Alkaline phosphatase

mechanism of action and implications for the treatment of LPS-mediated diseases

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mechanism of action and implications for the treatment of LPS-mediated diseases

Alkalische phosphatase

werkingsmechanisme en implicaties voor de behandeling van LPS-gemedieerde ziekten

(met een samenvatting in het Nederlands)

Proefschrift

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door

Alberdina Fiechter
geboren op 5 juni 1977, te Zwolle

Promotor Prof. dr. W. Seinen

Co-promotor Dr. M. Wulferink

So eine Arbeit wird eigentlich nie fertig,
man muß sie für fertig erklären,
wenn man nach Zeit und Umständen
das möglichste getan hat.

J.W. von Goethe, *Italienische Reise*, 1787

In herinnering aan opa Zwolle

Contents

Chapter 1

General introduction

Lipopolysaccharide

Lipopolysaccharide (LPS), also known as endotoxin, plays a major role in the development and/or aggravation of several multifactoral diseases, including sepsis, atherosclerosis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and multiple sclerosis (MS) [1-3]. It is a major constituent of the outer leaflet of the Gram-negative bacterial cell membrane and a single bacterial cell contains approximately 3.5 x 10^6 LPS molecules, which are essential for growth and stability of the bacterium [4]. A full length LPS molecule consists of four different parts: a lipid A moiety, an inner core, an outer core, and an O-antigen (Fig.1) [5].

The lipid A moiety of LPS is composed of two phosphorylated glucosamine saccharides linking at least six fatty acids. This part is the toxic component of the molecule, since injection of a chemically synthesized lipid A induces effects *in vivo* similar to an injection with a full length LPS molecule [6, 7]. The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A. Removal of one phosphate group results in the formation of monophosphoryl lipid A (MPLA), which has a significant reduced bioactivity compared to lipid A [8-10].

The inner core is the second part of the LPS molecule consisting of two or three 2-keto-3-deoxyoctonic acid (KDO) sugars and two or three heptose sugars. The minimal LPS structure that is produced by bacteria, which is required for growth and stability, consists of one KDO sugar and the lipid A moiety [5].

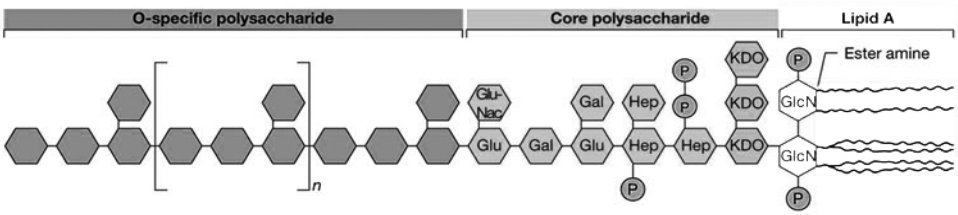

Figure 1: Schematic structure of LPS

The outer core contains common sugars with more structural diversity than the inner core. It is composed of three sugars with one or more sugars covalently bound as side chains.

The fourth part of the LPS molecule is the O-antigen, which is composed of a polymer of oligosaccharides with repeating units of three sugars. The structure of the repeating unit is characteristic for a bacterial strain within a serotype, which results in an important antigen. LPS molecules containing the O-antigen are denoted as Smooth-LPS (S-LPS), since presence of this part results in a smooth appearance of a bacterial colony on a plate. Bacteria expressing LPS without an O-antigen have a rough (R) appearance when grown on plate; hence the LPS molecule is called R-LPS.

LPS cell-signaling

Incorporated in the outer leaflet of the Gram-negative bacterial cell membrane, the LPS molecule is relatively non-toxic. When a dividing or dying bacterium spontaneously releases LPS into the mammalian circulation, it can interact with several proteins (LBP, CD14, TLR4, and MD2), which results in the induction of an inflammatory response by the host [11].

LBP

LPS, present in serum, can associate with several serum proteins like albumin, lactoferrin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and bacterial permeability-increasing protein (BPI) [12-16]. However, the most important protein present in serum that is able to bind LPS is LPS-binding protein (LBP), a glycosylated protein mainly synthesized in the liver by hepatocytes which production is increased in the acute phase of a Gram-negative bacterial infection [17]. The N-terminal domain of LBP binds the lipid A moiety of LPS with high affinity (Fig. 2) [18-21].

CD14

CD14 is produced by cells of the myeloid lineage (macrophages, monocytes, and PMNs) as a glycoprotein with a leucine-rich motif in its carboxyl-terminal region [22]. Two forms of CD14 are described: a membrane bound form (mCD14), attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, and a soluble form (sCD14), released from cells before GPI anchor addition can occur or after cleavage of the GPI anchor [23]. CD14 binds to the LBP-LPS complex (Fig. 2) [24]. However, sCD14 is able to bind directly to LPS without recognition of LPS by LBP [25]. The resulting sCD14-LPS complex can activate cells that are deficient in mCD14 expression [26].

Since CD14 lacks a transmembrane domain, an accessory receptor complex, TLR4/MD2, is required to initiate a cell signal transduction.

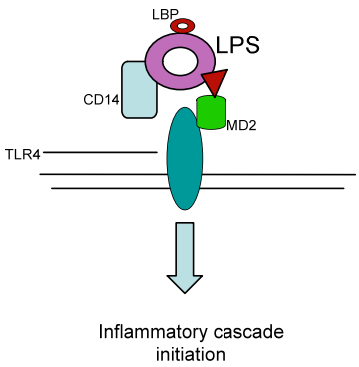

Figure 2: LPS-signaling cascade

TLR4/MD2 complex

Of the 11 Toll-like receptors (TLRs) in mammals known today, TLR4 is the primary TLR accountable for most LPS responses [27, 28]. TLR4 is a transmembrane protein with a leucine-rich motif in its extracellular domain. Associated with TLR4 is the soluble glycoprotein MD2 which is required for TLR4 activation [29]. LPS, concentrated by LBP and/or CD14, is delivered to the TLR4/MD2 complex on the cell

membrane, where the lipid A part of LPS is recognized by MD2 [30]. This results in a TLR4-induced signal transduction cascade in the cell (Fig. 2). In cells without a TLR4/MD2 receptor complex expression on the cell membrane, this complex resides intracellular, suggesting that LPS uptake is necessary, probably bound to sCD14 [31, 32]. This implies that TLR4 and MD2 are responsible for LPS signal transduction, but not for LPS internalization [33].

LPS-induced cell response

Once LPS interacts with the TLR4/MD2 receptor complex, the MyD88-dependent or MyD88-independent pathway is stimulated, which results in translocation of the nuclear factor κB (NFκB) to the nucleus [27]. NFκB activates promoters of genes coding for inflammatory mediators, such as cytokines and chemokines that play an important role in promoting the host's defense against an LPS insult, although extensive production can be harmful [34]. Every cell type responds to an LPS insult with the release of a different set of inflammatory mediators.

Major pro-inflammatory cytokines produced by mononuclear phagocytes after an LPS infection are TNF-α, IL-1β, and IL-6. TNF-α is considered to be an early-phase mediator and able to induce the production of IL-1β [35]. TNF-α and IL-1β share several biological activities. For instance, they activate neutrophils and upregulate production of adhesion molecules by endothelial cells, which results in adherence of neutrophils to blood vessels [36, 37]. High concentrations in the circulation of these two cytokines can lead to numerous pathophysiological effects, including vascular instability, myocardial depression, hypotension, fever, and multiple organ failure [38, 39]. Production of IL-6, a somewhat less potent pro-inflammatory cytokine than TNF-α and IL-1β, is induced by these two cytokines and displays some of the same pro-inflammatory actions as TNF-α and IL-1β [40]. In contrast, IL-6 exhibits anti-inflammatory effects by inhibiting the synthesis of TNF-α and IL-1β and inducing the release of soluble TNF receptor (sTNFR) and the IL-1 receptor antagonist (IL-1ra) [41]. Secretion of the anti-inflammatory cytokine IL-10 by LPS activation of lymphocytes,

mast cells, and mononuclear phagocytes results in a diminished pro-inflammatory cytokine production but a stimulated humoral and cytotoxic immune response [42].

The most important chemokine, IL-8, is derived primarily from mononuclear phagocytes, endothelial, and epithelial cells [43]. Secretion of IL-8 into the extracellular environment is induced by LPS, TNF-α, and IL-1β, and results in the chemoattraction, degranulation, and respiratory burst of neutrophils. It also stimulates neutrophil adherence to endothelial cells [44, 45].

Besides the induction of chemokine secretion, LPS, TNF-α, and IL-1β are also known to enhance the expression of inducible nitric oxide synthase (iNOS) in nearly all cell types [46]. Formation of iNOS results in the synthesis of nitric oxide (NO), which, at low concentrations, is an important mediator in vascular homeostasis and exerts a protective effect against LPS insults [47]. However, excessive production of NO can lead to hypotension and induction of apoptotic cell death [48].

All of the above mentioned inflammatory mediators serve a beneficial role in keeping homeostasis; yet, overexpression may be deleterious and therapies aiming at reducing the inflammatory response during an LPS insult receive much attention.

LPS-mediated diseases and anti-LPS therapies

Reducing LPS-induced effects have been primarily studied in the case of Gram-negative bacterial sepsis, which is characterized by an uncontrolled release of pro-inflammatory mediators of the host towards LPS. This response can ultimately develop to septic shock, the most severe form of sepsis that is associated with fever, hypotension, hypoperfusion, and multiple organ failure (MOF). The overall mortality in patients diagnosed with septic shock is more than 50%, indicating the importance in developing therapies for sepsis and septic shock [49, 50].

To date, the standard treatment for sepsis and septic shock aims at immediate resuscitation and stabilization. Patients are intubated and ventilated in case of respiratory failure, fluids and vasopressors are administered to restore blood pressure, and antibiotic treatment is initiated immediately [51]. Recently, several therapies have been explored that were directed against specific mediators in the pathogenic pathway

of sepsis and septic shock. Many focused on neutralizing or antagonizing the two most potent cytokines produced, TNF-α and IL-1β, or on inhibiting NO-synthesis. However, clinical trials did not succeed in showing a survival benefit, most likely because interfering with a single mediator is unlikely to alter the course of the disease [52, 53]. Intervention at the start of the LPS signaling cascade, thus neutralizing LPS, would therefore be most effective to reduce the harmful host response. Clinical trials using LPS-binding proteins or antibodies directed against LPS did not show a reduction in mortality in the treatment groups, probably because the used binding proteins and antibodies had low binding affinities for lipid A [54-56]. Several studies have shown that dephosphorylated lipid A is able to induce tolerance towards LPS, indicating that therapies aiming at directly reducing the toxicity of lipid A might therefore be another strategy to overcome the deleterious effects of LPS in sepsis and other LPS-mediated diseases [57-59].

Alkaline phosphatase

Alkaline phosphatase (AP) is an ubiquitous enzyme in the human body that can be present in four isoforms. There are three tissue-specific isoforms, *i.e*. placental (PLAP), germ cell (GCAP) and intestinal (IAP) AP and one tissue-nonspecific (TNSAP) isoform, the liver-bone-kidney (LBK) type. Genes encoding the tissue-specific APs are clustered on bands q34-q37 of chromosome 2, and their amino acid sequences share a 90-98% homology. TNSAP is 50% homologous to the tissue-specific APs and the gene maps to the distal short arm of chromosome 1, bands p34-p36.1 [60-66].

Post-translation, APs are modified by *N*-glycosylation and uptake of two zinc atoms and one magnesium atom in the active site, essential for enzymatic activity [67]. Addition of a glycosylphosphatidylinositol (GPI) anchor results in the attachment of the enzyme to the cell membrane, making it an ectoenzyme [68, 69]. Soluble AP can be present in the circulation after release from the cell membrane through the action of the GPI-specific phospholipase C [70, 71]. Being a glycoprotein, AP is clearly to be eliminated from the circulation by the asialoglycoprotein receptor (ASGPR) on hepatocytes [72]. Of the four isoforms, PLAP contains the most sialylated sugar

moieties and is therefore not recognized by the ASGPR, which results in a longer plasma retention time than GCAP, IAP, and TNSAP.

Besides the ability of all AP isoforms to catalyze the hydrolysis of phosphomonoesters at alkaline pH optima, thereby releasing inorganic phosphate and alcohol, every isoform serves a different biological function [73, 74]. PLAP is known to be important for the transfer of maternal IgG to the fetus and is a modulator of fetal growth, and IAP plays a role in lipid transport across the intestinal epithelium [75-77]. The importance of TNSAP in bone mineralization is shown in the case of the rare genetic disease hypophosphatasia. Mutations in the TNSAP gene result in a deficient enzyme leading to rickets or osteomalacia. The most severe form of the hypophosphatasia is caused by deactivating mutations in the TNSAP gene, which is lethal in infancy [78-81].

AP and LPS

AP is present throughout the body and abundantly expressed at sides where possible antigens, like LPS, may enter the circulation (*e.g.* lungs and gastrointestinal tract). Exposure of cells that form a barrier between the host's internal milieu and the external environment to LPS result in upregulation of AP, indicating that AP also serves a role in the natural defense system against an LPS insult [82, 83].

Since AP catalyzes the hydrolysis of phosphomonoesters and expression of AP is induced after an LPS insult, it is thought that this enzyme serves a role in LPS detoxification. As this molecule contains two phosphate groups in the lipid A moiety, one might be removed by AP, which results in the release of inorganic phosphate (P_i) and the formation of the dephosphorylated product monophosphoryl LPS (MPLPS) [84, 85]. MPLPS is 4 orders of magnitude less potent than LPS to induce an inflammatory response, probably because it is no longer recognized by one of the components of the LPS cell signaling cascade.

Several studies indicate the promising effects of AP against an LPS insult in a variety of animal models. A reduction in the inflammatory response induced by LPS could be observed in mice and piglets after treatment with PLAP or bovine intestinal alkaline phosphatase (BIAP) [86, 87]. Oral treatment of rats with LPS resulted in a prolonged

endotoxemia after inhibition of endogenous IAP [83]. In addition, the potential of AP on LPS-mediated diseases have been demonstrated in studies with Gram-negative bacteria insults and polymicrobial sepsis. In mice, infected with a lethal dose of Gram-negative bacteria, mortality was reduced after injection of PLAP or BIAP [87, 88]. Cytokine response and neutrophil influx in secondary peritonitis in mice was attenuated by BIAP [89]. Hepatic and pulmonary injury after liver ischemia-reperfusion with partial resection was reduced in rats treated with BIAP when compared to control animals, and administration of BIAP to sheep, injected with feces to mimic severe peritoneal sepsis, decreased IL-6 concentrations and prolonged survival time [90, 91].

All the above mentioned studies indicate that AP is able to reduce LPS induced insults, suggesting that this enzyme represents a new therapeutic drug in the treatment of LPS-mediated diseases.

Scope of this thesis

The first part of this thesis focuses on the effects of BIAP on an LPS insult *in vitro*. **Chapter 2** describes the use of three human epithelial cell lines to investigate the biological activity of BIAP in an *in vitro* system. Furthermore, the 'inflamed' *in vivo* situation was mimicked *in vitro* by exposing macrophages to epithelial cell supernatant previously incubated with LPS. In **Chapter 3**, the role of CD14 in detoxifying LPS by BIAP is tested.

In the second part of this thesis, studies are described that examine the effect of BIAP in two LPS-mediated diseases. The left anterior descending (LAD) coronary artery ligation model in mice was used to induce an acute myocardial infarction (AMI). BIAP was given i.v. as a prophylaxis and the inflammatory response in the acute phase after AMI was investigated (**Chapter 4**). In **Chapter 5**, experiments were performed to investigate if oral and rectal treatment with BIAP has beneficial effects in a dextran sulphate sodium (DSS)-induced colitis model in mice. Finally, the data described in this thesis are summarized and discussed (**Chapter 6**).

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Chapter 2

In vitro detoxification of LPS by bovine intestinal alkaline phosphatase (BIAP), a novel therapeutic drug for LPS-mediated diseases

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Abstract

Several investigators have demonstrated that bovine intestinal alkaline phosphatase (BIAP) attenuates the lipopolysaccharide (LPS)-induced inflammatory response in different animal models and to date the clinical development of the enzyme for the treatment of several LPS-mediated diseases (sepsis, inflammatory bowel disease (IBD) and coronary artery bypass grafting (CABG)) is pursued. Despite all the promising results obtained with BIAP *in vivo*, little is known about the mechanism by which it exerts its biological activity. An *in vitro* assay in which the inflammatory *in vivo* insult is mimicked would be an asset in exploring BIAP mechanisms and the co-factors that are involved.

To test the LPS-detoxifying capacity of BIAP *in vitro*, assays were performed in which mouse macrophages (RAW264.7) and human epithelial cells (T84, HEK293T and A-549) were incubated with LPS with or without BIAP, after which cell supernatants were screened for the presence of pro-inflammatory molecules. LPS-induced IL-8 production was significantly reduced in BIAP treated HEK293T and A-549 cells. Pre-incubation of RAW264.7 cells with BIAP showed a decrease in LPS-induced IL-6 and NO_x secretion. In addition, the inflamed environment was mimicked by incubating RAW264.7 cells with supernatant from epithelial cells stimulated with LPS with or without BIAP, thereby inducing inflammatory cytokine production. Incubation of LPS and BIAP on epithelial cells resulted in a significant reduction of TNF-α, IL-6 and NO_x secretion by RAW264.7 cells compared to LPS incubation alone.

In conclusion: The results of these studies clearly show that BIAP reduces LPS-induced production of pro-inflammatory molecules *in vitro*.

Introduction

Lipopolysaccharide (LPS) is a molecule present in the outer cell membrane of Gram-negative bacteria and consists of a variable repeating oligosaccharide chain (O-antigen) and an oligosaccharide core, covalently bound to lipid A [1, 2]. Lipid A contains two phosphate groups that are essential for the biological action of LPS [3, 4]. Removal of a single phosphate group from lipid A results in the formation of monophosphoryl lipid A (MPLA), which is non-toxic and able to induce tolerance towards LPS due to its antagonistic properties [5, 6].

Several acute and chronic diseases, for example Gram-negative sepsis, acute respiratory distress syndrome (ARDS) and ulcerative colitis (UC) are LPS-mediated and characterized by the excessive production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) and other inflammatory mediators (e.g. IL-8, NO_x) [7-9]. Many therapies against Gram-negative sepsis aimed at either the neutralization or antagonization of pro-inflammatory cytokines or at the neutralization of LPS by using anti-LPS antibodies or LPS-binding proteins [10-15]. However, none of these trials have been proven effective.

Next to a variety of isoform specific physiological roles of alkaline phosphatase (AP), it has been shown that AP is able to remove a single phosphate group from the lipid A moiety, thereby attenuating the toxicity of LPS [16-19]. Recently, several investigators have shown the promising therapeutic effects of AP in a variety of animal models. In mice lethally infected with gram-negative bacteria, human placental alkaline phosphatase (HPLAP) and bovine intestinal alkaline phosphatase (BIAP) were able to reduce mortality [20, 21]. Furthermore, BIAP attenuated LPS-induced TNF-α levels in piglets [21], hepatic and pulmonary injury after partial liver ischemia-reperfusion in rats treated with BIAP [22], cytokine response and neutrophil influx in secondary peritonitis in mice [23], and serum IL-6 concentration and mortality in septic sheep [24]. In addition, Koyama *et al.* (2002) showed that inhibition of endogenous intestinal alkaline phosphatase resulted in a prolonged endotoxemia in rats orally treated with LPS [25].

So far, it is not yet fully understood under which circumstances and conditions AP is able to detoxify LPS. This study focuses on the development of an *in vitro* assay that can be used to investigate these conditions and the possible co-factors that facilitate this. The effect of BIAP on LPS toxicity was tested in a direct activation assay using the murine macrophage cell line RAW264.7 and the human epithelial cell lines T84, A-549 and HEK293T. To represent the natural occurring response in which macrophages are attracted towards sites where inflammation occurs and respond to the inflammation-induced environmental factors, supernatant from epithelial cells stimulated with LPS with or without BIAP was given to macrophages and their response measured. It was observed that BIAP reduced the production of IL-8, TNF-α, IL-6 and NO_x induced by LPS on epithelial cells and macrophages.

Materials and methods

Bovine intestinal alkaline phosphatase

Bovine intestinal alkaline phosphatase (BIAP; GMP-processed, purity ALPIXG: 675 U/mg) was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of BIAP able to hydrolyse 1 μmole of p-nitrophenyl phosphate per minute at pH 9.6 at 25 ˚C.

Reagents and media

RPMI-1640, DMEM (4.5 g/l glucose), and DMEM/F-12 (1:1) media, all containing glutamax I, as well as penicillin, streptomycin, hepes, and sodiumpyruvate were from Invitrogen Corp. (Breda, The Netherlands). Fetal bovine serum (FBS) was obtained from Wisent Inc. (Quebec, Canada). Sulphanylamide, n-naphtylethylenediamine, lactate dehydrogenase (LDH) assay, and E. coli LPS O111:B4 (prepared by phenol extraction and dissolved in sterile phosphate-buffered saline), were from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). P-nitrophenyl phosphate (pNPP) was from Acros Organics (Geel, Belgium). Human IL-8, murine IL-6 and murine TNF-α ELISA kits were purchased from Biosource Europe SE (Nivelles, Belgium).

Cell culture

The murine macrophage cell line RAW264.7, and the human epithelial cell lines T84 (colorectal carcinoma) and HEK293T (embryonic kidney) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human epithelial cell line A-549 (lung carcinoma) was a kind gift from M.C. Dessing (AMC, Amsterdam). RAW264.7 and HEK293T cells were maintained in DMEM/F12 (1:1) medium. T84 cells were cultured in DMEM medium containing 4.5 g/l glucose, whereas A-549 cells were grown in RPMI-1640. All media were supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Medium for RAW264.7 and HEK293T cells also contained 15 mM hepes and was supplemented with 1 mM sodiumpyruvate. Cells were grown in a humidified incubator

at 37 ˚C and 5% CO_2. All epithelial cell lines were subcultured at confluence by employing trypsin-EDTA (Invitrogen Corp., Breda, The Netherlands). RAW264.7 cells were scraped using a rubber policeman.

Direct activation of macrophages and epithelial cells

For direct activation assays, RAW264.7 cells were plated into 24-wells plates (Nunc, Roskilde, Denmark) at a concentration of $2x10^5$ cells/ml and grown for 16 ± 2 hrs at 37 °C and 5% CO_2. Medium was refreshed and cells were incubated with 1 ml of medium containing 1 µg LPS for 24 hrs with or without 1 U BIAP. Epithelial cells were plated into 12-well plates (Nunc, Roskilde, Denmark) with 2 ml of medium. On reaching confluence, medium was refreshed and cells were incubated with 1 µg LPS/ml for 24 hrs with or without 1 U BIAP/ml. In other experiments, RAW264.7 cells were pre-incubated with 1 U BIAP/ml. After 2 hrs, cells were rinsed with PBS and subsequently incubated with 1 µg LPS/ml for 24 hrs. Control cells were incubated with 1 µg LPS/ml alone. All cell supernatants were collected after 24 hrs and stored at –70 ˚C until further use.

Indirect activation of macrophages

RAW264.7 cells were cultured and plated as previously described. Medium was removed and 800 µl of fresh medium was added. Supernatant from epithelial cells (200 µl), which were previously incubated with 1 µg LPS/ml with or without 1 U BIAP/ml for 2 hrs (T84 and HEK293T) or 24 hrs (A-549), was transferred to RAW264.7 cells. After an incubation period of 24 hrs, RAW264.7 cell supernatants were collected and stored at –70 ˚C until further use.

Cytokine assays

Commercially available ELISA kits were used to determine concentrations of murine IL-6 and TNF-α as well as human IL-8 in cell culture supernatants. Assays were performed according to the manufacturers' instructions.

Nitric oxide assay

Nitric oxide (NO_x) production was determined by means of the Griess method. In brief, 100 µl cell supernatant was incubated with 10 µl of a 10 mg/ml sulphanylamide solution in 5% phosphoric acid. After an incubation period of 10 minutes, 10 µl of a 1.39 mg/ml n-naphtylethylenediamine solution was added and plates were read at 540 nm.

BIAP-binding assay

Epithelial cells were grown in 12-wells plates until confluence was reached. RAW264.7 cells were seeded at a concentration of 5 x 105 cells/ml in 12-well plates and grown for 16 ± 2 hrs. Medium was replaced by fresh medium supplemented with 1 U of BIAP/ml and incubated for 2 hrs (T84, HEK293T and RAW264.7) or 24 hrs (A-549). After washing three times with PBS, binding of BIAP to cells during experiments was determined by measuring enzymatic activity of BIAP.

Enzymatic detection of BIAP

BIAP activity was detected enzymatically by incubating cells with 500 µl of an isotonic working solution containing 0.025 mM glycine, 8.62 mM $MgCl_2$ and 3.2 mM pNPP for 1 hr at 25 ˚C. The enzyme reaction was stopped by adding 500 µl of 2 M NaOH, after which the end product p-nitrophenol was quantitatively determined by measuring the extinction at 405 nm.

LDH release assay

It had to be determined whether or not the isotonic working solution had an effect on cell membrane permeability, to make sure that the measured BIAP activity was located extracellularly on the cell membrane. Therefore, LDH release was assessed in the supernatant after incubation of the cells. LDH content was determined according to the manufacturers' guidelines in aliquots from cell supernatant as well as in aliquots obtained after cell lysis. Lysed non-treated cells were included as a control for total

LDH activity. Cell membrane permeability was determined as the ratio of LDH activity recovered in the cell supernatant and total cell LDH content.

Statistical analysis

Data are depicted as mean ± S.E.M. Statistical analysis was performed using two-sided unpaired Student's *t*-test.

Results

Direct activation of macrophages and epithelial cells / direct bioassay

Bovine intestinal alkaline phosphatase (BIAP) was examined for its capacity to reduce LPS-induced macrophage and epithelial cell responses in vitro. To determine the optimal LPS response, cell lines were incubated for 24 hrs with different concentrations of LPS, ranging from 0.1-10 µg/ml in the presence or absence of 1 U BIAP/ml. LPS stimulated the production of IL-8 in a dose-dependent fashion in A-549 and HEK293T cells. Figure 1 shows IL-8 production by HEK293T cells as a representative parameter. Incubation of HEK293T cells with 1 µg LPS/ml and 1 U of BIAP/ml resulted in the same IL-8 production as 0.1 µg LPS/ml alone, implying that 1 U of BIAP/ml is able to reduce the effect of the 1 µg LPS/ml added by more than 90%. Therefore, all further *in vitro* assays were performed with an LPS concentration of 1 µg/ml.

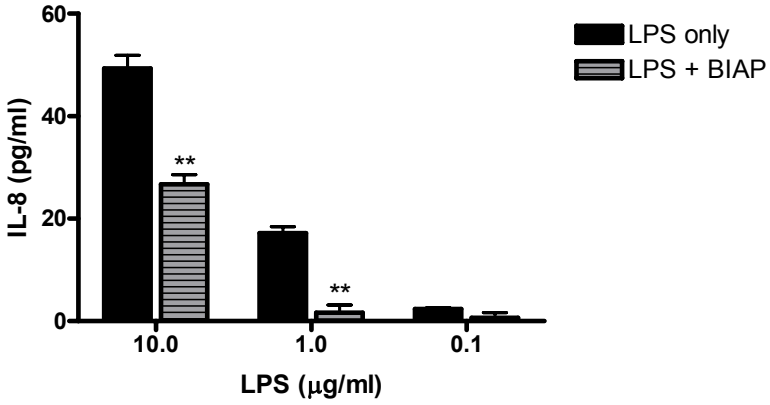

Figure 1: Dose-response curve of IL-8 production by HEK293T cells after exposure to LPS with or without BIAP. HEK293T cells were incubated with different concentrations of LPS for 24 hrs with or without 1 U BIAP/ml after which IL-8 production was determined. Incubation of HEK293T cells with LPS and BIAP resulted in a reduction of IL-8 production compared to LPS alone. Values are depicted as mean ± S.E.M. of 3 individual experiments and corrected for basal IL-8 production (** $P < 0.01$).

Incubation of A-549 cells with LPS and BIAP resulted in a 21% reduction in IL-8, whereas IL-8 secreted by HEK293T cells incubated under the same conditions showed a 42% reduction (Figure 2). T84 cells did not produce detectable amounts of IL-8.

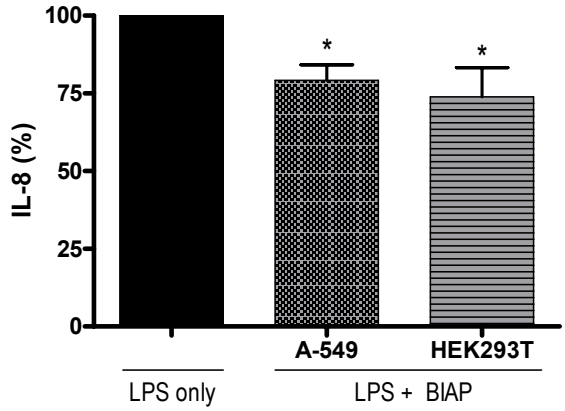

Figure 2: IL-8 production by A-549 and HEK293T cells after 24 hrs exposure to LPS with or without BIAP. A-549 and HEK293T cells were incubated with 1 µg LPS/ml for 24 hrs with or without 1 U BIAP/ml after which IL-8 production was determined. Incubation of A-549 and HEK293T cells with LPS and BIAP resulted in a reduction of IL-8 production compared to LPS alone. Cells treated with LPS alone are set at 100%. Values are depicted as mean ± S.E.M of 2 different experiments (* $P < 0.05$; *** $P < 0.001$).

Incubation of RAW264.7 cells with 1 µg LPS/ml with or without 1 U BIAP/ml for 24 hrs resulted in the secretion of the inflammatory parameters TNF-α, IL-6 and NO_x, which is shown in Figure 3. The simultaneous incubation of RAW264.7 cells with LPS and BIAP did not result in any decrease of cytokine- or NO_x levels. However, pre-incubation of RAW264.7 cells with 1 U BIAP for two hours, followed by washing with PBS and subsequent incubation of the cells for 24 hrs with 1 µg LPS/ml reduced IL-6 and NO_x levels by 17% and 30% respectively, but had no effect on TNF-α levels.

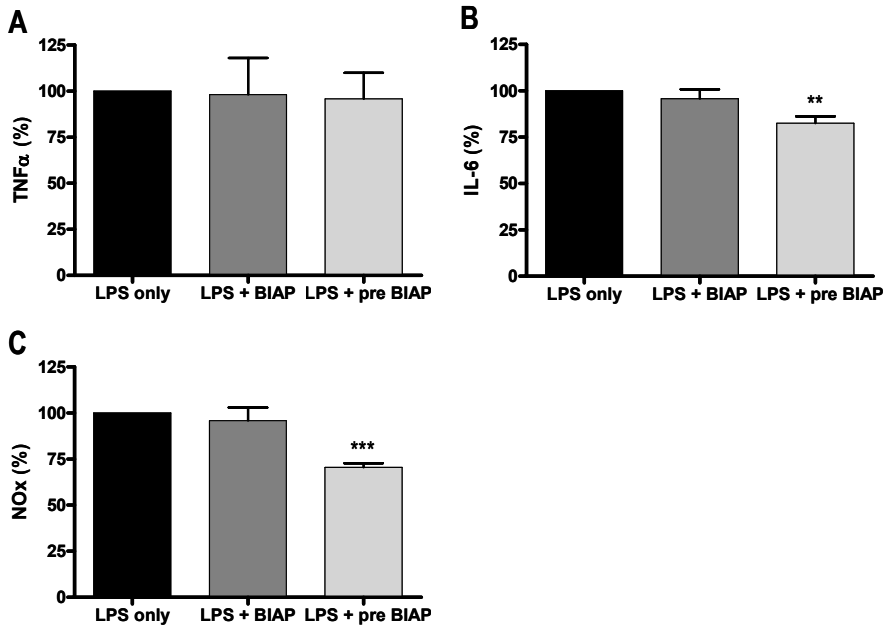

Figure 3: TNF-α production (A), IL-6 production (B) and NO_x production (C) by RAW264.7 cells after exposure to LPS with or without BIAP. RAW264.7 cells were incubated for 24 hrs with 1 µg LPS/ml alone, 1 µg LPS/ml and 1 U BIAP/ml, or 1 µg LPS/ml after pre-treatment with 1 U BIAP/ml. Incubation of RAW264.7 cells with LPS after pre-treatment with BIAP resulted in a decrease of IL-6 and NO_x production compared to LPS incubation alone. Cells treated with LPS alone are set at 100%. Values are depicted as mean ± S.E.M. of 3 individual experiments (** $P < 0.01$; *** $P < 0.001$).

Indirect activation of macrophages / indirect bioassay

To resemble the 'inflamed environment' in the *in vivo* LPS insult condition more closely, a culture model using epithelial cells and macrophages was developed. In this model epithelial cells were incubated with LPS with or without BIAP, followed by transfer of the supernatant to macrophages. After an additional 24 hrs incubation period on macrophages, inflammatory parameters were determined. Transfer of supernatants from LPS-stimulated epithelial cells to RAW264.7 cells resulted in the production of TNF-α, IL-6 and NO_x. In contrast, transfer of supernatants from epithelial cells simultaneously incubated with LPS and 1 U BIAP/ml to RAW264.7 cells resulted in a

significantly reduced secretion of TNF-α, IL-6 and NO_x, as is shown in Figures 4a-c. Incubation of LPS and BIAP on T84, A-549 or HEK293T cells resulted in a reduction in RAW264.7-cell secreted TNF-α of 44%, 29% and 17%, respectively (see Figure 4a). In addition, IL-6 secretion by RAW264.7 was reduced by 43%, 31% and 29% (see Figure 4b) whereas NO_x levels produced by RAW264.7 were reduced by 19%, 26% and 23% (see Figure 4c).

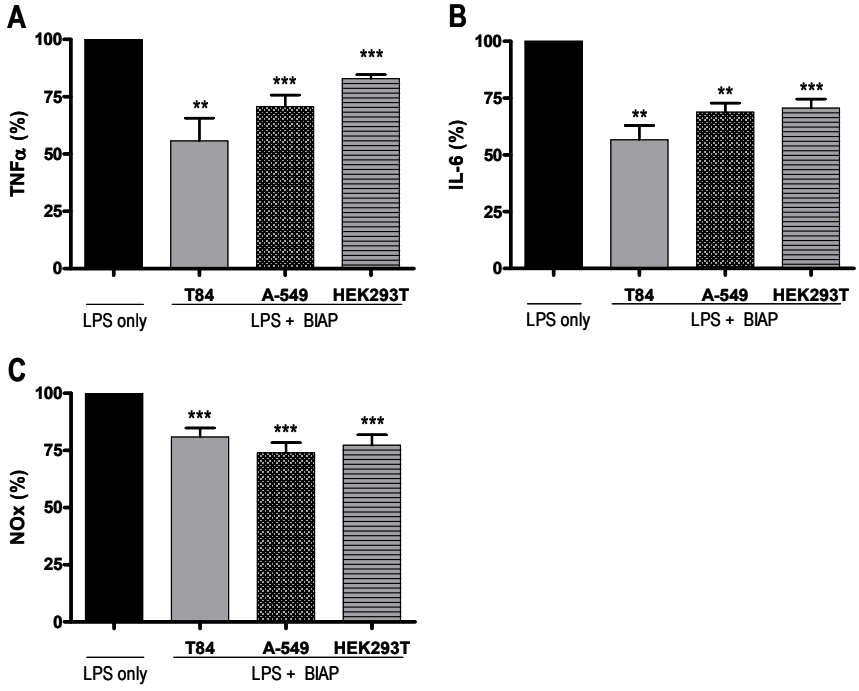

Figure 4: TNF-α production (A), IL-6 production (B) and NO_x production (C) by RAW264.7 cells after supernatant transfer of T84, A-549 and HEK293T cells exposed to LPS with or without BIAP. T84, A-549 and HEK293T cells were incubated with 1 µg LPS/ml for 2 hrs (T84 and HEK293T) or 24 hrs (A-549) with or without 1 U BIAP/ml after which supernatant was transferred to RAW264.7 cells. Incubation of T84, A-549 and HEK293T cells with LPS and BIAP resulted in a reduction of TNF-α, IL-6 and NO_x production by RAW264.7 cells compared to LPS alone. Cells treated with LPS alone are set at 100%. Values are depicted as mean ± S.E.M of at least 2 different experiments (** $P < 0.01$; *** $P < 0.001$).

BIAP-binding assay

To investigate the hypothesis that BIAP binds to the cells during the activation experiments, T84, A-549, HEK293T and RAW264.7 cells were incubated with 1 U BIAP/ml. After thorough rinsing with PBS, cells were incubated with an isotonic buffer containing pNPP. Hydrolysis of pNPP by (cell-associated) BIAP was visualized by measuring the end product p-nitrophenol. Figure 5 shows that BIAP activity on T84, A-549 and RAW264.7 cells incubated with BIAP was approximately 2 times higher than on cells not incubated with BIAP. For HEK293T cells incubated with BIAP, this increase was about 3.5. An LDH release assay indicated that incubation of cells with an isotonic buffer resulted in a marginal, and therefore negligible, increase in membrane permeability (data not shown). This, combined with the observation that pNPP does not penetrate the cell membrane [26], suggests that BIAP added to cultured epithelial and monocytic cells associates with these cells extracellularly.

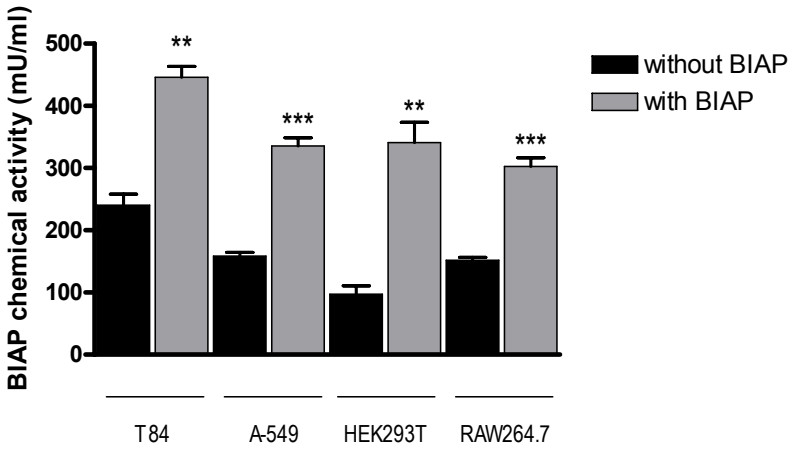

Figure 5: Binding of BIAP to T84, A-549, HEK293T and RAW264.7 cells. Cells were incubated with or without 1 U BIAP/ml for 2 hrs after which chemical activity of the enzyme was measured. Incubation of cells with BIAP showed a significant increase in chemical activity compared to basal conditions. Values are depicted as mean ± S.E.M. of 3 individual measurements (** $P < 0.01$; *** $P < 0.001$).

Discussion

Epithelial cells are present at sites where Gram-negative bacteria may enter the body (e.g. lung and gastro-intestinal tract), whereas macrophages are a part of the second line of defense in the body. Both cell types produce all kinds of inflammatory mediators upon LPS stimulation, and were therefore used to study the capacity of BIAP to detoxify LPS *in vitro*.

The effect of BIAP on LPS-induced cell responses was tested in a direct activation assay using three human epithelial cell lines T84 (colon), A-549 (alveolar) and HEK293T (kidney) and the murine macrophage cell line RAW264.7. Incubation of LPS and BIAP resulted in a significant decreased IL-8 production by A-549 and HEK293T cells. The effect of LPS with BIAP on IL-8 production was similar to that of approximately 10-40% of the LPS dose meaning that BIAP was able to detoxify up to 90% of the LPS present. Similar results were obtained by studies done by Koyama *et al.* (2002) which showed that rat intestinal AP (rIAP) had a valuable effect on endothelial cell viability when these cells were exposed to LPS [25]. Incubation of LPS and BIAP on RAW264.7 cells for 24 hrs did not have any reducing effect on cytokine or NO_x levels. In contrast, pre-incubation of RAW264.7 with BIAP followed by exposure to LPS resulted in decreased IL-6 and NO_x levels, while TNF-α levels were not affected. These results are in line with the experiments done by Aybay and Imir (1998) and Okemoto *et al*. (2006), showing that TNF-α induction by LPS and MPLA were similar when incubated on RAW264.7 cells [27, 28]. Since it is believed that AP dephosphorylates the lipid A part of LPS, these findings may also clarify our results. However, Bentala *et al.* (2002) reported that RAW264.7 cells incubated with MPLA were not activated to produce TNF-α [19]. These differences in observations remain to be elucidated.

In an 'inflamed' *in vivo* situation, epithelial cells produce several kinds of proteins that serve as chemo-attractants, like IL-8, MCP-1 and MIP-1. These chemo-attractants recruit macrophages and PMNs to the site of infection, resulting in the production of other inflammatory mediators. To more closely reflect this situation, indirect assays were performed in which supernatants from epithelial cells, stimulated with LPS with

or without BIAP, were transferred to RAW264.7 cells. Incubation of LPS with BIAP on epithelial cells resulted in decreased production of TNF-α, IL-6 and NO_x by RAW264.7 cells compared to incubation of LPS alone on epithelial cells. These results indicate that LPS was dephosphorylated and thereby detoxified by BIAP on epithelial cells.

An explanation for the striking difference in detoxifying capacity observed between simultaneous incubation of RAW264.7 with BIAP and LPS and pre-incubation of RAW264.7 with BIAP, followed by subsequent incubation with LPS might be found in the structure of the protein. BIAP is a glycosylphosphatidylinositol (GPI)-anchored protein and the presence of such a protein in an aqueous solution is energetically not favorable. Low and Zilversmit (1980) and Medof *et al.*(1996) reported that GPI anchored proteins bind to cell membranes [29, 30]. When BIAP is able to bind to the macrophage cell membrane before LPS stimulation, competition for LPS can occur between TLR4 and BIAP which results in a reduced production of inflammatory mediators. Since TLR4 expression is absent or down regulated on the epithelial cell surface, LPS needs to be internalized to induce an inflammatory response [31, 32]. This process is more time consuming than direct activation of macrophages by TLR4 signaling, generating the possibility for BIAP to dephosphorylate LPS.

To test the hypothesis that BIAP is able to bind to the cell membrane, experiments were performed in which RAW264.7 and epithelial cells were incubated with BIAP, after which cell-bound BIAP was visualized enzymatically. The results show that after BIAP incubation, the enzyme activity associated with cells is increased when compared to control cells. Experiments by DePierre and Karnovsky (1974) showed that the substrate used to enzymatically visualize AP, pNPP, does not penetrate the cell membrane [26]. This, together with the observation that incubation with an isotonic substrate solution does not result in increased membrane permeability, leads to conclude that BIAP was associated with the outside of the cell membrane, possibly by means of its GPI-anchor, thereby suggesting that dephosphorylation of LPS by BIAP is within the extra cellular environment.

Since AP is abundantly expressed by epithelial cells at sites where possible pathogens may enter the body (e.g. lung and intestine), this study clearly confirms a role for AP in reducing LPS-mediated cell responses by BIAP in different *in vitro* assays.

Acknowledgements

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Chapter 3

Bovine intestinal alkaline phosphatase (BIAP) inhibits the lipopolysaccharide (LPS)-induced IL-8 production by CD14-transfected human epithelial cells and the IL-6 production by human endothelial cells

submitted

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Abstract

Bovine intestinal alkaline phosphatase (BIAP) has been shown to exert an anti-inflammatory function in several *in vivo* models of LPS-mediated diseases by removing a phosphate group from the lipid A moiety of LPS, thereby reducing LPS toxicity. A previous study *in vitro* showed that BIAP was only able to detoxify LPS in a cell-based system, indicating that accessory molecules are probably involved in the biological activity of BIAP. Since CD14, a component of the LPS-signaling complex, colocalizes with endogenous AP at the cell membrane of epithelial cells and is found together with AP in granules of neutrophils, the role of CD14 in LPS detoxification by BIAP was investigated in this study. Therefore, HEK293T cells stably expressing CD14 (HEK293T/CD14) were incubated with LPS in the presence or absence of BIAP. IL-8 production by LPS-stimulated HEK293T/CD14 cells was reduced significantly in the presence of BIAP and blocking CD14 by using an anti-CD14 antibody resulted in the same reduction in the IL-8 response as BIAP addition. No additional effect of BIAP on IL-8 reduction was seen when cells were preincubated with an anti-CD14 antibody. A comparable result was obtained with HUVEC incubated with LPS. BIAP was unable to reduce the LPS-induced IL-6 production by HUVEC in the absence of sCD14. Furthermore, it was shown that BIAP prevented the binding and uptake of LPS by HEK293T/CD14 cells.

In conclusion, the obtained results in this study demonstrate that the ability of BIAP to reduce the inflammatory response in *in vivo* LPS-mediated disease models is dependent on the presence of CD14. Further studies are necessary to investigate the exact mechanism of action.

Introduction

Lipopolysaccharide (LPS), a component of the outer cell membrane of Gram-negative bacteria, is a potent stimulator of the innate immune system [1, 2]. The toxic part of LPS is located in its lipid A moiety which contains two phosphate groups essential for the biological action of the molecule [3]. Removal of one phosphate group results in the formation of a less toxic lipid A part [4-6].

In the last few years, it has been shown that epithelial and endothelial cells play a role in innate immune recognition of bacteria and their components. Stimulation of these cells by LPS is mediated by soluble CD14 (sCD14) present in the extracellular environment [7, 8]. LPS is bound by sCD14 with high affinity and the sCD14-LPS complex interacts intracellular with a signaling molecule, Toll-like-receptor 4 (TLR4) in association with the adapter molecule MD2, resulting in the production of chemokines and cytokines (e.g. IL-8 and IL-6) [9-12].

Alkaline phosphatase (AP), a nonspecific hydrolase for phosphomonoesters, is usually attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, providing enzymatic activity outside the cell [13]. The mammalian AP family consists of several isoforms of the enzyme, including the tissue-non-specific AP (liver-bone-kidney type) and the tissue-specific APs (intestinal, placental, and germ-cell type) [14]. High expression levels of AP can be found at sites were possible pathogens may enter the body, like the lung (tissue-non-specific AP) and the gastro-intestinal tract (intestinal AP).

Several investigators have shown promising therapeutic effects of AP on LPS toxicity. Studies by Bentala *et al.* (2002) and Verweij *et al.* (2004) showed that human placental alkaline phosphatase (HPLAP) is able to reduce mortality in mice infected with either LPS or a lethal doses of Gram-negative bacteria [15, 16]. Bovine intestinal alkaline phosphatase (BIAP) reduced inflammation in piglets injected with LPS and inhibition of endogenous intestinal alkaline phosphatase (IAP) resulted in a prolonged endotoxemia in rats after oral treatment with LPS [17, 18]. In studies with polymicrobial induced sepsis, BIAP attenuated cytokine response and neutrophil influx in secondary peritonitis in mice and reduced hepatic and pulmonary injury after

ischemia-reperfusion with combined with partial liver resection in rats [19, 20]. Decreased IL-6 concentrations and prolonged survival time was observed in BIAP-treated sheep injected with feces derived from their own intestine to mimic severe endotoxemia conditions [21].

Until now, the mechanism by which AP detoxifies LPS is not fully understood. It is believed that AP is able to remove one phosphate group from the lipid A part of LPS at physiological pH, thereby dephosphorylating and detoxifying LPS [22, 23]. Since AP interacts with LPS, it might be assumed that receptors of the innate immune system are involved in LPS recognition by AP. Detmers *et al.* (1995) showed that CD14 and AP share an intracellular compartment in neutrophils from which they move to the plasma membrane after exposure to appropriate agonists [24]. Recently, a colocalization of AP activity and CD14 expression was found in the kidney, liver, and intestine [25]. This might indicate that presence of CD14 is required for detoxification of LPS by AP. In line with these observations, we hypothesize that interactions between the CD14-LPS complex and AP exist, and that this interaction facilitates LPS detoxification by AP.

In this study, it was investigated whether BIAP modifies the activating properties of the CD14-LPS complex on epithelial and endothelial cells. For this purpose, the effect of BIAP on the production of IL-8 induced by the CD14-LPS complex on human embryonic kidney (HEK293T) cells and on the IL-6 production by human umbilical vein endothelial cells (HUVEC) was determined.

Materials and Methods

Bovine intestinal alkaline phosphatase

Bovine intestinal alkaline phosphatase (BIAP; GMP-processed, purity ALPIXG: 675 U/mg) was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of BIAP able to hydrolyze 1 µmole of p-nitro phenyl phosphate per minute at pH 9.6 at 25 ˚C.

Reagents

DMEM/F-12 (1:1) medium and MEM199 medium were from Invitrogen Corp. (Breda, The Netherlands) as well as penicillin, streptomycin, hepes, sodiumpyruvate and puromycin. Foetal bovine serum (FBS) was obtained from Wisent Inc. (Quebec, Canada). *E. coli* LPS O111:B4, FITC-labelled *E. coli* LPS O111:B4, endothelial cell growth factor (ECGF) and normal human AB serum (HS) were from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and heparin was from LEO Pharma B.V. (Breda, The Netherlands). Monoclonal mouse anti-human CD14 antibody (clone 134620) and isotype control IgG1 were from R&D systems (Wiesbaden, Germany). Human recombinant sCD14 was a kind gift from C. Schuett (Ernst-Moritz-Arndt University, Greifswald, Germany). Human IL-8 and IL-6 ELISA kits were purchased from Biosource Europe SE (Nivelles, Belgium).

Epithelial cell culture

The human embryonic kidney cell line HEK293T/CD14, stably expressing human mCD14, cloned into the pRc/RSV expression vector, was a kind gift from Y. Kruize (LUMC, Leiden, The Netherlands) and D.T. Golenbock (University of Massachusetts Medical School, Worcester, USA). HEK293T/CD14 cells were grown in DMEM/F12 (1:1) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mM hepes, 1 mM sodiumpyruvate and 5 µg/ml puromycin. Cells were cultured in a humidified incubator at 37 ˚C and 5% CO_2 and sub cultured at confluence by employing trypsin-EDTA.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were a kind gift from I. Kathmann (AMC, Amsterdam, The Netherlands). Cells were cultured in 1% gelatin-coated culture flasks (Greiner Bio-One, Frickenhausen, Germany) in MEM199 medium supplemented with 10% heat-inactivated FBS, 10% heat-inactivated HS, 100 U/ml penicillin, 100 µg/ml streptomycin, ECGF, and 5 U/ml heparin. HUVEC were cultured in a humidified incubator at 37 ˚C and 5% CO_2 and sub cultured at confluence by employing trypsin-EDTA. Only cells of the sixth passage were used.

Activation of epithelial cells

HEK293T/CD14 cells were seeded into 96-well culture plates (Nunc, Roskilde, Denmark). On reaching confluence, medium was refreshed and cells were incubated with medium containing 1 µg of LPS/ml with or without different concentrations of BIAP. After an incubation period of 24 hrs, cell supernatants were collected and stored at –70 ˚C until further use.

In other experiments, HEK293T/CD14 cells were seeded into 96-well culture plates and grown until confluency was reached. Medium was removed and replaced with medium without FBS. Cells were incubated with 10 µg/ml of anti-human CD14 antibody for 1 hr at 37 ˚C and 5% CO_2. One µg of LPS/ml or 1 µg of LPS/ml and 1 unit of BIAP/ml were added. Controls were performed with LPS alone and with LPS in the presence of BIAP. After 5 hrs of incubation at 37 ˚C and 5% CO_2, FBS was added to a final concentration of 10% and cells were left to incubate for 18 hrs at 37 ˚C and 5% CO_2. Cell supernatants were collected and stored at –70 ˚C until further use.

Activation of endothelial cells

HUVEC were seeded into 96-well culture plates coated with 1% of gelatin. On reaching confluence, medium was refreshed and cells were incubated with medium containing 1 µg of LPS/ml with or without different concentrations of BIAP. After an incubation period of 24 hrs, cell supernatants were collected and stored at –70 ˚C until further use.

In other experiments, HUVEC were seeded into 1% gelatin-coated 96-well culture plates and grown until confluency was reached. Medium was removed and replaced with medium without serum. Cells were incubated with 2 µg/ml of human sCD14 for 1 hr at 37 ˚C and 5% CO_2. One µg of LPS/ml alone or together with 1 unit of BIAP/ml were added. After 5 hrs of incubation at 37 ˚C and 5% CO_2, FBS was added and cells were left to incubate for an additional period of 18 hrs at 37 ˚C and 5% CO_2. Cell supernatants were collected and stored at –70 ˚C until further use.

Quantitation of IL-8 and IL-6 release by ELISA

Cell culture supernatants collected after 24 hrs were processed for IL-8 or IL-6 quantification by sandwich ELISA according to the manufacturers' instructions. Briefly, 96-well Maxisorp F8 plates (NUNC, Roskilde, Denmark) were coated overnight at 4 ˚C with 100 µl/well of 1 µg/ml monoclonal anti-IL-8 or anti-IL6 antibody diluted in 0.05 M carbonate buffer (pH 9.4) or PBS (pH 7.4) respectively and subsequently washed with wash buffer (PBS-0.05% [vol/vol] Tween-20). Anti-IL-8 antibody coated plates were blocked for 1 hr at 37 ˚C with 250 µl of PBS-0.05% (vol/vol) Tween-20 containing 1% (wt/vol) BSA. Anti-IL-6 antibody coated plates were blocked for 2 hrs at 37 ˚C with 300 µl of PBS containing 0.5% (wt/vol) BSA. Cell supernatants and standards were diluted in assay buffer (PBS-0.05% [vol/vol] Tween-20 containing 0.5% [wt/vol] BSA) and 50 µl was transferred to the wells which was immediately followed by a second biotin-conjugated polyclonal anti-IL-8 antibody (50 µl of a 0.2 µg/ml solution in assay buffer) or a biotin-conjugated polyclonal anti-IL-6 antibody (50 µl of a 0.16 µg/ml solution in assay buffer). Following an incubation period of 2 hrs at room temperature, plates were washed with wash buffer and wells were incubated with 100 µl of a 0.3 µg/ml Streptavidin-HRP solution in assay buffer for 45 min at room temperature after which plates were washed with wash buffer. Detection was performed with a 100 µl tetramethyl benzidine (TMB) solution at room temperature. After 10 min, the reaction was stopped with 50 µl of 2 M H_2SO_4 per well and absorbance was measured at 450 nm on a microplate reader. IL-8 and IL-6

concentrations were quantitated in comparison with a recombinant human IL-8 or IL-6 standard curve respectively.

Statistical analysis

Data are presented as the means ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni as *post hoc* test. Values of $P < 0.05$ were considered significant.

Results

Effect of BIAP on the IL-8 production by LPS stimulated HEK293T/CD14 cells

In order to determine if BIAP modulates the expression of IL-8 by LPS-stimulated HEK293T/CD14 cells, secretion of IL-8 in the presence of 1µg/ml of LPS and various amounts of BIAP was assessed by ELISA. As shown in Fig. 1, the exposure of cells to LPS resulted in IL-8 production. IL-8 secretion induced by 1 µg/ml of LPS was significantly decreased in the presence of 1 unit/ml of BIAP. These results demonstrate that BIAP interferes with IL-8 expression by HEK293T/CD14 cells induced by LPS.

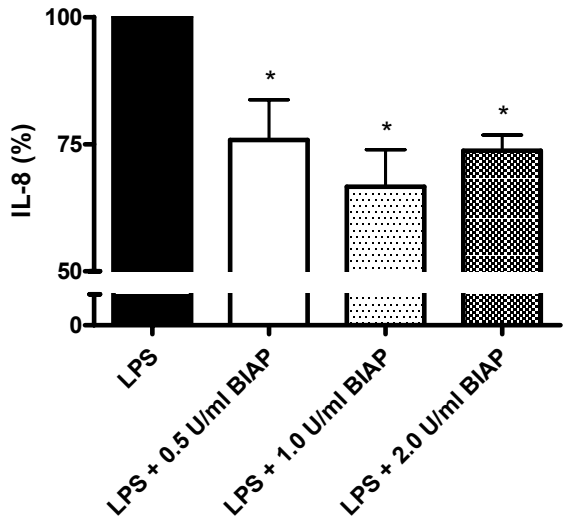

Figure 1: Effect of BIAP on IL-8 production by LPS stimulated HEK293T/CD14 cells in the presence of serum. HEK293T/CD14 cells were incubated for 24 hrs with 1 µg of LPS/ml alone or in the presence of different concentrations of BIAP. Data presented are means ± SEM, * $P < 0.05$.

Effect of BIAP on the IL-6 production by LPS stimulated HUVEC

To determine the effect of BIAP on the expression of IL-6 by LPS-stimulated HUVEC, secretion of IL-6 in the presence of 1 µg/ml of LPS and various amounts of BIAP was

assessed by ELISA. Fig. 2 shows that incubation of HUVEC with LPS resulted in production of IL-6. One and 2 unit/ml of BIAP significantly reduced IL-6 production induced by 1 µg/ml of LPS by approximately 25%.

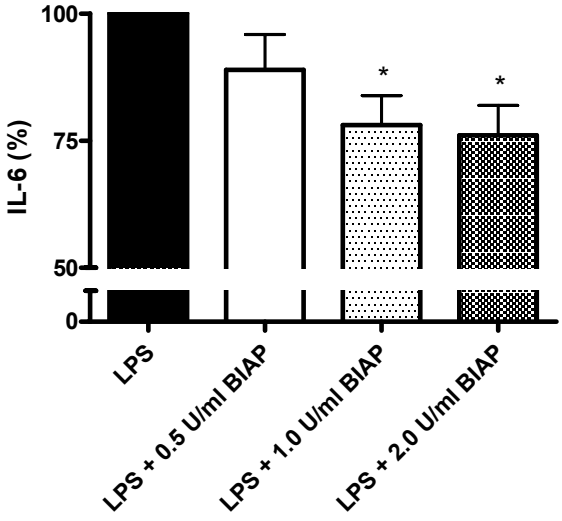

Figure 2: Effect of BIAP on IL-6 production by LPS stimulated HUVEC in the presence of serum. HUVEC were incubated for 24 hrs with 1 µg LPS/ml alone or in the presence of different concentrations of BIAP. Data presented are means ± SEM, * $P < 0.05$.

Necessity of CD14 on BIAP inhibition of LPS induced IL-8 production in HEK293T/CD14 cells

HEK293T/CD14 cells were incubated with 1 µg/ml of LPS and 1 unit/ml of BIAP with or without 10 µg/ml of anti-human CD14 antibody. IL-8 production was measured by ELISA. As shown in Fig. 3, IL-8 production was 30.7 ± 4.4% decreased in the presence of BIAP and LPS compared to LPS alone. Incubation of HEK293T/CD14 cells with anti-human CD14 antibody reduced the IL-8 production by LPS induction with 39.4 ± 6.7%. No additional effect of BIAP detoxification of LPS could be observed when HEK293T/CD14 cells were incubated with anti-human CD14 antibody.

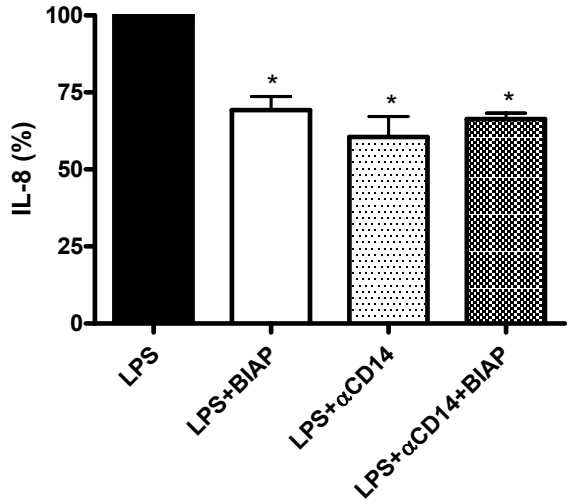

Figure 3: Effect of BIAP on IL-8 production by LPS stimulated HEK293T/CD14 cells in the presence of anti-human CD14 antibody. HEK293T/CD14 cells were incubated with anti-human CD14 antibody and subsequently stimulated with LPS in the presence or absence of BIAP for 5 hrs. After 5 hrs of activation, FBS was added to a final concentration of 10% and cells were incubated for an additional period of 19 hrs. Data presented are means ± SEM, * $P < 0.05$.

Necessity of CD14 on BIAP inhibition of LPS induced IL-6 production in HUVEC

Huvec were incubated with 1 µg/ml of LPS or 1 µg/ml of LPS and 1 unit/ml of BIAP with or without 2 µg/ml of human sCD14. IL-6 production was measured by ELISA. As shown in Fig. 4, BIAP had no effect on reducing LPS-induced IL-6 production. When coincubated with human sCD14, the IL-6 production by LPS-stimulated HUVEC was significantly increased compared to LPS alone (198.7 ± 23.8% *versus* 100%, respectively). In the condition where HUVEC were incubated with LPS and human sCD14, addition of BIAP resulted in a decrease of IL-6 production induced by the LPS-sCD14 complex by about 40% (198.7 ± 23.8% *versus* 133.8 ± 7.5%, respectively).

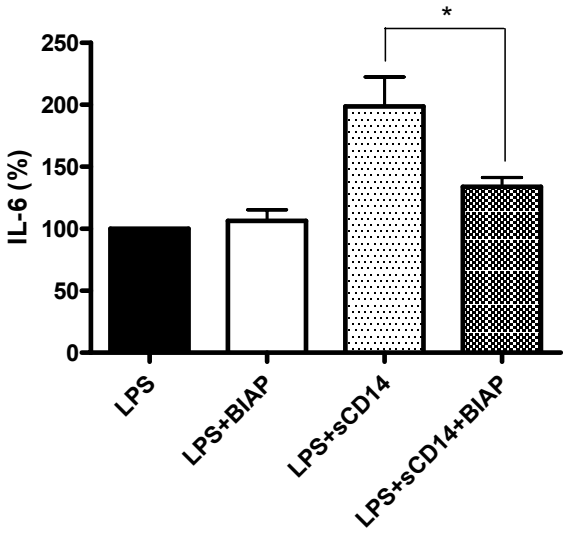

Figure 4: Effect of BIAP on IL-6 production by LPS stimulated HUVEC in the presence of human sCD14. HUVEC were incubated with human sCD14 and subsequently stimulated with LPS in the presence or absence of BIAP for 5 hrs. After 5 hrs of activation, FBS was added to a final concentration of 10% and cells were incubated for an additional period of 19 hrs. Data presented are means ± SEM, * $P < 0.05$.

Discussion

Numerous investigators have shown the effects of BIAP on attenuating activation of the innate immune system in several animal models of LPS-mediated diseases. It is believed that BIAP dephosphorylates the lipid A moiety of LPS which results in a four orders of magnitude less potent form to induce an inflammatory reaction, but, unfortunately, direct effects of BIAP on LPS detoxification *in vivo* cannot be assessed since the *Limulus* amoebocyte lysate (LAL) assay is unable to make a discrimination between LPS dephosphorylated LPS [26]. Yet, *in vitro* dephosphorylation of LPS by BIAP (measured by the release of free phosphate) could not be detected. A previous study demonstrated that BIAP was only able to detoxify LPS in a cell-based *in vitro* system, as determined by a reduction in cell-derived inflammatory mediators, indicating that accessory molecules are probably involved in the biological activity of BIAP (Fiechter *et al*., submitted). Since CD14, a component of the LPS-signaling complex (composed of TLR4, CD14, and MD2) colocalizes with endogenous AP at the cell membrane of epithelial cells and is found together with AP in granules of neutrophils, the role of CD14 in LPS detoxification by BIAP was investigated in this study [24, 25].

In the first part of this study, it was shown that BIAP had an effect on attenuating IL-8 production in LPS-activated HEK293T/CD14 cells. A similar reduction in IL-8 production was obtained with HEK293T cells transfected with CD14 and TLR4, suggesting that presence of TLR4 does not contribute to the LPS-detoxifying capacity of BIAP (data not shown). Comparable results were obtained with LPS-stimulated HUVEC since production of this cytokine was significantly decreased in the presence of BIAP. Excessive production of these inflammatory mediators *in vivo* can result in severe damage of the epithelial end endothelial barrier, and the observed effect of BIAP on reducing IL-8 and IL-6 production might be a beneficial treatment during an LPS insult.

Next, it was determined whether HEK293T/CD14 cells could still be stimulated by LPS in the absence of CD14 and if BIAP in this situation would be able to detoxify LPS. Blocking of CD14 resulted in a significant reduction of IL-8 production, but not a

total remission of the LPS-induced response. This is in contrast with several publications stating that HEK293T cells not transfected with any part of the LPS-signaling complex are unresponsive to LPS, although none examined the IL-8 production by HEK293T cells after 24 hours under these extreme LPS overload conditions. BIAP addition resulted in the same reduction in the IL-8 response as blocking of CD14 and no additional effect of BIAP on IL-8 reduction was seen when cells were preincubated with an antibody directed against CD14. LPS-stimulated HUVEC showed an almost two fold increase in IL-6 production when coincubated with sC14. Only in this condition BIAP was able to reduce the LPS-induced effects. To avoid the presence of endogenous CD14 contamination in the HUVEC experiments, only cells of the sixth passage were used as studies by Jersmannn *et al.* (2001) showed that HUVEC express CD14 on their cell membrane, but expression was significantly decreased with increasing numbers of culture passage [27].

The results obtained with HEK293T/CD14 cells and HUVEC indicate that presence of CD14 is essential for BIAP to detoxify LPS. A possible explanation for these findings can be that LPS, present in blood or other body fluids, will form micelles which are disrupted by CD14 thereby enabling BIAP to reach the otherwise concealed lipid A moiety of LPS. In the absence of CD14, LPS will stay in an aggregated state making it difficult for BIAP to dephosphorylate LPS. Furthermore, CD14 is needed to direct LPS to the cell surface were it can encounter BIAP, which, being a glycosyl-phosphatidylinositol (GPI)-anchored protein, will bind to the cell membrane since presence in an aqueous solution is energetically not favorable [28].

In conclusion, this study demonstrates that BIAP is able to inhibit the LPS-induced expression of IL-8 by HEK293T/CD14 cells and the IL-6 production by HUVEC. Although the exact mechanism of action was not made clear, the effect of BIAP depends at least on the presence of CD14. Since in the *in vivo* situation AP is abundantly present at sites where possible antigens (*e.g.* LPS) may enter the body these results imply that AP may serve a role in the natural defense system.

Acknowledgements

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Chapter 4

Bovine Intestinal Alkaline Phosphatase (BIAP) reduces inflammation after induction of acute myocardial infarction (AMI) in mice

submitted

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Abstract

Objective

There has been increasing evidence suggesting that lipopolysaccharide (LPS) may be an important activator of the innate immune system after acute myocardial infarction (AMI). Bovine intestinal alkaline phosphatase (BIAP) reduces inflammation in several LPS-mediated diseases by dephosphorylation of the lipid A moiety of LPS. The aim of this study was to investigate the effect of BIAP on reducing inflammation after AMI in Balb/c mice.

Methods

Just before permanent ligation of the left anterior descending (LAD) coronary artery to induce AMI, BIAP was administrated i.v. as a prophylaxis. After 4 hours, mice were sacrificed and the inflammatory response was assessed.

Results

AMI induced the production of IL-6, IL-1β, IL-10 and mMCP-1. BIAP treatment resulted in a significantly reduction of the pro-inflammatory cytokines IL-6, IL-1β and the chymase mMCP-1. No difference in the production of the anti-inflammatory cytokine IL-10 was observed between the control group and the BIAP-treated group.

Conclusion

In a mouse model of permanent LAD coronary artery ligation, BIAP diminishes the pro-inflammatory responses but does not have an effect on the IL-10 anti-inflammatory response in the acute phase after AMI.

Introduction

Lipopolysaccharide (LPS), an endotoxin present in the outer cell membrane of Gram-negative bacteria, is a potent stimulator of the innate immune response. When entering the circulation, LPS binds to the lipopolysaccharide-binding protein (LBP) which interacts with CD14, MD2, and Toll-like receptor 4 (TLR4) to start a signaling cascade leading to cytokine production [1-4].

Cardiogenic shock (CS), a severe form of heart failure (HF), is the leading cause of death among patients hospitalized with acute myocardial infarction (AMI). It is well known that AMI is associated with an increased inflammatory reaction [5]. There has been growing evidence suggesting that endotoxin is an important stimulus for this phenomenon. Decreased cardiac function reduces bowel perfusion, leading to hypoperfusion and ischemia of the intestinal mucosa. This results in increase of gut permeability, and subsequent translocation of endotoxin into the circulation [6, 7].

Several studies with HF patients, irrespective of etiology, have shown an increase of soluble CD14 (sCD14) in plasma, TLR4 expression on monocytes and increased levels of bacteria or endotoxin when compared to control groups [6, 8-12]. Taken together, these data lead to suggest that LPS is an important mediator in the observed inflammatory response after AMI.

There is increasing evidence that alkaline phosphatase (AP) is able to remove one phosphate group from the lipid A moiety of LPS, thereby dephosphorylating and detoxifying LPS [13, 14]. In mice, infected with a lethal dose of Gram-negative bacteria, mortality was reduced after injection of human placental alkaline phosphatase (HPLAP) or bovine intestinal alkaline phosphatase (BIAP) [15, 16]. A reduction in the inflammatory response induced by LPS could be observed in mice and piglets after treatment with HPLAP or BIAP [15, 17]. Oral treatment of rats with LPS resulted in a prolonged endotoxemia after inhibition of endogenous intestinal alkaline phosphatase (IAP) [18]. In addition, the potential effects of AP on LPS-mediated diseases have been demonstrated in animal studies with polymicrobial sepsis. Cytokine response and neutrophil influx in secondary peritonitis in mice was attenuated by BIAP [19]. Hepatic and pulmonary injury after liver ischemia-reperfusion with partial resection was

reduced in rats treated with BIAP when compared to control animals [20]. Studies performed by the group of J.-L. Vincent (Brussels, Belgium) with BIAP administration to sheep, injected with feces to mimic severe endotoxemia conditions, showed a decrease in IL-6 concentrations and a prolonged survival time [21].

In this study, the left anterior descending (LAD) coronary artery ligation was used as a model to induce an AMI in mice. The objective was to examine the potential effect of BIAP on reducing the pro-inflammatory response in the acute phase after AMI by its ability to detoxify LPS. Prior to LAD ligation, BIAP was used as a prophylaxis by i.v. administration. The resulting systemic inflammatory response was investigated.

Materials and methods

Bovine intestinal alkaline phosphatase

Clinical grade bovine intestinal alkaline phosphatase was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of BIAP able to hydrolyse 1 µmole of p-nitrophenyl phosphate per minute using a Tris-glycin buffer at pH 9.6 at 25 ˚C.

Myocardial infarction induction

Specific pathogen free (SPF) female BALB/c mice (23-27 gram) were purchased from Charles River (Sulzfeld, Germany) and were acclimatized for 1 week under barrier conditions in filter-topped macrolon cages with drinking water and standard food pellets *ad libitum*.

Myocardial infarction was induced as described previously [22]. Briefly, mice were anaesthetized by inhalation of a mixture of O_2 air and 4% isoflurane, endotracheally intubated, and mechanically ventilated. The LAD coronary artery was exposed via a left thoractomy and double ligated with an 8.0 prolene suture.

First, it had to be determined at which time point after AMI induction pro-inflammatory cytokine production could be detected. Therefore, mice were sacrificed 4, 6, and 24 hours after AMI induction and blood was collected (n=3 per time point). To examine the effect of BIAP, mice were divided into two groups: an AMI group treated with BIAP (n=4) and an AMI control group treated with vehicle alone (n=4). BIAP was injected into the tail vein just before anaesthezation as a single intravenous dose of 5 IU in 100 µl PBS (approximately 100 times above plasma levels). Control mice were injected with an equal volume of PBS. Mice were sacrificed and blood was collected. Heart, lung, liver and kidneys were removed and fixed in 4% para-formaldehyde in PBS.

The study was approved by the animal ethics committee of the Faculty of Veterinary Medicine, Utrecht University.

Determination of alkaline phosphatase activity

Five µl of serum was incubated for 60 minutes at 37 ˚C with 200 µl assay mix containing incubation buffer (0.025 M glycin/NaOH, pH 9.6), p-nitrophenyl phosphate and $MgCl_2$ at final concentrations of 1.25 and 2 mM respectively. The end product p-nitrophenol was quantatively determined by measuring the extinction at 405 nm.

Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples were centrifuged and serum was collected for determination of mouse IL-6, TNF-α, IL-1β and IL-10 protein concentrations by commercially available ELISA kits according to the manufacturers' protocol (IL-6 and TNF-α from Biosource, Etten-Leur, The Netherlands; IL-1β from R&D Systems, Abingdon, UK; and IL-10 from BD Biosciences, Erembodegem, Belgium). Mouse mast cell protease-1 (mMCP-1) ELISA was from Moredun (Midlothian, Scotland, UK) and performed according to the manufacturer's instructions.

Statistics

All data presented are mean ± SEM. Statistical analysis was performed using Student's *t*-test for unpaired data (GraphPad Prism). Values were considered significant when $P < 0.05$.

Results

Determination of the IL-6 response

One of the major pro-inflammatory cytokines produced in AMI patients is IL-6. First, it had to be determined at which time point after AMI in Balb/c mice IL-6 production could be detected. Therefore, mice underwent permanent ligation of the LAD coronary artery to induce AMI and were sacrificed at three different time points. Before operation, IL-6 concentration was below detection limit (< 4 pg/ml) (Fig. 1). Peak IL-6 serum levels were observed 4 hours after AMI. Elevated serum levels of IL-6 could still be detected 6 and 24 hours after AMI. Based on these results, mice were sacrificed 4 hours after AMI in the BIAP treatment experiments.

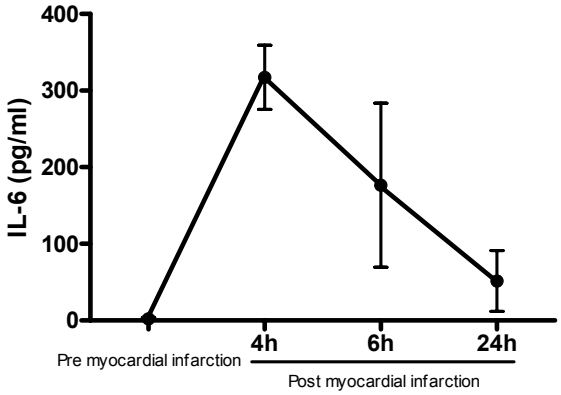

Figure 1: Production of the pro-inflammatory cytokine IL-6 after acute myocardial infarction. Mice were sacrificed at different time points and IL-6 production was determined (n = 3 per time point). Values are depicted as mean ± SEM.

AP activity

AP activity was determined in serum samples by measuring hydrolysis of p-nitrophenyl phosphate by AP. All mice that received BIAP had slightly elevated serum levels of AP activity 4 hours after AMI compared to control mice ($P < 0.05$), indicating that BIAP was still present in the circulation (Fig. 2).

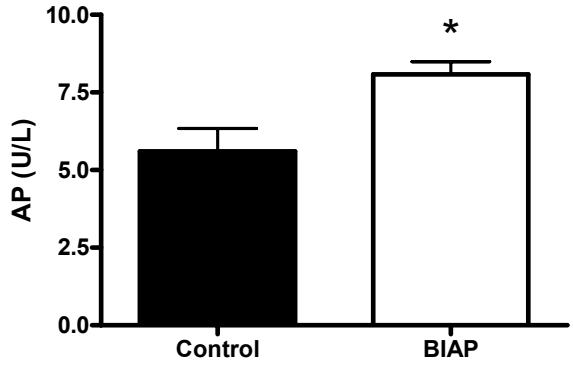

Figure 2: Alkaline phosphatase activity 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Cytokine response

Systemically elevated concentrations of IL-6, IL-1β, TNF-α, and IL-10 are observed in patients with AMI. Therefore, presence of these cytokines was determined. Before LAD coronary artery ligation, concentrations of the different cytokines were below detection levels. In contrast, 4 hours after AMI IL-6, IL-1β and IL-10 were excessively produced (Fig. 3). TNF-α production could not be determined at this time-point. Administration of BIAP resulted in a significant reduction of the pro-inflammatory cytokines IL-6 and IL-1β when compared to controls. IL-6 levels were reduced by approximately 40% and IL-1β by approximately 30%. No difference in the anti-inflammatory cytokine IL-10 production could be observed between the control group and the BIAP-treated group.

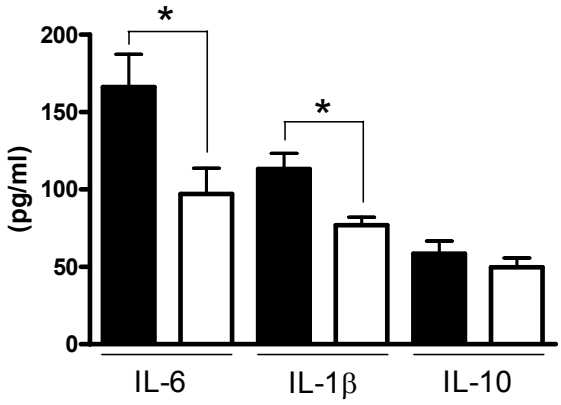

Figure 3: Effect of BIAP on the production of the pro-inflammatory cytokines IL-6 and IL-1β and on the anti-inflammatory cytokine IL-10 4 hours after acute myocardial infarction. Levels of IL-6, IL-1β, and IL-10 were determined using specific ELISA; (■) control mice and (□) BIAP-treated mice. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Mast cell activation

After AMI in mammals, mast cells are activated to release chymases. Activation of mast cells in mice can be measured by the release of the mouse mast cell protease-1 (mMCP-1) chymase. It was therefore interesting to see what the effect of BIAP administration would be on mast cell behavior. Serum levels of mMCP-1 were 14.7 ng/ml 4 hours after LAD coronary artery ligation. BIAP treatment reduced mMCP-1 levels in serum to 8.4 ng/ml (approximately 40%), implying a significant reduction in mast cell activation ($P < 0.05$) (Fig. 4).

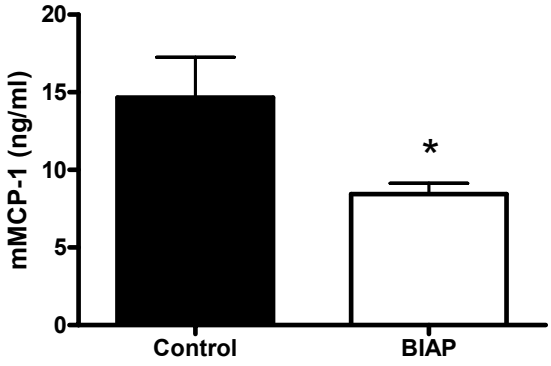

Figure 4: Effect of BIAP on the production of mMCP-1, 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Discussion

Cardiogenic shock (CS) is the major cause of death in patients hospitalized with acute myocardial infarction (AMI) [23]. AMI results in intestinal hypoperfusion, which leads to increased gut permeability. Consequently, translocation of endotoxin into the circulation occurs. There has been growing evidence that presence of endotoxin is responsible for the observed systemic inflammatory response after AMI and that this may play an important role in the onset of CS [6, 7, 12]. Reducing inflammation after AMI has received little attention in research, and a specific pharmacologic treatment to reduce the inflammatory response after AMI has yet to be introduced.

To date, several studies have demonstrated the potential therapeutic effect of BIAP on LPS-mediated diseases, and it was therefore interesting to examine the ability of BIAP on reducing inflammation after AMI [19-21]. Therefore, Balb/c mice received an i.v. injection of BIAP just before AMI induction by a permanent ligation of the LAD coronary artery.

Four hours after AMI, a significant reduction in the concentrations of the two most prominent pro-inflammatory cytokines present in serum in the acute phase after AMI, IL-6 and IL-1β, was observed when compared to vehicle controls. TNF-α concentration in serum, generally believed to be an early-onset proinflammatory cytokine, was below detection limit, suggesting that the chosen time point is not relevant to detect this cytokine in Balb/c mice after LAD coronary artery ligation. A reduction in pro-inflammatory cytokine production indicates a diminished systemic innate immune response, which may decrease myocardial dysfunction and reduce the development of CS after AMI [8]. BIAP treatment had no effect on IL-10 production. Since IL-10 is a potent anti-inflammatory cytokine, and several *in vivo* studies have shown its protective role in a variety of pathological states (e.g. colitis, hepatic ischemia/reperfusion (I/R), and myocardial I/R), a reduced production due to BIAP treatment would not be favorable [24-26].

Chymases are abundantly produced after AMI in mammals, and are known to be involved in the cleaving of angiotensin I (Ang I) to form angiotensin II (Ang II) [27, 28]. The excessive formation of Ang II, which is observed in the acute phase after AMI,

is arrhythmogenic, and several studies in different animal models have shown that decreasing Ang II formation by a specific chymase inhibitor contributes to a reduction in the mortality rate in the acute phase after AMI [29, 30]. Studies in rats revealed that production of the rat chymase MCP-2 (rMCP-2) is increased after stimulation of mast cells with LPS [31, 32]. Given that BIAP has an effect on decreasing LPS toxicity, the influence of BIAP on the formation of the mouse chymase mMCP-1 was determined. In BIAP-treated mice, mMCP-1 production was significantly reduced by approximately 40% when compared to vehicle-treated mice. This result indicates a reduction of Ang II formation and consequently a decrease in arrhythmias, which may improve cardiac function and reduce CS complication.

Direct effects of BIAP on LPS detoxification could not be determined in this study. Since it is believed that BIAP is able to detoxify LPS through dephosphorylation of the lipid A moiety, the *Limulus* amoebocyte lysate (LAL) assay cannot be used as it is unable to make a discrimination between lipid A and monophosphoryl lipid A (MPLA) [33]. Therefore, decreased activation of the innate immune response because of BIAP administration could not be linked to decreased LPS levels in this study, and thus the direct effect of BIAP on LPS could not be assessed. Nonetheless, the specific activity of HPLAP and BIAP against an LPS insult has been undoubtedly demonstrated *in vivo* [15, 17].

In conclusion, a single i.v. dose of BIAP reduced the production of the chymase mMCP-1 by mast cells and diminished the systemic pro-inflammatory cytokine response in the acute phase after AMI. Therefore, it is proposed that BIAP represents a novel therapeutic drug in attenuating the pro-inflammatory response after AMI thereby reducing the incidence of CS complication.

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Chapter 5

Bovine intestinal alkaline phosphatase (BIAP) reduces body weight changes and inflammation in a murine model of dextran sulfate sodium (DSS)-induced colitis

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Abstract

Inflammatory bowel disease (IBD) is characterized by inflammation of the bowel, possibly by an aberrant immune response to enteric bacteria and their components. Lipopolysaccharide (LPS), a component of the outer leaflet of Gram-negative bacteria, is thought to be important in aggravating the disease. As bovine intestinal alkaline phosphatase (BIAP) has been shown to exert anti-inflammatory effects in several different animal models of LPS-mediated diseases, we examined the potential therapeutic effect of BIAP in IBD. In this study, dextran sulfate sodium (DSS) was used to induce acute colitis in C57BL/6 mice. Oral treatment with BIAP started 4 days after start of the DSS treatment. Colitis severity was assessed by determination of body weight, colon length, and local inflammatory response (oedema, leukocyte infiltration, loss of crypts and goblet cells, and cytokines). Treatment with BIAP resulted in a significant reduction of body weight loss and TNF-α production. Furthermore, there was less shortening, histopathological damage and leukocyte infiltration of the colon in mice treated with BIAP. Present findings indicate that BIAP attenuates DSS-induced colitis and suggest that BIAP may be a promising therapeutic drug for the treatment of IBD.

Introduction

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by an excessive inflammation of the intestine. There has been growing evidence that IBD results from an abnormal response of the host to commensal enteric bacteria leading to activation of the mucosal immune system [1-4]. Blocking of the immune response to commensal bacteria or their components may therefore be interesting in ameliorating IBD.

Lipopolysaccharide (LPS), an endotoxin present in the outer leaflet of Gram-negative bacteria, is a potent stimulator of the inflammatory response. LPS is a complex molecule composed of a polysaccharide chain (O-chain) and a toxic lipid moiety (lipid A). The two phosphate groups of lipid A are essential for its immunostimulatory effect [5, 6]. Removal of one of the phosphate groups of lipid A results in the formation of mono-phosphoryl lipid A (MPLA), which is non-toxic and able to induce tolerance to endotoxin [7, 8]. Several animal models of intestinal inflammation have shown the role of intestinal bacteria in IBD development, and since many enteric bacteria are Gram-negative this supports a role for LPS in aggravating this disease.

A number of previous studies have demonstrated promising therapeutic effects of alkaline phosphatase (AP) on several LPS-mediated diseases, possibly because AP may dephosphorylate the lipid A moiety of LPS [9, 10]. For instance, recent studies have shown that bovine intestinal alkaline phosphatase (BIAP) and human placental alkaline phosphatase (HPLAP) reduced mortality in mice infected with a lethal dose of Gram-negative bacteria, and decreased inflammation in mice and piglets injected with LPS [11-13]. BIAP appeared also capable of attenuating cytokine response and neutrophil influx in secondary peritonitis in mice and reducing hepatic and pulmonary injury after liver ischemia-reperfusion with partial resection in rats [14, 15]. Furthermore, BIAP decreased IL-6 concentrations and prolonged survival time in sheep injected with feces [16]. As additional proof of the LPS-modulating potential of AP, inhibition of endogenous intestinal alkaline phosphatase (IAP) resulted in prolonged LPS-induced endotoxemia in rats [17]. Besides the there effects *in vivo*, BIAP also has anti-inflammatory effects *in vitro* by reducing IL-8 production by epithelial cells and IL-6

and TNF-α secretion by macrophages in response to LPS treatment (Fiechter *et al.*, submitted).

The combination of the suspected role of Gram-negative bacteria in IBD and the possible LPS-detoxifying potential of AP led to the hypothesis that AP could also be beneficial in the treatment of IBD. The present study shows the therapeutic effect of BIAP in dextran sulfate sodium (DSS) induced acute colitis in mice, a preclinical model for testing the efficacy of treatments for IBD.

Material and Methods

Mice and reagents

Female C57BL/6 mice, 10 weeks of age were purchased from Charles River (Sulzfeld, Germany). Mice were acclimatized for 4 weeks under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a temperature of 23 ± 2 °C, 50-55% relative humidity, and a 12 hrs light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the Faculty of Veterinary Medicine, University Utrecht. Clinical grade BIAP was obtained from Biozyme (Blaenavon, United Kingdom) and chemicals were from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

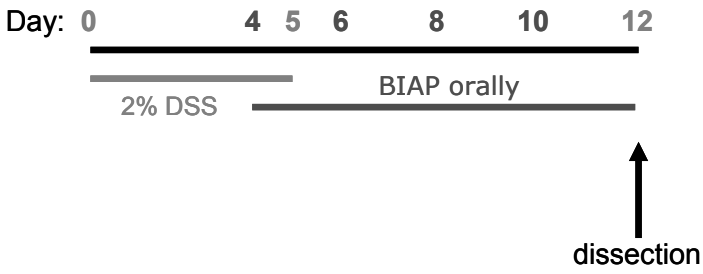

Figure 1: Study outline for DSS-induced acute colitis in C57BL/6 mice. Mice were provided with drinking water containing 2% (w/v) DSS during 5 days followed by 7 days of normal drinking water. Oral treatment with 100 U of BIAP started on day 4 and was continued until mice were sacrificed at day 12.

Induction of colitis and treatment with BIAP

Mice were divided in groups of 5 (vehicle treatment) or 10 (BIAP treatment) mice per treatment. To induce acute colitis, C57BL/6 mice were provided with drinking water containing 2% (w/v) DSS (MP Biomedicals, Aurora, OH; MW 35-50 kD, lot no 1359H) for five consecutive days, followed by normal drinking water. No differences in water consumption between treatment groups were observed. Treatment with BIAP started 4 days after initiation of DSS exposure. BIAP was administered

orally as a 100 U bolus dissolved in 100 µl vehicle (Tris-HCl buffer, pH 7.2) by gavage once a day. Vehicle-treated mice received 100 µl of vehicle alone (Fig. 1).

Determination of colitis severity

Body weight and behavior were examined daily during the experiment. Mice were sacrificed by cervical dislocation 12 days after the induction of colitis. The complete colon was dissected, colon length was measured with a ruler and feces were removed carefully. The colon was cut longitudinally, after which the full length of half of the colon was fixed in 4% phosphate-buffered formalin and embedded in paraffin. Routine 5 µm sections were prepared and stained with haematoxylin and eosin. Sections were evaluated in a blind coded fashion by two independent investigators and scored for oedema (none = 0; involved area less than 1/5 of the colon = 1; involved area up to half of the colon = 2; involved area more than half of the colon = 3), loss of crypts (no changes = 0; less than 1/5 = 1; up to half = 2; more than half = 3), loss of goblet cells (no changes = 0; less than 1/5 = 1; up to half = 2; more than half = 3), and infiltration of leucocytes (no infiltrations = 0; infiltrations mainly in mucosa in less than 1/5 of colon = 1; infiltrations in mucosa and submucosa up to half of the colon = 2; infiltrations in more than half of the colon = 3). The histological score was calculated as the sum of the scores for each category and resulted in a maximum score of 12 in most severe situation. Evaluation of independent investigators were averaged and resulted in a histological score for each parameter for each mouse.

Myeloperoxidase (MPO) activity

A biopsy of the dissected colon of 20 to 40 mg (exact weight was recorded) was snap-frozen in liquid nitrogen and stored at -20 °C until analysis. Tissue was thawed and homogenized by means of a Braun Potter in 500 µl of ice-cold lysis buffer (15 mM Tris-HCl pH 7.4, containing 300 mM NaCl, 2 mM $MgCl_2$, 1% triton X-100 and 1 µg/ml of the protease inhibitors Leupeptin A and Aprotinine). Homogenate was centrifuged for 7 min at 700 x g, supernatant was taken and centrifuged for another 10 min at 12000 x g after which 10 µl of the supernatant was diluted 5 times in 0.5%

hexadecyltrimethylammonium bromide (HETAB) in 50 mM potassium phosphate buffer pH 6.0. MPO activity was determined by adding 150 µl ready to use 3,3',5,5'-tetramethylbenzidine substrate (TMB) and increase in absorbance at 630 nm was recorded for at least 15 min. One unit of MPO activity was defined as a 0.001 increase in absorbance per 5 min, and the results were normalized for initial tissue weight.

Colon culture and cytokine analysis

A distal biopsy of the dissected colon of 30 to 50 mg (exact weight was recorded), was washed three times in ice-cold PBS, containing 40 mg/ml glucose, 100 IU/ml penicillin-streptomycin and 250 µg/ml fungizone, and incubated in 500 µl of RPMI1640 (Invitrogen Life Technologies, Paisley, Scotland) supplemented with 10% FCS (Greiner Bio-One B.V., Frickenhausen, Germany) for 24 hrs at 37 °C and 5% CO_2. Supernatant was collected and stored at -20 °C until analysis.

IL-12p40 and IFN-γ were determined by sandwich ELISA (BD Pharmingen, Erembodegem, Belgium). Highbond Costar 3590 plates were coated overnight at 4 ˚C with 2 µg/ml rat anti-mouse IL-12p40 in PBS or 2 µg/ml rat anti-mouse IFN-γ in carbonate buffer, washed with PBS/0.05% Tween-20 and blocked with PBS/0.05% Tween-20/1% BSA (IL-12p40/p70) or PBS/0.05% Tween-20/3% milk powder (IFN-γ) for 4 hrs at room temperature. Plates were washed and samples and standards were diluted in PBS/0.05% Tween-20/1% BSA and incubated overnight at 4 ˚C. After subsequent washing, plates were incubated with 0.5 µg/ml rat anti-mouse IL-12p40/p70 conjugate or 0.25 µg/ml rat anti-mouse IFN-γ conjugate in PBS/0.05% Tween-20/1% BSA for 1 hr at room temperature. Plates were washed and incubated with 0.1 µg/ml streptavidin-HRP (Sanquin, Amsterdam, The Netherlands) in PBS/0.05% Tween-20/1% BSA for 45 min at room temperature. After the final washes, 0.1 mg/ml TMB substrate was added and color development was stopped after 10-15 min with 2 M H_2SO_4. Absorbance was measured at 450 nm. TNF-α ELISA (Biosource, Nivelles, Belgium) and IL-10 ELISA (BD Pharmingen, Erembodegem, Belgium) were performed according to the instructions of the manufacturer. Cytokine production was normalized for weight of colon tissue used in culture.

Statistics

Data are presented as the groups mean ± standard error of the mean (SEM) and analyzed using two-sided unpaired Student's *t*-test (GraphPad Prism).

Results

Body weight and colon length

The most obvious clinical symptom in DSS-induced colitis is loss of body weight. A marked weight loss was observed from day 4 onward and reached a maximum at day 8 in the vehicle-treated group (18.9% ± 2.6%). Oral administration of BIAP resulted in a significant reduction of body weight loss (10.9% ± 2.2% on day 9) (Fig. 2A).

Decrease in colon length is a marked indicator in DSS colitis. Dissection of the colon at day 12 revealed a difference in colon length between the two groups: the colon length of the BIAP-treated group was significantly longer than the vehicle-treated group (7.7 ± 0.2 cm *versus* 6.7 ± 0.3 cm respectively) (Fig. 2B).

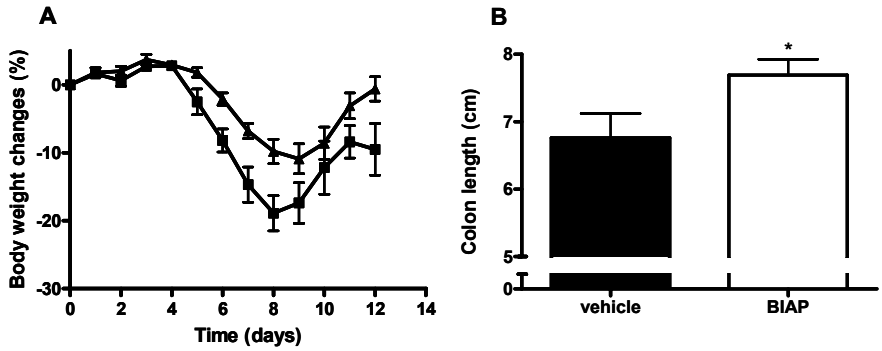

Figure 2: Oral BIAP treatment facilitates accelerated recovery from body weight loss (A) and attenuates shortening of the colon (B) in DSS-induced colitis. Mice were provided with drinking water supplied with DSS for 5 days. Daily BIAP treatment started at day 4 and was continued until mice were sacrificed at day 12; (A) (■) DSS-exposed mice (n = 5) and (▲) BIAP-treated DSS-exposed mice (n = 10). Values are depicted as means ± SEM. * $P < 0.05$ significantly different from vehicle-treated mice.

Histopathology

Microscopical examination of colon sections showed a marked inflammation in the vehicle-treated group, characterized by crypt lesions, loss of goblet cells and oedema formation. Increased numbers of neutrophilic granulocytes were found scattered

throughout the mucosa and submucosa and especially in areas which showed lesions. Next to this, lymphocyte infiltrations (cryptopatches) were present more frequently in inflamed colon. Oral treatment with BIAP significantly reduced leukocyte infiltration, improved crypt architecture and resulted in less oedema formation and loss of goblet cells (Fig. 3 and Fig. 4).

Colon levels of MPO were also examined; BIAP treatment showed a trend towards a reduction compared to the vehicle-treated group. MPO levels were reduced from 16330 ± 3703 to 13190 ± 3077 U/g colon (data not shown).

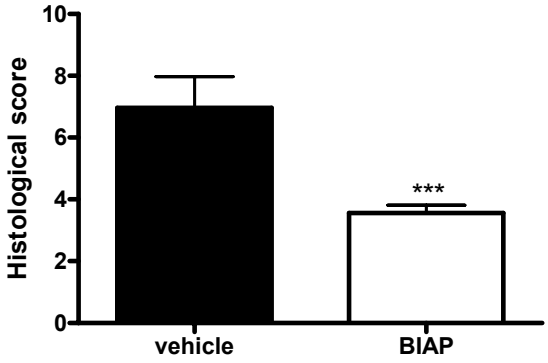

Figure 3: Oral BIAP treatment reduces histological severity scores. The total colon inflammatory score was characterized by the degree of oedema, loss of crypts, loss of goblet cells, and leucocyte inflitration, resulting in a total score of 12. Each value expresses the mean ± SEM of 5-10 mice. *** $P < 0.001$ significantly different from vehicle-treated mice.

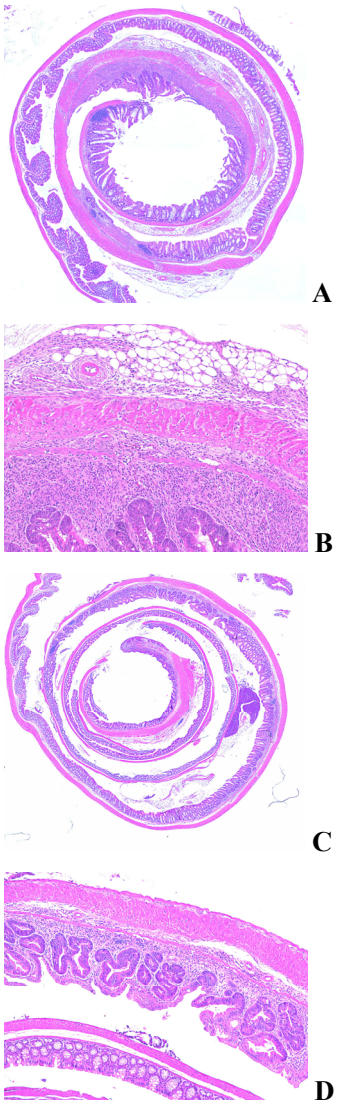

Figure 4: BIAP administered orally reduces colon inflammation. Representative tissue sections are of a vehicle-treated mouse (A and B), and a BIAP-treated mouse (C and D). Paraffin-embedded sections were stained with hematoxylin-eosin. Objective magnification A and C x4; B x20; D x10.

Cytokine response

Production of cytokines in the colon was determined by incubating colon biopsies in RPMI1640 for 24 hours. The pro-inflammatory cytokines TNF-α, IL-12p40 and IFN-γ were excessively produced 12 days after the onset of colitis as well as the anti-inflammatory cytokine IL-10 (Fig. 5). Treatment with BIAP resulted in a significant reduced TNF-α production when compared to treatment with vehicle. BIAP administration showed a trend towards a reduction in IL-12p40 and IFN-γ levels compared to the vehicle-treated group. No effect of BIAP could be seen on the production of the anti-inflammatory cytokine IL-10.

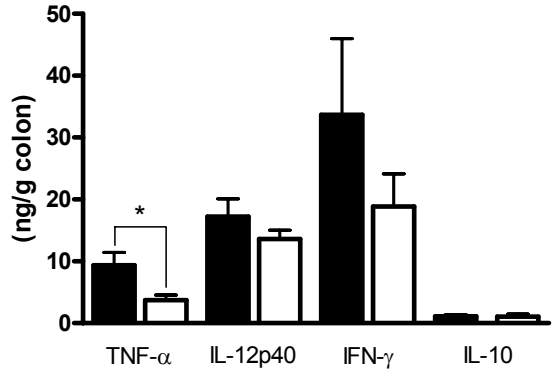

Figure 5: Oral treatment with BIAP downregulates pro-inflammatory cytokine release from cultured colon parts in DSS-treated animals, but has no effect on the production of the anti-inflammatory cytokine IL-10. Levels of TNF-α, IL-12p40, IFN-γ, and IL-10 were determined using specific ELISA, and normalised for grams of colon tissue cultured, (■) vehicle-treated mice and (□) BIAP-treated mice. Each value expresses the mean ± SEM of 5-10 mice. * $P < 0.05$ significantly different from vehicle-treated mice.

Discussion

In recent years there has been growing evidence that IBD results from an abnormal response of the host to commensal enteric bacteria leading to activation of the mucosal immune system [3, 18]. Blocking the innate immune response to bacteria or their components (e.g. LPS) may therefore be interesting in ameliorating IBD. BIAP has been shown to exert anti-inflammatory effects in several different animal models of LPS-mediated diseases, and it was therefore interesting to examine the potential therapeutic effect of BIAP in IBD.

In the present study, the effect of BIAP on colitis in C57BL/6 mice was examined. Mice were exposed to DSS for 5 days to induce acute colitis and treatment with BIAP started on day 4, resembling a possible early therapeutic treatment in patients.

In the control group treated orally with vehicle, induction of acute colitis by DSS resulted in a profound body weight loss from day 4 onward after initiation of colitis, which is consistent with results obtained by Melgar *et al.* (2005) and Aharoni *et al.* (2006) [19, 20]. Lowest body weight was reached at day 8. Oral BIAP treatment showed a significant reduction in body weight loss, indicating a protection against DSS-induced colitis. The beneficial effects of BIAP on body weight loss were accompanied by a less decrease in colon length, and less histopathological damage and leukocyte infiltration confirming a decrease in inflammation.

Oral treatment with BIAP also had an effect on the pro-inflammatory cytokine secretion in the colon. TNF-α production was significantly reduced in colon cultures from mice treated with BIAP. Apart from its effect on TNF-α production, BIAP showed a trend towards reduction of secretion of the p40 subunit of IL-12 and IL-23, both pivotal pro-inflammatory cytokines in colitis [21, 22]. Melgar *et al.* (2005) showed a remarkable reduction in p40 subunit production at day 12 in DSS-treated mice, suggesting it to be of less relevance at this time point [19]. It is possible that we therefore did not find significant differences in p40 production. Although not significant, production of the Th1 cytokine IFN-γ was reduced in BIAP-treated mice, indicating a decreased activation of the adaptive immune system. We did not observe any difference in production of the anti-inflammatory cytokine IL-10 between vehicle

and BIAP-treated mice, despite the fact that IL-10 is considered a key cytokine in colon homeostasis based on the increased susceptibility of IL-10-KO mice to colitis [23]. This is in line with the observations of van Veen *et al.* (2006) who detected BIAP-induced decreases in pro-inflammatory cytokine production but could not detect IL-10 increase in a liver-resection and ischemia model [15].

Interestingly, a protection against DSS-induced colitis was also observed in mice when BIAP was administered rectally. Mice received BIAP via an enema every other day to keep discomfort to a minimum. BIAP-treated animals showed a less decrease in body weight and a significant reduction in TNF-α production. No effect of BIAP on colon length and morphology could be observed. Remarkably, less reduction in body weight was observed and less TNF-α was produced in the colon of mice treated rectally with vehicle compared to mice receiving vehicle via the oral route. Enema application possibly results in removing commensal enteric bacteria from the gut, thereby already reducing activation of the mucosal immune system. However, rectal BIAP treatment was still able to further reduce DSS-induced effects.

The above mentioned results are all indicatives of LPS detoxification by BIAP, ending up in less activation of the innate immune response. However, direct effect of BIAP on LPS dephosphorylation could not be assessed in this study, since the *Limulus* amoebocyte lysate (LAL) assay cannot discriminate between LPS and dephosphorylated LPS [24]. Another possibility to assess LPS dephosphorylation is by measurement of phosphate release, but this can only be used *in vitro*. Nevertheless, the positive effects of several isoforms of AP towards detoxification of LPS have been clearly demonstrated in mice and piglets [11, 12].

Pilot studies analyzing the pharmacokinetics in our model revealed that after a single oral dose of BIAP approximately 10% of the administered BIAP reached the colon in its enzymatically active form (unpublished data). It is conceivable that BIAP lost part of its activity by passing through the stomach, due to low pH sensitivity. However, oral administration of 100 U of BIAP, which results in 10 U of active BIAP reaching the colon, should be more than sufficient to decrease LPS-mediated inflammation in DSS-induced colitis, since studies by van Veen *et al.* (2005) showed that an i.v. injection of

0.15 U/gram body weight was able to reduce inflammation in a secondary peritonitis model in mice [14].

In conclusion, treatment with BIAP of a DSS-induced acute colitis in mice results in a reduction of body weight loss and colon shortening, and attenuates the inflammatory response. BIAP therefore represents a promising therapeutic tool for the treatment of IBDs, like UC and Crohn's disease.

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Chapter 6

Summary and general discussion

Summary

Alkaline phosphatase (AP) is an ubiquitous enzyme in the human body that can be present in four isoforms. There are three tissue-specific AP isoforms, *i.e*. placental (PLAP), germ cell (GCAP) and intestinal (IAP) and one tissue-nonspecific isoform, TNSAP also called liver-bone-kidney (LBK) type [1-7]. Besides the ability of all AP isoforms to catalyze the hydrolysis of phosphomonoesters at alkaline pH optima, thereby releasing inorganic phosphate and alcohol, every isoform serves a different biological function [8-10].

At sites where possible antigens, like lipopolysaccharide (LPS), may enter the circulation, AP is abundantly present (*e.g.* lungs and gastrointestinal tract). Exposure of cells that form a barrier between the host's internal milieu and the external environment to LPS result in upregulation of AP, implying that AP may serve a role in the natural defense system [11, 12]. In fact, AP is thought to remove one of two phosphate groups from the lipid A moiety of LPS, resulting in inorganic phosphate (P_i) release and the formation of non-toxic dephosphorylated monophosphoryl LPS (MPLPS) [13, 14].

In the last decade, several studies have indicated the effects of different isoforms of AP against an LPS insult or against LPS-mediated diseases in a variety of animal models, suggesting that this enzyme represents a new therapeutic drug in the treatment of LPS toxicity [15-20].

Experiments described in this thesis were performed to obtain more insight in the mechanism by which AP is able to detoxify LPS. Furthermore, experiments focused on the use of AP as a treatment in two LPS-mediated diseases, acute myocardial infarction and ulcerative colitis.

Several studies have shown the promising therapeutic results of bovine intestinal alkaline phosphatase (BIAP) on LPS insults *in vivo* but so far none has explored the effects of BIAP on reducing LPS-mediated pro-inflammatory mediator production *in vitro*, let alone how LPS and dephosphorylated LPS are differentially recognized and reacted to by macrophages, epithelial or endothelial cells. Since AP is abundantly

expressed by epithelial cells at 'portes d'entrees' and macrophages play a role in the second line of defense, these cells were used to investigate in **Chapter 2** the capacity of BIAP to detoxify LPS in an *in vitro* assay. The 'inflamed' *in vivo* interplay between epithelium and innate immune system was mimicked by exposing macrophages to epithelial cell supernatant previously incubated with LPS with or without BIAP. The results of these studies clearly showed that BIAP reduced the LPS-induced inflammatory response by epithelial cells, as measured by production of IL-8, and a diminished production of IL-6 and NO_x by macrophages. Incubation of macrophages with LPS and BIAP only resulted in a reduced pro-inflammatory mediator production when cells were preincubated with BIAP. Incubation of epithelial cells and macrophages with BIAP alone revealed that this exogenous glycosyl-phosphatidylinositol (GPI)-anchored enzyme bound to the cell membrane.

Having established that BIAP detoxifies LPS *in vitro*, and that we were not able to detect direct dephosphorylation of LPS by BIAP (as measured by the release of free phosphate) in a cell-free environment, it was therefore interesting to assess the possible role of accessory molecules necessary for BIAP to detoxify LPS. Since we found that BIAP binds to the cell membrane and other studies showed the colocalization of endogenous AP with CD14, we focused in **Chapter 3** on the possible involvement of CD14 in detoxification of LPS by BIAP [21, 22]. HEK293T cells, stably expressing CD14 (HEK293T/CD14) were incubated with LPS in the presence or absence of BIAP. It was shown that IL-8 production by LPS-stimulated HEK293T/CD14 cells was reduced significantly in the presence of BIAP and blocking CD14 by using an anti-CD14 antibody resulted in the same reduction in the IL-8 response as BIAP addition. No additional effect of BIAP on IL-8 reduction was seen when cells were preincubated with an anti-CD14 antibody. A comparable result was obtained with HUVEC incubated with LPS. BIAP was unable to reduce the LPS-induced IL-6 production by HUVEC in the absence of sCD14.

It has been shown that after an acute myocardial infarction (AMI) the production of pro-inflammatory mediators is elevated and it is proposed that this is due to impaired perfusion of the mucosal epithelium of the gut which results in translocation of LPS

from the intestine into the circulation. In **Chapter 4**, the left anterior descending (LAD) coronary artery ligation model in mice was used to induce AMI, with BIAP given i.v. as prophylaxis. In the acute phase after AMI, the pro-inflammatory response was reduced in mice receiving BIAP as indicated by a reduction in IL-6 and IL-1β production. Moreover, mouse mast cell protease-1 (mMCP-1) production was decreased in BIAP-treated mice, indicating less activation of mast cells, and a reduction in Angiotensin II (Ang II) formation.

There is growing evidence that inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), results from an abnormal response of the host to commensal enteric bacteria or their components (e.g. LPS), which leads to activation of the mucosal immune system. In **Chapter 5**, the use of BIAP as an early treatment in dextran sulfate sodium (DSS)-induced acute colitis in mice, a preclinical model for testing the efficacy of treatments for IBD, was investigated. Oral (by gavage) or rectal (by enema) administration of BIAP resulted in a significant reduction of DSS-induced body weight loss and TNF-α production in the colon. Furthermore, there was less shortening, histopathological damage and leukocyte infiltration of the colon in mice treated orally with BIAP.

General discussion and further perspectives

Since AP catalyzes the hydrolysis of phosphomonoesters (thereby releasing inorganic phosphate and alcohol) it is believed that the capacity of this enzyme to detoxify LPS is through dephosphorylation of the lipid A moiety of LPS. Direct dephosphorylation of LPS can be measured by means of a phosphate (P_i) release assay and studies by Bentala *et al.* (unpublished data) have shown a P_i increase after incubation of LPS with PLAP *in vitro*. Until now, we have not been able to repeat these observations when using BIAP instead of PLAP. Detoxification of LPS by BIAP was only observed when incubated in a cell-based *in vitro* system as determined by a reduced production of cell-derived pro-inflammatory mediators (**Chapter 2**). Furthermore, the experiments in this chapter which demonstrated that BIAP binds to the cell membrane of epithelial cells and macrophages could explain the previous observations by Beumer *et al.* (2003),

who showed that the distribution phase of i.v. administered BIAP in piglets results in a biphasic clearance from plasma divided in a fast initial phase and a slower second phase [16]. Binding to and subsequently dissociation from the cell membrane of the endothelial cell implicate a much longer biological half-life than expected from serum-samples taken in the preclinical and clinical pharmacokinetic studies. This effect might even be enhanced by the observation that, as shown in this thesis, BIAP exert its LPS-detoxifying effect when bound to cell membrane of epithelial cells and macrophages.

The fact that BIAP was only able to detoxify LPS in a cell-based *in vitro* system indicates that accessory molecules are probably involved in the biological activity of BIAP. Since CD14, a component of the LPS-signaling complex, colocalizes with endogenous AP at the cell membrane of epithelial cells and is found together with AP in granules of neutrophils, the role of CD14 in LPS detoxification by BIAP was investigated [21, 22]. It was shown that presence of CD14 is necessary for BIAP to detoxify LPS, although the exact mechanism of action was not made clear (**Chapter 3**). A possible explanation for this result can be that LPS, present in blood or other body fluids, will form micelles which are disrupted by CD14 (as shown in studies using LPS concentrations found in plasma under septic shock conditions) enabling BIAP to reach the otherwise concealed lipid A moiety of LPS. In the absence of CD14, LPS will stay in an aggregated state making it unable for BIAP to dephosphorylate LPS. Additional studies are needed to elucidate this. Furthermore, the role of the other components of the LPS-signaling complex, TLR4 and MD2, were not assessed. Fitzgerald *et al.* (2004) proposed that a region of MD2 act as cationic interaction site for the two phosphate groups of lipid A [23]. It is possible that removal of one of these phosphate groups by BIAP reduces the affinity of MD2 for LPS, thereby inhibiting signal transduction through TLR4. In Figure 1, the proposed mechanism of action of AP on cells expressing TLR4 and MD2 on their cell membrane is depicted. Since AP is an ectoenzyme, it is believed that the preferred site of action is within the extracellular environment. Epithelial and endothelial cells on which cell membrane expression of TLR4 and MD2 is absent or downregulated probably internalize LPS already dephosphorylated by AP.

One major disadvantage of animal derived proteins used therapeutically in humans is the immune-recognition and subsequent immune reactions against these foreign proteins. Indeed, preclinical safety in rodents showed high titers of anaphylactic IgG1 antibodies directed towards BIAP, endangering the further development of the bovine derived protein for systemic therapeutical use in humans.

However, since humans consume daily bovine products in their food, it was not expected that BIAP-related side effects occur after repeated BIAP administration because humans are orally tolerated. Indeed, mice, which develop anaphylaxis after multiple i.v. challenge with BIAP, did not show any anaphylactic signs and elevated levels of BIAP-specific IgG1 after oral tolerance induction. Furthermore, phase I safety studies in humans showed that a single i.v. administration of high doses of BIAP (up to 3100-fold above plasma level) did not result in specific anti-BIAP antibody induction nor result in acute or sub-acute toxicity. These studies indicate that BIAP administration is safe and the observed positive effects of BIAP in several LPS-mediated diseases in animal models implies that this enzyme can be beneficial in reducing LPS toxicity in humans.

Having established this, a Phase IIa clinical trial with severe sepsis patients was initiated. Preliminary results show a significant higher 90 days survival rate in the BIAP-treated group of 18% compared to the placebo-treated group. So far, the only approved drug for sepsis treatment is activated protein C (APC) which reduces overall mortality in severe sepsis patients by 6%. Since APC exerts an anticoagulation and anti-inflammation effect (by inactivating factors Va and VIIIa) in a late stage of sepsis, whereas BIAP interferes with one of the initiators (LPS) of sepsis at the beginning of the inflammatory cascade, a combined therapy might represent a beneficial approach in this highly complicated disease.

Besides sepsis, another disease suggested to be LPS-mediated is for instance heart failure (HF). It is proposed that the inflammatory response in patients after HF, irrespective of etiology, is due to a systemic LPS increase. The positive effects of i.v. administration of BIAP on reducing the pro-inflammatory response and on Ang II formation after induction of one form of HF in mice, AMI, implicated that this enzyme might also be applicable to patients with other causes of HF (**Chapter 4**). In addition to

this, surgery under narcosis also leads to intestinal hypoperfusion resulting in inflammation comparable to that observed in HF patients. From this point of view, a Phase II clinical trial has currently started focusing on BIAP-mediated reduction of post-surgical ischemia-reperfusion damage in patients undergoing coronary artery bypass grafting (CABG).

In contrast to AMI, which as its name already implies is a relatively acute disorder; colitis is distinguished by chronic inflammation of the colon, indicating that treatment of this persistent disease involves regular administration of medicine. Frequent i.v. dosage of any drug is not desirable, which has been the chosen route for BIAP administration in previous studies and the above mentioned AMI study. To avoid this and apply BIAP locally to the infected site, it was given orally to mice treated with DSS. Although DSS-induced colitis parameters were significantly reduced by BIAP, thereby showing for the first time intervention of BIAP in a chronic disease mediated by LPS, the administration was not optimal since only 10% of orally given BIAP was detectable in the colon in its active form (**Chapter 5**). Increasing the local bioavailability of BIAP in the colon can be reached by the usage of enteric coated tablets which are resistant to the low pH and proteolytic enzymes in the stomach. The production of these tablets is currently under development.

Even though safety studies with BIAP have shown that a single i.v. administration does not result in adverse side effects, to further decrease the risk of immunogenicity the use of a human, in stead of a bovine AP is preferred. Several studies have already shown the positive effects of human placental alkaline phosphatase (HPLAP) administration to mice injected with LPS or *E. coli* [15, 17]. While the specific enzyme activity (units/mg) *in vitro* of HPLAP is lower than that of BIAP, the half-life time in blood is much higher ($t_{½\ HPLAP}$ = several days *versus* $t_{½\ BIAP}$ = several minutes), making it a suitable AP for the treatment of conditions in which multiple LPS insults are expected over a prolonged period, whereas BIAP can be used in an acute LPS-induced disease or as a prophylaxis at surgeries with a high risk of bacterial infection.

Besides the fact that large amounts of APs derived from human tissues are difficult to obtain, the down stream process (DSP) resulting in a highly purified and safe end product is expensive and labor intensive. Therefore, production of a recombinant AP in

a mammalian, preferably a human cell-system to get the correct glycosylation, important to obtain a biologically active enzyme, seems to be an ideal alternative. Recombinant production of different isoforms of human origin may also provide more insight in the specific requirements and mechanism of action for each isoform. This may help to develop specific isoforms for specific diseases requiring acute or chronic, systemic or local therapeutic effects.

In conclusion, this thesis demonstrates that LPS-induced inflammation can be reduced by BIAP in an *in vitro* cell-based system as well as in an AMI and colitis experimental model *in vivo*. The current data, coupled with BIAP's safety record, justifies the development of BIAP for the treatment of LPS-mediated diseases in humans.

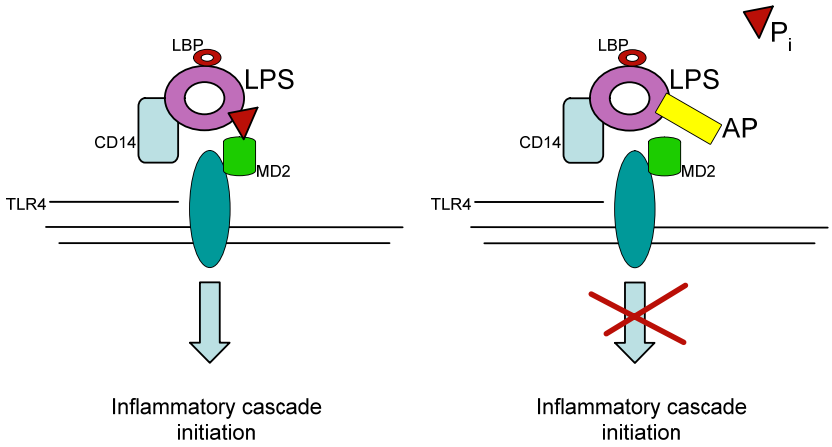

Figure 1: Proposed mechanism of action of AP at the cell membrane of cells expressing the TLR4/MD2 complex.

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Chapter 7

Nederlandse samenvatting

Lipopolysaccharide (LPS), ook wel endotoxine genaamd, is een antigeen die een belangrijke rol speelt in de ontwikkeling en/of verergering van verschillende multifactoriële ziekten zoals sepsis, atherosclerosis, ziekte van Crohn en colitis ulcerosa. Het is een belangrijk bestanddeel van het celmembraan van Gram-negatieve bacteriën (één bacterie bevat ongeveer 3,5 x 10^6 LPS moleculen) en is essentieel voor zijn groei en stabiliteit. Wanneer een Gram-negatieve bacterie zich deelt of wordt vernietigd (door bijvoorbeeld antibiotica) komt spontaan LPS vrij. Als dit gebeurt in het menselijke lichaam kan het interacteren met verschillende eiwitten die aanwezig zijn in de circulatie wat resulteert in activering van cellen en voornamelijk degene die behoren tot het immuunsysteem. Deze geactiveerde immuuncellen produceren allerlei ontstekingsmediatoren, zoals cytokines, chemokines en vrije radicalen, die een nuttige rol spelen in het behoud van de homeostase van het menselijke lichaam tijdens een infectie met LPS.

Een van de belangrijkste LPS-gemedieerde ziekten is Gram-negatieve sepsis welke zich kenmerkt door een ongecontroleerde productie van ontstekingsmediatoren door de gastheer na het vrijgekomen van LPS. Sepsis kan zich in een kort tijdsbestek ontwikkelen tot septische shock welke als symptomen koorts, hypotensie en hypoperfusie heeft en wat leidt tot het falen van de nieren, lever en longen. Tot nu toe richt de standaardprocedure voor patiënten met sepsis en septische shock zich op het verkrijgen van een stabiele situatie. Daartoe wordt direct gestart met een antibioticum-behandeling en worden patiënten geïntubeerd en geventileerd in het geval van longfalen en vocht en bloeddrukverhogers toegediend om de bloeddruk te herstellen. Desondanks komen nog steeds meer dan 50% van de patiënten die gediagnosticeerd zijn met septische shock te overlijden wat aangeeft dat het verkrijgen van een therapie tegen sepsis en septische shock van groot belang is.

Klinische studies die zich bezig houden met het verminderen van LPS-geïnduceerde effecten in patiënten met sepsis of septische shock richten zich voornamelijk op het onschadelijk maken van één van de vele geproduceerde ontstekingsmediatoren. Vele concentreren op het neutraliseren of antagoniseren van de twee belangrijkste cytokines, TNF-α en IL-1β, of op de inhibitie van NO-synthese, een vrije radicaal. Desalniettemin hebben deze studies niet het gewenste effect op de verbetering van overlevingskansen

laten zien, waarschijnlijk omdat het onschadelijk maken van een enkele ontstekingsmediator niet voldoende is. Interventie met de veroorzaker LPS is vermoedelijk de meest doeltreffende manier om sepsis en septische shock te behandelen. Toch toonden LPS-bindende antilichamen en eiwitten ook geen vermindering in mortaliteit aan, vermoedelijk omdat deze een te lage affiniteit hadden voor het toxische gedeelte van LPS, lipid A.
In de afgelopen decennia hebben verscheidende *in vivo* studies laten zien dat wanneer een van de twee fosfaatgroepen van lipid A chemisch verwijderd werd, dit zogenaamde gedefosforyleerde lipid A ongeveer 10.000 keer minder potent was om een ontstekingsreactie te veroorzaken dan lipid A zelf. Dit geeft aan dat een therapie die gericht is op het direct verminderen van de toxiciteit van lipid A een nieuwe strategie kan zijn om de schadelijke effecten van LPS in verschillende LPS-gemedieerde ziekten te overwinnen.

Alkalische fosfatase (AF) is een alomtegenwoordig enzym in het menselijke lichaam en komt voor in vier verschillende isovormen, te weten placentair, geslachtscel, intestinaal en lever-been-nier-type AF. Het is voornamelijk aanwezig op plekken waar mogelijke antigenen, bijvoorbeeld LPS, in de circulatie terecht kunnen komen zoals de longen en het gastro-intestinale systeem. Blootstelling van cellen die een barrière vormen tussen het interne milieu van de gastheer en de externe omgeving aan LPS leidt tot opregulatie van AF wat impliceert dat dit enzym een mogelijke rol speelt in het immuunsysteem. Aangezien AF de hydrolyse van fosfaatesters hydrolyseert werd in de jaren ’90 van de vorige eeuw verondersteld dat dit molecuul ook in staat zou kunnen zijn om enzymatisch een van de twee fosfaatgroepen van het lipid A gedeelte van LPS te verwijderen waardoor lipid A wordt gedefosforyleerd en LPS wordt gedetoxificeerd. De laatste 10 jaren hebben een aantal *in vivo* studies het effect van uit de intestine van runderen (bovien intestinaal alkalisch fosfatase; BIAF) afkomstig AF tegen een LPS insult aangetoond zoals in studies met muizen en varkens waarin de door LPS-geïnduceerde ontstekingsreactie significant minder was in dieren die behandeld waren met BIAF. Ook diermodellen van buikvliesontsteking en leverschade lieten zien dat toediening van BIAF een vermindering gaf van de ontstekingsreactie en lever- en

longschade wat impliceert dat dit enzym een mogelijk nieuw medicijn kan zijn in de behandeling van LPS-gemedieerde ziekten.

Experimenten welke beschreven zijn in dit proefschrift zijn uitgevoerd om meer inzicht te krijgen in het werkingsmechanisme van BIAF. Daarnaast werd er aandacht besteed aan de behandeling met BIAF van twee ziekten waarbij LPS een mogelijke rol speelt: het acute myocardinfarct (AMI) en colitis ulcerosa (CU).

Tot nu toe hebben alle studies met BIAF zich gericht op het effect van dit enzym tijdens een LPS insult *in vivo*. Aangezien humaan AF voornamelijk tot expressie wordt gebracht door epitheelcellen (cellen die de buitenste laag van organen bekleden) en samen met macrofagen (cellen die behoren tot het immuunsysteem) als een van de eerste celtypen in aanraking komen met schadelijke invloeden vanuit de omgeving werden deze cellen in **hoofdstuk 2** gebruikt om de LPS detoxificerende capaciteit van BIAF *in vitro* te onderzoeken. De behaalde resultaten laten zien dat BIAF in staat is om de LPS-geïnduceerde productie van de chemokine IL-8 door colon-, long- en nierepitheelcellen als de cytokine IL-6 en de vrije radicaal NO door macrofagen te verminderen. Daarnaast werd aangetoond dat BIAF zich met behulp van een anker aan de cel bindt.

Gezien het feit dat BIAF in een celvrije omgeving niet in staat is om LPS te detoxificeren en dat dit wel het geval is zodra er cellen aanwezig zijn, rees de vraag of er misschien additionele moleculen nodig zijn voor BIAF om LPS te detoxificeren. Aangezien er in hoofdstuk 2 werd aangetoond dat BIAF aan de cel bindt en andere studies de co-lokalisatie van endogeen AF en het eiwit CD14 hebben laten zien, werd in **hoofdstuk 3** gekeken naar de mogelijke rol van CD14 in de detoxificatie van LPS door BIAF. Humane nierepitheelcellen die CD14 tot expressie brengen werden daartoe geïncubeerd met LPS met of zonder BIAF. De LPS-geïnduceerde productie van IL-8 was significant verminderd in de aanwezigheid van BIAF. Blokkering van CD14 door middel van een antilichaam gericht tegen CD14 zorgde ervoor dat er geen effect van BIAF meer waarneembaar was. Een vergelijkbaar resultaat werd verkregen met

humane endotheelcellen afkomstig uit de navelstrengader. BIAF was niet in staat om de door LPS-geïnduceerde IL-6 productie door deze cellen te reduceren in de afwezigheid van CD14. Dit betekent dat, om LPS te detoxificeren, BIAF afhankelijk is van de aanwezigheid van CD14.

Na een AMI neemt de productie van ontstekingsmediatoren enorm toe. Er wordt verondersteld dat dit komt doordat onder anderen de perfusie van het gastro-intestinale systeem is verminderd, wat resulteert in translocatie van LPS van het lumen van de intestinum naar de circulatie. In **hoofdstuk 4** wordt een muizenmodel gebruikt waarbij de linker kransslagader van de muis werd geligeerd om een AMI te veroorzaken. BIAF werd intraveneus toegediend als profylaxis. In de acute fase na inductie van een AMI was de ontstekingsreactie significant verminderd in muizen die behandeld waren met BIAF. Bovendien waren bepaalde immuuncellen, mastcellen genaamd, veel minder geactiveerd in met BIAF behandelde muizen waardoor er minder angiotensine II, een hormoon dat vaatvernauwend werkt, wordt gevormd.

Er is toenemend bewijs dat ontstekingsziekten van het gastro-intestinale systeem, waaronder CU, een gevolg is van een abnormale respons van de gastheer op commensale darmbacteriën of op één van hun bestanddelen (bijvoorbeeld LPS) wat leidt tot activatie van immuuncellen in het slijmvlies van de darmwand. In **hoofdstuk 5** wordt de orale en rectale toediening van BIAF vier dagen na de inductie van een dextraan sulfaat sodium (DSS)-geïnduceerde acute CU in muizen, een preklinisch model om de effectiviteit van behandelingen voor CU te testen, onderzocht. Orale en rectale toediening van BIAF resulteerde in een vermindering van DSS-geïnduceerde gewichtsafname en TNF-α productie in het colon. Bovendien was het colon minder verkort en was er een afname in histopathologische schade en leukocyt infiltratie in het colon van muizen die oraal behandeld waren met BIAF.

In **hoofdstuk 6** worden de verkregen resultaten die in dit proefschrift beschreven staan samengevat en bediscussieerd. De algemene conclusie is dat de door LPS-geïnduceerde ontstekingsreactie door BIAF gereduceerd kan worden zowel in een *in vitro* celsysteem

als wel in een experimenteel model van AMI en CU *in vivo*. De verkregen data gekoppeld aan het feit dat BIAF in veiligheidsstudies in de mens geen bijverschijnselen lieten zien, rechtvaardigt de ontwikkeling van BIAF voor de behandeling van LPS-gemedieerde ziekten.

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Curriculum vitae

Alberdina Fiechter werd geboren op 5 juni 1977 te Zwolle. In 1993 behaalde zij haar MAVO diploma aan de Jhr. Mr. de Savornin Lohman te Hilversum, en in 1995 en 1997 behaalde zij respectievelijk haar HAVO en VWO diploma aan het Alberdingk Thijm College te Hilversum. In 1998 begon zij aan haar studie Biologie en Medisch Laboratoriumonderzoek aan de Hogeschool Utrecht. In het laatste studiejaar liep zij stage bij het departement Pharmaceutical Proteomics, Utrecht Institute of Pharmaceutical Sciences, Universiteit Utrecht te Utrecht, waar zij werd begeleid door Dr. R. Oosting. Aansluitend op het behalen van haar diploma in 2002 is zij begonnen als AIO aan het onderzoek welke beschreven staat in dit proefschrift. Dit onderzoek was een samenwerking tussen het Institute for Risk Assessment Sciences (IRAS), Universiteit Utrecht te Utrecht en AM-Pharma B.V. te Bunnik en werd begeleid door Prof. dr. W. Seinen en Dr. M. Wulferink.