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Intestinal Alkaline Phosphatase Prevents Antibiotic-Induced Susceptibility to Enteric Pathogens

Sayedna Nasrin Alam, MD^{1,*}, Halim Yamine, MD^{1,*}, Omeed Moaven, MD¹, Rizwan Ahmed, MD¹, Angela K. Moss, MD¹, Brishti Biswas, BS¹, Nur Muhammad, MD¹, Rakesh Biswas, BS¹, Atri Raychowdhury¹, Kanakaraju Kaliannan, MD¹, Sathi Ghosh, MD¹, Madhury Ray, MD¹, Sulaiman Hamarneh, MD¹, Soumik Barua¹, Nondita S. Malo¹, Atul K. Bhan, MD², Madhu S. Malo, MD, PhD¹, and Richard A. Hodin, MD¹

¹Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

²Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Abstract

Objective—To determine the efficacy of oral supplementation of the gut enzyme intestinal alkaline phosphatase (IAP) in preventing antibiotic-associated infections from *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) and *Clostridium difficile*.

Summary background data—The intestinal microbiota plays a pivotal role in human health and well-being. Antibiotics inherently cause dysbiosis, an imbalance in the number and composition of intestinal commensal bacteria, which leads to susceptibility to opportunistic bacterial infections. Previously, we have shown that IAP preserves the normal homeostasis of intestinal microbiota and that oral supplementation with calf IAP (cIAP) rapidly restores the normal gut flora. We hypothesized that oral IAP supplementation would protect against antibiotic-associated bacterial infections.

Methods—C57BL/6 mice were treated with antibiotic(s) +/- cIAP in the drinking water followed by oral gavage of *S. Typhimurium* or *C. difficile*. Mice were observed for clinical conditions and mortality. After a defined period of time mice were sacrificed and investigated for hematological, inflammatory and histological changes.

Results—We observed that oral supplementation with cIAP during antibiotic treatment protects mice from infections with *S. Typhimurium* as well as *C. difficile*. Animals given IAP maintained their weight, had reduced clinical severity and gut inflammation, and improved survival.

Correspondence and reprint request: Madhu S. Malo, MD, PhD, Department of Surgery, Massachusetts General Hospital, Jackson §12, 55 Fruit Street, Boston, MA 02114, USA. Telephone: (617) 726 1956, Fax: (617) 726 3114, mmalo@partners.org.

*SNA and HY contributed equally.

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Conclusion—Oral IAP supplementation protected mice from antibiotic-associated bacterial infections. We postulate that oral IAP supplementation could represent a novel therapy to protect against antibiotic-associated diarrhea (AAD), *C. difficile*-associated disease (CDAD), and other enteric infections in humans.

Keywords

Antibiotic-associated diarrhea; bacterial pathogenesis; *Clostridium difficile*-associated disease; inflammation; microbial homeostasis; salmonellosis

Introduction

Antibiotics have been developed to treat pathogenic bacterial infections; however, susceptibility of antibiotic-treated hosts to opportunistic enteric pathogenic infections is an unfortunate consequence of this treatment.¹ It is believed that the destruction of the normal commensal flora by antibiotics allows pathogenic bacteria to more easily infect the host. For example, streptomycin-treated mice can be infected with a very small number of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (<10 colony forming units, CFU), whereas approximately one million CFU are required to infect untreated control animals. In 1971, Waaij et al.² demonstrated that antibiotic treatment made mice more susceptible to colonization by *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Recent work by Sekirov et al.³ demonstrated that antibiotic-mediated alterations of the intestinal microbiota in mice were dose-dependent, and the greater the perturbation, the greater was the susceptibility to *S. Typhimurium*. Chen et al.⁴ developed a mouse model for *Clostridium difficile*-associated disease (CDAD) and found that oral gavage of 10⁵ CFU of *C. difficile* precipitated severe CDAD in antibiotic-treated mice resulting in death within 3 days. On the contrary, the same CFU of *C. difficile* failed to cause disease in mice not exposed to an antibiotic. These observations of increased susceptibility to pathogens in animals exposed to antibiotics are also relevant to humans. Recently, Glynn et al.⁵ conducted a population-based, case-control study using data from the Food-borne Diseases Activity Surveillance Network (Foodnet) during 1996–1997 and found that prior antibiotic treatment (during the 4 weeks preceding illness onset) increases the risk of sporadic infections with multidrug-resistant *S. Typhimurium*.

Extensive epidemiological studies have shown that antibiotic-associated diarrhea (AAD), an unwanted consequence of an antimicrobial therapy, is due to changes in the composition and function of the commensal intestinal flora with the consequential overgrowth of opportunistic pathogenic bacteria, especially *Clostridium difficile*^{6–8}.

We have recently shown that the brush-border enzyme intestinal alkaline phosphatase (IAP) preserves the normal homeostasis of intestinal microbiota.⁹ Furthermore, oral administration of calf IAP (cIAP) along with an antibiotic rapidly restores the gut flora that would otherwise be lost due to the antibiotic treatment, indicating a prebiotic (compound that promotes commensal bacterial growth) role for IAP. Further, we showed that oral IAP supplementation dramatically reduced colonization with *S. Typhimurium* in mice. Based on these observations we developed the hypothesis that the maintenance and/or rapid restoration of normal gut flora in the setting of antibiotic administration would prevent pathogenic bacterial infection by competing for ‘food and shelter (mucosal attachment sites)’. In this paper we provide evidence that co-administration of IAP during an antibiotic therapy protects mice from the enteric pathogens *S. Typhimurium* and *C. difficile*.

Methods

For detailed information please see the on-line Supplemental Data File.

Animals

Mice (*Mus musculus* C57BL/6) were bred at the Massachusetts General Hospital (MGH) Animal Facility (Boston, MA). Eight week old female mice were housed in Biosafety Level 2 (BL2) room in hard top cages with five animals per cage. Mice were maintained in a temperature-controlled room (22 to 24°C) with a 12-h light/12-h dark diurnal cycle with food and water *ad libitum*. The IACUC at MGH reviewed and approved all the animal experiments.

Bacterial Culture

Clostridium difficile strain VPI 10463 (ATCC 43255) stock suspension was grown in Difco cooked meat media and incubated anaerobically for 48 h at 37°C. Streptomycin-resistant *Salmonella enterica* serovar Typhimurium SL1344 (NCTC 13347) was grown in BHI media and Hektoen plates containing streptomycin (100 µg/ml) in ambient air at 37°C overnight.

Salmonella Infection

Groups of mice (n = 5) received streptomycin (5 mg/ml) in their drinking water. Simultaneous with streptomycin, one group received calf IAP (cIAP, 200 U/ml) in the drinking water (cIAP⁺ Group) while the other group (cIAP⁻ Group) received the 'vehicle for cIAP' (50 mM KCl, 10 mM tris-HCl [pH 8.2], 1 mM MgCl₂, 0.1 mM ZnCl₂, 50% Glycerol) in the drinking water. Antibiotic (streptomycin) treatment continued for 3 days, and 2 days after discontinuation of antibiotic, mice received approximately 5,000 CFU of *Salmonella* Typhimurium by oral gavage. cIAP/vehicle treatment continued for further 5 days when the experiment was terminated.

Induction of *C. difficile* Colitis

Similar to the strategy for infecting mice with *Salmonella* Typhimurium (see above) groups of mice (n = 5) received streptomycin +/- cIAP in their drinking water. This combination was given for 3 days then on day 4, streptomycin was discontinued but IAP/vehicle was continued. Also on day 4, animals from both groups received intra-peritoneal injections of clindamycin (10 mg/kg body weight). On day 6, IAP/vehicle was discontinued and both groups received approximately 1,000 CFU of *C. difficile* by oral gavage. The protocol for antibiotics and *C. difficile* dosage was determined through repeated experiments and this final protocol was repeated twice with reproducible results. In addition, IAP activity was monitored in the stool in order to verify similar intake of the enzyme in the animals that received it.

Clinical Score

To assess the clinical condition, the rating scale developed by Lankowski and Hohmann¹⁰ was followed (also see Supplemental Data File). Clinical scoring was performed by a single blinded researcher.

Interleukin-1beta (IL-1β) Assay

The ELISA kit from eBioscience was used to quantitate IL-1β levels in blood samples and colonic segments.

Intestinal Alkaline Phosphatase (IAP) Assay

IAP activity was determined by hydrolyzing p-nitrophenyl phosphate (pNPP) as previously described^{9, 11} (also see Supplemental Data File).

C. difficile Toxin Assay

Stool samples were collected on ice and the toxin assay was done using the Premier Toxins A & B kit from Meridian Bioscience (Cincinnati, OH).

Mouse Colon Histology

Hematoxylin and Eosin (H&E) slides were made from different sections of the colon and graded by a single blinded pathologist following the protocol as previously described¹² (also see Supplemental Data File).

Myeloperoxidase (MPO) Assay

The MPO assay was performed using o-dianisidine dihydrochloride method as previously described^{13–15} (also see Supplemental Data File).

Colon Organ Culture

One centimeter of the colonic segment was collected and cultured following the protocol as previously described¹⁶ (also see Supplemental Data File).

Results

Oral Supplementation with IAP Reduces Intestinal Colonization and Systemic Translocation of *Salmonella* Typhimurium

Mice (C57BL/6) were treated with streptomycin +/- cIAP followed by oral gavage with *S. Typhimurium*. The animals were then sacrificed 5 days after *S. Typhimurium* infection and tissues were examined for colonization and translocation. We observed that the colonic luminal content of the control cIAP⁻ mice had approximately 25-fold more *S. Typhimurium* compared to the cIAP⁺ animals (4.5E+09 +/- 1.9E+09 vs. 1.9E+08 +/- 8.5E+07 CFU/g, respectively). Similarly, the cecal content of the cIAP⁻ group also had approximately 20-fold higher *S. Typhimurium* count compared to the cIAP⁺ group (1.7E+10 +/- 2.7E+09 vs. 9.0E+08 +/- 3.0E+08 CFU/g, respectively) (Figure 1A).

To determine whether IAP also affects mucosal colonization, we measured the number of *S. Typhimurium* attached to the mucosa. The colonic mucosa of the cIAP⁻ group had approximately 10-fold more *S. Typhimurium* compared to the cIAP⁺ group (4.2E+05 +/- 1.3E+05 vs. 4.4E+04 +/- 5.1E+03 CFU/g, respectively). Similarly, cecal mucosa of the cIAP⁻ group contained nearly 4-fold more *S. Typhimurium* count compared to the cIAP⁺ group (4.9E+05 +/- 1.4E+05 vs. 1.2E+05 +/- 1.4E+05 CFU/g, respectively) (Figure 1B).

Figure 1C shows the data on systemic translocation of *S. Typhimurium* from the gut to mesenteric lymph nodes (MLN), liver and spleen. The MLN of the cIAP⁻ group had about 3-fold more *S. Typhimurium* compared to that of the cIAP⁺ group (1.8E+05 +/- 1.4E+05 vs. 5.7E+04 +/- 3.2E+04 CFU/g tissue, respectively). The liver of the cIAP⁻ animals had about 7-fold more *S. Typhimurium* compared to the liver of the cIAP⁺ animals (2.5E+05 +/- 2.4E+05 vs. 3.7E+04 +/- 6.7E+03 CFU/g tissue, respectively). Although a higher number of *S. Typhimurium* translocated to the spleen in the cIAP⁻ compared to the cIAP⁺ mice, the difference was not statistically significant (4.7E+05 +/- 3.2E+05 vs. 4.0E+05 +/- 2.5E+05 CFU/g tissue, respectively). In a separate set of experiments with a different antibiotic, mice were treated with amoxicillin +/- cIAP followed by *S. Typhimurium* gavage and we found a

higher number of *S. Typhimurium* translocated to the MLN, liver and spleen in the cIAP⁻ animals compared to the animals of cIAP⁺ group, all of the differences being statistically significant (data not shown).

Oral Supplementation with IAP Improves the Clinical Condition of *S. Typhimurium*-Infected Mice

We recorded the daily individual weights of *S. Typhimurium*-infected mice and a line graph showing the percentage change in the weights of each group of animals is presented in Figure 2A. The animals in the cIAP⁺ group experienced normal weight gain, whereas those in the cIAP⁻ group suffered significant weight loss. In addition, we assessed the clinical conditions of the mice daily, following a blinded protocol, and calculated the clinical scores based on Lankowski and Hohmann.¹⁰ As shown in Figure 2B, from Day 3 on, the differences in clinical score between the two groups were statistically significant.

To determine the local inflammatory status in the gut we measured the myeloperoxidase (MPO) activity in different segments of the intestine. The MPO activity in most intestinal segments was similar between the groups, except in the cecal tissue where the MPO activity was significantly higher in the cIAP⁻ mice (3.92 $\pm$ 0.35 vs. 2.88 $\pm$ 0.17 units/g cecal tissue, respectively, see Figure 2C). Finally, we also analyzed a variety of hematological parameters and found no statistically significant difference in the WBC, differential, and platelet counts, but did observe lower hemoglobin levels in the cIAP⁻ compared to cIAP⁺ mice (9.87 $\pm$ 1.63 vs. 12.40 $\pm$ 0.47 g/dl blood, respectively, see Table 1).

IAP Supplementation Rapidly Restores the Gut Flora Lost Due to a Multiple Antibiotic Regimen

We next explored the effects of oral IAP supplementation on *C. difficile*-associated disease (CDAD). We modified an established model of CDAD⁴ by using only 2 antibiotics, streptomycin (5 mg/ml drinking water) and clindamycin (10 mg/kg body weight, intra-peritoneal injection). Prior to oral gavage with *C. difficile* we determined the effects of IAP on the restoration of the gut flora after discontinuation of the two antibiotics. Consistent with our previous report,⁹ IAP was able to rapidly restore the gut flora as determined by plating of stool samples aerobically on MacConkey (Figure 3A) and BHI agar plates (Figure 3B). The MacConkey agar plate selects for the Gram-negative bacteria *Enterobacteriaceae*, e.g., *E. coli*. In the case of cIAP⁺ animals, we observed the growth of approximately 2.17E+06 $\pm$ 1.26E+06 CFU/g stool on the MacConkey agar plates on Day 6, and most of these CFU were *E. coli* colonies. Interestingly, the stools of the cIAP⁻ group contained no Gram-negative bacteria on Day 6. The BHI plate allows the growth of most types of bacteria, and as expected, we observed that the stool of both groups produced CFU on these plates, however, the stools of the cIAP⁺ mice contained approximately 10-fold more bacteria compared to the stools of cIAP⁻ animals (6.27E+06 $\pm$ 3.32E+06 vs. 6.43E+05 $\pm$ 2.97E+05 CFU/g stool, respectively). Although these data are statistically significant, it is important to note that the majority of the intestinal flora is anaerobic, and aerobes, as determined here, comprise a very small portion of total bacteria and thus can only be considered a surrogate marker of total bacterial composition and number. In order to verify that the mice took in similar amounts of IAP, we assayed the stool IAP activity levels in the treatment group and found that it was similar between the animals within that group (data not shown). In addition, we measured daily water consumption during the antibiotic treatment period to ensure similar intake by the different groups and no significant differences were found (data not shown).

IAP Supplementation Improves the Clinical Condition of *C. difficile*-Infected Mice

After antibiotic treatment we exposed the mice to approximately 1,000 CFU of *C. difficile* via oral gavage. We monitored the weights of each animal daily and observed that while the cIAP⁺ group mostly maintained their original weight, the cIAP⁻ animals had significant loss of weight within a few days (approximately 15% on day 4) (Figure 4A). We also observed the clinical conditions of the mice following a blinded protocol and calculated clinical scores as described above. Figure 4B shows that there was no significant difference between the two groups for the first 2 days; however, from day 3 the animals in the control group started getting sick while all the animals in the cIAP⁺ group stayed healthy. This difference was even more marked on days 4 and 5.

We then measured the levels of *C. difficile* toxins (A & B) in the stools of individual animals. Figure 4C shows the clearance rate of toxins from the two groups of mice. It is evident that the cIAP⁺ group rapidly cleared their toxins (by Day 5, all the animals were free of toxins), whereas all of the animals in the cIAP⁻ group still had toxins in their stool. In a separate set of experiments using 8 IAP-treated mice compared to 9 controls, IAP supplementation led to an improvement in survival, most evident on day 6, when almost 80% of the cIAP⁻ animals died compared to only 25% of the animals in the cIAP⁺ group.

IAP Supplementation Minimizes Intestinal Mucosal Injury in *C. difficile*-Infected Mice

We evaluated the status of intestinal injury in both groups of mice by histological analyses (H&E staining) of different colonic segments (histological analyses were carried out by a pathologist who was blinded to the groups). Inflammatory scoring was assigned based on an established protocol.¹² Figure 5A shows that all the colonic segments in the cIAP⁻ group had more inflammatory injury compared to the respective colonic segments of the cIAP⁺ group. The proximal colonic segments had the most injury and the degree of inflammation decreased in the more distal regions. The differences in the histological scores among the respective colonic segments of the two groups were statistically significant. Figures 5B and 5C show representative sections of the distal colon from cIAP⁻ and cIAP⁺ animals, respectively, demonstrating more extensive colonic mucosal injury in the cIAP⁻ mice (see epithelial disruption (e)).

IAP Supplementation Prevents Inflammatory Changes

We performed organ culture of the colonic segments of the animals and measured IL-1 β levels in the culture media. We found that IL-1 β levels were approximately 10-fold higher in the cIAP⁻ compared to cIAP⁺ mice (1,229.71 $\pm$ 99.34 vs. 138.15 $\pm$ 24.61 pg/ml culture media, respectively) and this difference was statistically significant (Figure 6). We also analyzed the hematological parameters of the cIAP⁻ and cIAP⁺ mice and found that there was no statistically significant difference in the counts of WBC, neutrophils or platelets (Table 1). However, similar to the case of *S. Typhimurium* infection, we observed lower hemoglobin levels in the cIAP⁻ mice compared to the cIAP⁺ animals (13.70 $\pm$ 0.66 vs. 14.83 $\pm$ 0.56 g/dl blood, respectively), and the difference was statistically significant (Table 1).

Discussion

The intestinal microbiota plays a pivotal role in maintaining human health and well-being.^{9, 17–20} Dysbiosis, defined as imbalances in the number and composition of intestinal microbes, is considered to be the cause or consequence of various diseases, including AAD,^{21, 22} CDAD,²³ salmonellosis,^{1, 3} AIDS,²⁴ diabetes,²⁵ and obesity.²⁶ Antibiotics cause dysbiosis by definition, since their purpose is the killing of bacteria; however, the type and extent of such dysbiosis obviously depends on the microbial targets of the specific

antibiotic, suggesting the probable existence of antibiotic-specific dysbiotic profiles of intestinal microbiota. At present, little is known about the specific dysbiotic profile that occurs with a given antibiotic treatment.

In this paper, we assessed the efficacy of intestinal alkaline phosphatase (IAP) in the prevention of the short-term consequences of antibiotic-induced dysbiosis, specifically Salmonellosis and CDAD. IAP is a brush-border enzyme that is exclusively produced by the enterocytes of the proximal small intestine, and the enzyme is secreted into the intestinal lumen.²⁷ Physiologically, IAP exerts many functions, including detoxifying bacterial toxins,²⁸ dephosphorylating ATP,²⁹ limiting fat absorption,³⁰ regulating host-microbial interaction and gut mucosal defense,^{31, 32} maintaining local gut immunity,³³ and preserving intestinal microbial homeostasis.⁹ The pharmacological properties of exogenous IAP include prevention of sepsis,³⁴ peritonitis,¹⁵ and bacterial pathogenesis.⁹ IAP has also been used to treat inflammatory bowel disease in animal models as well as in humans.^{14, 35} Because IAP is a naturally occurring gut enzyme, it can be safely administered orally and does not appear to be associated with any side effects.

Based on the described pre-biotic and anti-inflammatory properties of IAP, we predicted that IAP would not only prevent bacterial pathogenesis, but also ameliorate inflammatory symptoms due to infection. Indeed, in the present study, we demonstrated the efficacy of oral IAP supplementation in preventing antibiotic-induced susceptibility of mice to *S. Typhimurium* and *C. difficile* infections, two major global health problems. *Salmonella enterica* are Gram-negative bacteria that cause typhoid fever and gastroenteritis.³⁶ *S. enterica* serovar Typhi (*S. Typhi*) is mostly responsible for typhoid fever, while non-typhoidal *Salmonella* (NTS) strains cause food poisoning called Salmonellosis. It was estimated that in the year 2000 the global burden of typhoid cases was 21.7 million, out of which 217,000 patients died.³⁷ NTS Salmonellosis affects approximately three billion people world-wide and causes 200,000 deaths every year.³⁸ In the United States approximately 1.4 million *Salmonella* infections occur and 600 people die annually.⁵ The estimated costs of medical care and loss of productivity due to *Salmonella* infection ranges from \$0.5–\$2.3 billion dollars/year in the USA.⁵

Clostridium difficile-associated disease (CDAD) is already a major health problem in developed countries, and is more recently emerging in developing countries.^{6–8, 39} *C. difficile* infection alone is responsible for approximately 3 million cases of diarrhea and pseudomembranous colitis annually in the United States, and the mortality rate varies from 1 to 2.5%.^{23, 40} Infection rates for *C. difficile* are reported to be approximately 10% after 2 weeks of hospitalization, but may reach 50% after 4 or more weeks.⁴⁰ In addition, the incidence of *C. difficile* infection is 20 to 100 times higher in patients greater than 60 years of age than in patients 10 to 20 years of age.²¹ Hospitalized patients with *C. difficile* infections have a death rate of 4% to 10%.⁴⁰ A conservative estimate of the annual US cost for CDAD management is \$3.2 billion dollars.⁴¹

We believe that the beneficial effects of IAP seen in this study are likely related to the ability of this enzyme to rapidly restore the commensal gut microflora in the context of antibiotic treatment. Previously work from our laboratory did not show any direct effect of IAP on multiple bacterial species (including *Salmonella*) when grown together in vitro.⁹ In addition, It is also important to note that in the *C. difficile* experiments, IAP administration was stopped prior to the administration of *C. difficile*, such that IAP did not directly interact with the bacteria and/or its toxins. As such, we believe that protection by IAP against antibiotic-induced infections is due to its ability to rapidly restore the normal commensal gut flora, rather than a direct inhibitory effect on the bacteria themselves. It is likely that the

normal commensal flora creates a healthier environment within the gut that is less favorable to pathogenic bacterial growth.

The beneficial impact of the IAP supplementation was seen in regard to the relative resistance to infection by the two pathogens, *Salmonella* and *C. difficile*. The decreased inflammation within the colon, as evidenced by histology and blunted IL-1 β response in organ culture, reflects the lesser degree of colitis, resulting in higher hemoglobin levels due to less blood loss in the stool.

In conclusion, we believe that oral supplementation with IAP during antibiotic treatment may represent a novel way to prevent bacterial infections due to *Salmonella*, *C. difficile*, and perhaps other enteric pathogens in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAD	antibiotic-associated diarrhea
CDAD	<i>Clostridium difficile</i> -associated disease
cIAP	calf intestinal alkaline phosphatase
IAP	intestinal alkaline phosphatase
LPS	lipopolysaccharides

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Figure 1.

Oral Supplementation with cIAP Reduces Intestinal Colonization and Systemic Translocation of *Salmonella* Typhimurium. Groups of mice (n = 5) were co-treated with streptomycin (5 mg/ml) +/- cIAP (200 U/ml) in the drinking water for 3 days. Two days after discontinuation of streptomycin, mice were gavaged with 5,000 colony forming units (CFU) of *S. Typhimurium*. cIAP treatment continued for another 5 days (total 10 days). Mice were sacrificed 5 days after bacterial gavage and CFU in different tissues were determined. Values are expressed as mean +/- SEM. *, p < 0.05 (Student's t test). (A) *S. Typhimurium* CFU in the luminal content of colon and cecum. (B) *S. Typhimurium* CFU in

the mucosa of colon and cecum. (C) *S. Typhimurium* CFU in the mesenteric lymph nodes (MLN), liver and spleen.

Figure 2.

Oral IAP Supplementation Improves Clinical Conditions in Mice Infected with *S. Typhimurium*. For the description of animals see Figure 1 and also Methods. Mice were observed for weight and clinical conditions for 5 days after bacterial gavage. For determining myeloperoxidase (MPO) activity cecal tissue was homogenized by sonication and supernatant was collected after centrifugation followed by MPO assay. MPO activity was expressed as average units/gm cecal tissue $\pm$ SD. (A) Percentage change in weight of mice infected with *S. Typhimurium* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). *, $p < 0.05$ (Student's *t* test). (B) Clinical score of mice infected with *S. Typhimurium* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). ***, $p < 0.001$ (Student's *t* test). (C) MPO activity in the cecal tissue of mice infected with *S. Typhimurium* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). *, $p < 0.05$; ***, $p < 0.001$ (Student's *t* test).

Figure 3.

Oral Supplementation with cIAP Rapidly Restores the Normal Gut Flora in Mice after Discontinuation of Treatment with Multiple Antibiotics. Groups of mice were co-treated with antibiotics (streptomycin 3 days orally followed by a single intraperitoneal injection of clindamycin on day 4) +/- cIAP (200 U/ml). Individual stool samples were grown on MacConkey agar plates (A) and Brain Heart Infusion (BHI) agar plates (B). Please note that on days 2 to 5 animals from both groups did not grow any bacterial colonies from the collected stool samples indicating the effectiveness of antibiotic treatment. Values are expressed as average CFU/gm stool +/- SEM. *, $p < 0.05$; ***, $p < 0.001$ (Student's t test).

Figure 4.

Oral cIAP Supplementation Improves Clinical Conditions in Mice Infected with *C. difficile*.

For the description of animals see Figure 3 and also Methods. Groups of mice were co-treated with antibiotics (streptomycin 3 days orally followed by a single intraperitoneal injection of clindamycin on day 4) +/- cIAP (200 U/ml). On day 6, IAP/vehicle was discontinued and both groups received approximately 1,000 CFU of *C. difficile* by oral gavage. Weight, clinical conditions and the presence of *C. difficile* toxins in the stool were monitored daily. (A) Percentage change in weight of mice infected with *C. difficile* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). *, $p < 0.05$ (Student's t test). (B) Clinical score of mice infected with *C. difficile* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). **, $p < 0.01$; ***, $p < 0.001$ (Student's t test). (C) Clearance of toxins in mice infected with *C. difficile* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). **, $p < 0.01$ (Student's t test).

Figure 5.

Oral Supplementation with cIAP Minimizes Colonic Injury in *C. difficile*-Infected Mice. For the description of animals see Figure 3 and also Methods. Groups of mice were co-treated with antibiotics (streptomycin 3 days orally followed by a single intraperitoneal injection of clindamycin on day 4) +/- cIAP (200 U/ml). On day 6, IAP/vehicle was discontinued and both groups received approximately 1,000 CFU of *C. difficile* by oral gavage. Mice were sacrificed 4 days after *C. difficile* infection. Hematoxylin and Eosin (H&E) staining was performed on slides from different segments of the colon and the mucosa examined. Lesions were graded based on an established grading system.¹² (A) Histological score of mice

infected with *C. difficile* and treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻). Note: The lower the score the better is the outcome. *, $p < 0.05$; **, $p < 0.01$ (Student's t test). (B) Representative histological slide of distal colon of *C. difficile*-infected mice not treated with cIAP (-IAP). (C) Representative histological slide of distal colon of *C. difficile*-infected mice treated with cIAP (+IAP). Structures: e, epithelial disruption; i, inflammatory infiltrate; Re, re-epithelialization. Magnification: 200 $\times$. Note: Extensive epithelial disruption (e) is evident in the untreated mice (-IAP).

Figure 6.

Oral Supplementation with cIAP Minimizes Inflammation. Mice were treated with cIAP (cIAP⁺) or without cIAP (cIAP⁻) for 5 days and then infected with *C. difficile*. For the description of animals see Figure 3 and also Methods. Animals were sacrificed 4 days after *C. difficile* infection. Colonic segments were obtained and organ culture performed. Using an ELISA kit, proinflammatory cytokine interleukin-1beta (IL-1 β) levels were determined in the colon organ culture media. Values are expressed as pg/ml culture media $\pm$ SD. ***, p < 0.001; (Student's t test).

Table 1

Oral Supplementation of Intestinal Alkaline Phosphatase (IAP) Maintains Higher Hemoglobin Levels in *Salmonella* Typhimurium and *Clostridium difficile*-Infected Mice.

Infectious organism	Mice (C57BL/6)	WBC (10 ⁵ /μl)	Granulocytes (10 ³ /μl)	Platelets (10 ³ /μl)	Hemoglobin (g/dl)
<i>Salmonella</i> Typhimurium	cIAP ⁺ Group	2.24 ± 0.83	1.47 ± 0.58	56.40 ± 15.50	12.40 ± 0.47 *
	cIAP ⁻ Group	2.25 ± 0.96	1.77 ± 0.85	50.00 ± 17.84	9.87 ± 1.63
<i>Clostridium difficile</i>	cIAP ⁺ Group	2.60 ± 0.81	1.23 ± 0.46	281.75 ± 49.82	14.83 ± 0.56 *
	cIAP ⁻ Group	2.76 ± 0.33	1.42 ± 0.33	311.20 ± 54.76	13.70 ± 0.66

cIAP⁺ Group (n = 5) received calf IAP (cIAP, 200 U/ml = 20 μl/ml) in the drinking water; control cIAP⁻ Group (n = 5) received vehicle for cIAP (20 μl/ml) in the drinking water (see Methods). Values are expressed as mean ± SD.

* p < 0.05 (Student's t test).