Effects of intestinal alkaline phosphatase on intestinal barrier function in a cecal ligation and puncture (CLP)-induced mouse model for sepsis

Original Article

Effects of intestinal alkaline phosphatase on intestinal barrier function in a cecal ligation and puncture (CLP)-induced mouse model for sepsis.

Running title: Alkaline phosphatase and the gut barrier

Authors

Philip Plaeke[1,2], Joris G De Man[1,2], Annemieke Smet[1,2], Surbhi Malhotra-Kumar[3], Isabel Pintelon[4], Jean-Pierre Timmermans[4], Sara Nullens[1,2], Philippe G Jorens[1,2,5], Guy Hubens[6,7], Benedicte Y De Winter[1,2]

Affiliations

1. Laboratory of Experimental Medicine and Pediatrics (LEMP), University of Antwerp, Antwerp, Belgium
2. Infla-Med Research Consortium, University of Antwerp, Antwerp, Belgium
3. Department of Medical Microbiology, University of Antwerp, Antwerp, Belgium
4. Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium
5. Department of Intensive Care Medicine, Antwerp University Hospital, Edegem (Antwerp), Belgium
6. Department of Abdominal Surgery, Antwerp University Hospital, Edegem (Antwerp), Belgium
7. Antwerp Surgical Training, Anatomy and Research Centre (ASTARC), University of Antwerp, Antwerp, Belgium

Corresponding Author

Prof. Dr. Benedicte De Winter
University of Antwerp
Laboratory of Experimental Medicine and Pediatrics (LEMP)
Building T, T232
Universiteitsplein 1
2610 Wilrijk (Antwerp)
Belgium

E-mail: benedicte.dewinter@uantwerpen.be

Word Count: 5207

Abstract

Background

Sepsis is a severe pathological condition associated with systemic inflammation, intestinal inflammation and gastrointestinal barrier dysfunction. Intestinal alkaline phosphatase (IAP) has been demonstrated to detoxify lipopolysaccharide, an important mediator in the pathophysiology of sepsis. We investigated the effect of treatment with IAP on intestinal permeability, intestinal inflammation, and bacterial translocation.

Methods

OF-1 mice were divided into 4 groups (n=12/group), undergoing either a sham or cecal ligation and puncture (CLP) procedure to induce sepsis. Mice received IAP or a vehicle intraperitoneally 5 min prior to the onset of the CLP or sham procedure, which was repeated every 12 hours for 2 consecutive days. After two days, *in vivo* intestinal permeability, intestinal inflammation, and bacterial translocation were determined.

Key Results

CLP-induced sepsis resulted in significantly more weight loss, worse clinical disease scores, bacterial translocation, and elevated inflammatory cytokines. Intestinal permeability was increased up to 5-fold ($p<0.001$). IAP-activity was significantly increased in septic animals. Treatment with IAP had no effect on clinical outcomes but reduced the increased permeability of the small intestine by 50% ($p=0.005$). This reduction in permeability was accompanied by a modified gene expression of claudin-1 ($p=0.025$), claudin-14 ($p=0.035$), and interleukin 12 ($p=0.015$). A discriminant analysis showed that treatment with IAP is linked to modified mRNA levels of several tight junction proteins and cytokines.

Conclusions and Inferences

Treatment with IAP diminished CLP-induced intestinal barrier disruption, associated with modified expression of several cytokines and claudins. Nevertheless, this effect did not translate into better clinical outcomes in our experimental setup.

Keywords: Sepsis, Intestinal Alkaline Phosphatase, Intestinal Permeability, Bacterial Translocation, Tight Junction Proteins

Key Points:

- Sepsis damages the intestinal barrier and causes intestinal inflammation. Since intestinal alkaline phosphatase (IAP) detoxifies bacteria-derived endotoxins, this study assessed its role in a mouse model for sepsis.
- IAP reduced intestinal barrier impairment, which was mediated by altered expression of several tight junction proteins and cytokines. Nevertheless, clinical outcomes or inflammation were not influenced.
- IAP is a potential target for barrier protection and could have clinical importance in disorders characterized by disturbed intestinal permeability, including sepsis.

Abbreviations

CLP – Cecal Ligation and Puncture

IAP – Intestinal Alkaline Phosphatase

LPS – Lipopolysaccharide

CRP – C-reactive protein

FITC – Fluorescein Isothiocyanate

IL – Interleukin

ZO – Zonula Occludens Protein

TNAP – Tissue-nonspecific alkaline phosphatase

Introduction

Sepsis is a pathological condition caused by a dysregulated inflammatory response, usually as a reaction to an infectious agent. This inflammatory response is progressive, partially self-maintained by the immune system and can rapidly switch between a pro-inflammatory and an immunosuppressive state[1]. Sepsis is associated with high morbidity and mortality with, apart from antibiotics, limited therapeutic options currently available[2].

The gastrointestinal tract has been hypothesized to play an important role in the pathophysiology of sepsis. During sepsis, systemic inflammation can cause gastrointestinal inflammation via the influx of proinflammatory mediators, activation of inflammatory cascades, hypoperfusion and ischemia-reperfusion injury[3]. Besides, intestinal barrier dysfunction, characterized by tight junction disruption, epithelial cell death, and loss of the mucus layer, occurs during sepsis. This phenomenon clears the way for enteropathogens and proinflammatory molecules like lipopolysaccharide (LPS) released from certain gram-negative bacteria to permeate through the intestinal barrier into the bloodstream, thus amplifying the already ongoing systemic inflammation.

Recent data suggest a role for intestinal alkaline phosphatase (IAP), a brush border-bound alkaline phosphatase subtype solely expressed by enterocytes, in sepsis[4]. Although IAP is ubiquitously expressed in the gastrointestinal tract, its distribution is variable with the highest expression levels in the duodenum and IAP expression levels decreasing over the length of the intestine. Among many other functions, IAP has been documented to be involved in the regulation of duodenal bicarbonate secretion[5, 6] and in lipid absorption by the intestinal epithelium[7-14]. However, the role of IAP seems to encompass more than just these physiological functions, and IAP has been shown to be of importance in the response to different pathological conditions as well. Through its dephosphorylating activity, IAP is able to detoxify LPS derived from gram-negative bacteria by degrading LPS into the less biologically active monophosphoryl-LPS. It hereby loses its potency to activate the Toll-like receptor 4 driven proinflammatory pathway[15-21]. Moreover, recent studies have linked IAP to the maintenance

of the healthy microbiome, induction of mucosal tolerance and even expression of tight junction proteins[17, 22-25]. Several *in vivo* animal studies confirmed the potential of IAP to detoxify LPS, improving survival rates and reducing the systemic release of several proinflammatory cytokines in several models of sepsis or inflammation[17, 20, 26-32].

In humans, the effect of IAP on acute kidney injury during sepsis has been investigated in three phase II clinical trials[33-35]. In the first two trials, treatment with IAP improved renal parameters, and decreased serum CRP and interleukin (IL)-6 levels, but had no effect on mortality. In a more recent large multicenter trial, the improvement of short-term kidney function could not be confirmed[35].

Given the important role of the gastrointestinal tract in the pathophysiology of sepsis and the promising effects of IAP treatment on acute kidney injury during sepsis, we here studied the effect of a combined preventive and curative treatment with IAP on intestinal permeability, bacterial translocation, and inflammation in a validated experimental animal model for sepsis.

Materials and methods

Animals

Eight week old male OF1 mice (Oncins France 1, outbred mice, average weight 40.0 ±5g, Charles River Laboratories, Ecully, France) were housed in a controlled environment in the animal facility of the University of Antwerp. Mice were kept at standardized conditions, with a temperature of 21 °C, 40-60% humidity, a 12h light-dark cycle, and with *ad libitum* access to water and regular chow throughout the experiment. After arrival in the animal facility, mice were allowed to acclimatize for 7-10 days before the onset of the experiments. All experiments received approval from the ethical committee of the University of Antwerp (File number ECD2016-68). Weight loss above 15% was considered as a humane endpoint for this experiment.

Cecal Ligation and Puncture (CLP) Procedure

The cecal ligation and puncture technique (CLP) is considered the gold standard for inducing a polymicrobial, intra-abdominal sepsis, and has already been fully characterized immunologically at our lab[36]. Briefly, mice were anesthetized with a mixture of ketamine (60 mg kg^{-1}, Pfizer, New York City, NY, USA) and xylazine (10 mg kg^{-1}, Bayer, Leverkusen, Germany). Following a midline laparotomy, the cecum was identified and ligated with a silk 5-0 thread at 50% of its length. Subsequently, the ligated cecum was punctured once through-and-through with a 21G sterile needle to bring the cecal endolumen in continuity with the abdominal cavity. Afterwards, the abdomen was closed in two layers with a 5-0 nonabsorbable nylon suture (Ethilon, Ethicon, Somerville, NJ, USA) and mice were allowed to recover while their temperature was kept constant. Sham-operated mice underwent a similar protocol, however, in these mice the cecum was solely manipulated, but not ligated nor punctured after performing the laparotomy.

Treatment and experimental protocol

Mice were randomized into 4 different treatment groups (n=12/group): group 1, sham procedure and vehicle treatment; group 2, sham procedure and IAP treatment; group 3, CLP procedure and vehicle treatment; group 4, CLP procedure and IAP treatment. All mice underwent a three-day experimental protocol (Figure 1). On the first day mice underwent either the sham or CLP procedure. Calf IAP (10,000 IU ml^{-1}, New England Biolabs, Ipswich, MA, USA) was intraperitoneally administered twice a day at a dose of 1 IU g^{-1} mouse. The first dose was given 5 min. prior to the surgical procedure, and subsequent doses were administered every 12h (Figure 1). The vehicle (saline 0.9%) was administered at similar intervals and at an equal volume (10 µl g^{-1} mouse). The dose of 1 IU g^{-1} mouse was determined based on preliminary experiments and previous literature data, with this dose of 1 IU g^{-1} having the most significant influence on permeability[27]. IAP activity of the stock and treatment solution was confirmed with a pNPP assay (SensoLyte Phosphatase Activity Assay Kit, Anaspec Inc., Fremont, CA, USA).

During the whole experiment, mice were clinically monitored with a validated clinical disease score as published by Nullens *et al.,* and weight loss occurring after the induction of sepsis was registered daily[37]. Body temperature was measured using an infrared thermometer (Braun No Touch NTF3000, Braun, Kronberg, Germany), as described by Mei et al.[38]. Core abdominal temperatures were measured 48 hours after the sham or CLP procedure in the space between the liver and diaphragm. Buprenorphine (0.05 mg kg^{-1}, Merck Sharp & Dohme, Kenilworth, NJ, USA) and a glucose solution, at a combined volume of 1 ml, were administered subcutaneously twice a day for analgesia and fluid resuscitation, as recently recommended to do in animal sepsis models[39].

An additional experiment was conducted to determine the influence of the CLP-model and of the treatment with IAP on food intake, water intake, urine output, and stool production. In this experiment, 20 male OF1 mice (n=5/group), were housed in metabolic cages, otherwise undergoing an identical experimental protocol as described above.

Measurements of intestinal permeability

On the third day of the experiment (meaning 48h following the procedure), intestinal permeability, bacterial translocation, and the effects on gene expression of cytokines and tight junctions were determined. Intestinal permeability was assessed *in vivo* two days after the sham/CLP procedure by measuring the dissemination of a 4 kDa Fluorescein Isothiocyanate Dextran (FITC-Dextran) from the intestinal lumen into the blood. In short, mice were anesthetized with a ketamine (90mg kg^{-1}) and xylazine (10mg kg^{-1}) solution and placed in a supine position. After opening the abdomen, the ileum was ligated with a silk 5-0 thread proximally of the ileocecal junction to isolate the small intestine. One hundred microliter of a 40 mg ml^{-1} FITC-Dextran solution (Sigma-Aldrich, Saint Louis, MO, USA) was injected directly into the ligated ileum, just proximal of the ligation. Subsequently, the abdomen was closed and mice were kept anesthetized while monitoring the body temperature. One hour later, the abdomen was reopened and blood was drawn by means of a terminal cardiac puncture. Blood was centrifuged (5min, 10,000g) and the FITC-Dextran concentration was measured with a fluorescence

spectrophotometer (Excitation 485 nm; Emission 518 nm). Fluorescence levels were subsequently transformed into serum concentrations using a linear standard curve.

Gene expression of tight junction proteins was measured with reverse-transcriptase polymerase chain reaction (RT-PCR) on samples of the terminal ileum. For this, the most distal part of the terminal ileum, adjacent to the caecum, was collected, transferred to RNAlater (Sigma Aldrich, Saint-Louis, MO, USA), and stored in -80 °C until analysis. Total RNA was extracted using the Isolate II RNA Mini Kit (Bioline – Meridian Life Sciences, Memphis, TN, USA). The concentration and quality of the RNA were evaluated using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Subsequently, RNA was converted to cDNA by reverse transcription using the SensiFast™ cDNA synthesis kit (Bioline – Meridian Life Sciences, Memphis, TN, USA). Relative gene expression was then determined by SYBR Green RT-qPCR using the SensiFAST™ SYBR Hi-ROX Kit (Bioline – Meridian Life Sciences, Memphis, TN, USA) on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Altered gene expression of the following tight junction proteins was measured in the terminal ileum: *claudin-1, -2, -3, -4, -5, -7, -14, -18, occludin, and zonula occludens (ZO) protein 1 and 3.* Primer sequences are shown in Supplementary Table 1. RT-qPCR reactions were performed in duplicate and involved an initial DNA polymerase activation step for 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension for 1 min at 60°C. Analysis and quality control were performed using Qbase plus software (version 3.0, Biogazelle, Gent, Belgium)[40]. Relative expression of the target genes was normalized to the expression of the housekeeping genes *Gapdh*, *18s rRNA* and *Hprt1*, and expressed as CNRQ (calibrated normalized relative quantities) values for each sample.

Histology on terminal ileum samples was performed on specimens collected during the euthanasia and fixed in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (ThermoFisher Scientific, Waltham, MA, USA). Immunohistochemical staining for claudin-1 was performed using the anti-claudin-1 antibody (ab15098; Abcam, Cambridge, United Kingdom) and Alexa Fluor 546 donkey anti-

rabbit IgG as secondary antibody (ThermoFisher Scientific, Waltham, MA, USA). Nuclei were stained with DAPI (not visualized on the image). Images were taken with a Leica TCS SP8 confocal laser scanning microscope (Leica-microsystems, Wetzlar, Germany) using a White Light Laser at 20x magnification.

Measurements of inflammation and bacterial translocation

Intestinal inflammation at the protein level was determined using cytometric bead array. Terminal ileum samples were collected, stored on ice, and mechanically homogenized in a Tris-HCL (1M) EDTA (0.5M) buffer with 0.5% Tween-20 and protease inhibitor (cOmplete protease inhibitor, Roche, Basel, Switzerland). Samples were centrifuged at 16.000 g for 10 minutes and the supernatants were stored at -80°C until analysis. The cytometric bead array was performed in accordance with the manufacturer's instructions. In short, supernatant of mechanically homogenized ileal tissue was suspended with antibody-coated fluorescent beads (BD Biosciences, Franklin Lakes, NJ, USA) and fluorescence was measured using flow cytometry (Accuri, BD Biosciences, Franklin Lakes, NJ, USA) to simultaneously determine levels of different cytokines. Cytokines measured at the protein level were Interleukin (IL)-1ß, -6, -10, -12, and Tumor Necrosis Factor Alpha (TNFα). Quantification of changes in gene expression of *Il-1ß, Il-6, Il-8, Il-10, Il-12a, Il-18,* and *Tnf* was performed with RT-PCR as described above.

Bacterial translocation was determined by culturing homogenized mesenteric lymph nodes, homogenized liver tissue, blood, and abdominal lavage fluid. In short, abdominal lavage fluid was collected after rinsing the abdomen with 2 ml of isotonic saline, allowing the saline to remain within the peritoneal cavity for at least 30 seconds. Blood was taken centrally through a terminal cardiac puncture while keeping the diaphragm intact to prevent contamination of the peritoneal cavity. Mesenteric lymph nodes were aseptically taken out of the mesentery adjacent to the ascending colon and near the ileocecal valve, hence collecting the first station of lymph draining from the ligated caecum. Lymph nodes were collected without opening any abscess, bowel or causing an

intraperitoneal bleeding. Mesenteric fat was carefully removed from the lymph nodes. Liver tissue was aseptically collected from both the left and right liver lobe. Both the mesenteric lymph nodes and the liver tissue were transferred to 5 ml of cooled phosphate-buffered saline as transport medium, and subsequently mechanically homogenized through a 40 µm nylon cell strainer using a 10 ml syringe plunger. Afterwards, all samples were plated onto blood agar and incubated at 37 °C and 5 % CO_2. After 24 hours cultures were examined for direct growth and the first identification with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) was performed if colonies were already present at that time. Afterwards, culture enrichment was added and cultures were followed up to another 48 hours before final identification of cultured strains with MALDI-TOF. Bacterial translocation was considered to have occurred in case enteropathogens were demonstrated with MALDI-TOF[41].

IAP activity and IAP-gene expression

In mice, IAP activity is mainly expressed by two genes, *Akp3* and *Alpi,* the latter also known as *Akp6*. While both genes encode functioning IAP, spatial expression of both genes in the gastrointestinal tract is quite different: *Akp3* expression is mainly restricted to the duodenum, while *Alpi* is found in the whole intestine[42, 43]. The effect of sepsis and IAP treatment on IAP gene expression at the terminal ileum was determined with RT-PCR as described above. Expression of *Alpl*, the gene encoding for tissue-nonspecific alkaline phosphatase (TNAP), was determined to measure the synthesis of TNAP in the terminal ileum.

Activity of IAP was spectrophotometrically determined in supernatants of homogenized terminal ileum samples, using a commercially available pNPP reaction-based assay kit according to the manufacturer's instructions (SensoLyte Phosphatase Activity Assay Kit, Anaspec Inc., Fremont, CA, USA). End-point activity was determined 30 min. after incubation with pNPP at 405nm. To discriminate IAP from tissue-nonspecific alkaline phosphatase (TNAP), the same pNPP assay was repeated, but 50

mMol of L-Phenylalanine was added to the assay[44]. Because L-phenylalanine is an inhibitor of IAP, addition of this amino acid allows a measurement of all non-IAP related alkaline phosphatase activity.

Statistical analysis

Weight loss is expressed as the percentage of weight lost compared to the body weight at baseline. Results of the FITC-Dextran permeability assay and changes in gene expression are presented as the relative difference and standard error of the mean (SEM) with the vehicle-treated sham group acting as the reference group. Culture results are presented as the number and percentage of cultures testing positive for enteropathogens and this for every treatment group. Data were statistically analyzed with a two way ANOVA with LSD post-hoc testing or Student's t-test when appropriate. Levene's test was used to assess the equality of variances when required. Weight loss data were analyzed with a linear repeated measures regression. Point-biserial correlation (r_{pb}) analysis and the Chi-Square test were used to analyze bacterial translocation. To multivariately determine whether treatment groups could be discriminated based on subsets of cytokines or tight junction proteins, a discriminant analysis was performed. The results of these discriminant analyses are displayed as the most influential variables in the different discriminant functions. This data is supplemented by the strongest pooled within-group correlations between the studied discriminating cytokines/tight junction proteins and the canonical discriminant functions. A p-value of 0.05 was considered statistically significant. Statistical analysis was performed with SPSS (IBM, Chicago, IL, version 23.0) and results were graphically visualized with Graphpad Prism software (Graphpad Software v7, La Jolla, CA, USA).

Results

Effects of sepsis and IAP on clinical outcomes

At baseline, no significant differences in body weight were observed between the different treatment arms and all mice scored zero on the clinical disease score. CLP or sham surgery resulted in an initial weight loss in all mice who underwent these procedures (Figure 2). Yet, while this weight loss flattened out or even weight regain occurred in the sham-operated mice, progressive weight loss persisted in mice with CLP-induced sepsis. Already within the first 24 hours after surgery, the CLP procedure resulted in significantly lower body weight ($p<0.001$) compared to mice that underwent the sham procedure. On the morning of the terminal experiments, sham- and CLP-operated mice had lost on average 4.00% (SEM ±0.90) and 10.50% (SEM ±0.53) of their original body weight, respectively. Treatment with IAP had no significant effect on weight loss in either sham-operated mice or septic mice (Figure 2).

On the evening before the final experiments, CLP-induced sepsis was associated with significantly higher clinical disease scores (0.04 ±0.04 vs. 4.63 ±0.23, $p<0.001$). Treatment with IAP slightly reduced these clinical disease scores, but this effect was not statistically significant ($p=0.124$).

Effects of the CLP procedure and treatment with IAP on metabolic parameters are displayed in Table 1. Food and water intake, as well as urine output and stool production, were all significantly decreased because of the CLP procedure. These parameters were, however, unaffected by the treatment with IAP. Throughout the experiment, body temperatures were followed, but no significant differences between the different groups were observed.

As fluid resuscitation was meticulously provided, no mortality was observed following the surgical procedure and during the 2-day follow-up.

Effects on Intestinal permeability

Following the CLP procedure, the permeability of the small intestine for 4kDa FITC-dextran increased 3-5-fold compared to the corresponding sham groups (305 ng ml^{-1} ±52 vs. 2097 ng ml^{-1} ±462 respectively, $p<0.001$, Figure 3). Treatment with IAP significantly reduced the permeability of the small intestine for 4kDa FITC-dextran in septic mice compared to vehicle-treated septic mice (FITC-dextran

concentrations 1050 ng ml^{-1} ±188 vs. 2097 ng ml^{-1} ±462, p=0.005; Figure 3).

RT-PCR analysis demonstrated a modified expression for *claudin-2* (p=0.005), *claudin*-4 (p=0.003), *claudin-14* (p=0.023), *claudin-18* (p=0.043) and *occludin* (p<0.001) in the ileum of septic mice due to the CLP procedure (Table 2). Two-day treatment with IAP enhanced the gene expression of *claudin-1* (p=0.025) and *claudin-14* (p=0.035), but not the other tight junction proteins in the small intestine in both sham and CLP-operated mice. Confocal immunohistochemical staining for claudin-1 demonstrated that the CLP procedure only had a very mild impact on the morphology of the intestinal barrier and on the apical expression pattern of claudin-1 when comparing both sham groups (Figure 4a & 4b) with the vehicle-treated CLP group (Figure 4c). In addition, the claudin-1 staining supports our observations of a comparable gene expression of claudin-1 after CLP treatment and a higher gene expression after IAP treatment, given the moderately but visibly higher staining of claudin-1 at the apical epithelial surface in the IAP-treated CLP group (Figure 4d) compared to the vehicle-treated CLP group which is possibly linked to the intestinal permeability changes observed.

Although the relative gene expression of individual tight junction proteins was not significantly modified when analyzed in a univariate manner, when combined, discriminant analysis revealed *claudin-14* (correlation between the expression levels of claudin 14 and the canonical discriminant function: 0.353), *claudin-5* (0.346), *claudin-1* (0.331), *occludin* (0.317), and *claudin-4* (-0.354) to be good predictors to differentiate sham from CLP-operated mice, and mice treated with vehicle from IAP-treated mice (Figure 5b). Based on the gene expression of all tight junction proteins in this experiment, 89.2% of all mice could be correctly classified into the different treatment groups. In addition, 88.9% of the septic mice treated with IAP, which had a different expression profile of tight junction proteins, could be assigned to the appropriate groups (Figure 5b).

Effects on bacterial translocation

While bacterial translocation to the mesenteric lymph nodes, blood, abdominal cavity, and liver tissue was rarely observed in mice that underwent the sham procedure, its incidence was highly increased

following the CLP procedure (Figure 6). Cultures of mesenteric lymph nodes, liver tissue, abdominal lavage fluid and hemocultures were positive for the presence of enteropathogens in 100.00% (n=12/12), 75.00% (n=9/12), 91.67% (n=11/12), 41.67% (n=5/12), of vehicle-treated septic mice, respectively. In absolute numbers, translocation of enteropathogens was less frequently encountered when IAP had been administered, but these differences did not reach statistical significance. The majority of enteropathogens identified with MALDI-TOF consisted of *Escherichia coli* and *Enterococcus faecalis*. For a detailed overview of all bacteria belonging to the gut microbiome that were identified with MALDI-TOF, we refer to the supporting material – Supplementary Table 2.

Permeability of the small intestine for 4kDa FITC-Dextran and the presence of enteropathogens in the mesenteric lymph nodes (299 ng ml^{-1} ±83 vs. 2325 ng ml^{-1} ±532, p=0.002; r_{pb}=0.477, p=0.002), liver tissue (510 ng ml^{-1} ±144 vs. 2530 ng ml^{-1} ±636, p=0.002; r_{pb}=0.480, p=0.001), and abdominal lavage fluid (450 ng ml^{-1} ±170 vs. 2208 ng ml^{-1} ±540, p=0.007; r_{pb}=0.416, p=0.007) were significantly correlated (Supporting information – Figure 1).

Effects on Intestinal inflammation

CLP-induced abdominal sepsis significantly altered the gene expression of several cytokines (Table 2). The intestinal expression levels of *Il-1ß* (p=0.001), *Il-6* (p=0.002), *Tnfα* (p<0.001), *Il-10* (p<0.001), *Il-18* (p<0.001) were significantly increased and expression of *Il-8* was decreased (p=0.036) in septic animals. When analyzed univariately, treatment with IAP had no significant effect on the expression of these or the other cytokines studied, except for *Il-12* (p=0.015), whose expression was significantly enhanced (Table 2).

A discriminant analysis showed that the combination of *Il-18* (0.673), *Il-10* (-0.643), *Tnfα* (0.374), *Il-1ß* (0.297), and *Il-6* (0.276) predicted best whether mice had undergone a sham or CLP procedure. *Il-12* (-0.463), *Tnfα* (-0.420), *Il-10* (-0.322), and *Il-6* (0.357) emerged as the most important determinants for identifying mice receiving IAP treatment (Figure 5a). The cytokine profiles of 79.2% of mice that

underwent a CLP procedure were sufficiently distinct to differentiate IAP-treated from vehicle-treated mice, indicating an effect of IAP on intestinal inflammation.

At the protein level, elevated cytokine levels of IL-1ß (+88.14%, p=0.001), IL-6 (+62.51%, p<0.001), and TNFα (+72.98%, p<0.001) were observed after the induction of sepsis (Table 3). On the other hand, no effects on IL-10 and IL-12 were seen. IAP had no effect on cytokine levels at the protein level.

IAP activity and IAP gene expression

Following the induction of sepsis, total alkaline phosphatase activity at the terminal ileum increased significantly from 49 ±5 to 64 ±5 ng ml^{-1} (p=0.002) and further to 74 ±8 ng ml^{-1} after administration of IAP to septic mice (Figure 7). TNAP activity was limited in both sham groups but doubled in both CLP groups (p<0.001). Levels of TNAP were similar between both sham groups and the two CLP groups, therefore no effect of treatment with IAP was observed. IAP activity remained steady across all 4 groups, although the fraction of IAP in the total alkaline phosphatase activity decreased due to the increase in TNAP activity. The IAP activity slightly increased in the IAP-treated CLP group, but this effect was not statistically significant.

In contrast to the IAP activity, gene expression of *Akp3* and *Alpi* decreased 5-fold (p<0.001) and 2-fold (p<0.001) following the CLP procedure, but was not affected by IAP treatment (Table 2). Gene expression of TNAP, encoded by the *Alpl* gene, did not demonstrate any difference between any of the 4 treatment groups (Table 2; supporting information – Figure 2).

Discussion

Sepsis is a severe pathology associated with high morbidity and mortality[45, 46]. During sepsis, the gastrointestinal tract reinforces the already ongoing complex interplay between pro- and anti-inflammatory mediators. Our results demonstrate increased intestinal permeability, a significantly

higher occurrence of bacterial translocation and modified gene expression of cytokines and tight junction proteins following the induction of sepsis by a CLP procedure. These changes are in accordance with earlier observations in the literature and suggest the dissemination of pathogens to the entire body through the vascular and lymphatic systems, in response to an initial proinflammatory trigger[47, 48]. The complexity of the inflammatory response to sepsis seen in humans, which is typically characterized by a dynamic pattern of concomitantly elevated levels of both pro- and anti-inflammatory mediators, is also reflected in our model.

After the administration of IAP to septic mice in a combined preventive and curative set-up, the increased permeability of the small intestine for FITC-dextran was reversed by almost 50% compared to the vehicle-treated septic mice. Additionally, IAP administration enhanced the gene expression of IL-12 in the intestine of control and septic mice. IL-12 is a proinflammatory cytokine involved in the response to microbiota and microbial products by stimulating the production of other cytokines and enhancing the cytotoxic response of natural killer cells and T-lymphocytes[49]. Our results further revealed increased mRNA expression levels of *claudin-1* and *claudin-14* following IAP administration. While claudin-1 has been recognized as a main structural tight junction protein in the intestine, the link between claudin-14 and intestinal permeability is unclear[50]. Recently, claudin-14 has been associated with vascular integrity and its expression in the intestine could be influenced by a probiotic *E. coli* strain[51, 52]. A link between IAP and claudin-14, however, has not been described to date. We propose that the enhanced expression of intestinal claudin-1 and claudin-14 may underlie the beneficial effect of IAP on intestinal permeability to FITC dextran.

However, the beneficial effect of IAP on intestinal permeability appeared to be caused not only by changes in the gene expression of *Il-12*, *claudin-1,* and *claudin-14*. Our results also showed that the mRNA levels of several other cytokines and tight junction proteins tended to be altered in IAP-treated mice. Although the differences in gene expression for many of these cytokines and tight junction proteins individually appear to be small, suggesting limited direct physiological consequences, we hypothesized that the CLP model and treatment with IAP presumably altered several mediators at the

same time. In order to observe these differences and to identify potential profiles linked with IAP, we clustered cytokines and tight junctions proteins using a discriminant analysis. This analysis attempts to assess whether combinations of tight junction proteins and cytokines selected for this study could predict group membership to the IAP treatment and sham/CLP status, and thus identify cytokine and tight junction protein profiles. This analysis clearly showed that the different groups in this study could be distinguished from one another following this approach. We could identify selective tight junctions (occludin, claudin-1, claudin-4, claudin-5 and claudin-14) and cytokines (TNFα, IL-1ß IL-6, IL-10 and IL-12) which together act as the main predictors for the presence of sepsis and for the beneficial effect of IAP. Our results, therefore, indicate that IAP treatment influenced the ongoing inflammatory profiles and had a multilevel impact on the intestinal barrier, rather than causing modifications in individual proteins as one main driving force for the barrier dysfunction.

Our experiments demonstrate that endogenous alkaline phosphatase activity was increased in the intestine during CLP-induced sepsis, which was mainly caused by an increase in TNAP activity. This increased total alkaline phosphatase activity is presumably beneficial during inflammation and sepsis, because of the ability of alkaline phosphatase to detoxify LPS. Although IAP activity did not significantly increase during sepsis, its activity remained very stable during sepsis. Remarkably, this is in contrast with the IAP gene expression (*Akp3* and *Alpi*), which was significantly lower following the CLP procedure, independent of IAP treatment. We hypothesized that this decreased gene expression of IAP-encoding genes could be related to a negative feedback system caused by the increased alkaline phosphatase activity. Yet, if this hypothesis was valid, we would expect that the gene expression would be further modified by IAP treatment, resulting in even lower expression levels in the IAP-treated animals. Instead, no differences were observed between both groups. More likely, the loss of IAP gene expression in the ileum could also be related to the downregulation of the IAP synthesis based on the reduced food intake during sepsis, as confirmed in our metabolic measurements. It is known that intestinal levels of IAP vary depending on the nutritional status and location in the gastrointestinal tract, with malnourishment and starvation lowering the synthesis of new IAP[53]. Finally, IAP gene

expression could also be reduced in the CLP groups because of sepsis-caused epithelial damage resulting in epithelial cells losing their ability to produce IAP.

Increased production elsewhere (e.g. more proximal) in the alimentary tract might help to preserve the IAP activity, compensating for the decreased gene expression at the terminal ileum. Also, this raised alkaline phosphatase activity appears not to be significantly influenced by exogenous IAP, as IAP activity was not significantly increased in IAP-treated septic mice compared to vehicle-treated septic mice. Moreover, because IAP is eliminated quite rapidly from the peritoneal cavity, it is unlikely that we would have observed any direct activity of the exogenous IAP, 12 hours after the last administration[27]. Therefore we have no evidence to support a direct effect of exogenous IAP on the endogenous IAP synthesis or activity.

The beneficial effect of IAP treatment on intestinal permeability during sepsis was not associated with a positive effect on clinical outcomes, nor did IAP therapy result in a statistically decreased bacterial translocation of enteropathogens to the mesenteric lymph nodes, liver tissue, blood or abdominal cavity. In contrast to our results, IAP has been shown to reduce bacterial translocation in multiple studies with different experimental models. One study, using an IAP knockout model, demonstrated a higher incidence of spontaneous bacterial translocation to the mesenteric lymph nodes and bacterial counts tended to be higher when IAP was absent in the intestinal mucosa[19]. Similarly, a higher tendency for bacterial translocation to the liver and lungs was demonstrated in a model of peritonitis, which was induced by injection of abdominal lavage fluid coming from mice with a CLP-induced sepsis[54]. In that study, bacterial translocation could be reduced with respectively 65% and 79% following oral IAP administration in a similar dose, but without the preventive administration[54]. The reason for the discrepancies between the results of these studies and our results is presumably multifactorial. Firstly, these models are characterized by different underlying pathophysiological mechanisms (e.g. ischemia-reperfusion and peritonitis), in which the trigger for the sepsis is not continuously present. Additionally, bacterial translocation in our study was measured two days after the induction of sepsis, at which time point the exogenously administered IAP could still be insufficient

to prevent virulent enteropathogens from migrating through the already severely impaired intestinal barrier.

Some limitations and assumptions of our study design should be considered. First, we opted to administer IAP in a combined preventive and curative set-up as a maximal treatment strategy, although it might be clinically more relevant to study the curative administration of IAP only. We also chose to administer IAP intraperitoneally, while oral administration might be more ideal, allowing it to directly interfere with endoluminal pathogens and the LPS they produce. Unfortunately, during abdominal sepsis, and also in our murine model, efficient oral distribution of IAP to the different bowel segments was prevented by paralytic ileus. In addition, IAP was administered at only 4 time points, a period of treatment that might have been insufficiently long enough to significantly alter the gene expression of cytokines and tight junctions or to invoke beneficial effects on clinical outcomes. Finally, because our experiments were focused on intestinal permeability, bacterial translocation, and intestinal inflammation, we did not investigate the influence of IAP on 7-14 day mortality and survival. However, mortality in our CLP model has been extensively studied at our lab and can be limited by providing sufficient fluid resuscitation, as has been done in this study[36]. Additionally, a beneficial effect of IAP when administered intraperitoneally has already been demonstrated in the study by Ebrahimi et al.[27].

In conclusion, IAP has been demonstrated to be a promising detoxifying molecule against LPS produced by enteropathogens. Our results show an ameliorative effect of IAP on the increased intestinal permeability following CLP-induced sepsis. This is associated with a direct beneficial effect of IAP treatment on the gene expression of *Il-12*, *claudin*-1 and *claudin-14*, but was also related to changes in the expression of several other cytokines and tight junction proteins. Therefore, this study demonstrates the ability of IAP to interfere with the intestinal barrier during sepsis.

Acknowledgements

The authors wish to thank Petra Aerts, Dominique De Rijck, Ilse Goolaerts, Kitty Gys, Angelika Jürgens, Christine Lammens, Rita Van Den Bossche, Marleen Vinckx, and Lieve Vits for their assistance with the experiments and histological analysis.

Authors' contributions

Conception and design of the research study: PP, BDW, JDM, SN; Statistical Analysis of the data: PP; Data collection: PP, IP; Supervision: BDW, JDM, AS, GH, PJ, JPT; Providing the resources and materials: BDW, JDM, JPT; Visualization of the data: PP, IP, JPT; Drafting of the manuscript: PP, BDW, JDM; Critical revision and editing of the manuscript: BDW, JDM, AS, PJ, GH, SN, JPT, IP, PP; Final approval of the version submitted: PP, BDW, JDM, AS, PJ, GH, JPT, IP, JPT.

Conflict of Interest Statement

Competing Interests: the authors have no competing interests

Funding

This work was supported by a grant from the Flemish Institute for Research (FWO, grant number G.028615N to BDW) and a grant of the University of Antwerp (TOP grant, Grant Number PS ID Antigoon 35018, 2018-2021 to BDW). The Leica SP 8 (Hercules grant AUHA.15.12) microscope was funded by the Hercules Foundation of the Flemish Government.

Conference presentations

A part of this work was presented at the 2017 Neurogastroenterology meeting organized by the European Society of Neurogastroenterology and Motility (Cork, Ireland), and the abstract was published as Plaeke P et al., Abstract 7, Neurogastroenterology and Motility August 2017; Volume 29, supplement 2[55].

References

1. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent J-L. Sepsis and septic shock. *Nature Reviews Disease Primers*. 2016;2:16045.

2. Howell MD, Davis AM. Management of sepsis and septic shock. *JAMA*. 2017;317(8):847-8.

3. Deitch EA. Bacterial translocation or lymphatic drainage of toxic products from the gut: What is important in human beings? *Surgery*.131(3):241-4.

4. Fawley J, Gourlay DM. Intestinal alkaline phosphatase: a summary of its role in clinical disease. *J Surg Res*. 2016;202(1):225-34.

5. Akiba Y, Mizumori M, Guth PH, Engel E, Kaunitz JD. Duodenal brush border intestinal alkaline phosphatase activity affects bicarbonate secretion in rats. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(6):G1223-33.

6. Mizumori M, Ham M, Guth PH, Engel E, Kaunitz JD, Akiba Y. Intestinal alkaline phosphatase regulates protective surface microclimate pH in rat duodenum. *J Physiol*. 2009;587(Pt 14):3651-63.

7. Economopoulos KP, Ward NL, Phillips CD, Teshager A, Patel P, Mohamed MM, Hakimian S, Cox SB, Ahmed R, Moaven O, Kaliannan K, Alam SN, Haller JF, Goldstein AM, Bhan AK, Malo MS, Hodin RA. Prevention of antibiotic-associated metabolic syndrome in mice by intestinal alkaline phosphatase. *Diabetes, obesity & metabolism*. 2016;18(5):519-27.

8. Narisawa S, Huang L, Iwasaki A, Hasegawa H, Alpers DH, Millan JL. Accelerated fat absorption in intestinal alkaline phosphatase knockout mice. *Mol Cell Biol*. 2003;23(21):7525-30.

9. Nakano T, Inoue I, Koyama I, Kanazawa K, Nakamura K, Narisawa S, Tanaka K, Akita M, Masuyama T, Seo M, Hokari S, Katayama S, Alpers DH, Millan JL, Komoda T. Disruption of the murine intestinal alkaline phosphatase gene Akp3 impairs lipid transcytosis and induces visceral fat accumulation and hepatic steatosis. *Am J Physiol Gastrointest Liver Physiol*. 2007;292(5):G1439-49.

10. Kaliannan K, Hamarneh SR, Economopoulos KP, Nasrin Alam S, Moaven O, Patel P, Malo NS, Ray M, Abtahi SM, Muhammad N, Raychowdhury A, Teshager A, Mohamed MM, Moss AK, Ahmed R, Hakimian S, Narisawa S, Millan JL, Hohmann E, Warren HS, Bhan AK, Malo MS, Hodin RA. Intestinal

alkaline phosphatase prevents metabolic syndrome in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(17):7003-8.

11. Malo MS. A high level of intestinal alkaline phosphatase is protective against type 2 diabetes mellitus irrespective of obesity. *EBioMedicine*. 2015;2(12):2016-23.

12. Lalles JP. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr Rev*. 2010;68(6):323-32.

13. Lallès J-P. Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. *Reviews in Aquaculture*. 2019;0(0).

14. Lalles JP. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutrition reviews*. 2014;72(2):82-94.

15. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*. 1999;274(16):10689-92.

16. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. 2008;42(2):145-51.

17. Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe*. 2007;2(6):371-82.

18. Bentala H, Verweij WR, Huizinga-Van der Vlag A, van Loenen-Weemaes AM, Meijer DK, Poelstra K. Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. *Shock*. 2002;18(6):561-6.

19. Goldberg RF, Austen WG, Jr., Zhang X, Munene G, Mostafa G, Biswas S, McCormack M, Eberlin KR, Nguyen JT, Tatlidede HS, Warren HS, Narisawa S, Millan JL, Hodin RA. Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(9):3551-6.

20. Koyama I, Matsunaga T, Harada T, Hokari S, Komoda T. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clinical biochemistry*. 2002;35(6):455-61.

21. Poelstra K, Bakker WW, Klok PA, Kamps JA, Hardonk MJ, Meijer DK. Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol*. 1997;151(4):1163-9.

22. Malo MS, Alam SN, Mostafa G, Zeller SJ, Johnson PV, Mohammad N, Chen KT, Moss AK, Ramasamy S, Faruqui A, Hodin S, Malo PS, Ebrahimi F, Biswas B, Narisawa S, Millan JL, Warren HS, Kaplan JB, Kitts CL, Hohmann EL, Hodin RA. Intestinal alkaline phosphatase preserves the normal homeostasis of gut microbiota. *Gut*. 2010;59(11):1476-84.

23. Liu W, Hu D, Huo H, Zhang W, Adiliaghdam F, Morrison S, Ramirez JM, Gul SS, Hamarneh SR, Hodin RA. Intestinal alkaline phosphatase regulates tight junction protein levels. *J Am Coll Surg*. 2016;222(6):1009-17.

24. Chen KT, Malo MS, Beasley-Topliffe LK, Poelstra K, Millan JL, Mostafa G, Alam SN, Ramasamy S, Warren HS, Hohmann EL, Hodin RA. A role for intestinal alkaline phosphatase in the maintenance of local gut immunity. *Digestive diseases and sciences*. 2011;56(4):1020-7.

25. Yang Y, Millan JL, Mecsas J, Guillemin K. Intestinal alkaline phosphatase deficiency leads to lipopolysaccharide desensitization and faster weight gain. *Infection and immunity*. 2015;83(1):247-58.

26. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *The Journal of pharmacology and experimental therapeutics*. 2003;307(2):737-44.

27. Ebrahimi F, Malo MS, Alam SN, Moss AK, Yammine H, Ramasamy S, Biswas B, Chen KT, Muhammad N, Mostafa G, Warren HS, Hohmann EL, Hodin RA. Local peritoneal irrigation with intestinal alkaline phosphatase is protective against peritonitis in mice. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract*. 2011;15(5):860-9.

28. Su F, Brands R, Wang Z, Verdant C, Bruhn A, Cai Y, Raaben W, Wulferink M, Vincent JL. Beneficial effects of alkaline phosphatase in septic shock. *Critical care medicine*. 2006;34(8):2182-7.

29. van Veen SQ, van Vliet AK, Wulferink M, Brands R, Boermeester MA, van Gulik TM. Bovine intestinal alkaline phosphatase attenuates the inflammatory response in secondary peritonitis in mice. *Infection and immunity*. 2005;73(7):4309-14.

30. Riggle KM, Rentea RM, Welak SR, Pritchard KA, Jr., Oldham KT, Gourlay DM. Intestinal alkaline phosphatase prevents the systemic inflammatory response associated with necrotizing enterocolitis. *The Journal of surgical research*. 2013;180(1):21-6.

31. Mohamed M, Economopoulos KP, Patel P, Muhammad N, Moaven O, Moss AK, Hamarneh SR, Teshager A, Kaliannan K, Abtahi SM, Alam SN, Malo NS, Tao Q, Malo MS, Hodin RA. Tu1620 Intestinal alkaline phosphatase is an endogenous anti-inflammatory factor. *Gastroenterology*.144(5):S-808.

32. Bender B, Baranyi M, Kerekes A, Bodrogi L, Brands R, Uhrin P, Bosze Z. Recombinant human tissue non-specific alkaline phosphatase successfully counteracts lipopolysaccharide induced sepsis in mice. *Physiological research*. 2015;64(5):731-8.

33. Pickkers P, Heemskerk S, Schouten J, Laterre P-F, Vincent J-L, Beishuizen A, Jorens PG, Spapen H, Bulitta M, Peters WHM, van der Hoeven JG. Alkaline phosphatase for treatment of sepsis-induced acute kidney injury: a prospective randomized double-blind placebo-controlled trial. *Critical Care*. 2012;16(1):R14-R.

34. Heemskerk S, Masereeuw R, Moesker O, Bouw MP, van der Hoeven JG, Peters WH, Russel FG, Pickkers P. Alkaline phosphatase treatment improves renal function in severe sepsis or septic shock patients. *Critical care medicine*. 2009;37(2):417-23, e1.

35. Pickkers P, Mehta RL, Murray PT, Joannidis M, Molitoris BA, Kellum JA, Bachler M, Hoste EAJ, Hoiting O, Krell K, Ostermann M, Rozendaal W, Valkonen M, Brealey D, Beishuizen A, Meziani F, Murugan R, de Geus H, Payen D, van den Berg E, Arend J, Investigators S-A. Effect of human recombinant alkaline phosphatase on 7-day creatinine clearance in patients with sepsis-associated acute kidney injury: A Randomized Clinical Trial. *JAMA*. 2018;320(19):1998-2009.

36. Nullens S, De Man J, Bridts C, Ebo D, Francque S, De Winter B. Identifying therapeutic targets for sepsis research: a characterization study of the inflammatory players in the cecal ligation and puncture model. *Mediators of inflammation*. 2018;2018:5130463.

37. Nullens S, Staessens M, Peleman C, Plaeke P, Malhotra-Kumar S, Francque S, De Man JG, De Winter BY. Beneficial effects of anti-interleukin-6 antibodies on impaired gastrointestinal motility, inflammation and increased colonic permeability in a murine model of sepsis are most pronounced when administered in a preventive setup. *PLoS ONE*. 2016;11(4):e0152914.

38. Mei J, Riedel N, Grittner U, Endres M, Banneke S, Emmrich JV. Body temperature measurement in mice during acute illness: implantable temperature transponder versus surface infrared thermometry. *Scientific Reports*. 2018;8(1):3526.

39. Osuchowski MF, Ayala A, Bahrami S, Bauer M, Boros M, Cavaillon J-M, Chaudry IH, Coopersmith CM, Deutschman CS, Drechsler S, Efron P, Frostell C, Fritsch G, Gozdzik W, Hellman J, Huber-Lang M, Inoue S, Knapp S, Kozlov AV, Libert C, Marshall JC, Moldawer LL, Radermacher P, Redl H, Remick DG, Singer M, Thiemermann C, Wang P, Wiersinga WJ, Xiao X, Zingarelli B. Minimum Quality Threshold in Pre-Clinical Sepsis Studies (Mqtipss): An international expert consensus initiative for improvement of animal modeling in sepsis. *Shock*. 2018;50(4):377-80.

40. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol*. 2007;8(2):R19-R.

41. Joly Condette C, Elion Dzon B, Hamdad F, Biendo M, Bach V, Khorsi-Cauet H. Use of molecular typing to investigate bacterial translocation from the intestinal tract of chlorpyrifos-exposed rats. *Gut Pathog*. 2016;8:50-.

42. Yang Y, Wandler AM, Postlethwait JH, Guillemin K. Dynamic evolution of the LPS-detoxifying enzyme intestinal alkaline phosphatase in zebrafish and other vertebrates. *Frontiers in immunology*. 2012;3:314-.

43. Yang Y, Millán JL, Mecsas J, Guillemin K. Intestinal alkaline phosphatase deficiency leads to lipopolysaccharide desensitization and faster weight gain. *Infection and immunity*. 2015;83(1):247-58.

44. Matsushita M, Irino T, Kamiyama K, Muramoto Y, Kawaguchi T, Nakano T, Komoda T. Evaluation of a method for measuring tissue non-specific alkaline phosphatase activity in healthy subjects. *Annals of clinical biochemistry*. 2007;44(Pt 6):544-8.

45. Mayr FB, Yende S, Angus DC. Epidemiology of severe sepsis. *Virulence*. 2014;5(1):4-11.

46. Angus DC, Wax RS. Epidemiology of sepsis: an update. *Critical care medicine*. 2001;29(7 Suppl):S109-16.

47. Yoseph BP, Klingensmith NJ, Liang Z, Breed ER, Burd EM, Mittal R, Dominguez JA, Petrie B, Ford ML, Coopersmith CM. Mechanisms of intestinal barrier dysfunction in sepsis. *Shock*. 2016;46(1):52-9.

48. de Jong PR, González-Navajas JM, Jansen NJG. The digestive tract as the origin of systemic inflammation. *Critical Care*. 2016;20(1):279.

49. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual review of immunology*. 1995;13:251-76.

50. Lu Z, Ding L, Lu Q, Chen YH. Claudins in intestines: Distribution and functional significance in health and diseases. *Tissue barriers*. 2013;1(3):e24978.

51. Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke J-D, Serino M, Tilg H, Watson A, Wells JM. Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology*. 2014;14(1):189.

52. Baker M, Reynolds LE, Robinson SD, Lees DM, Parsons M, Elia G, Hodivala-Dilke K. Stromal Claudin14-heterozygosity, but not deletion, increases tumour blood leakage without affecting tumour growth. *PLoS ONE*. 2013;8(5):e62516.

53. Hamarneh SR, Mohamed MMR, Economopoulos KP, Morrison SA, Phupitakphol T, Tantillo TJ, Gul SS, Gharedaghi MH, Tao Q, Kaliannan K, Narisawa S, Millán JL, van der Wilden GM, Fagenholz PJ, Malo

MS, Hodin RA. A novel approach to maintain gut mucosal integrity using an oral enzyme supplement. *Annals of Surgery*. 2014;260(4):706-15.

54. Wang W, Chen S-W, Zhu J, Zuo S, Ma Y-Y, Chen Z-Y, Zhang J-L, Chen G-W, Liu Y-C, Wang P-Y. Intestinal alkaline phosphatase inhibits the translocation of bacteria of gut-origin in mice with peritonitis: mechanism of action. *PLoS One*. 2015;10(5):e0124835-e.

55. Plaeke P, De Man J, Gys K, Lammens C, Malhotra S, Jorens P, Hubens G, De Winter B. Conference abstratct - Effects of intestinal alkaline phosphatase on intestinal permeability and bacterial translocation in an experimental model for sepsis. *Neurogastroenterology & Motility*. 2017;29(Supplement 2):5-6.

Tables

Table 1 – Sepsis and IAP treatment induced changes in metabolic parameters.

Groups (n=5/group):	Sham + Vehicle	Sham + IAP	CLP + Vehicle	CLP + IAP
Metabolic parameter				
Food Intake (g/24 hrs)	2.85 ±0.82	3.77 ±0.46	0.70 ±0.30*[a]	1.10 ±0.40*[a]
Water intake (g/24hrs)	3.44 ±1.00	3.98 ±0.63	1.49 ±0.37*	1.62 ±0.39*
Urine output (g/24hrs)	1.88 ±0.13	1.51 ±0.41	0.61 ±0.08*	0.92 ±0.13*
Stool production (g/24 hrs)	1.28 ±0.36	2.06 ±0.36	0.32 ±0.15*	0.62 ±0.24*
Core body temperature (°C)	37.00 ±0.48	35.92 ±0.58	36.10 ±0.47	35.70 ±0.37

Summary of the food and water intake and output as observed in 20 OF1 mice, housed in metabolic cages. Core body temperature was measured in the space between the liver and diaphragm. Results are displayed as the input/output in grams over a time span of 24 hours ±standard error of the mean. Statistical analysis was performed with two-way ANOVA . * indicates a significant effect ($p<0.05$) of CLP. [a]$p<0.001$. No significant effects of treatment with IAP on metabolic parameters were observed.

Table 2 – Effects of the CLP procedure and treatment with IAP on the gene expression of inflammatory cytokines and tight junction proteins.

Gene (Protein)	Sham + Vehicle	Sham + IAP	CLP + Vehicle	CLP + IAP
Tight junction proteins				
Cldn1 (Claudin-1)	1.27 ±0.16	1.55 ±0.23#	1.34 ±0.28	2.42 ±0.43#
Cldn2 (Claudin-2)	1.14 ±0.14	1.17 ±0.12	0.79 ±0.08*	0.84 ±0.12*
Cldn3 (Claudin-3)	1.04 ±0.06	1.06 ±0.04	0.91 ±0.05	0.98 ±0.06
Cldn4 (Claudin-4)	1.01 ±0.06	1.10 ±0.05	0.91 ±0.08*	0.98 ±0.06*
Cldn5 (Claudin-5)	1.05 ±0.11	1.25 ±0.10	0.86 ±0.12*	0.88 ±0.13*
Cldn7 (Claudin-7)	1.07 ±0.06	1.07 ±0.05	1.05 ±0.04	1.06 ±0.07
Cldn14 (Claudin-14)	1.13 ±0.24	2.37 ±0.84#	2.53 ±0.43*	5.28 ±1.56#*
Cldn18 (Claudin-18)	1.72 ±0.66	6.02 ±3.34	8.90 ±4.43*	14.45 ±4.97*
Ocln (Occludin)	1.22 ±0.17	1.26 ±0.19	0.68 ±0.10*	0.69 ±0.10*
Tjp1 (ZO1)	1.02 ±0.05	1.11 ±0.02	1.05 ±0.04	1.07 ±0.05
Tjp3 (ZO3)	1.05 ±0.06	1.03 ±0.03	1.08 ±0.05	1.14 ±0.07
Inflammatory Cytokines				
Il1b (IL-1ß)	1.19 ±0.19	1.15 ±0.23	10.28 ±3.73*	7.45 ±2.35*
Il6 (IL-6)	1.27 ±0.27	1.36 ±0.29	8.84 ±3.26*	5.68 ±1.56*
Cxcl15 (IL-8)	1.31 ±0.18	1.52 ±0.35	1.01 ±0.20*	0.77 ±0.19*
Il10 (IL-10)	1.09 ±0.15	1.06 ±014	1.80 ±0.21*	2.29 ±0.34*
Il12a (IL-12)	2.11 ±0.52	2.27 ±0.48#	1.14 ±0.20	2.70 ±0.48#
Il18 (IL-18)	1.08 ±0.11	1.13 ±0.11	3.07 ±0.32*	2.91 ±0.33*
Tnf (TNFα)	1.17 ±0.19	1.17 ±0.13	2.03 ±0.30*	2.85 ±0.47*
Intestinal Alkaline Phosphatase encoding genes				
Akp3	2.60 ±0.40	3.24 ±0.46	0.50 ±0.09*	0.65 ±0.10*
Alpi	1.72 ±0.15	1.95 ±0.17	0.80 ±0.16*	0.99 ±0.15*
Alpl	1.00 ±0.14	1.05 ±0.12	1.05 ±0.14	1.11 ±0.14

Analysis of mRNA levels after quantification with rt PCR on terminal ileum samples. The expression of each gene was normalized using QBase plus and expressed as a CNRQ value, with *Gapdh*, *18s rRna* and *Hprt1* as housekeeping genes. For a list of the used primers, see Supplementary Table 1. Statistical analysis was performed with two-way ANOVA followed by one-way ANOVA and LSD post-hoc testing when appropriate. Results are displayed as the mean relative gene expression ± standard error of the mean (SEM). N= 12 mice/group. * indicates a significant effect ($p<0.05$) of CLP. # indicates a significant effect ($p<0.05$) of IAP.

Table 3 – Effects of the CLP procedure and treatment with IAP on the synthesis of inflammatory cytokines at the protein level.

Protein	Sham + Vehicle	Sham + IAP	CLP + Vehicle	CLP + IAP
Cytokines (pg ml^{-1})				
IL-1ß	6.46 ±1.00	8.35 ±2.05	54.48 ±23.06*	44.06 ±14.07*
IL-6	5.06 ±0.66	12.38 ±5.88	218.22 ±81.82*	137.26 ±33.77*
IL-10	3.21 ±0.44	3.68 ±0.75	4.12 ±0.85	4.23 ±0.88
IL-12	1.62 ±0.26	1.19 ±0.23	1.62 ±0.29	1.31 ±0.31
TNFα	7.49 ±0.60	7.47 ±1.06	28.53 ±7.71*	21.43 ±4.99*

Analysis of inflammatory cytokines at the protein level in the small intestine as determined with Cytometric Bead Array. Statistical analysis was performed with two-way ANOVA followed by one-way ANOVA and LSD post-hoc testing when appropriate. Results are displayed as the concentration of cytokine (pg ml^{-1}) ± standard error of the mean (SEM). N= 12 mice/group. * indicates a significant effect ($p<0.05$) of CLP. No significant effects of treatment with IAP on cytokine levels were detected.

Figure Legends

Figure 1 – Experimental Protocol

Overview of the experimental protocol. Mice received IAP or vehicle starting 5 min prior to the onset of the CLP or sham procedure and this therapy was repeated after the procedure every 12 hours for 3 consecutive times. Throughout the duration of the experiment, mice were followed clinically using a validated clinical disease score and by monitoring the body weight on days 1, 2 and 3. On the third day, *in vivo* intestinal permeability, bacterial translocation, mRNA and protein levels of cytokines and tight junction proteins were determined.

Figure 2 - Effects of the CLP procedure and treatment with IAP 1 IU g^{-1} on clinical outcomes.

Effects of treatment with IAP on clinical disease scores and weight loss in mice undergoing a CLP or sham procedure. Clinical disease scores (A) are displayed as the absolute scores. The majority of the mice that underwent sham surgery scored zero. In contrast, the CLP procedure caused significantly higher sepsis-related symptoms. Weight loss (B) is represented as the % weight loss compared to baseline, which was measured shortly before sham/CLP surgery. CLP caused a significant decrease in body weight compared to sham, but without an effect of treatment with IAP. N= 12 mice/group. *indicates $p<0.05$ between CLP groups and the matching sham groups.

Figure 3 – Effects of the CLP procedure and treatment with IAP 1 IU g^{-1} on intestinal permeability measured *in vivo* in the small intestine.

Effects of the CLP procedure and treatment with IAP on small intestinal permeability measured with 4 kDa FITC-Dextran. Data are presented as mean relative serum FITC concentrations ± SEM with the vehicle-treated sham group acting as the reference group. Statistical analysis with two-way Analysis of Variance and LSD post-hoc testing. N= 12 mice/group. * indicates significant difference ($p<0.05$)

from the respective sham group. # indicates significant difference ($p<0.05$) from the CLP + Vehicle group.

Figure 4 – Immunofluorescent microscopy for Claudin-1 in terminal ileum samples.

Immunofluorescent staining of claudin-1 on the apical epithelium of the terminal ileum in the sham + vehicle (a), sham + IAP (b) and CLP + vehicle (c) groups. The CLP procedure had a very mild influence on the morphology of the intestinal epithelium, as seen in the CLP + vehicle (c) group. A moderately but visibly higher staining of claudin-1 at the apical epithelial surface was observed in the IAP-treated CLP group (Figure 4d) compared to the vehicle-treated CLP group. Images were taken on a Leica TCS SP8 confocal laser scanning microscope (Leica-microsystems, Wetzlar, Germany) using a White Light Laser at 20x magnification. Scale bars = 100 µm, L= Intestinal lumen.

Figure 5 – Discriminant analysis of gene expression of cytokines and tight junction proteins

Discriminant analysis used to predict classification into the different treatment groups (sham/CLP & Vehicle or IAP) in a multivariate matter, based on modifications in the gene expression of cytokines and tight junction proteins. Dots represent individual mice. The group centroid is the average of each group projected on function 1 (x-axis) and function 2 (y-axis).

Graph A demonstrates the ability to differentiate mice belonging to the different groups based on altered cytokine expression. *IL-1ß, -6, -8, -10, -12, -18*, and *TNFα* were included in this analysis. Function 1 (horizontal axis) mainly consists of predictors for discrimination between sham and CLP. In this function, *IL-18*, *IL-1ß* and *IL-10* were the most predictive and hence most altered cytokines as a result of sepsis. Function 2 (vertical axis) mainly provides predictors for discriminating between vehicle/IAP treatment. This function was mostly influenced by *IL-12, IL-6* and *TNFα*. Nevertheless, *TNFα, IL-6, IL-10* and *IL-1ß* were significant predictors in both functions. Cytokine profiles could not

distinguish sham groups from each other. Overall, 62.5% of mice were classified correctly. This percentage increased to 79.2% in the CLP groups.

Similarly graph B demonstrates whether groups could be identified based on modified gene expression of tight junction proteins (*claudin-1, -2, -3, -4, -5, -7, -14, -18; ZO-1, ZO-3,* and *occludin*). Function 1 (horizontal axis) depended mainly on *claudin-4*, *claudin-14* and *claudin-1*, while *claudin-5, occludin* and *claudin-2* were the most important predictors in function 2 (vertical axis). However, most of these tight junction proteins were instrumental in differentiating both sham from CLP and vehicle from IAP, indicating that sepsis and IAP influenced multiple tight junction proteins.

Figure 6 – Bacterial translocation of enteropathogens to the mesenteric lymph nodes, blood, liver and abdominal cavity.

Translocation of gastrointestinal tract derived enteropathogens to the mesenteric lymph nodes (A), blood (B), liver (C), and abdominal cavity (D), demonstrating the effect of CLP-induced sepsis and treatment with IAP on bacterial translocation. Results are expressed as the % of positive cultures, as determined with MALDI-TOF after enrichment. The absolute number of positive cultures is displayed below each group. Enteropathogens considered in the analysis: *Escherichia coli, Enterococcus faecalis, Enterococcus cloacae, Enterococcus gallinarum, Enterococcus Hirae*. Statistical analysis with a chi-square test comparing CLP groups to the corresponding controls and logistic regression for additional analyses. N= 12 mice/group. * indicates $p<0.05$.

Figure 7 – Total alkaline phosphatase activity, tissue-nonspecific alkaline phosphatase activity (TNAP) and IAP activity in terminal ileum samples.

AP, Alkaline Phosphatase; TNAP, Tissue-Nonspecific Alkaline Phosphatase; IAP, Intestinal Alkaline Phosphatase. Overview of the total alkaline phosphatase activity, TNAP and IAP activity in terminal

ileum samples. The graph displays the total alkaline phosphatase activity (blue dashed line), composed of a fraction IAP and TNAP. Fractions represent the percentage of total AP which is IAP (fraction IAP) or TNAP (fraction TNAP). Fractions and absolute activity levels are displayed below the graph.

CLP-induced sepsis causes a significant increase in total alkaline phosphatase activity and TNAP activity. Although the fraction of IAP decreased, the absolute amount of IAP activity remained stable, or even increased in the IAP-treated CLP group. Data presented as the activity measured in ng ml-1 ±Standard error of the Mean. Statistical analysis with two-way Analysis of Variance. N= 12 mice/group. *indicates significant difference ($p<0.05$) from the respective sham group.

Figure Legends for the supporting information

Supporting Figure 1 - Correlation between culture results and intestinal permeability (determined with 4 kDa FITC-dextran.

Point-biserial correlation (r_{pb}) analysis of the FITC-Dextran concentration (logistic transformation) with culture results of mesenteric lymph nodes, liver tissue, blood and peritoneal lavage fluid. Positive culture results were correlated with higher FITC-Dextran concentrations, except for the hemoculture results. r_{pb} = correlation coefficient for the point biserial correlation analysis.

Supporting Figure 2 – Gene expression of *Alpl*, the gene encoding murine tissue-nonspecific alkaline phosphatase (TNAP)

Gene expression of *Alpl*, the gene encoding tissue-nonspecific alkaline phosphatase, in the ileum. Data represents the relative gene expression (CNRQ), normalized to *Gapdh*, *18s rRNA*, *Hprt1* housekeeping genes. No significant differences were observed between the 4 study groups. Two-way ANOVA was used for the statistical analysis.