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The lipopolysaccharide structure affects the detoxifying ability of intestinal alkaline phosphatases

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Abstract

Lipopolysaccharide (LPS) is one of the most potent mediators of inflammation. In swine husbandry, weaning is associated with LPS-induced intestinal inflammation, resulting in decreased growth rates due to malabsorption of nutrients by the inflamed gut. A potential strategy to treat LPS-mediated disease is administering intestinal alkaline phosphatase (IAP). The latter can detoxify lipid A, the toxic component of LPS, by removal of phosphate groups. Currently, 183 LPS O-serotypes from *E. coli* have been described, however, comparative experiments to elucidate functional differences between LPS serotypes are scarce. In addition, these functional differences might affect the efficacy of LPS detoxifying enzymes. Here, we evaluated the ability of four LPS serotypes (O26:B6, O55:B5, O111:B4 and O127:B8) derived from *Escherichia coli* to trigger the secretion of pro-inflammatory cytokines by porcine PBMCs. We also tested the ability of three commercially available IAPs to detoxify these LPS serotypes. The results show that LPS serotypes differ in their ability to trigger cytokine secretion by immune cells, especially at lower concentrations. Moreover, IAPs displayed a different detoxification efficiency of the tested serotypes. Together, this study sheds light on the impact of LPS structure on the detoxification by IAPs. Further research is however needed to elucidate the LPS serotype-specific effects and their implications for the development of novel treatment options to alleviate LPS-induced gut inflammation in weaned piglets.

Keywords Lipopolysaccharides, Alkaline phosphatases, Intestine, Detoxification

Background

Weaning represents a critical and stressful period of life, which can perturb the complex network of epithelial cells and immune cells present within the gut. These perturbations impair gut function and immunity, resulting in a growth lag of the piglets [1]. A key factor in this post-weaning growth lag is the underdeveloped gut due to early weaning, leading to a reduced ability of piglets to digest nutrients and raise appropriate immune responses. This immature gut is susceptible to gut inflammation. Many bacterial components such as flagellin, bacterial DNA or peptidoglycans can play a role in the development of intestinal inflammation [2]. However, one of the most important mediators of gut inflammation is lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria [3, 4]. For example, intraperitoneal injection of LPS to post-weaning piglets leads to decreases in daily weight gain and feed intake [5–7]. Therefore, research into novel treatments to prevent or alleviate LPS-mediated intestinal inflammation is crucial to maintain gut health of piglets.

LPS is a strongly immunogenic molecule consisting of three regions: lipid A, the core oligosaccharide and the

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O-antigen. The former two regions are quite conserved and are necessary for survival of the bacteria, whereas the O-antigen is a repeating oligosaccharide chain which contains most of the variation among different bacterial species. It forms the basis for the O-serotype classification of *Escherichia coli* [8]. LPS is toxic to most animals and is frequently used to mimic intestinal inflammation. Its toxicity stems from the lipid A region which is recognized by the CD14/MD-2/TLR4 complex [9]. After dimerization, the intracellular domain of TLR4 activates two potential signalling cascades, either the myeloid differentiation factor 88 (MyD88) or TLR domain adaptor interferon-β inducing (TRIF) pathway, leading to the nuclear translocation of NF-κB, which initiates a potent immune response characterized by the production of inflammatory cytokines such as IL-6 and TNFα [10]. The two phosphoryl groups of lipid A are key for this recognition and toxicity, as dephosphorylation turns lipid A into a TLR4 antagonist [11, 12]. As such, enzymatic dephosphorylation of LPS as a detoxifying strategy has gained a lot of interest as a potential treatment of LPS-induced diseases.

Intestinal alkaline phosphatases (IAPs) are small intestinal brush border enzymes secreted by enterocytes into the intestinal lumen and its surrounding blood vessels. IAPs function as regulators of lipid absorption and gut homeostasis by controlling mucosal inflammation through detoxification of bacterial molecules such as LPS and flagellin through dephosphorylation [13, 14]. Recent studies have focussed on IAP as a potential treatment for LPS-mediated inflammatory diseases such as ulcerative colitis and sepsis [15–18].

Although many studies have focussed on LPS-mediated diseases, not much is known about the effect of different serotypes on immune cell activation. A recent study showed that human neutrophils responded to LPS O128:B12 but not to the other four tested *E. coli*-derived LPS serotypes by releasing neutrophil extracellular traps (NETs) [19]. It is often speculated that variable responses are linked to differences in the used LPS serotypes and hence linked to structural differences, though research on this topic is scarce [20–25]. While the structure of the lipid A moiety and the core oligosaccharide is well known, the exact structure of each described serotype is difficult to elucidate due to the large variation of the O-antigen. Furthermore, many modifications to lipid A have been described. Unchanged lipid A contains two phosphate groups and six acyl chains and strongly binds to TLR4. Potential modifications include transfer or deletion of acyl chains or the modification of phosphate groups. These alterations to the LPS structure are used by bacteria to avoid detection by the host immune system [26–28]. For example, it was recently shown that O-acylation of the phosphate groups of lipid A protected them from cleavage by calf IAP due to steric hindrance [29].

Due to the importance of LPS-induced inflammation in swine husbandry and since pig models are frequently used in the study of sepsis due to their similarities with human physiology and immune system [30–32], the present study aimed to evaluate the ability of different LPS serotypes to activate porcine blood mononuclear immune cells and whether these different serotypes are differently detoxified by IAP.

Materials and methods

Isolation of porcine peripheral blood mononuclear cells

Pigs were purchased from the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) and kept as blood donor in BSL-2 animal units (22.7 m^2) in an enriched environment. The units were cleaned daily. Animals had access to water and feed ad libitum and their general health was monitored on a daily basis. Circadian rythm was maintained with artificial light. At 36 weeks of age, the animals were euthananised by an intravenous injection of sodium pentobarbital 20% (60 mg/2.5 kg bodyweight, Kela) to collect tissues used in experiments unrelated to this study. Porcine peripheral blood mononuclear cells (PBMCs) were isolated as described [33]. In brief, blood was taken on heparin from the jugular vein of pigs, aged 11 to 36 weeks (mixed sex, Belgian land race). PBMCs were isolated by density gradient centrifugation using Lymphoprep (Axis-shield, Oslo, Norway). Erythrocytes were lysed in ammonium chloride solution. The resulting PBMC fraction was washed twice in ice cold PBS + 1 mM EDTA and the viability was confirmed by exclusion of the vital dye Trypan blue. PBMCs were cultured at 1×10^6 cells/well in a 96-well plate (Corning, NY, USA) in leukocyte medium (RPMI-1640 (Gibco, Waltham, MA, USA), fetal bovine serum (FBS, 10%, Gibco), sodium pyruvate (1 mM, Gibco), L-glutamine (2 mM, Gibco), non-essential amino acids (1%, Gibco), penicillin (100 IU/ml)—streptomcyin (100 µg/ml) (P/S, Gibco) and kanamycin (100 µg/ml, Gibco)) for 2h at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were then stimulated with the bacterial stimulants for 24h under the same conditions. Next, the cell-free supernatant was collected and stored at -20 °C until analysis.

Pre-treatment of bacterial stimulants with intestinal alkaline phosphatases

LPS serotypes O26:B26, O55:B5, O111:B4 or O127:B8 (Sigma, Saint Louis, MO, USA, 3 µg/ml) or flagellin (Invivogen, San Diego, CA, USA, 3 µg/ml) were incubated with or without bovine IAP (Sigma, P0114 or A2356) or calf IAP (Sigma) (50 µg/ml) at 40°C for 4 h while

gently rocking. The reaction mixture contained 100 mM HEPES, 10 mM Mg^{2+} and 1 mM Zn^{2+}. After incubation the reaction mixtures were stored at -20°C until use.

Cytokine enzyme-linked immunosorbent assay (ELISA)

The secretion of the proinflammatory cytokines TNFα and IL-6 in cell-free supernatants was measured using porcine-specific DuoSet enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The relative secretion was calculated by the following formula:

$$Relativedecrease = \left(1 - \frac{[sample] - [negativecontrol]}{[positivecontrol] - [negativecontrol]}\right) * 100\%$$

Statistical analysis

Statistical analysis was performed using Prism 7 software (Graphpad Software, CA, USA). Significance was assessed using the Friedman test, with Dunn's test for multiple comparisons correction. A $p < 0.05$ was considered statistically significant.

Results

To evaluate whether the LPS serotype affects the ability of LPS to activate porcine PBMCs, these were stimulated with four different LPS serotypes at different concentrations for 24h and the TNFα secretion was measured by ELISA (Fig. 1).

All LPS serotypes induced TNFα secretion by PBMCs at the tested concentrations as compared to the control. Although at higher concentrations no differences were observed between the different serotypes, at lower concentrations the LPS serotypes differed in their ability to induce TNFα secretion by PBMCs. This difference was most noticeable at 1 ng/ml between serotype O26:B6 and O127:B8. Over all concentrations, the secretion of TNFα induced by LPS O26:B6 was significantly different from LPS O127:B8 and a trend was noticed between LPS O55:B5 and LPS O127:B8 ($p = 0.08$).

Next, we assessed whether the observed differences in the ability of LPS serotypes to trigger TNFα secretion by PBMCs might translate to a variable detoxification of these serotypes by IAP. To test this, LPS serotypes were incubated with three commercial IAPs (bovine IAP P0114 (BIAP P), A2356 (BIAP A) or calf IAP (CIAP)) for 4h. The IAP-treated LPS serotypes were then administered to PBMCs and their ability to induce secretion of pro-inflammatory cytokines by PBMCs was evaluated to assess the efficiency of LPS detoxification. Interestingly, the tested IAPs are not able to detoxify all LPS serotypes to a similar degree (Fig. 2).

Although not significant, the data hint at a higher potential of CIAP and BIAP A to detoxify LPS as shown by the induced TNFα secretion. BIAP P shows the least effect on LPS-induced TNFα secretion (on average a 14% decrease), while CIAP and BIAP A show comparable amounts of detoxification (29% and 28%, respectively). The heatmap in Fig. 2F summarizes the relative decrease in TNFα secretion induced by the enzymatic detoxification. The different LPS serotypes also showed a different susceptibility to IAP dephosphorylation. LPS serotype O55:B5 and O111:B4 are more susceptible to IAP detoxification based on TNFα secretion (average of 25% and 37% decrease, resp.) as compared to LPS O26:B6 (16%) and LPS O127:B8 (17%). Interestingly, the decrease in

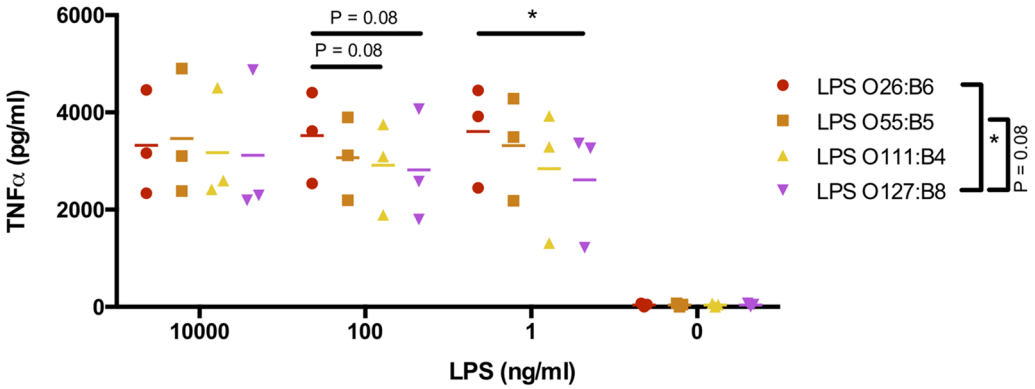

Fig. 1 Different LPS serotypes stimulate TNFα secretion in porcine PBMCs. PBMCs were stimulated with different LPS serotypes at the indicated concentrations. After 24h, the supernatants were collected and the TNFα concentration was determined via ELISA. Data are presented as the mean (horizontal line) of three biological replicates. *, $p < 0.05$. All tested serotypes significantly induced TNFα secretion compared to the control ($p < 0.01$) and significant differences were noted between LPS serotypes over all concentrations (Δ, $p < 0.05$)

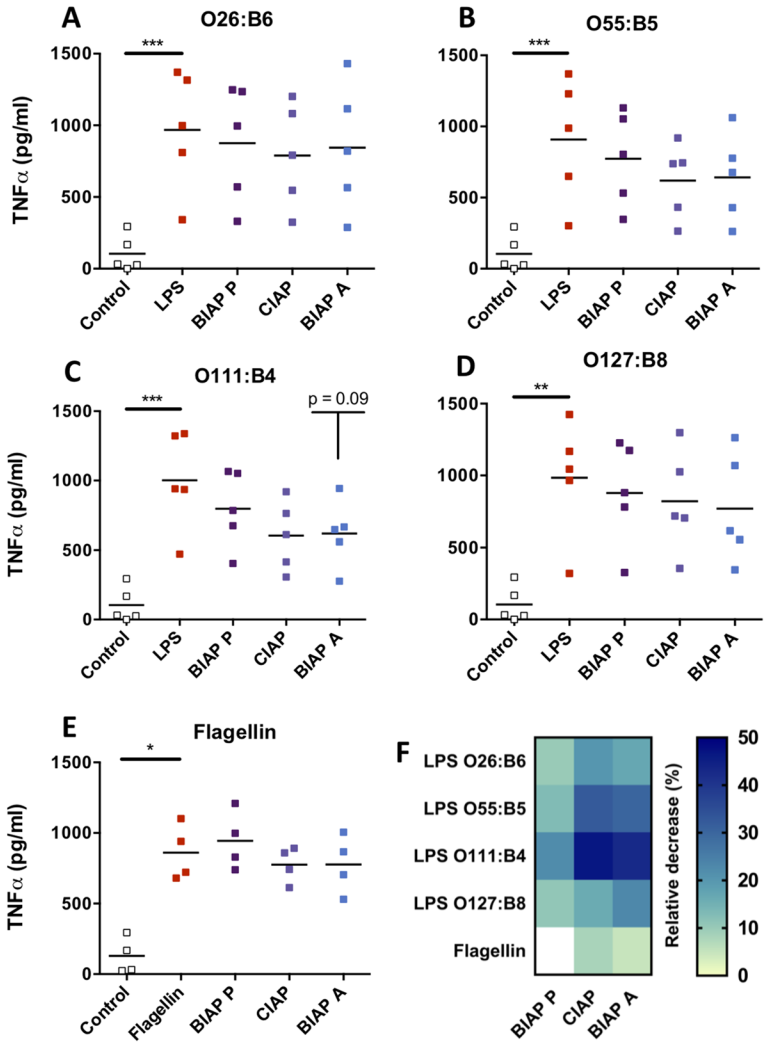

Fig. 2 TNFα secretion of porcine PBMCs stimulated with IAP pre-treated LPS. LPS was treated with different IAPs and added to PBMCs for 24h (LPS conc. = 10 ng/ml). Supernatants were collected and analysed via TNFα ELISA. **A**-**D** TNFα secretion. The horizontal line represents the mean of independent biological replicates ($n = 5$). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. **E** Relative decrease in TNFα secretion. Mean of independent biological replicates ($n = 4$). BIAP P = Bovine IAP P0114. CIAP = Calf IAP. BIAP A = Bovine IAP A2356

TNFα secretion was significantly different in LPS O26:B6 compared to LPS O55:B5 when treated with CIAP or BIAP A ($p < 0.05$) and when comparing LPS O111:B4 to LPS O127:B8 treated with CIAP ($p < 0.05$). This further shows that LPS serotypes are differentially affected by IAP. Flagellin found in motile bacteria is recognised by TLR5. As such, it can stimulate the host immune system and trigger inflammation [34]. Similar to LPS, PBMCs were incubated with flagellin either treated with IAP or not. Untreated flagellin induced a clear TNFα secretion (Fig. 2E). However, unlike LPS, flagellin was not susceptible to IAP detoxification based on TNFα secretion.

Similar to TNFα, another pro-inflammatory cytokine secreted by PBMCs upon LPS stimulation is IL-6. Figure 3 shows that all LPS serotypes significantly increased IL-6 secretion by PBMCs. The relative decrease in IL-6 secretion induced by IAP treatment is summarized in a heatmap (Fig. 3F). Similar to the TNFα responses, BIAP P showed the least effect on the LPS-induced IL-6 secretion. Only an average decrease of 23% was observed.

CIAP and BIAP A treatment of the LPS serotypes resulted in a relative decrease in IL-6 secretion of 31% and 33%, respectively. Like the secretion of TNFα, LPS serotypes also showed a different susceptibility to IAP detoxification based on IL-6 secretion. PBMC stimulation with IAP-treated LPS O111:B4 and LPS O55:B5 resulted in a decreased IL-6 secretion of 36% and 31%

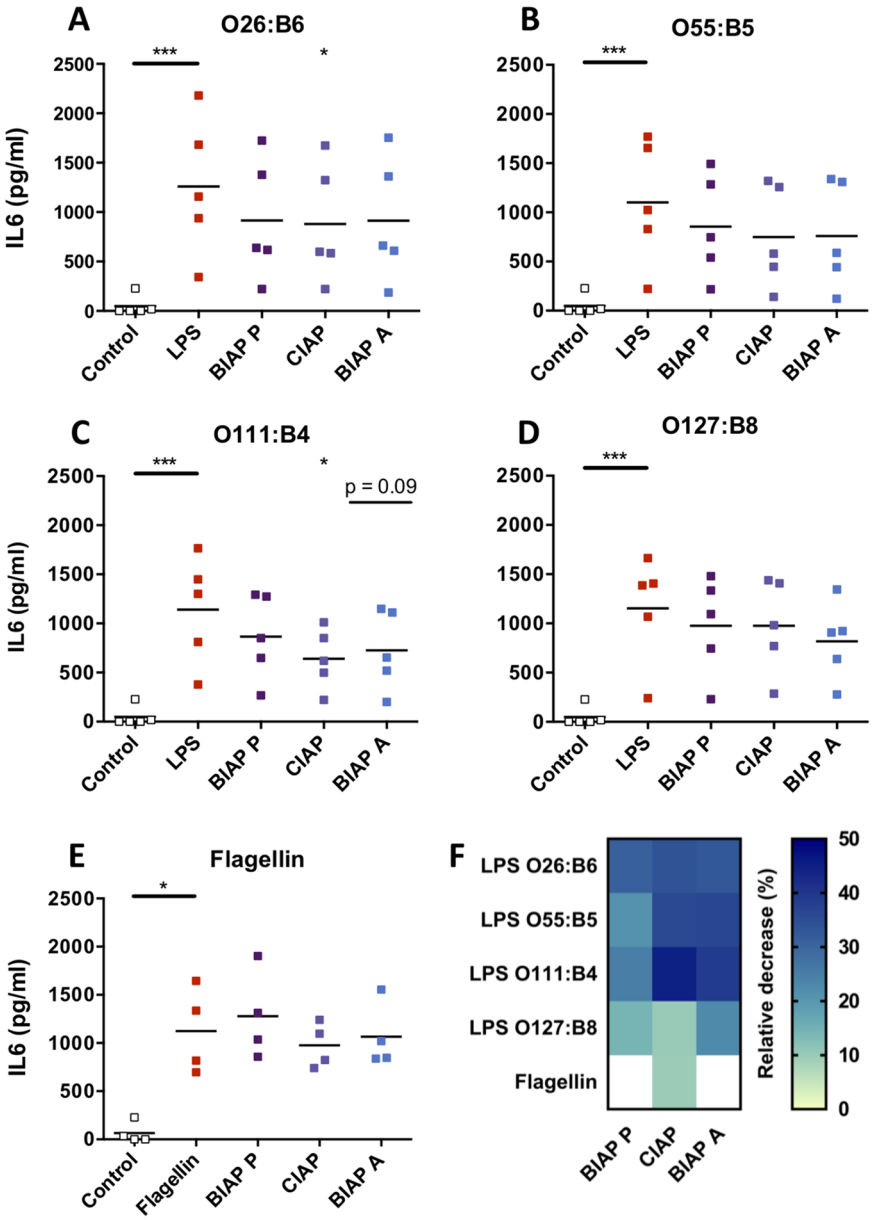

Fig. 3 IL-6 secretion of porcine PBMCs induced by IAP pre-treated LPS. LPS was treated with different IAPs and added to PBMCs for 24h (LPS conc. = 10 ng/ml). Supernatants were then collected and analysed via IL-6 ELISA. **A**-**D** IL-6 secretion. The horizontal line represents the mean of independent biological replicates ($n = 5$). *, $p < 0.05$; ***, $p < 0.001$. **E** Relative decrease in IL-6 secretion. Mean of independent biological replicates ($n = 4$). BIAP P = Bovine IAP P0114. CIAP = Calf IAP 79390. BIAP A = Bovine IAP A2356

as compared to the untreated LPS serotypes, respectively. Interestingly, IAP-treated LPS O26:B6 showed a similar decrease in IL-6 production unlike the TNFα response ($p < 0.05$). However, similar to the TNFα secretion, LPS O127:B8 was less affected by IAP treatment. Contrary to the TNFα response, CIAP could significantly decrease the LPS-induced IL-6 secretion by treatment of LPS O26:B6 and LPS O111:B4. The IL-6 secretion thus confirms the effect of different serotypes on the detoxification efficiency of IAPs. Here, the relative decrease in IL-6 secretion differed significantly between BIAP A-treated LPS O26:B6 and LPS O111:B4 ($p < 0.05$). Likewise, the relative decrease in IL-6 secretion differed significantly between CIAP-treated LPS O127:B8 and LPS O55:B5 and between CIAP-treated LPS O127:B8 and LPS O111:B4 ($p < 0.05$). Untreated flagellin also induced IL-6 secretion in stimulated PBMCs (Fig. 2E). However,

like the TNFα secretion, IAP treatment had no effect on the ability of flagellin to induce IL-6 secretion.

Discussion

LPS is the main structural component of the cell wall of Gram-negative bacteria and is well known for its potent induction of inflammation. Upon recognition of LPS by the MD-2/TLR4 complex, signalling factors such as NF-κB and IRF3 induce the expression of pro-inflammatory cytokines like TNFα, IL-6 and IL-1β [10, 35]. In the present study, we found that different LPS serotypes have a different ability to induce TNFα secretion by porcine PBMCs. Data on the correlation between the structural differences of LPS serotypes and immune cell modulation has been reported in human and murine cells but not in porcine immune cells. For instance, in a recent study, the immune responses of human neutrophils induced by different LPS serotypes were not only bacterial species-specific but also serotype-specific, as *E. coli*-derived LPS O128:B12 and LPS extracted from *Pseudomonas aeruginosa* were able to induce the formation of neutrophil extracellular traps, contrary to the other tested serotypes isolated from *E. coli* or *Salmonella enterica* [19]. It was suggested that the O-antigen sugar composition was responsible for this effect. In a study focusing on murine preterm labor induced by LPS it was also speculated that different responses to LPS serotypes were due to a different modulation of TLR4 recognition by variances in the O-antigen [25]. Indeed, the O-antigen accounts for most of the variability between LPS serotypes. However, many modifications to the basic lipid A structure have also been described and are used by bacteria to evade host immunity, colonize new niches or facilitate toxin secretion [27, 28, 36]. As the degree of acylation and phosphorylation are crucial for LPS recognition by MD-2/TLR4, many bacteria modify the number or length of acyl-chains and phosphate groups of their LPS. Also a change in bacterial surface charge or antibacterial peptide resistance can be achieved in this manner. Although many studies have focussed on specific lipid A modifications and their effects on the bacteria, data on the structure of different LPS serotypes is scarce and the serotype-specific effects of LPS on the host immune system remain elusive.

Indeed, gathering new insights on LPS-mediated gut inflammation is crucial to develop novel strategies to prevent or treat LPS-induced inflammation. Intestinal alkaline phosphatases have gained attention to treat inflammatory diseases, due to their ability to dephosphorylate different bacterial components, such as LPS and flagellin. For example, exogenous IAP has been used for experimental treatment of severe ulcerative colitis in both mice and humans [15, 17, 18]. Moreover, in swine husbandry, IAP has been suggested as a feed additive as a novel way to improve gut health of piglets during the post-weaning period [37]. Here, we showed that different commercially available IAP enzymes have varying efficiencies in detoxifying a variety of LPS serotypes by dephosphorylation. BIAP P seemed the least effective at LPS detoxification as compared to CIAP and BIAP A, which could decrease the LPS-induced cytokine secretion in PBMCs to a similar extent. The degree of LPS and flagellin detoxification noticed in the present experiments was also much lower than reported in other studies. On average 16%-37% less TNFα was secreted by porcine PBMCs when LPS was treated with different IAPs. Other studies conversely, reported higher efficiencies with a similar detoxification protocol. Chen et al. [14] reported a 50% decrease in CXCL-8 secretion by LPS O111:B4 in HT29 cells after only 2h of LPS treatment. Interestingly, the detoxification effect was only perceived at higher LPS concentrations. Also, flagellin was treated for 16h to induce a 40% decrease. Hwang et al. [18] reported the strongest effect of IAP on LPS. In this study, murine macrophages were incubated with IAP for 24h and stimulated with LPS O127:B8 for 4h. It was shown that the highest tested IAP concentration could decrease the secretion of TNFα and IL-6 by 75% and 85%, respectively. Of note, mice are quite resilient to LPS-induced inflammation compared to pigs and humans, requiring much higher LPS concentrations to activate immune cells [30, 31]. Interestingly, a recent study identified three porcine IAP isoforms in the small intestine [38]. Further research might explore whether these porcine IAP isofroms also exhibit a varying ability to detoxify different LPS serotypes. Moreover, to our knowledge serotype-specific differences in LPS-induced cytokine secretion has not yet been reported in a porcine immune cells. A serotype-specific effect on IAP dephosphorylation was also noticed, as the *E. coli*-derived LPS serotypes O55:B5 and O111:B4 seemed more susceptible than LPS O26:B6 and LPS O127:B8. As mentioned above, many modifications exist which affect LPS recognition. However, as the structural differences between these serotypes have not been elucidated, it is difficult correlate potential modifications with the observed functional differences.

Further research should be conducted to elucidate the specific LPS serotype structures, possible modifications on the core molecule and their relation to biological activity. Nevertheless, the few available studies together with our results highlight the importance of serotype-specific effects in LPS research. Although IAPs have been proven to be a valuable tool to treat LPS-induced inflammation, our data call for more research to gain further insight in serotype-specific effects of LPS on the host immune system.

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Authors' contributions
BV, MW and BD conceived the idea and designed the research. BV performed the experimental work. BV, MW, EC and BD analyzed the data. BV wrote the manuscript with contributions from EC and BD. All authors reviewed the manuscript before submission. All authors contributed to the article and approved the submitted version.

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Availability of data and materials
All data generated during this study are included in this article.

Declarations

Ethics approval and consent to participate
The animal study was reviewed and approved by Ethical Committee of the Faculties of Veterinary Medicine and Bioscience Engineering of Ghent University (EC 2017–121). All methods were carried out in accordance with relevant guidelines and regulations and were reported according to the ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
Not applicable.

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