Detoxification of LPS by alkaline phosphatase
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Chapter 2
General Introduction
Lipopolysaccharide (LPS) is a toxic compound and a component of the cell wall of Gramnegative bacteria. LPS is an important pathogenic factor in sepsis. Its role in other diseases like liver fibrosis, cholestasis, hepatitis B & C, obstructive jaundice and inflammatory bowel disease (1-5) is now increasingly recognized. Although LPS plays an important role in all these diseases, its dominant role in Gramnegative sepsis is most often studied and generally known. Sepsis can occur when LPS levels in the blood become elevated, for instance due to a decreased LPS clearance in the liver or infiltration of LPS into the circulation across the intestinal wall. Elevated serum LPS levels can lead to a systemic inflammation and a massive activation of the immune system. This in turn will lead to the production of high levels of cytokines, lipid mediators and reactive oxygen species. In addition, blood coagulation is abnormal in sepsis and blood clotting in small blood vessels may lead to multiple organ failure and even death if vital organs are affected. In all LPS-mediated diseases, many different mediators, especially cytokines, are involved. Most therapies against these diseases are therefore based on eliminating particular cytokines and thereby attempting to shut down the inflammatory response. But as there are so many processes and mediators involved, in particular during sepsis, elimination of one mediator is unlikely to be sufficient.

The aim of this chapter is to give an overview of the diseases in which LPS is thought to play an important role, in particular in the onset or progression of the disease. The role of LPS in these processes will be described as well as some of the therapies that are currently used for these disorders. In addition, the potential role of the LPS-dephosphorylating enzyme alkaline phosphatase (AP), as a new therapeutic agent in LPS-mediated diseases, will be discussed.

Lipopolysaccharide
Lipopolysaccharide (LPS) or endotoxin is a major component of the cell-wall of Gramnegative bacteria. It covers about 30 to 40 % of the bacterial cell surface. LPS is an amphiphilic molecule composed of a prominent sugar chain and several fatty...
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acids. Each strain of bacteria shows a somewhat different composition of the LPS molecule, but the gross structure of LPS molecules is quite similar in the majority of Gramnegative bacteria. Each molecule can be divided in a lipid A part, an inner core, an outer core and a long polysaccharide chain (Fig. 1). The hydrophilic polysaccharide chain contains many heptoses and anionic KDO (2-keto-3-deoxy-octonate) sugars. The KDO-sugars link the hydrophilic polysaccharide chain to a hydrophobic lipid portion, lipid A, which anchors the LPS molecule into the bacterial outer membrane. The lipid A part is the most conserved part of the LPS molecule and is generally composed of two glucosamines to which two phosphate molecules and four to six fatty acids are bound (6). The lipid A moiety and particular the two phosphate groups in this part of the LPS molecule largely determine its toxicity (7; 8).

![Figure 1: Schematic representation of the structure of LPS (picture from http://glycoforum.gr.jp/science/word/immunity/IS-A01E.html). A full color version of this figure can be found on page 169.](image)

In vivo, multiple molecules are involved in the activation of cells by LPS. In order to activate cells, LPS first binds to the LPS-binding protein (LBP) in plasma. LBP is a 60-kDa acute phase protein that is mainly produced by hepatocytes in the liver but also in lower amounts by respiratory type II epithelial cells in the lung (9). Normal serum LBP levels in humans vary between 2 and 20 µg/ml (9-12), but LBP levels rise quickly to 100-200 µg/ml (12) during an acute phase response for instance caused by an inflammation.
After binding to LBP, the LPS-LBP complex binds to CD14, a 55-kDa glycoprotein. There are two forms of the CD14 molecule, a membrane-bound form and a non-membrane bound form that is present in serum. The membrane-bound form of the CD14 receptor is mainly expressed by macrophages, monocytes and neutrophils (13; 14) and is attached to the membrane via a glycosyl-phosphatidyl inositol (GPI) anchor. Activation of cells that do not express membrane-bound CD14, e.g. endothelial cells, epithelial cells, dendritic cells, fibroblasts, smooth muscle cells and vascular endothelium (15; 16), can occur via soluble CD14 (sCD14) present in serum. sCD14 is found in the serum of healthy individuals, but similar to LBP, levels rise quickly during a massive systemic inflammatory response like sepsis (17). Together, LPS, LBP and sCD14 form a complex. For a long time, people were puzzled by how this LPS-LBP-CD14 complex was able to initiate intracellular signals as CD14 does not have an intracellular signal transduction moiety.

A few years ago, a family of receptors was discovered that showed homology with Toll receptors in Drosophila (18). This receptor family was called the toll-like receptor family and in human currently this receptor family consists of 10 members (19), which have a variety of functions in innate immunity. The toll-like receptors (TLR) recognize pathogen-associated molecular patterns, which allows the immune system to sense molecules that are present in many pathogens. One member of the toll-like receptor family, the toll-like receptor 4 (TLR4), appeared to be the LPS-receptor (20). This TLR4 forms a LPS-signaling complex with another molecule that also appeared to be essential for LPS signaling, the myeloid differentiation-2-protein (MD-2) (21-23). The intracellular part of the toll-like receptor is responsible for the many intracellular signaling events after binding of the LPS-LBP-CD14 complex to the TLR4-MD-2 complex. Mice bearing mutations in the TLR4 receptor (24) or MD-2 (25; 26) appeared to be hyporesponsive to LPS, thereby confirming the crucial role of the TLR4 and MD-2 in LPS-signaling.

After binding of the LPS-LBP-CD14 complex to the TLR4-MD-2 complex, intracellular signaling is triggered. Several intracellular pathways are activated (27), for instance the NFκB, the AP-1, the IRF3 and IRF5 and the p38-MAPK pathways (15; 19).
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Figure 2: Binding of Gramnegative and Grampositive bacteria to cells and intracellular pathways activated by LPS and MDP. Figure from Bendtzen (Institute for Inflammation Research, Rigshospitalet Univ Hosp, Copenhagen), 2004. LPS, lipopolysaccharide; LBP, LPS-binding protein; PG, peptidoglycan; MDP, muramyl dipeptide; TLR4, toll-like receptor 4; MD-2, myeloid differentiation protein 2; LRR, leucine-rich repeat; TIR, Toll/IL-1 receptor homology domain; IRAK, IL-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; NFkappaB, nuclear factor kappa B; IkappaB, inhibitory kappa B proteins.

All these pathways lead to the production of many cytokines and effectormolecules. Upon LPS stimulation, macrophages start producing high amounts of pro-inflammatory cytokines such as Il-1, TNFα and IL-6 but also IL-12, IL-15 and IL-18 (15; 28). The release of pro-inflammatory cytokines in turn triggers other events like production of more cytokines, lipid mediators (prostaglandins, leukotrienes and platelet activating factor), reactive oxygen species like superoxide anion (O$_2^-$), the hydroxide radical (OH) and nitric oxide (NO) and upregulation of adhesion molecules, enabling neutrophils to migrate into tissues. These mediators and adhesion molecules are mainly produced by macrophages but also neutrophils and endothelial cells contribute to their rapid rise in inflamed tissue.

TNFα appears to be a crucial cytokine which is not only produced by macrophages, but also serves as a potent activator of macrophages that are not yet activated. Upon activation, all these macrophages start to produce IL-1β and IL-6,
which promotes coagulation in small blood vessels. Downregulation of three endogenous anticoagulant proteins, i.e. antithrombin, protein C and tissue factor inhibitor, further contributes to the pro-coagulant state of sepsis (15). Disturbed coagulation is one of the major characteristics of sepsis and may lead to disseminated intravascular coagulation (DIC), multiple organ failure due to hypoperfusion and ischemia and eventually death when the function of crucial organs has been largely violated.

Besides cytokines, also lipid mediators are released by macrophages upon LPS stimulation. Lipid mediators like platelet activating factor (PAF) and several products of the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism, like prostaglandin E2, thromboxane A2 and leukotriene C4, are formed (28). These factors are responsible for the activation of vascular endothelium and regulation of the vascular tone and high levels may lead to vascular damage (15).

Finally, reactive oxygen species (ROS) are produced by macrophages upon LPS stimulation. Low concentrations of ROS are beneficial and enhance the antimicrobial killing capacity of macrophages, on the other hand, high concentrations of ROS cause tissue damage and high levels of NO are associated with vasodilatation and hypotension (29).

Together, an excess of cytokines, protease release and ROS-production in tissues, perturbed coagulation, vasodilatation and hypotension all contribute to the development of multiple organ failure (Fig. 3) after systemic activation of the immune system by LPS in the circulation. Obviously, these events can also occur locally as part of the normal defense system. However, the systemic activation of all these events may result in a life-threatening disease.
Figure 3: The sepsis cascade: from LPS-binding towards fulminated sepsis. Scheme from Cohen, Nature, 2002 (15).

**Role of LPS in disease**

**Systemic diseases**

As outlined above, systemic LPS can elicit a systemic inflammatory response syndrome (SIRS), which is characterized by fever, diffuse intravascular coagulation, shock, multiple organ failure and eventually death. At present, sepsis is still the most important cause of death in intensive care units and the 10th cause of death overall in the United States, accounting for about 215,000 deaths a year in this country alone (30). Common causes of elevated LPS levels in the blood are open wounds i.e. in the case of burns or trauma’s, chronic inflammation of the intestines like in Crohn’s disease and ulcerative colitis, the use of antibiotics, a decreased liver function and abdominal surgery.
Normally LPS levels in humans are very low, ranging between 4 and 10 pg/ml (31-33). The main organ responsible for keeping the LPS concentration at a low level is the liver. LPS is removed from the circulation mainly by Kupffer cells (34) and likely also partly by endothelial cells as binding of LPS to scavenger receptors on Kupffer cells and endothelial cells was reported (35). The internalized LPS is modified by these cells and subsequently released from them and exposed to hepatocytes that take up the compound and secrete the modified LPS into the bile (36). However, apart from this, hepatocytes themselves also directly remove LPS from the circulation. This is mostly done by endocytosis of chylomicrons and lipoproteins such as low density lipoproteins (LDL), high density lipoproteins (HDL) and very low density lipoproteins (VLDL), that are all capable of binding LPS (See references in Rensen et al (37)). If the LPS-removing capacity of the liver is reduced and an excess of LPS cannot be removed from the portal circulation, the levels in the general blood circulation rise and a systemic inflammatory reaction may occur. People with a progressive liver disease like fibrosis thus have an increased chance of developing elevated endotoxin levels in the blood due to a decreasing detoxifying capacity of the liver.

**IBD**

Inflammatory Bowel Disease (IBD) is a general name for diseases that are due to inflammation within the intestinal wall. In the Western world, approximately 3 million people are suffering from IBD (38), making it an important chronic disease. The 2 most well-known examples of IBD are Crohn’s disease (CD) and ulcerative colitis (UC).

CD usually affects the lower part of the small intestine, i.e. the part of the ileum closest to the colon, but can in principle affect any part of the gastro-intestinal tract. In contrast to CD, UC is limited to the colon and is most often located in the descending part of the colon and in the rectum. Another difference between CD and UC is that CD affects all layers of the intestine whereas UC only affects the intestinal lining. Common symptoms of IBD are abdominal pain, diarrhea, sometimes fatigue and weight loss.

It is believed that the inflammation of the damaged gut is the result of an inappropriate ongoing activation of the mucosal immune system triggered by
components of the normal luminal flora (39). When the sub-epithelial layers of the intestinal wall are exposed to LPS due to damage, macrophages residing in these layers start to produce cytokines and chemoattractants (40), leading to the migration of high amounts of neutrophils to the site of damage and local activation. Production of high amounts of ROS by accumulated neutrophils will evoke local damage and further enhance inflammation, which sometimes leads to systemic effects. During relapses of CD and UC, some patients are diagnosed with elevated serum levels of LPS (3-5) and cytokines like TNFα, IL-1β, IL-6 and IL-17 (41; 42). Chronic inflammation may result in further damage to the intestinal barrier and an increased permeability of the mucosa, enabling LPS to leak across the intestinal membrane into the circulation. For CD even bacterial translocation across the intestinal barrier has been reported (43; 44).

Despite their high prevalence, the etiology of CD and UC is still unclear. Due to many epidemiological studies it has become clear that genetic predisposition plays an important role in the susceptibility of people to develop these chronic inflammatory diseases. Some genes are now linked to this predisposition.

Mutations in the caspase recruitment domain (CARD15) gene (See figure 2), also called nucleotide-binding oligomerization domain (NOD2) gene, are associated with a higher susceptibility to Crohn’s disease (45-48). The exact function of the NOD2 gene, which is present in macrophages (49), epithelial cells (50), Paneth cells (51), granulocytes and dendritic cells (52), is still not fully elucidated. At first it was thought that the NOD2 might function as an intracellular LPS receptor, but recent work of Inohara et al. and Girardin provided evidence that NOD2 functions as an intracellular sensor of bacterial peptidoglycan. Not LPS but muramyl dipeptide (MDP, see figure 2), which is a component of peptidoglycan (a membrane compound of many bacteria), appears to be the compound that is recognized by NOD2 (53; 54).

Also certain mutations in the “LPS-receptor”, i.e. the toll-like receptor 4, are associated with a higher susceptibility for IBD. The Asp299Gly TLR4 polymorphism for instance is detected twice as much in CD patients compared to healthy controls (55). Besides mutations in the TLR4, which seem to predispose for IBD, also the altered expression of the TLR4 in inflamed intestinal tissue most likely contributes to the pathogenesis of diseases like CD and UC. Recent studies
have shown that the expression of the TLR4 is upregulated in inflamed intestinal tissue (56). This might elicit a response on LPS, which is abundantly present in the intestinal lumen.

Finally, mutations in the CD14 receptor are found to occur significantly more often in CD patients compared to controls (57). The discovery that mutations in receptors involved in LPS and peptidoglycan signaling, e.g. CD14, TLR4 and NOD2, seem to predispose for IBD, strongly emphasizes that an aberrant detection of bacterial products in the intestine is a very important factor in the development of IBD. It might result in a chronic immune response to bacteria and bacterial products that are part of the natural gut microflora.

Liver diseases

Fibrosis
Liver fibrosis is a disease which can be the result of many underlying different diseases and disorders like cholestasis, chronic viral infections (hepatitis B and C), parasitic disease e.g. schistomiasis, metabolic disorders (e.g. lipid and glycogen storage disorders, Wilson’s disease and hemochromatosis), cancer growth, immune deficiencies (autoimmune diseases like primary sclerosing cholangitis and primary biliary sclerosis), persistent liver inflammation (sarcoidosis) and environmental chemicals, e.g. alcohol (58; 59). Alcohol abuse is a well-known cause of liver fibrosis by inducing steatohepatitis which will ultimately result in hepatocyte damage and scar tissue formation. In Western society, today also obesity, insulin resistance and hyperlipidemia are important risk factors for developing liver fibrosis as a result of non-alcoholic steato-hepatitis (NASH) (60). NASH is regarded as a typical welfare disease and as more and more people become obese or diabetic, it can be expected that the number of people suffering from NASH will increase in the coming years.

Liver fibrosis is a chronic disease that is characterized by excessive production of extra-cellular matrix (ECM). Healthy liver cells are gradually replaced by ECM, which results in a continuous decrease of liver function. Hepatic stellate cells (HSC) are the most important cells in the fibrotic process as these cells are the main
producers of the ECM components. The development of liver fibrosis may take 15 to 20 years before it reaches the end-stage of liver failure, cirrhosis. Patients with liver cirrhosis are vulnerable for infections and Gramnegative sepsis may occur in these patients. One reason is the decreased capacity of the liver to remove LPS from the circulation. Another reason is the increased intestinal permeability, in particular occurring in patients in which the fibrosis is due to alcohol abuse (61). Alcohol causes mucosal injury in the upper part of the gastrointestinal tract resulting in an increased intestinal permeability and increased endotoxin levels in the portal system (62; 63). In addition, excessive amounts of alcohol also result in a decreased intestinal motility, resulting in bacterial overgrowth in the gut of Gramnegative bacteria (59). The increased intestinal permeability and bacterial overgrowth may result in further LPS leakage (64; 65) or bacterial translocation. The latter has been demonstrated in cirrhotic rats (66) and cirrhotic patients (67; 68). LPS leakage and bacterial translocation both contribute to elevated portal blood levels of LPS. The excessive amount of gut-derived endotoxin will lead to the activation of Kupffer cells in the liver and thus to the onset of an inflammatory reaction in the liver and aggravation of the fibrotic process (63). Moreover, several reports have described that an enhanced sensitivity of the liver to LPS caused by increased expression of CD14 on Kupffer cells, also serves as an important contributor to the inflammatory reaction in the liver (69-71). The fact that CD14-deficient mice showed only minimal pathological changes upon chronic alcohol exposure when compared with wild-type mice, further emphasizes the importance of CD14 in alcohol-induced liver injury (72).

Recently, several studies have reported that HSC might contribute in another way to the progression of liver fibrosis. HSC are well-known as producers of ECM in fibrosis, but current reports showed that HSC are also capable of releasing pro-inflammatory cytokines, thereby contributing to the inflammatory component of fibrosis. In addition, Brun et al demonstrated that murine HSC expressed receptors for bacterial endotoxin and that HSC develop a strong pro-inflammatory phenotype upon LPS stimulation (73). These results confirm an earlier study of Paik et al in which the presence of molecules participating in LPS-signaling, e.g. CD14, TLR4 and MD-2, were demonstrated on activated human HSC (74). In
addition, they also showed that LPS stimulation of these activated HSC resulted in activation of the NF-κB pathway.

Involvement of LPS-signaling molecules in the progression of the process of fibrogenesis was also demonstrated by Isayama et al. They showed in a murine model of fibrosis caused by experimental cholestasis that LBP- and CD14-knock-out mice showed less fibrosis and liver damage (75).

Hepatitis B and C and alcoholic hepatitis
Viral infections with hepatitis B and C and alcoholic hepatitis are three of the most well-known causes of liver fibrosis. In 2000, it was estimated by the World Health Organization that world-wide approximately 350 and 170 million people were infected with hepatitis B and C respectively. Numbers of patients suffering from alcoholic liver disease are not clear as a majority of these patients experiences no signs of disease.

As the liver is the major LPS-removing organ in the body and as the liver function is impaired during hepatitis B and C and alcoholic hepatitis, endotoxemia may occur in patients suffering from these diseases (1; 2; 31; 76; 77). Lin et al demonstrated that chronic hepatitis patients did not have significantly elevated endotoxin levels in the blood. Yet, very significant elevations of endotoxin levels could be detected in chronic hepatitis patients with an acute exacerbation of the disease (77). A decreased phagocytotic capacity of polymorphonucleocytes and Kupffer cells, which are the major cells responsible for removing LPS and whole bacteria from the circulation, and a reduced antibacterial activity of T-cells (78) contribute to the development of endotoxemia during viral hepatitis. An increased intestinal permeability may further contribute to elevated LPS levels in alcoholic liver disease (61; 63). The effects of elevated LPS levels during hepatitis may be further enhanced by the increased levels of sCD14 in chronic hepatitis B (79) and C patients (78; 79). In addition, sCD14 and C-reactive protein levels were higher in patients with cirrhotic livers than in patients with non-cirrhotic livers (79). In patients suffering from alcoholic hepatitis, also elevated levels of LPS-binding protein (LBP) were detected in the acute phase of the disease (31).
Obstructive jaundice
Obstructive jaundice is a liver disease in which the bile flow into the intestine has been decreased or stopped due to obstruction of bile duct(s). Obstructive jaundice is characterized by a high post-operative morbidity of around 10% (80). A common post-operative complication of obstructive jaundice is renal failure, which is often associated with portal and/or systemic endotoxemia (80). Also in the pre-operative state of obstructive jaundice, portal and systemic endotoxemia are thought to be an important factor in the pathophysiology of the disease (81; 82). Clinical studies revealed that elevated serum levels of LPS in obstructive jaundice may have several causes. First, it has been shown that during obstructive jaundice, the capacity of the reticuloendothelial system (RES) of the liver to remove LPS or whole bacteria from the circulation (83; 84) has been decreased. Second, enhanced absorption of endotoxin from the intestinal lumen due to a decreased or abrogated gastrointestinal bile flow has been reported (85). In addition, also increased intestinal permeability during cholestasis has been mentioned as a reason for elevated serum LPS levels (82; 86; 87) and bacterial translocation. Oral administration of bile acids reduced this increase in endotoxin absorption (80; 85; 88) and bacterial translocation (88), thus emphasizing the importance of bile flow in preventing endotoxin absorption.

Available therapies

Sepsis
There are many experimental anti-sepsis therapies, most of which are not very successful in patients or only effective in animal models. As sepsis is a very complex disease in which many mediators and pathways are involved, elimination of one mediator or inhibition of a single pathway seems often insufficient. Cytokines and especially TNFα and IL-1 play an important role in the onset of sepsis and the development of septic shock. High levels of these cytokines are associated with increased mortality (89). Several clinical trials have therefore been performed with anti-inflammatory agents like anti-TNF antibodies, IL-1 receptor antagonists and soluble TNF receptors, but also with antibradykinin agents,
platelet activating factor acetylhydrolases and antiprostaglandin agents (90). However, only very small improvements have been seen and sometimes even adverse effects were found, as shown by Fisher et al in a study with a soluble TNF receptor (91).

Besides the production of cytokines, sepsis also leads to activation of the coagulation cascade. Endothelial cells activated by pro-inflammatory cytokines like TNFα, IL-1 and IL-6, start releasing tissue factor, leading to the activation of factor VII and thus initiation of the coagulation cascade and the formation of thrombin. This may lead to blood clotting in small blood vessels and occlusion of these vessels resulting in disseminated intravascular coagulation (DIC). The result of DIC is organ dysfunction which may eventually lead to multiple organ failure and death. Therapies aimed at the impaired coagulation cascade are anti-coagulant therapies like antithrombin III (AT-III), tissue factor pathway inhibitor and activated protein C (90). Activated protein C has, as part of the protein C pathway, also an important role in counterbalancing the fibrinolytic pathway and also seems to directly influence neutrophil activity. To date, only this activated protein C (Xigris®) has shown to be a partly successful therapeutic strategy for sepsis by significantly, but modestly, improving survival, in certain groups of patients (92; 93).

The therapies mentioned so far, are mainly focused on eliminating mediators of the inflammatory cascade. Few therapies are directed against the initiator of the cascade, the LPS molecule itself. Some therapies with anti-endotoxin antibodies have been explored, but no significant effects were detected then (94; 95).

Besides the already mentioned therapies, there are also new experimental therapies explored in clinical trails that are currently ongoing. In previous studies, we demonstrated that alkaline phosphatase (AP) is able to detoxify LPS by dephosphorylation (96-99). The applicability of this concept as a therapy is currently tested in two different clinical trials, each dealing with a different LPS-mediated disease. In one trial, the effect of AP on the clinical outcome of septic patients is examined and in the second trial, the effect of AP on intestinal inflammation in ulcerative colitis is assessed. An overview of the various anti-sepsis therapies that have been explored is given in table 1.
Table 1: Overview of clinically tested therapeutic compounds directed at different mediators of the sepsis-cascade. Based on information of amongst others Deans and Riedemann (90; 100).

<table>
<thead>
<tr>
<th>Target</th>
<th>Therapeutic compound</th>
<th>Reference</th>
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<tr>
<td></td>
<td>Soluble TNF receptors</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td>IL-1 receptor antagonist</td>
<td>(102)</td>
</tr>
<tr>
<td>Lipid mediators</td>
<td>Platelet activating factor (PAF) receptor antagonists</td>
<td>(103)</td>
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<td></td>
<td>Platelet activating factor acetylhydrolase (PAF-AH)</td>
<td>(104)</td>
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<tr>
<td></td>
<td>Cyclooxygenase inhibitor (ibuprofen)</td>
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</tr>
<tr>
<td></td>
<td>Bradykinin receptor antagonist</td>
<td>(106)</td>
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<tr>
<td></td>
<td>Thromboxane A2 inhibitor</td>
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</tr>
<tr>
<td></td>
<td>Thromboxane synthase inhibitor</td>
<td>(108)</td>
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<td>Nitric oxide (NO)</td>
<td>N(omega)-nitro-l-arginine methylester (L-NAME)</td>
<td>(109)</td>
</tr>
<tr>
<td></td>
<td>N(G)-monomethyl-L-arginine (L-NMMA)</td>
<td>(110)</td>
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<td>Lipopolysaccharide</td>
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<td>HA-1A human monoclonal antibody to endotoxin</td>
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<td>Glucocorticoids (hydrocortisone and equivalents)</td>
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<td></td>
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<tr>
<td>CD14</td>
<td>Anti-CD14 monoclonal antibody (IC14)</td>
<td>(118)</td>
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</table>

Liver fibrosis

Liver fibrosis is a disease as complex as sepsis. Both diseases have in common that no effective, curative therapy has been found to date. Despite many clinical trials that have been performed, liver fibrosis still cannot be reversed, leaving only liver transplantation as the final option for fibrotic patients. The fact that liver fibrosis can be the result of liver injury with very different etiologies, makes the development of therapeutics more complicated.

As the hepatic stellate cell (HSC) plays a central role in the progression of liver fibrosis, most experimental therapies for liver fibrosis are directed at the HSC. If HSC become activated, they transform from a quiescent vitamin A storing cell into a proliferative, fibrogenic and contractile myofibroblast, which starts to produce extra-cellular matrix (ECM) components. Many therapies are directed at interfering
at different pathways of this process: avoiding activation of quiescent HSC, downregulation of the activation of HSC, neutralizing proliferative, fibrogenic, contractile and/or pro-inflammatory responses of stellate cells, promotion of apoptosis of activated HSC or increasing the degradation of the produced ECM (58).

Avoiding HSC-activation might be achieved by anti-inflammatory agents, like corticosteroids, that prevent Kupffer cells from producing profibrogenic cytokines like TGF\(\beta\) and TNF\(\alpha\) and other mitogenic compounds like platelet derived growth factor (PDGF). In avoiding HSC-activation, anti-LPS therapies might come in view as recent studies have indicated that LPS can directly activate HSC (74). The increased leakage and reduced removal of LPS from the circulation during fibrosis, may indicate an important role for LPS and hence for anti-LPS drugs during fibrosis. As LPS is one of the most potent activators of Kupffer cells, preventing LPS from reaching the Kupffer cell might also help avoiding HSC activation.

**Hepatitis B and C**

Liver fibrosis induced by Hepatitis B and C may be inhibited by removal of the inciting stimulus. In recent years, many successes have been obtained in this area. Since 1999, the standard therapy for hepatitis C has been a combined therapy of interferon-alpha 2a (Roferon-A\(\text{\textregistered}\)) and ribavirin (119), a nucleoside analog that inhibits the replication of many RNA and DNA viruses. In recent years, the standard therapy for hepatitis C is a combination therapy consisting of pegylated interferons, like pegylated interferon-\(\alpha\)-2a (Pegasys\(\text{\textregistered}\)) or peginterferon-\(\alpha\)-2b (Pegintron\(\text{\textregistered}\)), combined with ribavirin (119), which enhances the effect of the pegylated interferons (120).

Today, several antiviral orally-administered nucleoside analogs like lamivudine, adefovir, dipivoxil and entecavir are used in the treatment of hepatitis B. In addition, new antiviral nucleoside analogs like telbuvidine, tenofovir and emtricitabine are currently clinically tested (121). The most successful treatment for chronic hepatitis B though, is treatment with pegylated interferon-\(\alpha\)-2a (Pegasys\(\text{\textregistered}\)) without a nucleoside analog. Pegylated interferon-\(\alpha\)-2b is in clinical development for treatment of hepatitis B (121). Despite the fact that elevated serum levels of LBP and sCD14 have been reported during hepatitis and endotoxemia occasionally
occurs in hepatitis patients, suggesting a role for LPS in the disease, no anti-LPS strategies are being explored for these diseases now.

**Inflammatory Bowel Disease**

Several therapies are used for the treatment of inflammatory bowel diseases (IBD). The chosen therapy depends on the localization, the severity and the nature of the disease. Mild to moderate disease activity is often treated with 5-aminosalicylic acid compounds like sulfalazine, olsalazine and mesalamine (122). 5-aminosalicylic acid is a topically active anti-inflammatory compound that reduces the inflammation in the intestinal mucosa. As bacteria are thought to play an important role in IBD, antibiotics are also examined as therapeutics in IBD. Most research about the use of antibiotics is performed in patients suffering from Crohn’s disease (CD) (123). Antibiotics like metronidazol have shown to be quite effective in CD affecting the perianal part of the colon. Also ciprofloxacin has been reported to have beneficial effects in CD. Patients who are suffering from more severe symptoms or patients not responding to 5-aminosalicylic acid compounds are often treated with corticosteroids like prednisone or hydrocortisone. Although corticosteroids are already used for a long time in IBD-treatment protocols, these compounds unfortunately sometimes cause side effects, which can make them inappropriate for chronic treatment. Possible side-effects are acne, severe mood changes, adrenal insufficiency, hypertension, bone loss and visual changes (124). If corticosteroid treatment does not ameliorate the disease or if too many side-effects are encountered, other immunosuppressants may be used. Common immunosuppressants used in IBD are azathioprine, 6-mercaptopurine, cyclosporine and methotrexate.

Besides these more or less established therapies, also several new therapies have been developed recently, amongst them a variety of cytokine-based therapeutics (125). One of these therapeutics is infliximab (Remicade®). Infliximab, an antibody composed of human and mouse components that binds and neutralizes TNFα, was originally developed as an anti-sepsis agent. Unfortunately, infliximab did not improve clinical outcome in trials with septic patients (126), but in contrast to this lack of beneficial effects in sepsis, infliximab proved to be quite beneficial in other
diseases in which high TNFα levels play an important role, like rheumatoid arthritis (127) and CD (128; 129). Ever since, infliximab has shown to have significant beneficial effects in many patients. Another cytokine which was thought to play an important role in inflammatory bowel disease is interleukin-10 (IL-10). IL-10 is an anti-inflammatory cytokine which inhibits several functions of macrophages, monocytes and dendritic cells (130). The thought that interleukin-10 might play an important role in IBD arose after the discovery that IL-10 knock-out mice developed spontaneously chronic enterocolitis (131). Patient trials though did not show the effects that were expected, which might be due to the complex pleiotropic functions of IL-10, exerting both anti- and pro-inflammatory effects (See references in Stokkers et al) (125).

Another compound that has been tested for its potential use as a therapeutic compound in IBD is the short-chain fatty acid butyrate. Butyrate is produced in large amounts in the colon when dietary saccharides that escaped enzymatic breakdown in the small intestine, are fermented by anaerobic bacteria in the colon. Products of this fermentation process are mainly short-chain fatty acids (SCFA), of which butyrate is the most interesting one regarding its effects on the colon. In the colon, butyrate serves as the primary energy source for colonic epithelium but also regulates epithelial cell proliferation and differentiation in the colonic crypts. Moreover, butyrate displayed anti-inflammatory effects (132), which led to the hypothesis that butyrate might be beneficial during ulcerative colitis (UC) (133). The mechanism behind the anti-inflammatory effects of butyrate are not fully elucidated yet, but Segain et al demonstrated in human biopsies and human lamina propria mononuclear cells that butyrate inhibited NFκB activation, which resulted in a lower production of inflammatory cytokines like TNFα, IL-6 and IL-1β (134). In addition, Luhrs et al, showed that butyrate ameliorates inflammation in UC by inhibiting the IκB mediated translocation of NFκB to the nucleus in lamina propria macrophages (135). So far, evidence from clinical trials using butyrate in UC is still limited to a few studies (135-137). The studies are hampered by the fact that when using enemas, the disease has to be located distal to the splenic flexure (133). New oral formulations of butyrate might help to overcome this problem in the future.
Alkaline phosphatase

Alkaline phosphatase (AP) is an endogenous enzyme present in several organs, for instance liver, lung, kidney, intestine and placenta. AP is a 120 kDa dimeric orthophosphoric monoester phosphohydrolase that hydrolyzes many substrates at a high pH. It is a membrane-bound enzyme, which is linked to the cell membrane by a glycosylphosphatidylinositol anchor enabling the enzyme to fulfill its function outside the cell.

There are several isoenzymes of AP: the liver/bone/kidney (LBK), the intestinal, the placental and the placental-like or germ-cell isoenzymes. The intestinal and placental isoenzymes are so-called organ-specific enzymes and are in healthy persons only expressed in intestinal and placental tissue. In contrast, the LBK-isoenzyme, also called tissue non-specific alkaline phosphatase, is not only expressed in liver, bone and kidney tissue but also in neutrophils (138; 139), endothelial cells and fibroblasts and consequently in nearly all organs of the body. The isoenzymes of AP share structural homology (140), especially the intestinal, placental and germ-cell AP, which are clustered on the long-arm of chromosome 2, which are up to 90 to 98% homologous (141). The other isoenzyme, the liver/bone/kidney isoenzyme, is located on chromosome 1 and is only 50% homologous to the other three isoenzymes (141). In contrast to humans, rats display two isoenzymes of the intestinal alkaline phosphatase (142).

For a long time, the only known physiological function of AP was a role in bone formation (143; 144). This role has already been described in 1923 by Robison. In his study in which he describes his theory of calcification, he mentioned “a bone enzyme” with a pH optimum of 9. At a later stage this enzyme was called AP. In addition, he described in this study that the function of the “bone-enzyme” is liberating phosphates from organic phosphates. The liberated phosphates subsequently form an insoluble precipitate with calcium ions and hence bone material is formed. Since then, AP has been used as a marker for bone formation by osteoblasts, the bone forming cells. But as it is present in large amounts in other organs, particularly in the intestine and brush border of renal tubules, already in 1947 Lorch anticipated that it was likely that the enzyme had another physiological
function (145). This completely other function was discovered in 1997 by Poelstra et al, who demonstrated that the enzyme AP was able to dephosphorylate and detoxify LPS (97; 98). Removal of one of the 2 phosphates of the lipid A part of LPS resulted in a non-toxic lipid A moiety (96-98). These findings were extended and confirmed by studies of Bentala et al, Verweij et al and Beumer et al (96; 99; 146). Dephosphorylation of LPS by AP seems relevant in several tissues with high AP activity, e.g. the small intestine and the liver.

Several diseases may lead to an altered expression of AP isoenzymes. For instance, placental alkaline phosphatase (plAP) has been detected in various types of cancer, e.g. testis, ovary, pancreas, colon, lymph tissue, stomach and bladder cancer (147), and is used as a tumor marker. It can be expected that the enzyme has other functions in these tissues, but this remains to be elucidated.

Until now, data about the regulation of endogenous AP expression are scarce. Several AP-inducing agents are known, for instance sodium butyrate (148-151), retinoic acid (152; 153) and IL-6 (154).

Sodium butyrate, as mentioned earlier, is a short-chain fatty acid that is produced in large amounts by colonic bacteria. It is known as a strong inducer of placental-like or germ-cell alkaline phosphatase, which is often present in tumors (155). As described in paragraph 2.3, butyrate also ameliorates symptoms of ulcerative colitis. Studies of Chakravortty et al and Wu et al (156; 157) demonstrated that this is associated with inhibition of IκB-degradation, which prevents NFκB-translocation to the nucleus and thereby activation of several genes, e.g. iNOS, TNFα and IL-8. In contrast, Ogawa et al did not find any effect of butyrate on IκB-degradation and NFκB-activation in human intestinal microvascular endothelial cells (132). Although it is known that butyrate induces germ-cell AP and AP is abundantly present in the intestine, no studies have reported whether butyrate might also induce intestinal AP.

Vitamin A or retinoic acid is another compound capable of increasing AP expression in a variety of cell lines, e.g. cultured human oral cancer cells (158), murine preosteoblastic cells (159), human ovarian carcinoma 3AO cells (160), intestinal Caco-2 cells (161) and rat small intestinal epithelial crypt IEC-6 cells.
Moreover, retinoic acid is known as a key regulator in the process of bone formation and LBK-AP is the most important enzyme involved in this process. Orimo et al demonstrated that retinoic acid regulates AP expression via a retinoic acid responsive element in the promoter region of the AP gene (162). Interestingly, HSC release their vitamin A upon activation during the fibrogenic process. Despite the fact that there are quite a few AP-inducers, a final common pathway for all these inducers to enhance AP activity is not known yet.

**AP expression during disease**

**Sepsis**

Several recent papers, of amongst others our group, have reported about the beneficial effect of administration of AP during sepsis (96; 99; 146; 163; 164). Administration of high amounts of AP increases the LPS-dephosphorylating capacity. As administration of “extra” AP results in a protective effect during sepsis, one could anticipate that probably endogenous AP levels have also changed during sepsis. This hypothesis is confirmed by several studies in which elevated AP levels during sepsis (165; 166) and bacteraemia (167) have been found. Nevertheless, elevated AP levels due to hepatic complications accompanying sepsis are more often reported. Sepsis also can affect the liver function and common hepatic complications occurring during sepsis are for instance cholestasis and jaundice (168-170).

**Liver fibrosis**

In normal human and rat livers, AP activity is only found at the hepatocyte canalicular membrane. In cholestatic livers, this activity profoundly changes. In rats subjected to bile duct ligation, AP activity was found not only at the canalicular membrane but also at the sinusoidal membrane of hepatocytes (171). An identical change in pattern of AP activity in the liver was found in human cholestatic livers. Livers of patients suffering from primary biliary cholangitis (PBC) displayed AP activity at both the canalicular and sinusoidal membrane
Furthermore, they also reported high AP activity in the cytoplasm of bile duct epithelial cells (172).

In addition, cholestatic liver diseases like PBC and primary sclerosing cholangitis (PSC) are also accompanied by significant changes in serum AP levels (172; 173). In fact, serum AP levels are used as a marker to assess disease activity. In contrast to cholestatic liver diseases, hepatitis C patients did not show any change in hepatic AP expression compared to control livers (172).

**Inflammatory bowel disease**

AP is normally expressed in high amounts by the epithelial cells of the small intestine and in small amounts by epithelial cells of the colon. If the gut epithelial layer is damaged, LPS from the bacteria colonizing the gut cannot be detoxified by AP and a subsequent exposure of sub-epithelial cell layers to this LPS may initiate a local inflammation. Damage to the AP-bearing epithelial layer of the intestine thus may be an important factor in the perpetuation of the inflammatory process in IBD.

Chronic inflammatory bowel diseases in some cases also lead to changes in serum AP levels. A common complication of inflammatory bowel diseases, and especially ulcerative colitis, is PSC. PSC ultimately results in biliary cirrhosis and is characterized by elevated serum AP levels (173).

**Other diseases**

Besides the diseases already mentioned in this paragraph, there are several other diseases displaying aberrant AP levels in tissue and / or serum.

Several bone diseases are characterized by very high AP levels, so called hyperphosphatasia or very low AP levels called hypophosphatasia. Examples of diseases which are accompanied by hyperphosphatasia are Paget’s disease and hereditary hyperphosphatasia. Hereditary hypophosphatasia has also been described and it is known that the low serum AP levels which are characteristic for this disease, are caused by a structural abnormality in the tissue non-specific AP enzyme, which is due to mutations in the gene (174; 175). Besides for bone diseases, AP levels are also used as a marker for rheumatoid arthritis. One of the hallmarks of rheumatoid arthritis are elevated AP levels in serum (176).
Besides bone diseases, also some tumors express abnormal levels of the enzyme AP. Especially elevated levels of placental alkaline phosphatase are measured in tumors of different origin (147). The cause of the AP elevations in these diseases may be the enhanced inflammatory activity leading to release of AP from neutrophils, enhanced activity of fibroblasts or osteoblasts that release AP, or aberrant transcription of embryonic genes in tumors.

This thesis
This chapter has given an overview of what is known about AP and LPS-mediated diseases, with a special focus on sepsis, IBD and liver fibrosis. In addition, the possible role of endogenous and exogenous AP in these diseases has been described. Sepsis, IBD and liver fibrosis are examples of three well-known diseases in which LPS plays an important role in the onset or progression. Of note, as shown in the previous paragraph, AP levels deviate from standard values in a lot more diseases. Yet, not much is known about the mechanism behind the endogenous regulation of AP activity. More knowledge about this process may lead to new possibilities for therapeutic interventions in LPS-mediated diseases. We therefore explored the factors that regulate endogenous AP activity (chapters 3 and 4).

To date, the only LPS-mediated disease in which AP had shown a significant therapeutic effect is sepsis. In mice, rats, pigs and sheep, significant effects of AP on survival and several inflammatory parameters were found (96; 99; 146; 163). So far, this concept has only been tested in sepsis, but in other diseases in which an excess of LPS is initiating inflammatory processes, administration of AP might also result in beneficial effects. For that reason, the second research topic of this thesis was elaborating the knowledge on the therapeutic effects of AP in sepsis (chapters 3 and 5) and examining its applicability in another disease in which LPS plays a fundamental role, IBD (chapter 6).
Reference List


69. Lukkari TA, Jarvelainen HA, Ononen T, Kettunen E, Lindros KO. Short-term ethanol exposure increases the expression of Kupffer cell CD14 receptor and lipopolysaccharide binding protein in rat liver. Alcohol Alcohol 1999;34:311-319.

70. Kono H, Wheeler MD, Rusyn I, Lin M, Seabra V, Rivera CA,


81. Clements WD, Erwin P, McCaigue MD, Halliday I, Barclay GR,


General Introduction


149. Khan KN, Tsutsumi T, Nakata K, Kato Y. Sodium butyrate


