Chapter 1

General introduction and outline of the thesis

General introduction

Infectious diseases are a major cause of morbidity and mortality worldwide (1). Improved hygiene, the introduction of antibiotics and vaccination programs have successfully reduced the morbidity and mortality of many infectious diseases. However, the increasing incidence of antibiotic resistance and the enhanced risk of worldwide spreading due to increasing travelling stress the importance of improving treatment against infectious diseases. To achieve this, expanding our understanding of the host responses to microbial invasion is mandatory. This thesis is focussed on the role of CD44 and osteopontin (OPN) during primarily pulmonary bacterial infection and inflammation.

Pulmonary immune response

The alveolar membrane is the largest surface of the body in contact with the outside environment, and is continuously exposed to respiratory pathogens such as bacteria and viruses. In the upper respiratory tract physical mechanisms like coughing and sneezing are used as a first line of defense against potential pathogens. In case microbes enter the terminal airways, they can cause pneumonia, one of the most common infectious diseases and the most frequent source of sepsis (2). Innate immune cells like respiratory epithelial cells, in a normal alveolus covered with a surfactant layer, and resident alveolar macrophages (AM) are the first cells to encounter pathogens in the alveoli (Figure 1, reviewed in (3)). The innate immune system is able to detect pathogens via a limited number of pattern-recognition receptors (PRR) which recognise conserved motifs that are expressed by pathogens but are absent in higher eukaryotes (4). Among other receptor families such as scavenger receptors, complement receptors, Fc-receptors, C-type lectins and Nucleotide-binding oligodimerization domain proteins (NOD)-like receptors, Toll-like receptors (TLRs) have a central role as PRR in the initiation of cellular innate immune responses. Upon recognition of a pathogen, lung epithelial cells will secrete cytokines and chemokines, and antimicrobial peptides that have microcidal activity or inhibit bacterial growth. AM are able to recognize, bind and phagocytose pathogens and subsequently kill them intracellularly. These cells can also secrete several mediators including cytokines and chemokines that mediate recruitment and activation of neutrophils from the circulation. These cells are potent phagocytes and critical for the effective elimination of pathogens. In addition, AM can phagocytose apoptotic neutrophils and thereby contribute to the resolution of pneumonia. In a number

of murine pneumonia models depletion of AM or neutrophils resulted in increased lethality and worsened outcome of the disease indicating the significant role of these cells during pulmonary infection (reviewed in (5)). In addition to rapid initiation of the nonspecific inflammatory reaction, the innate immune response is thought to orchestrate the adaptive immune response that primarily consists of T-cell and B-cell responses that provide specific memory of infection (6).

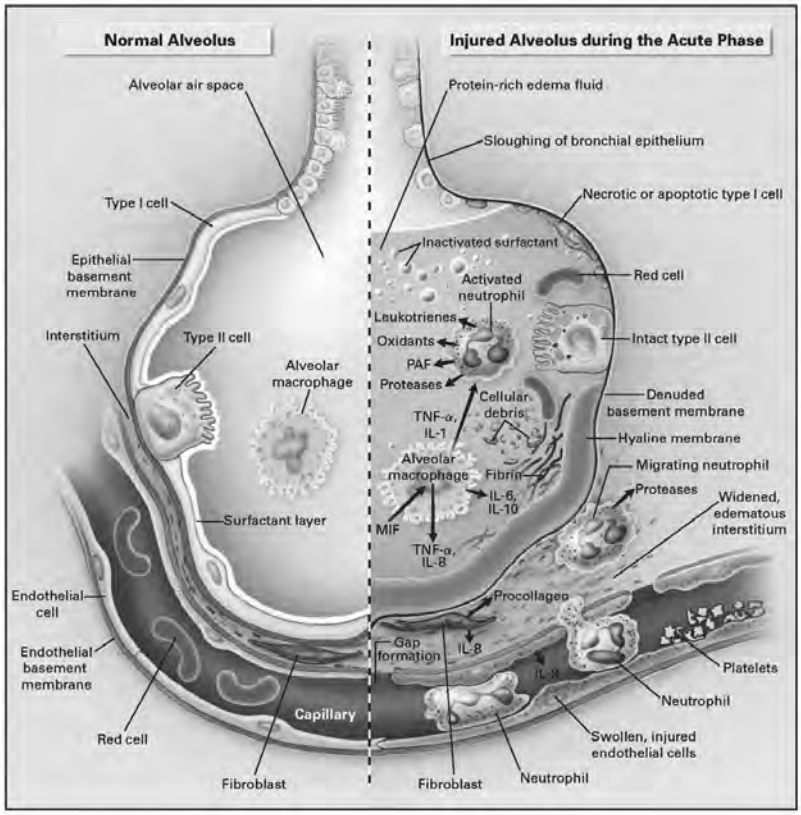

Figure 1. Depicted is a normal (left side) and inflamed (right side) alveolus. In the acute phase, there is sloughing of both the bronchial and alveolar epithelial cells, with the formation of protein-rich hyaline membranes on the denuded basement membrane. In the air space, an alveolar macrophage is secreting cytokines, including interleukin (IL)-1, IL-6, IL-8, and IL-10, and tumor necrosis factor (TNF)-α, which act locally to stimulate chemotaxis and activate neutrophils. Neutrophils are shown adhering to the injured capillary endothelium and marginating through the interstitium into the air space, which is filled with protein-rich edema fluid. Neutrophils can release oxidants, proteases, leukotrienes, and other proinflammatory molecules, such as platelet-activating factor (PAF). The influx of protein-rich edema fluid into the alveolus has led to the inactivation of surfactant. Adapted from (7).

CD44

The transmembrane glycoprotein CD44 was initially described in 1981 as a hyaluronic acid (HA) binding molecule (8) and as a homing receptor for lymphocytes (9). In the following decades, the diversity in structure and function of CD44 became clear. The single CD44 gene consists of 20 exons and encodes for a large variety of CD44 proteins. All isoforms share an extracellular N-terminal domain (coded by exon 1-5) which binds HA (10), and a C-terminal domain (coded by exon 16-20), which contains a transmembrane domain and an intracellular cytoplasmic domain (11). The cytoplasmic tail of CD44 interacts with proteins of the cytoskeleton including actin, ankyrin, ezrin, radixin and moesin (12-14), and connection of CD44 with the cytoskeleton was hypothesised to regulate HA binding to CD44. The standard form of CD44 is expressed by most tissues and consists of the structures encoded by exon 1-5 and 16-20 only (15). By alternative splicing of the 10 variable exons that are present in mice (exon v1-v10) many isoforms can be generated that differ in their extracellular domain (11). In addition to alternative splicing CD44 is altered by posttranslational modifications including N- and/or O-linked glycosylation and addition of glycosaminoglycan chains (Figure 2) (16, 17). Shedding of CD44 from cellular membranes results in soluble CD44 (sCD44). So far little is known about the function of sCD44 (18, 19). sCD44 is found in the circulation of normal humans (20) and mice (21), and the serum levels of sCD44 often correlate with inflammatory state and tumor progression (21-24).

CD44 functions and ligands

CD44 is widely expressed on a variety of cell types, including leukocytes and parenchymal cells (26, 27). This molecule has been implicated to mediate important physiological and pathological processes, such as cellular adhesion and migration by mediating cell-cell and cell-matrix interactions, cellular survival by affecting apoptosis and proliferation, and cytokine and chemokine responses (reviewed in (28, 29)).

The main ligand of CD44 is HA, a glycosaminoglycan that is a component of the extracellular matrix (Figure 3). Under physiologic conditions HA exists as a high molecular weight polymer of disaccharides that has a restricted tissue distribution and is mainly found in connective tissues such as cartilage and joints. However, during tissue injury and inflammation HA undergoes dynamic degradation resulting in accumulation of low molecular weight fragments (30). Binding of HA to CD44

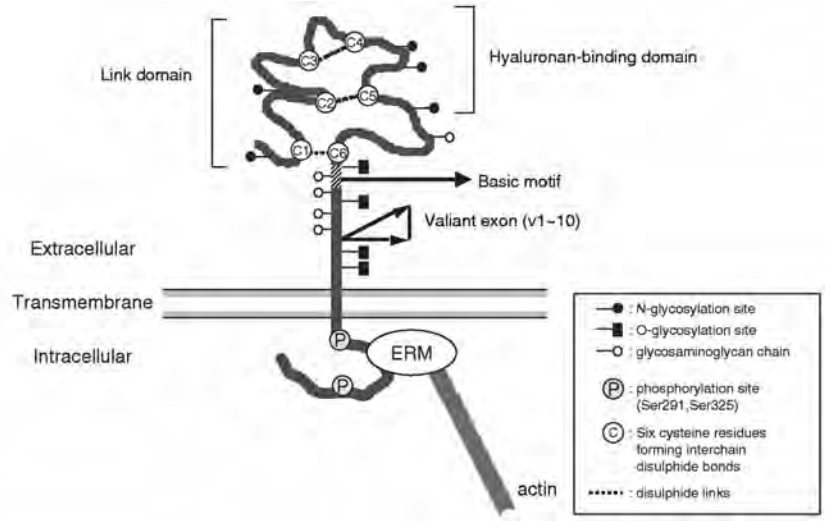

Figure 2. Structure of CD44 protein. CD44 type I transmembrane protein is composed of extracellular, transmembrane, and cytoplasmic domains. The globular region of CD44 in the extracellular amino-terminal domain has homology with the cartilage link protein, which is known as "link domain." Six cysteine residues that form interchain disulphide bonds confer stability on the link domain. The amino-terminal globular domain contains hyaluronan-binding motifs, which are in the link domain, and a basic motif that is outside the link domain. The extracellular domain is highly glycosylated. The stem structure of CD44 can be enlarged by the alternatively spliced variant exons (v1-v10) of CD44. The carboxy-terminal cytoplasmic domain interacts with ERM (ezrin, radixin, and moesin) proteins, which link to the actin cytoskeleton. From (25)

is strictly regulated and dependent on the cell type, state of cell activation and differentiation (31-33). HA may interact differentially with various cells and produce distinct biological effects depending on the molecular weight. HA fragments induce chemokine gene expression (34) and NF-κB activation (35) in AM, and promote angiogenesis (36), whereas high molecular weight HA does not. However, several HA induced effects appeared only partly CD44 dependent. In the past few years, it has become clear that TLRs may warn the host of danger in general by the ability to recognise endogenous mediators released during injurious processes (37). HA fragments are now known to additionally signal through TLR2 and TLR4, thereby affecting NF-κB activation and induction of inflammatory gene expression, cell survival and migration (38-41). Interestingly, recent studies have shown that CD44 positively regulates the induction of negative regulators of TLR signalling including IL-1R-associated kinase (IRAK)-M (42, 43). Moreover, CD44 is known to bind, internalise and degrade HA into inactive disaccharides (26, 44, 45), thereby clearing it from the extracellular compartment.

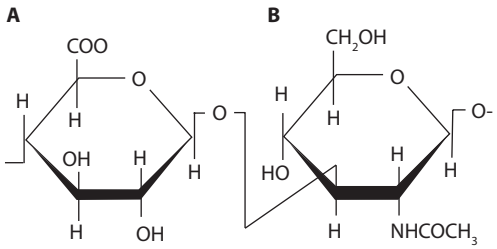

Figure 3. Hyaluronic acid is composed of repeating disaccharide units containing glucuronic acid (A) and *n*-acetylglucosamine (B). From (54).

OPN, the second major ligand of CD44, is a secreted phosphorylated glycoprotein (Figure 4) (46) that is expressed by a broad range of tissues and cells (47-49). Originally considered a bone matrix protein, OPN is now known to regulate inflammation, tissue remodeling and cell survival (49-51). It has been implicated as an important component of inflammation during both innate and adaptive immunity by mediating inflammatory cell differentiation, maturation and migration, and cytokine production (47, 49-53).

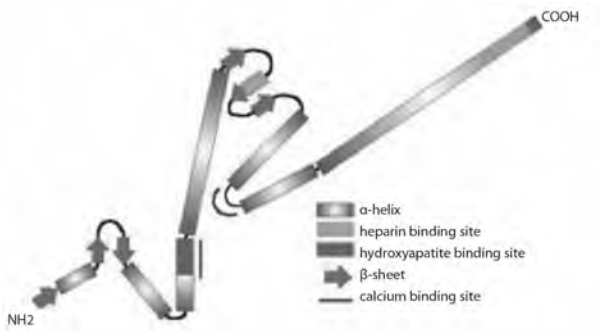

Figure 4. Osteopontin.

CD44 and osteopontin in lung disease

Under normal conditions CD44 is expressed by AM and pulmonary epithelial cells (55-57), and during inflammation CD44 expression in the lung can be upregulated (55, 58). Inflammatory cell recruitment is an important process during pulmonary inflammation (59), and CD44 is expressed on inflammatory, as well as endothelial and epithelial cells (60). Indeed, several studies suggest a central function for CD44 during sterile or infectious lung inflammation. CD44 on murine neutrophils is an

E-selectin ligand (61) and important for polarization and directed migration of those cells (62). Therefore, it can be anticipated that cell influx into the lung would be impaired in the absence of CD44; however, others suggested a negative regulation of epithelium-neutrophil interactions via CD44 (63). During ozone-induced airway hyperresposiveness inflammatory cell influx into the lungs was dependent on HA and CD44, and thus impaired in CD44 knockout (KO) mice (64). On the other hand, in a model of acute pulmonary lung inflammation induced by intratracheal lipopolysaccharide (LPS) treatment, neutrophils appeared earlier and remained enhanced in numbers in bronchoalveolar lavage fluid (BALF) from CD44 KO mice when compared to neutrophil numbers in BALF of wild-type (WT) mice (42), whereas during a low dose aerosol LPS challenge neutrophil numbers were lower in CD44 KO mice (65). Similarly, enhanced neutrophil recruitment in the absence of CD44 has been reported for *Mycobacterium* (*M*.) *tuberculosis* infection (66) and *Escherichia (E.) coli* induced pneumonia (67). In addition, CD44 has been shown to affect macrophage trafficking towards the lung. CD44 KO mice demonstrated increased macrophage numbers in their BALF during lung inflammation elicited by intrapulmonary delivery of LPS or bleomycin (42, 68), whereas decreased pulmonary macrophage numbers were found in CD44 KO mice after aerosol challenge with a low dose of LPS (65), during ozone-induced airway hyperresponsiveness (64) and tuberculosis (66). Moreover, cell migration and tissue damage are additionally affected by cytokine and chemokine release, a process that can also be influenced by CD44 (42). Taken together, the net effect of CD44 on pulmonary inflammation appears to be dependent on the stimulus, the severity of tissue damage and the predominant cell type involved in the inflammatory response. In line with the function of CD44 to take up and degrade HA (26), CD44 KO mice showed elevated levels of HA during sterile pulmonary inflammation (induced either by ozone (64), LPS (42), or bleomycin (68)), which was accompanied by sustained accumulation of inflammatory cells.

Patients suffering from diverse pulmonary diseases, including interstitial pneumonia, tuberculosis, silicosis and sarcoidosis, displayed enhanced OPN expression in their lungs as compared to healthy controls (69-71, 72, 73). In addition, patients with idiopathic pulmonary fibrosis demonstrated increased OPN levels in BALF (74). A possible protective role for OPN in mycobacterial infection was suggested by the observation that the extent of OPN protein expression in pathological lymph nodes from *M. bovis* BCG or *M. avium* infected patients was inversely correlated with disseminated infection and death (75). Furthermore, plasma OPN levels were

dramatically elevated in patients with interstitial pneumonia (76), in patients with sepsis of whom almost half suffered from pneumonia as the primary site of infection (77), and in patients with tuberculosis in which levels declined after successful therapy (78, 79). Moreover, a functional role for OPN has been described in several experimental models of non-infectious lung disease, including allergy and asthma (80, 81, 82), acute respiratory distress syndrome (73), granulomatous disease (83) and fibrosis (84, 85). However, knowledge on the functional role of OPN during pulmonary (bacterial) infection is limited.

Infection and inflammation models studied in this thesis

Respiratory tract infections

Infections of the respiratory tract are the 7th most prevalent cause of mortality in the United States (86, 87). According to the acquisition of pneumonia and the pathogens involved, community-acquired pneumonia (CAP) can be distinguished from nosocomial pneumonia. The pre-dominant pathogen responsible for CAP is *Streptococcus (S.) pneumoniae*, which is responsible for up to 60% of the cases. *S. pneumoniae* is a gram-positive diplococcus, of which the virulence is mainly determined by its capsule (88, 89). Nosocomial pneumonia usually occurs in patients with preexisting conditions and can be induced by various pathogens. *Klebsiella (K.) pneumoniae* is a gram-negative opportunistic rod-shaped bacterium that accounts for about 12% of hospital-acquired pneumonia cases (90, 91). Melioidosis is an important cause of severe sepsis in Southeast Asia and Northern Australia caused by the aerobic gram-negative soil-dwelling bacillus *Burkholderia* (*B.*) *pseudomallei* (92, 93). Infection is thought to occur by cutaneous inoculation or inhalation. More than half of patients with melioidosis presents with pneumonia associated with bacterial dissemination to distant organs (94), and all-cause mortality is as high as 50% in Northeast Thailand where the majority of reported cases occur (95). One of the most dramatic manifestations of chronic lung infection and inflammation is tuberculosis. This disease, caused by infection with the acid fast rod *M. tuberculosis*, is a serious threat to mankind. One-third of the world's population is infected with the tubercle bacillus and tuberculosis is responsible for two million deaths each year (96, 97). Upon infection with this pathogen healthy individuals develop a strong T-helper 1 (Th1) response which is able to contain the infection in granulomas and prevent active disease. However, *M. tuberculosis* bacilli are thereby not eradicated from the lungs and remain a potential danger to the infected individual (97).

Hyperoxia induced lung injury

Patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) almost invariably are given supplemental oxygen to maintain tissue oxygenation (98, 99). However, prolonged exposure to high oxygen concentrations can worsen or induce lung damage in already injured or previously healthy lungs (100). The mechanisms underlying hyperoxia induced lung injury are beginning to be understood. The inflammatory response to hyperoxia is dominated by the recruitment of neutrophils into the bronchoalveolar space and likely mediated by the generation of reactive oxygen species (98, 100). However it has become clear that neutrophils are not absolutely required for the induction of lung injury during hyperoxia (101-103).

Peritonitis

Peritonitis is most often caused by the presence of bacteria in the otherwise germ-free peritoneal cavity and is predominantly due to perforation of intestines. An immediate and adequate host defense is necessary to contain and kill the pathogen as due to the site of infection bacteria can quickly spread via the circulation, resulting in systemic inflammation and sepsis (2, 104). Like in the lung, upon bacterial appearance in the peritoneal cavity, resident peritoneal macrophages release chemoattractant factors to recruit circulating neutrophils and monocytes to kill the pathogen (105). *E. coli* is the most frequently isolated causative pathogen in peritonitis, which is responsible for 60% of the intraabdominal infections (106, 107).

Aim and outline of this thesis

The general aim of the research described in this thesis was to obtain more insight into the role of CD44 and OPN during infection and inflammation. The principal subject of investigation was pulmonary inflammation and (myco)bacterial respiratory tract infections. The first part of this thesis focuses on the role of CD44 during (pulmonary) inflammation and infection. **Chapter 2** describes the contribution of CD44 to hyperoxia induced lung injury. The function of CD44 during *E. coli* induced abdominal sepsis is reported in **chapter 3**. The role of CD44 in the acute and resolution phase during *S. pneumoniae* or *K. pneumoniae* induced pneumonia is described in **chapter 4** and **chapter 5**. TLRs are known to be important for pathogen recognition during pneumonia (108), and negative regulators of TLR-mediated immune responses are critical to attenuate TLR signaling and thereby prevent overwhelming inflammation (109). **Chapter 6** describes the role of the negative TLR regulator IRAK-M in the host

response during gram-negative and gram-positive pneumonia. In the next part of this thesis we report on the role of OPN during respiratory tract infections. The contribution of OPN to the host response during pneumonia caused by *K. pneumoniae* or *S. pneumoniae* infection is described in **chapter 7** and **chapter 8**. OPN levels in the circulation are enhanced in patients with sepsis, of whom almost half suffered from pneumonia as the primary site of infection (77), and in patients with tuberculosis (78, 79). Therefore, we investigated the role of OPN during severe gram-negative sepsis induced by *B. pseudomallei* infection and during chronic *M. tuberculosis* infection, which is described in **chapter 9** and **chapter 10**.

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