non-selective cationic channel that opens to form a cytolytic pore when exposed to micromolar concentrations of extracellular ATP (eATP). P2X7 activity therefore controls Tfh cell abundance in Peyer’s patches (PPs): Resistance of Tfh cells to ATP-mediated cell death by deletion of P2X7 enhances germinal center (GC) reactions. As eATP is produced in large quantities by the intestinal microbiota, this directly dampens immunity against enteric pathogens and oral vaccines. We show that ATP released by intestinal bacteria permeates the intestinal epithelium and can be found at high concentrations in hepatic portal blood. Eliminating this eATP, via administration of apyrase, dramatically improves the induction of specific IgA in response to either Salmonella infection or an inactivated oral vaccine. We could not measure any adverse effects of altered anti-microbiota immunity secondary to oral apyrase administration, suggesting that apyrase application is safe. Moreover, these enhanced immune responses provide superior protection from secondary infection.

Results
ATP released by microbiota affects Tfh cells in PPs via P2X7.
In the small intestine and portal vein of specific pathogen free (SPF) mice, we measured micromolar concentrations of eATP that was detected at much lower levels in germ-free (GF) mice or in other circulatory districts (Fig. 1a, b). To address the contribution of the epithelium to eATP in the small intestine, we induced epithelial regeneration in the ileum by starvation and refeeding, as described. In the presence of bacteria, the variations in epithelial turnover by starvation and re-feeding corresponded to undistinguishable concentrations of ileal eATP. In the absence of bacteria, starvation did not affect the percentage of proliferating cells. However, the concentration of ileal ATP was dramatically reduced with respect to SPF mice with comparable amount of proliferating epithelial cells, suggesting that the great majority of eATP measured in the ileal lumen is of bacterial origin (Supplementary Figure 1a, b). Therefore the microbiota generates high levels of eATP that can penetrate into the intestinal epithelium and draining blood. We cannot exclude that fungi, archaea, and protozoa might also contribute to the eATP present in the intestinal lumen. Consistent with other reports, eATP was detectable in cultures from different bacterial strains isolated from ilea of our mouse colony (Fig. 1c) and could be acutely exacerbated by vancomycin/ampicillin/metronidazole (VAM) treatment (Fig. 1d, e). In vivo VAM administration resulted in an acute significant increase of eATP in the ileum and portal vein blood (Fig. 1f). In wild type (WT), but not P2x7−/− mice, VAM administration-induced enhanced phosphatidylycerine (PS) exposure in Tfh cells from PPs (Fig. 1g and Supplementary Figure 1c, d), suggesting bacteria-derived eATP can modulate high-affinity SlgA response. Antibiotic treatment can be contraindicated in acute bacterial gastrointestinal infections due to negative effects on microbiota recovery. This data further suggest that antibiotic treatment may negatively affect the induction of T-cell dependent intestinal immunity in these infections.

Enhanced SlgA response by depletion of eATP.
The IgA response to E. coli is dependent on Tfh cells in PPs and is significantly enhanced in P2x7−/− mice, suggesting that P2X7 activity can affect the T-cell dependent SlgA response. To address whether depletion bacteria-derived ATP could influence T cell-dependent IgA responses via P2X7, we used a recombinant E. coli strain (E. coli:pApyr) carrying an expression plasmid for Shigella flexneri’s periplasmic ATP-diphosphohydrolase (apyrase) (Supplementary Figure 2a–d). The supernatant of E. coli:pApyr cultures showed ATP-degrading activity that was absent in E. coli:pBAD28 (Fig. 2b); its fractionation resulted in the recovery of apyrase activity within outer membrane vesicles (OMVs), suggesting that the enzyme was released in the extracellular space (Fig. 2a, c). Notably, eATP was undetectable in cultures of E. coli:pApyr, indicating that apyrase efficiently degraded ATP (Fig. 2d). To address whether bacteria-derived ATP could selectively limit SlgA responses in the small intestine of normally colonized animals, we administered E. coli:pApyr or E. coli:pBAD28 as control, to SPF mice by orogastric gavage (Supplementary Figure 3b). Administration of E. coli:pApyr resulted in the increase of Tfh cells in the PPs concomitant to reduced Annexin V staining in flow cytometry, suggesting degradation of bacterial ATP reduced Tfh cell death via P2X7 receptor (Fig. 3a). As expected, we observed a poor SlgA response to E. coli in mice gavaged with E. coli:pBAD28. However, anti-E. coli IgA was significantly increased in mice gavaged with E. coli:pApyr (Fig. 3b, c), despite identical intestinal E. coli load (Supplementary Figure 3a), indicating that abrogation of ATP release by bacteria results in the development of high-affinity IgA responses. The analysis of IgA in intestinal washes from mice gavaged with the two E. coli transformants on different bacterial species revealed lack of detectable reactivity (Supplementary Figure 4a). Moreover, intestinal IgA from untreated and immunized mice stained an analogous percentage of commensals from WT mice (Supplementary Figure 4b), indicating the absence of epitope-spreading to resident micro-biota members.

To further address the role of ATP released by bacteria in modulating the SlgA response, we monitored endoluminal ATP after orogastric administration of E. coli:pBAD28 and E. coli:pApyr in mice maintained with Chloramphenicol and Ampicillin (CA) (a bactericidal mix active on endogenous flora but not on CA-resistant E. coli:pBAD28 and E. coli:pApyr) or Penicillin/Streptomycin/Vancomycin (PSV) (bactericidal on both endogenous flora as well as E. coli transformants) in drinking water (Fig. 3d). Oral gavaging with E. coli:pBAD28 in mice maintained in PSV as compared to CA resulted in a significant increase of endoluminal ATP because of bacterial lysis (Fig. 3e). Notably, the analysis of anti-E. coli IgA after multiple gavaging in this setting showed that the increase in eATP concomitant to E. coli:pBAD28 gavaging in the presence of PSV correlated with reduced anti-E. coli IgA with respect to the group treated with non-bactericidal CA (Fig. 3f). In contrast, in mice colonized with E. coli:pApyr, ATP degradation by apyrase in both treatment groups (Fig. 3e) resulted in undistinguishable anti-E.coli IgA response (Fig. 3f). These data further show that an increased release of ATP by bacteria corresponds to a reduced generation of specific IgA.

Enhancement of specific SlgA by Salmonella vaccine with apyrase.
In the streptomycin mouse model of non-typhoidal
salmonellosis\textsuperscript{17}, oral infection with \textit{Salmonella enterica} serovar Typhimurium (S.Tm) leads to GALT colonization and systemic dissemination of bacteria, as originally shown with \textit{S. enteriditis}\textsuperscript{18}. To address whether apyrase expression in live-attenuated S.Tm could increase the specific SIgA response and confer enhanced protection from infection by a virulent strain, we generated an attenuated S.Tm strain (ATCC 53648) carrying either pBAD28 (S.Tm\textsuperscript{pBAD28}) or apyrase-bearing pHND10 (S.Tm\textsuperscript{pApyr}) (Supplementary Table 1). As observed with \textit{E. coli} pApyr, ATP was undetectable in culture medium of apyrase-expressing S.Tm\textsuperscript{Apyr} (Fig. 4a). Notably, Tfh and GC B cells as well as plasma cells secreting IgA specific for \textit{Salmonella} LPS were all significantly increased in mice immunized with S.Tm\textsuperscript{pApyr} (Fig. 4b, c and Supplementary Figure 5b-d), as was the concentration of anti-\textit{Salmonella} IgA in intestinal wash (Fig. 4d).

Fig. 1 Bacterial origin of intestinal ATP. a ATP concentration in the lumen of ileum from SPF and GF mice, bile, urine, and serum from SPF mice. b ATP concentration in serum from portal, jugular, inferior caval veins, and heart. c ATP concentrations in culture medium (bars) and cell growth (OD\textsubscript{600}) of the indicated bacterial species isolated from the small intestine of SPF mice. d Flow cytometry of ileal bacteria either maintained in culture medium (LB) or treated with VAM, for membrane damage (DIBAC\textsuperscript{3}+DAPI\textsuperscript{+} cells, upper dot plots) and cell death (SybrGreen\textsuperscript{+}DAPI\textsuperscript{+} cells, lower dot plots). e ATP concentrations (upper panel) and ileal bacteria growth (lower panel) in untreated (LB) and VAM-treated cultures. f ATP concentration in ileum and portal vein of SPF mice at 3 h after orogastric gavage with PBS or VAM. g Representative histograms and statistical analysis of Annexin V\textsuperscript{+} cells within Tfh cells from PPs of WT (C57BL/6) and P2rx7\textsuperscript{−/−} mice at 3 h after gavage with PBS or VAM. The boxplots show median and upper and lower quartiles. The extreme lines show the highest and lowest value. The boxplot is overlaid with the visualization of single observations. Two-tailed Mann-Whitney U-tests. **p < 0.01. One representative experiment out of at least three is shown.

Effective protection from \textit{Salmonella} infection by S.Tm\textsuperscript{pApyr}. SlgA protects the host from invasion by S.Tm or other enteropathogens by limiting the interaction of bacteria with the gut epithelium\textsuperscript{19,20}. In the non-typhoidal salmonellosis model, both engrafted growth and classical agglutination, requiring high-affinity IgA to cross-link dividing and colliding bacteria, are the main protective effects\textsuperscript{3}. As only non-clumped bacteria can
approach the intestinal epithelium and invade into host tissues, this has the effect of hugely reducing the infectious burden in the intestine. We first addressed whether the enhanced IgA responses observed after vaccination with S.Tm\(^{pApyr}\) corresponded with enhanced aggregation of \textit{Salmonella} in the gut lumen. Vaccinated mice were therefore orally infected with 1:1 mixture of GFP-enhanced \textit{Salmonella} and bacterial growth (OD\(_{600}\)) over time for S.\textit{Typhimurium} (Fig. 4e), and fewer S. Typhimurium swimming in the cecal crypts (Fig. 4f).

Correspondingly, when challenge infections were carried out with fully virulent S. Typhimurium (S.Tm\(^{WT}\)) in S.Tm\(^{pApyr}\) vaccinated mice, disease parameters, including histopathological score, fecal Lipocalin 2 (LCN2) and GALT infection, were all significantly decreased as compared to controls or S.Tm\(^{pBAD28}\)-vaccinated mice (Fig. 5a–c). \textit{Salmonella} colonizing systemic compartments (e.g. spleen and liver) follows a GALT-independent route of infection (presumably via blood circulation)\(^{22}\). Accordingly, infection with S.Tm\(^{WT}\) determines increased permeability of the gut-vascular barrier that is reflected by translocation of FITC-dextran from the intestinal lumen into the bloodstream and liver\(^{23}\). Vaccinated mice were significantly more resistant to blood absorption of FITC-dextran administered via orogastric route with respect to non-vaccinated animals and immunization with S.Tm\(^{pApyr}\) conferred enhanced protection (Fig. 5d). Moreover, S.Tm\(^{WT}\) CFUs were significantly reduced in the liver and spleen of these mice (Fig. 5e). These results indicate that immunization with apyrase-expressing bacteria confers improved protection from S.Tm systemic spreading.

Protection by S.Tm\(^{pApyr}\) depends on T-cell-dependent SIgA.

To control for effects of immunization that occur independently of adaptive immunity, we immunized recombinase-1 deficient (\textit{Rag-1}\(^{−/−}\)) mice with S.Tm\(^{pApyr}\) or S.Tm\(^{pBAD28}\). In \textit{Rag-1}\(^{−/−}\)/ mice there was no difference in susceptibility to \textit{Salmonella} infection between non-vaccinated or either vaccinated mouse group (Supplementary Figure 6a–e). To directly address the role of SIgA in conferring enhanced protection by vaccination with S.Tm\(^{pApyr}\), we performed the same immunization-challenge protocol in mice with deletion of the \textit{Igh}\(^{−/−}\) gene. These \textit{Igh}\(^{−/−}\) mice cannot produce recombinated variable regions of Ig heavy chains and have no detectable Ig. Analogously to \textit{Rag-1}\(^{−/−}\)/ mice, both non-immunized \textit{Igh}\(^{−/−}\)/ mice and \textit{Igh}\(^{−/−}\)/ mice vaccinated with either S.Tm transformants were equally susceptible to \textit{Salmonella} infection (Supplementary Figure 6f–j), supporting a crucial role for SIgA in controlling the local infection and systemic spreading of the pathogen.

We carried out experiments to mechanistically link the abrogation of ATP to the loss of signaling via P2X7 on Tfh cells.
We therefore immunized P2rx7−/− mice with S.TmPBAD28 or S. TmPApyr. Whereas S.TmPApyr-induced enhanced IgA responses in WT littermates, P2rx7−/− mice generated analogous amounts of S.Tm-specific SIgA after immunization with live-attenuated S. TmBAD28 or S. TmPApyr (Supplementary Figure 7a). Accordingly, P2rx7−/− mice were equally protected from local and systemic infection with virulent S.Tm irrespective of the immunization strain (Supplementary Figure 7b–d).

**Improved response to inactivated oral vaccines by apyrase.** We tested the ability of apyrase to enhance the induction of IgA by inactivated oral vaccines3,24 (Supplementary Figure 8a). We generated these vaccines by treating pure-cultured bacteria with 1% peracetic acid (PA)—a strong oxidizing agent. Notably, no increase of extracellular ATP was detected in the intestine of GF mice upon oral administration of inactivated bacteria (Supplementary Figure 8b). The oxidative treatment also abolished the function of the apyrase enzyme, resulting in identical IgA priming to a vaccine constructed from the empty-vector-carrying strain (Supplementary Figure 8b). The oxidative treatment also abolished the function of the apyrase enzyme, resulting in identical IgA priming to a vaccine constructed from the empty-vector-carrying strain (Supplementary Figure 8b). 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**Fig. 4** Enhanced anti-*Salmonella* SIgA by apyrase. **a** ATP concentrations in culture medium (bars) and bacterial growth (OD<sub>600</sub>) over time for *S*. *Tm*<sup>BAD28</sup> and *S*. *Tm*<sup>Apyr</sup>. **b** Quantification of Tfh and GC B cells and **c** representative contour plots with statistical analysis of plasma cells specific for *S*. *Tm* LPS in PP s 48 h after orogastric infection with *S*. *Tm*<sup>WT</sup> in non-immunized mice (CTRL) and mice immunized with *S*. *Tm*<sup>BAD28</sup> or *S*. *Tm*<sup>Apyr</sup>. **d** Intestinal anti-*S*. *Tm* IgA titer in non-immunized mice (CTRL) and mice immunized with *S*. *Tm*<sup>BAD28</sup> or *S*. *Tm*<sup>Apyr</sup>. **e** Representative 2D (upper panels, scale bar: 5 µm) and 3D (lower panels, scale bar: 5 µm) images, and statistical analysis of bacterial clumping in live cecal content from mice vaccinated with *S*. *Tm*<sup>BAD28</sup> or *S*. *Tm*<sup>Apyr</sup>, 8 h after orogastric administration of a 1:1-mix of mCherry- and GFP-tagged *S*. *Tm*<sup>Att</sup> (10<sup>7</sup> CFU). CTRL, non-vaccinated mice. **f** Images from two-photon confocal microscopy of caeca and 3D rendering of crypts from E-cadherin-mCFP mice 18 h after infection with 10<sup>7</sup> CFU of GFP-tagged *S*. *Tm*<sup>Att</sup>. Infected mice were either non-vaccinated (CTRL) or vaccinated with the indicated *S*. *Tm* transformant. Graph shows the statistics of total fluorescence inside the crypts per unit of internal area. The boxplots show median and upper and lower quartiles. The extreme lines show the highest and lowest value. The boxplot is overlaid with the visualization of single observations. Kruskal–Wallis with Dunn’s post-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. One representative experiment out of at least three is shown.
The effectiveness of inactivated vaccines was dependent on the extent of colonization. These results suggest that a dominant inhibitory effect of the microbiota is eATP production, as antibody titers induced by the inactivated vaccine in GF mice are similar to those induced by the inactivated vaccine plus apyrase in colonized animals (Fig. 6d, g). This secretory response correlates with effective protection from subsequent infection (Fig. 6e, f, h). Therefore, inclusion of recombinant apyrase into inactivated oral vaccines greatly improves the responsiveness to these preparations without any major side-effects.

Discussion
The purinergic signaling system, which uses ATP and related nucleotides as signaling molecules, plays pleiotropic roles in regulating physiological and pathological responses in virtually all mammalian tissues. This intercellular communication...
Fig. 6 Apyrase enhances the induction of IgA by inactivated oral vaccines. SPF and GF mice were untreated (CTRL) or immunized with PA-S. Tm transformants together with crudely-purely apyrase (APY extract) or mock extract (mock extract) where indicated, pretreated with streptomycin, infected with S. TmWT (10⁸ CFU i.g.) and analysed 24 h later. a Intestinal lavage IgA titer and b pathogen loads (CFU) in mLN, liver and spleen in SPF mice either non-immunized (CTRL) or immunized with PA-S. Tm pBAD28 or PA-S. Tm pApyr. c Representative H&E sections of the cecum from SPF mice at 24 h post infection and statistical analysis of histopathological scores. Star: submucosal edema; white arrow: neutrophils aggregates; black arrow: epithelial defects; arrowhead: goblet cells. Scale bar: 50 µm. d Intestinal anti-S. Tm IgA titer, e pathogen loads (CFU) in mLN, liver and spleen and f statistical analysis of histopathological scores in non-immunized mice (CTRL) and mice immunized with PA-S. Tm pBAD28 conditioned with the indicated extract. g Intestinal lavage IgA titer and h pathogen loads (CFU) in mLN, liver and spleen in GF mice either non-immunized (CTRL) or immunized with PA-S. Tm pBAD28 or PA-S. Tm pApyr. The boxplots show median and upper and lower quartiles. The extreme lines show the highest and lowest value. The boxplot is overlaid with the visualization of single observations. Kruskal–Wallis with Dunn’s post-test: *p < 0.05, **p < 0.01, ***p < 0.001. One representative experiment out of two is shown.
modality emerged very early in evolution. In the endosymbiotic relationship between α-proteobacteria and the archeon, from which the eukaryotic cell originated, ATP released by mitochondria (i.e. α-proteobacteria) evolved also as a signaling molecule to communicate mitochondrial fitness. Bacteria release ATP via mechanosensitive channels. Proteobacteria, in particular, were shown to secrete elevated amounts of ATP that were modulated by adherence to different surfaces. Indeed, ATP release as well as ATP breakdown enzymatic systems are present in all kingdoms of life (Supplementary Figure 2a). The observation that ATP released by bacteria limits T cell abundance via P2X7 receptor and ensures controlled T-dependent SIgA responses in the small intestine indicates eATP can act as an inter-kingdom signalling molecule. This regulatory pathway plays a crucial role in shaping a beneficial gut ecosystem for host metabolism. On the other hand, it contributes to the relative resistance of the intestinal adaptive immune system to generating high-affinity SIgA upon oral immunization. Overcoming this insensitivity has typically required the use of live-attenuated oral vaccines, which encompass significant safety risks. Transient subversion of bacterial ATP-mediated control of Tfh cells by apyrase could be exploited to enhance T cell-dependent SIgA response and limiting intestinal inflammation, at the same time eliciting more effective and protective SIgA against enteropathogens. Our experiments demonstrate that this strategy is safe and generates potent protective immune responses.

Methods

Mice. Animal experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the cantonal Veterinary (C57BL/6J, P2X7−/− (B6.129P2-P2x7tm1Gai(lf)), Rag2−/−, H−/−, and E-cadherin−/− (B6.129P2(Cg)-Cdhl1tm1Cle/lf)). Mice were bred in the SPF facility at the Institute for Research in Biomedicine, Bellinzona, Switzerland. C57BL/6J GM mice were maintained in flexible film isolators at the Clean Animal Facility, University of Bern, Switzerland. Where indicated, mice were treated with an antibiotic association containing Metronidazole (2.5 mg), Ampicillin (2.5 mg), and Vancomycin (1.25 mg) (VAM) in 200 μl per mouse by oral gavage. For analysis of epithelial turnover, mice were starved for 36 h with bedding chips and drinking water. After starvation, mice were re-fed for 24 h and then sacrificed for analysis. The re-feeding period was set to begin at 8:00 in all experiments. Where indicated, mice were pretreated for 15 days with VAM. In all experiments, up to five mice were housed per cage in a 12-h light-12-h dark cycle.

Determination of the ileal volume. To calculate the ileal volume, the last 5 cm of the terminal ileum were excised from 8-week-old female mice and fixed in neutral buffered formalin (16 h at 4 °C). After fixation samples were dehydrated (70% ethanol, two changes, 1 h each; 80% ethanol, one change, 1 h; 95% ethanol, one change, 1 h; 100% ethanol, three changes, 1.5 h each; xylene, three changes, 1.5 h each), embedded in paraffin and then cut at micrometre to the desired thickness (6 μm). Ten sections spanning the 5 cm were obtained and stained with hema-toxilin and eosin (H&E). The internal areas of sections were calculated by ImageJ and the mean value (base) multiplied for 5 cm (height) (Supplementary Figure 9).

Quantification of ATP. For quantification of ileal ATP, intestinal content was collected by lavage with 10 ml of intestinal wash buffer (PBS, 0.5 M EDTA, Soybean trypsin inhibitor, PMSF), spun and filtered (0.22 μm) to remove any bacteria-sized contaminants and immediately frozen in dry ice. ATP concentration in the intestinal washes was multiplied for the dilution factor to obtain the actual endoluminal ATP concentration (Supplementary Figure 9). Bile and urine were collected from gallbladder and bladder through puncture with a 34G needle. For quantification of ATP secreted by commensal bacteria in culture, intestinal content was plated on BH agar and cultured for 16 h at 37 °C. Single colonies were picked and cultured in BH broth or LB. To quantify the ATP production during bacterial growth, the bacterial culture supernatant was collected at different O.D., centrifuged and filtered (0.22 μm). For quantification of ATP in serum, inferior caval, jugular and portal veins, and heath were exposed and blood collected through puncture with a 34G needle. Emulsified sera were discharged. The extracellular ATP concentration was evaluated by bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin according to the manufacturer’s protocol (Life Technologies Europe B.V.).

OMVs isolation. OMVs were isolated from 250 ml of LB cultures and E. coliBAD28 and E. coliK12 were grown to late-exponential phase (O600~0.8–1.0) and removed from culture supernatant by centrifugation. The collected supernatants were filtered (0.22 μm) and concentrated using the Vivaspin 20 concentrators, molecular weight cutoff 50-kDa (GE Healcare), to eliminate free apyrase (~27 kDa) from the medium. The collected concentrates were then centrifuged at 270,000 g for 3 h at 4 °C to yield crude OMV’s preparations that were resuspended in PBS and tested for apyrase activity.

Apyrase activity test. To test the apyrase activity in bacterial supernatants, OMVs and intestinal washes, samples were incubated with 50 μM ATP for 30 min at room temperature. ATP concentration was evaluated by a bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin according to the manufacturer’s protocol (Life Technologies Europe B.V.). The ATPase activity of the samples was expressed as the percentage of non-degraded ATP.

Antibodies and flow cytometry. The following mAbs were purchased from BD Biosciences: biotin conjugated anti-CD45 (clone: 28G, Cat: 551960, dilution 1:50), phycoerythrin (PE) conjugated anti-ICOS (clone: 7E.17G9, Cat: 552146, dilution 1:200), PE conjugated anti-CD11b (clone: 28-1-2, Cat: #553714, dilution 1:100) and PE conjugated anti-Fas (clone: Jo2, Cat: #553258, dilution 1:200). Allophycocyanin (APC) conjugated anti-B220 (clone: RA3-6B2, Cat: #103212, dilution 1:200), APC-Cy7 conjugated anti-CD19 (clone: 6D5, Cat: #115530, dilution 1:200), APC-Cy7 conjugated anti-CD4 (clone: 12G5, Cat: #103215, dilution 1:200) and APC-eFluor780 anti-LPS (Sigma-Aldrich) with Hydrazide-Biotin reagent (Pierce Biotechnology) according to the manufacturer’s instructions. Cells were stained with 30 mg ml−1 biotinylated LPS, FITC-anti-IgA, PE-anti-CD138 antibodies at 4 °C for 45 min and then with Alexa Fluor 647 labeled streptavidin. Annexin V staining was performed in the presence of FACS shield and Annexin-V binding buffer (50 mM HEPES, pH 7.3, 140 mM NaCl, 8 mM EDTA, Soybean trypsin inhibitor, PMSF), spun and centrifuged (0.22 μm). For infection experiments, S.Tm WT (SL1344 wild-type clone SB300) or the respective mutants were cultured on LB containing the appropriate antibiotic (1 μg ml−1) for 18–24 h, diluted 1:23 at 37 °C, diluted 1:20 and sub-cultured for 3 h in 0.3 M NaCl supplemented LB without antibiotics. The bacterial strains used in the study are listed in Supplementary Table 1.

Determination of specific antibody titers by flow cytometry. Specific antibody titers in mouse intestinal washes were measured by flow cytometry. Intestinal contents were collected by lavages with 5 ml of intestinal wash buffer (PBS, 0.5 M EDTA, Soybean trypsin inhibitor, PMSF), spun and filtered (0.22 μm) to remove any bacteria-sized contaminants. Bacterial targets were resuspended at a density of 107 bacteria ml−1. Intestinal washes were serially diluted with PBS, and 25 μl of bacterial suspensions were incubated with 50 μl of wash containing the appropriate antibody (1 μg ml−1) for 30 min at 4 °C. After washing, bacteria were incubated for 1 h with monoclonal FITC-anti-mouse IgA and then resuspended in 2% paraformaldehyde in PBS for acquisition on a FACS Canto device. FlowJo software (TreeStar, Ashland, OR) was used to determine the total IgA concentration in an undiluted aliquot of the same intestinal wash sample used for analysis in flow cytometry. Median fluorescence intensities (MFI) were plotted against antibody concentrations for each sample (log2 scale) and 4-parameter logistic curves fitted using Prism (Graphpad, La Jolla, CA). Titers were calculated from these curves as the inverse of the antibody concentration giving an above-background signal. The concentration of total antibody titer required to achieve a given MFI (for example = 500) was calculated by re-arrangement of the fitted 4-parameter logistic equation for each sample. As this value is low where a strong antibody response is present, the inverse of this value was plotted. Thus titers are calculated as the inverse total antibody concentration required to achieve a given MFI. The antibody value chosen as “above background” necessarily varies between experiments due to the flow cytometer settings, but is constant within any one analysis.
Treatment of bacterial cultures with antibiotics. Ampicillin (2.5 μg ml\(^{-1}\))
vancomycin (1 μg ml\(^{-1}\)), metronidazole (1 μg ml\(^{-1}\)) were added to intestinal bac-
terial culture when OD\(_{600}\) reached 0.5 value. At different times after addition of
antibiotics, bacterial cultures were spun and supernatants collected in a sterile tube.
ATP concentration was evaluated by bioluminescence assay in filtered supernatants
(see above).

Production of peracetic acid killed vaccines. To produce PA killed vaccines, bacteria
grown for 16 h to late stationary phase were collected by centrifugation and
resolved with an aqueous solution of H\(_2\)O\(_2\) (30%) and PAA (6%) at a ratio of 1:1 to
resulting in a PA concentration of 10\(^{10}\) μg ml\(^{-1}\) to 10\(^{11}\) μg ml\(^{-1}\). The PA
kill solution was added to bacterial cultures spun at 1500g for 5 min and incubated
for 30 min at 37°C. Bacterial suspensions were centrifuged and filtered to remove
bacterial debris.

Preparation of periplasmic extract. E. coliBAD28 and E. coliAPY were prepared as
described above and collected by centrifugation. After washing, bacteria were
resuspended (10\(^{10}\) CFU ml\(^{-1}\)) in PBS with 30 mM Tris-HCl (pH 8.0), 4 mM
dEAT, 1 mM PMSF, 20% sucrose and 0.5 mg ml\(^{-1}\) lysozyme and incubated 2 min
at 37°C. MgCl\(_2\) (10 mM final) was added to the bacterial solution and incubation
was continued for 1 h at 37°C. At the end of the incubation period bacterial
suspensions were centrifuged at 11,000×g for 10 min at 4°C and supernatants
were stored (periplasmic extract).

Oral vaccination protocols. For vaccination with E. coli transformants, E. coliP-
BAD28 and E. coliAPY were collected by centrifugation, washed in sterile PBS and
10\(^{10}\) CFU ml\(^{-1}\) administered to mice by orogastric gavage. The procedure was repeated
three times for three different days and mice were sacrificed at day 22 or 28 (Supple-
mentary Figure 3b). For vaccination with S.Tm transformants, S.TmpBAD28 and S.
TmpAPY were collected by centrifugation, washed in sterile PBS and 5 × 10\(^{9}\)
CFUs administered to mice by orogastric gavage. The procedure was repeated every 3
days for 3 weeks and mice were sacrificed at day 22 or 28 (Supplementary Figure 3b).

Challenge infections with S. Typhimurium. Mice were pretreated with 1 g kg\(^{-1}\)
streptomycin sulfate in sterile PBS by gavage. Twenty-four hours later, S.Tm\(_{11}\)
(10\(^{8}\) CFU 0.1 ml\(^{-1}\) PBS) were gavaged into the stomach. For determination of total
bacterial loads, homogenates of PPs, mLN, spleen and liver collected at 24 and 48 h
after infection, were plated on MacConkey agar plates containing 50 μg ml\(^{-1}\)
mnisms to culture plates.

Live confocal microscopy of cecal content. Vaccinated or control mice were
pretreated with 0.8 kg \(^{-1}\) ampicillin sodium salt in sterile PBS by gavage. Twenty-
four hours later, mice received 10\(^{8}\) CFUs of a 1:1 mix of mCherry(pFPV25.1) and
GFP-(pM985) expressing avirulent S.Tm. For imaging, cuvette content was gently
diluted to 1:10 w/v in sterile PBS containing 6 μg ml\(^{-1}\) chloramphenicol, avoiding
heavy mixing. The suspension (200 μl) was transferred to 35 mm dish, 14 mm
glass diameter, poly-D-lysine coated Petri dish (MatTek Corporation) and imaged using
a Leica TCS SP5 confocal microscope with a x100/1.44 NA oil immersion objective
(HCX PL APO CO x100/1.44 oil). Individual bacteria were visually scored as planktonic, whereas aggregates of equal or more than three bacteria were
colored as clumps.

Multiphoton microscopy and analysis. Vaccinated or control E-cadherin-mCFP
mice were pretreated with 0.8 kg \(^{-1}\) ampicillin sodium salt in sterile PBS by gavage.
After 24 h, mice received 10\(^{7}\) CFUs of S.Tm\(_{11}\) expressing GFP con-
stitutively. After 18 h, the infected animals were sacrificed and the whole-ceca
collected for 2-photon analysis. Deep tissue imaging was performed on a custo-
mized two-photon platform (TrimScope, LaVision BioTec)\(^{35}\). The objective used
was a Nikon Apo 0.85×, 10.0W IR Corrected. The fluorescent signal has been separated using a custom configuration of detecting PMTs equipped with a set of
dichroic mirror and selective bandpass filters for the Blue, Green, and Red channels
(respectively detecting the fluorescence in the range of 450 nm–495 nm, 500
nm–550 nm, 600 nm–635 nm). 3D reconstructions of the whole ceca shown in
Fig. 4f and Supplementary Figure 10 were performed by acquiring a z-stack with a
step between slices of 3 μm for a total depth of 120 μm. Images were analysed using
FIJI software\(^{36}\) with a custom-developed macro to automate image processing:
the internal area of crypts was segmented by applying a threshold on the intensity of
the red autofluorescence (intensity above 1 = 1500 a.u.). Within the identified ROI
(in the red channel) the green particles above the threshold (intensity above 1 =
in the green channel) were detected and total fluorescence was measured by
summing the single particles fluorescence intensity along the entire z-stack. The
obtained total intensity was normalized by the total internal area of the crypts
(obtained by summing up the single slice areas found in the red channel). 3D
renderings shown in Fig. 4f and Supplementary Figure 10 were made with Clear
Volume plugin of FIJI software\(^{37}\).

Histological evaluation of Salmonella-induced typhlitis. Ceca from all animals
were examined at necropy, fixed in 10% neutral buffered formalin for at least 48 h
prior to embedding in paraffin and stained with H&E. Pathological scores were
determined in a blinded manner using a scoring scheme, which takes into consi-
deration the severity of submucosal edema (scores 0–3), neutrophilic infiltration
into the lamina propria (scores 0–4), loss of goblet cells (scores 0–3), and epithelial
damage (scores 0–3)\(^{17,38}\).

Phylogenetic tree of apyrases. Protein sequences were retrieved from GenBank
by searching for the terms “apyrase” and “ectonucleotide triphosphate diphos-
phohydrolase”. Putative and partial sequences were excluded from the analysis.
Protein sequences were aligned using MUSCLE\(^{39}\). The phylogenetic trees were
inferred by using FastTree\(^{40}\).

Statistical analysis. Where two groups of data were compared, analysis was
performed using GraphPad Prism 7.02 for Windows (http://www.graphpad.com).

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