university of groningen / faculty of mathematics and natural sciences

THE TOLL ROAD TOWARDS NGAL EXPRESSION

Exploring the Relationship Between Activation of Toll-Like Receptors and Expression of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Inflammatory Diseases

BACHELOR THESIS

Pathophysiology Research

Irene Zijlstra, S2506149
Bachelor Life Science and Technology
Faculty of Mathematics and Natural Sciences
University of Groningen, Groningen, The Netherlands
Supervised by asst. prof. E.R. Popa and dr. J. Moser
Date: 24 June 2016

REVIEW: THE TOLL ROAD TOWARDS NGAL EXPRESSION

Exploring the Relationship Between Activation of Toll-Like Receptors and Expression of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Inflammatory Diseases

I. Zijlstra

Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

Abstract

Members of the lipocalin protein family are involved in the regulation of innate immune responses. One family member of particular interest is neutrophil gelatinase-associated lipocalin (NGAL): NGAL is known for its classical function to limit bacterial growth during infection by binding to bacterial siderophores. Furthermore, NGAL might have an anti-inflammatory effect, since it can be detected in a large number of inflammatory conditions in different organ systems. Regulatory mechanisms for expression of NGAL during inflammation and tissue damage are poorly understood. One potential regulatory mechanism might be mediated by Toll-like receptors (TLRs). In this review, the relationship between TLR activation and induction of NGAL expression will be investigated. The potential molecular signalling pathway of TLR activation to NGAL expression will be highlighted as well.

TLRs are activated by exogenous pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs), which can be present during inflammatory conditions. An important transcription factor that is activated in the TLR activation signalling pathway is nuclear factor κB (NF-κB). Since the *LCN2* gene, coding for NGAL, includes an NF-κB binding site in its promoter region, NF-κB might be one of the crucial factors in regulation of NGAL expression by TLR activation.

In different inflammatory conditions in a large number of organ systems, including the central nervous system, respiratory, gastrointestinal, hepatic and renal system, it was found that NGAL expression was significantly increased due to activation of TLRs by specific PAMPs or DAMPs. It was confirmed that activation of TLR2, TLR3 and TLR4 has a crucial role in induction of NGAL expression in inflammatory diseases.

Important functions of NGAL include the contribution to an anti-inflammatory response during bacterial infections by binding to siderophores, thus preventing bacterial growth and dissemination through the body. Furthermore, apoptosis seems to be regulated partly by NGAL, whereas NGAL inhibits necrosis. Stimulation of apoptosis and inhibition of necrosis also contributes to an anti-inflammatory state.

In conclusion, this review describes that activation of TLRs induces expression of NGAL during inflammatory conditions. NF-κB activation is the crucial factor in TLR-mediated NGAL expression.

Contents

Introduction

Lipocalins are a large and expanding family of proteins. Members of this family have great structural and functional diversity. It has been shown that lipocalins are involved in the regulation of innate immune responses (Flower, 1996; Flo et al., 2004). One member of the lipocalin family of particular interest is neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2 (LCN-2) in human or 24p3 protein in mouse (Cowland et al., 1997; Mårtensson et al., 2014), since its expression is markedly increased during inflammatory conditions. (Nasioudis et al., 2015).

NGAL is known for its classical function to limit bacterial growth during infection (Flo et al., 2004). For growth, most bacteria require an iron concentration that is higher than the concentration of freely available iron in the host. To solve this deficit, bacteria secrete siderophores, which are high-affinity iron-binding proteins. Due to this high affinity, siderophores can steal iron from host iron-binding proteins such as lactoferrin or transferrin. By binding to siderophores, NGAL prevents the bacterial iron acquisition, and thus the bacterial growth (Nasioudis et al., 2015).

This classical function already implies an important role for NGAL during infectious inflammation. NGAL is also known as an acute-phase protein (APP), since its plasma levels are rapidly elevated during inflammatory stimulation. Furthermore, NGAL has an anti-inflammatory function and prevents ongoing tissue damage (Flower, 1996). NGAL can be found during both infectious and non-infectious inflammatory conditions in a large number of organs and tissues, including the central nervous system, lungs, stomach, small intestine and colon, liver and kidneys (Jha et al., 2015; Nasioudis et al., 2015).

Regulatory mechanisms for NGAL expression during inflammation and tissue damage are poorly understood (Paragas et al., 2011). One potential regulatory mechanism might be mediated by Toll-like receptors (TLRs). TLRs are a family of pattern recognition receptors (PRRs) that play a crucial role in detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). When these components are recognised by TLRs, several signal transduction pathways are initiated. These signal transduction pathways trigger the expression of genes that control innate immune responses (Takeda et al., 2005). Since it is known that TLRs mediate innate immune responses and that NGAL is involved in inflammation and immune responses as well, TLRs might play a role in the regulation of NGAL expression.

In this review, I will investigate the relationship between TLR activation and induction of NGAL expression. The potential molecular signalling pathway of TLR activation leading to NGAL expression will be highlighted as well.

Toll-like receptors in innate immunity: structure, localisation and ligands

The innate immune system provides the first line of protection against pathogens or tissue damage during inflammation. Activation of TLRs on immune cells and other tissue specific cells, such as hepatocytes in the liver or tubular epithelial cells in the kidney (Anders et al., 2004), mediate this protection by initiating innate immune reactions (Abbas et al., 2012).

TLR4 was the first mammalian TLR to be discovered. Subsequently, several other proteins that were related to TLR4 were identified (Takeda et al., 2005). Currently, the mammalian TLR family consists of at least 13 members (Kawai et al., 2009), of which TLR1 to TLR9 are functional in both human and murine species (Takeda et al., 2005).

The TLRs belong to the group of type I integral membrane glycoproteins. TLRs are characterised by leucine-rich repeat motifs and cystine-rich motifs, which are involved in ligand binding. The Toll/IL-1 receptor (TIR) homology domain of a TLR is located on its cytosolic tail and is essential for intracellular signal transduction (Abbas et al., 2012).

The cellular localisation of a TLR determines the subfamily to which it belongs. The

first subfamily consists of TLR1, TLR2, TLR4, TLR5 and TLR6, which are exclusively expressed on the surface of host cells. The second subfamily TLRs are localised in intracellular vesicles, for instance the endosomes, lysosomes or on the endoplasmatic reticulum (ER) and consists of TLR3, TLR7, TLR8 and TLR9 (Kawai et al., 2009; Abbas et al., 2012).

Each member of the TLR family binds to specific PAMPs or DAMPs. Since PAMPs are only expressed in pathogens, but not in host cells, TLRs can discriminate between self and non-self (Kawai et al., 2009; Takeda et al., 2005). Bacterial lipopeptides are recognised by TLR1, TLR2 and TLR6. Bacterial peptidoglycan, the bacterial cell wall forming component, is another ligand for TLR2. The exogenous PAMP lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is recognised by TLR4. Bacterial flagellin is the microbial ligand for TLR5. Viral double stranded RNA (dsRNA) is the ligand for TLR3, single stranded RNA (ssRNA) of viruses is the specific structure recognised by both TLR7 and TLR8. At last, unmethylated CpG DNA is recognised by TLR9 (Takeda et al., 2005; Abbas et al., 2012). The structure and localisation of TLR family members and their specific PAMP ligands are shown in figure 1.

As mentioned earlier, TLRs are also involved in recognition of DAMPs. DAMPs are endogenous molecules which are located in the cell, but become extracellular when they are released from damaged or necrotic cells. Thus, DAMPs signal cell damage when they are sensed by TLRs. Important DAMPS in initiation of an innate immune reaction are heat shock proteins (HSPs) and high-mobility group box 1 (HMGB1), which is a DNA-binding protein with functional roles in transcription and DNA repair. When the localisation of HSPs and HMGB1 becomes extracellular due to tissue damage, they can activate TLR signalling in dendritic cells and macrophages. HSPs and HMGB1 are ligands for both TLR2 and TLR4. The roles of other TLRs in recognition of DAMPs remains unclear (Vabulas et al., 2001; Asea et al., 2002; Takeda et al., 2005; Abbas et al., 2012).

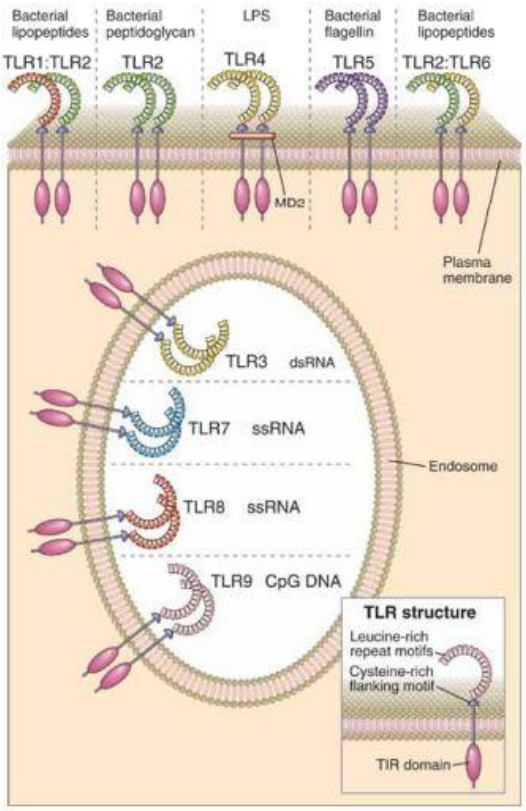

FIGURE 1: The structure and cellular localisation of TLR family members and their specific PAMP ligands (Abbas et al., 2012).

Signalling pathways of Toll-like receptors

When TLRs are activated by their ligands, expression of several genes involved in immune responses is triggered. Recognition of the PAMP or DAMP ligands by a TLR first results in either homodimerisation or heterodimerisation. All TLRs form homodimers, except for TLR1 and TLR6, which form heterodimers with TLR2. This dimerisation triggers the activation of the TLR signalling pathways, that originate at the TIR domain (Takeda et al., 2005). Subsequently, TIR domain-containing adaptor proteins, such as MyD88 and TRIF, are recruited. These adaptor proteins recruit and activate various protein kinases, which leads to activation of different transcription factors (Abbas et al., 2012; Takeda et al., 2004).

Two important transcription factors that are involved in regulation of gene expression are nuclear factor κB (NF-κB) and interferon re-

sponse factors (IRFs). NF-κB promotes gene expression of molecules required for inflammatory responses, such as pro-inflammatory cytokines, chemokines and endothelial adhesion molecules. IRFs stimulate gene expression of type I interferons (IFNs), which are essential for anti-viral immune responses (Abbas et al., 2012; Takeda et al., 2004). This TLR signalling pathway is illustrated in figure 2.

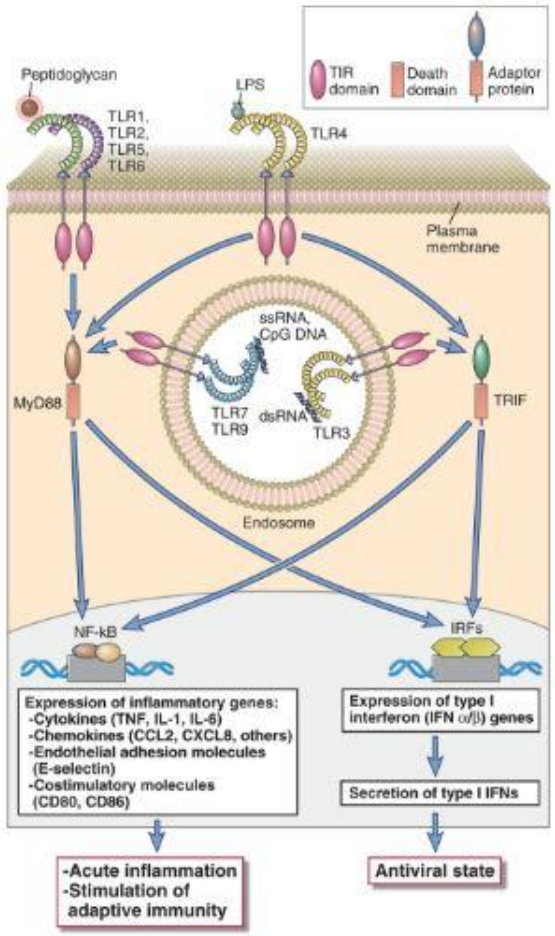

FIGURE 2: Overview of the molecular TLR signalling pathway towards gene expression of molecules involved in an innate immune response (Abbas et al, 2012). TLRs activate adaptor molecules MyD88 and TRIF, which stimulate transcription factors NF-κB and IRFs, leading to expression of inflammatory molecules and an antiviral state.

LCN2 gene transcription due to Toll-like receptor activation

It has been observed that transcription of the *LCN2* gene, coding for NGAL, was markedly increased in immune cells that were activated by LPS (Flo et al., 2004). This enhanced gene expression might be due to activation of TLRs, since several studies state that NGAL expression is partly regulated by NF-κB (Iannetti et al., 2008; Zhao et al., 2013; Nasioudis et al., 2015; Jha et al., 2015). The *LCN2* gene has a 3696 base pair coding region, including an NF-κB binding site in its promoter region (Cowland et al., 1997; Nasioudis et al., 2015). As described, NF-κB is one of the various transcription factors that are activated in TLR stimulation (Abbas et al., 2012). Moreover, pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-17 and tumor necrosis factor (TNF)-α might be involved in stimulation of NGAL expression by induction of NF-κB as well (Cowland et al., 2003; Karlsen et al., 2010; Nasioudis et al., 2015).

Other intracellular signalling molecules that might play a role in the regulation of *LCN2* gene expression are CCAAT/enhancer binding protein (C/EBP), signal transducer and activator of transcription (STAT) proteins and extracellular signal-regulated kinases (ERKs). However, C/EBPζ, a member of the C/EBP family, was identified as an inhibitor of the *LCN2* gene transcription (Iannetti et al., 2008; Jha et al, 2015).

It is found that TLRs stimulate *LCN2* gene transcription mainly via C/EBPs and NF-κB activation. On the other hand, pro-inflammatory cytokines regulate *LCN2* gene transcription by either C/EBPs, NF-κB, ERKs or STATs. An overview of those intracellular, molecular signalling pathways is shown in figure 3.

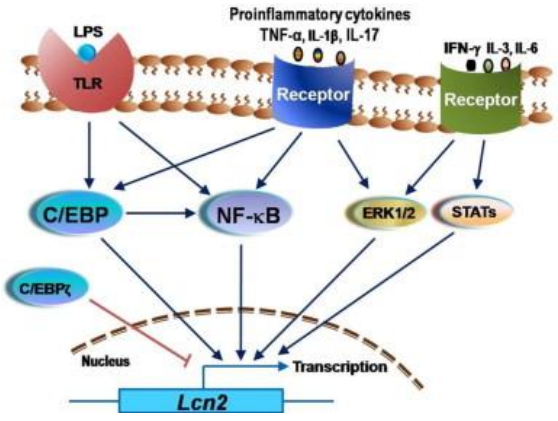

FIGURE 3: Molecular pathway towards NGAL expression by induction of the LCN2 gene (Adapted from Jha et al., 2015). C/EBP, NF-κB, ERK1/2 and STATs are believed to stimulate expression of NGAL, in contrast to C/EBPζ that inhibits transcription of the LCN2 gene.

Since TLRs are activated in inflammatory diseases and in tissue injury or damage (Abbas et al., 2012) and *LCN2* gene expression is found to be positively regulated by TLR activation (Flo et al., 2004; Nasioudis et al., 2015), the specific roles of NGAL in inflammatory diseases or tissue injury can be further investigated.

Regulation of NGAL expression by Toll-like receptors in various inflammatory diseases

Now a potential molecular signalling pathway of TLR activation towards NGAL expression is described, this relationship will be further explored. Several studies found an increased expression of NGAL in different pathological, inflammatory conditions in various organ systems, which might be a consequence of TLR activation. The findings of these studies will be discussed in this section.

Central nervous system

Next to production and secretion of NGAL by immune cells as a component of the innate immune system, it was suggested that NGAL is produced by the choroid plexus in the central nervous system (CNS). The major roles for NGAL in the CNS include prevention of infection and mediation of neuroinflammation and related diseases (Jha et al., 2015).

NGAL was identified as an inducer of chemokine expression in microglia, astrocytes, neurons and endothelial cells in the CNS (Lee et al., 2011). These chemokines regulate migration and activation of astrocytes and microglia in response to LPS exposure. Impaired migration of those cell types was observed in *LCN2* knock-out mice (Jha et al., 2015). Since microglia have a protective role against injury or pathogens in the CNS (Filiano et al., 2015), this result supports the defensive function of NGAL in the CNS.

However, this protective role of NGAL in neuroinflammation and injury in the CNS can be questioned, because another study reported that NGAL has detrimental effects in these conditions (Rathore et al., 2011). NGAL is increased in neuroinflammation and injury and contributes to loss of neurons and astrocytes. This suggests that NGAL contributes to neurological disorders such as neurodegeneration, instead of protection against those conditions (Jha et al., 2015).

NGAL levels in the cerebrospinal fluid (CSF), produced in the choroid plexus, are significantly higher during inflammatory conditions (Jha et al., 2015), such as acute meningitis (Nasioudis et al., 2015). TLRs may contribute to these elevated NGAL levels, since the PAMPs, for instance bacterial or viral components in acute meningitis, are recognised by the TLRs. As described, this activation induces expression and secretion of NGAL. These findings suggest that NGAL is a strong potential candidate for use as biomarker in screening and diagnosis of bacterial infection, since the CSF is a sterile compartment under healthy conditions (Jha et al., 2015; Nasioudis et al., 2015).

Respiratory system

TLRs contribute to the development of airway inflammation. Activation of TLRs mediates T-lymphocyte differentiation, cytokine production and activation of eosinophils (Iwamura et al., 2008). NGAL expression may be stimulated as well due to TLR activation in the respiratory system.

NGAL is constitutively present within tracheal goblet cells and type II pneumocytes in healthy lung tissue (Nasioudis et al., 2015). Those cell types express certain TLRs as well (Thorley et al., 2007). In a mouse model of bacterial pneumonia initiated by *Escherichia coli* (*E. coli*), expression of NGAL was significantly increased in those cell types. Tracheal goblet cells and type II pneumocytes, together with migratory neutrophils, contribute to elevated levels of NGAL at the site of infection. In *LCN2* knock-out mice, higher bacterial load of *E. coli* was observed. This suggests a crucial role of NGAL in the clearance of the bacteria and prevention of its dissemination, by binding to bacterial siderophores (Wu et al., 2010; Nasioudis et al., 2015).

However, another study states that NGAL deactivates macrophages and worsens the outcome of *Streptococcus pneumoniae* (*S. pneumoniae*) derived pneumonia (Warszawska et al., 2013). Since the bacterial clearance was also impaired in this study, the difference in findings could be explained by the bacterial class involved in pneumonia. It is possible that *S. pneumoniae* studied by Warszawska et al. developed an alternative mode of iron acquisition that does not rely on siderophores, in contrast to *E. coli* studied by Wu et al. (Nasioudis et al., 2015). Therefore, bacterial growth was not limited in the study of Warszawska et al. by NGAL.

In another murine model, acute endotoxemia was induced using LPS, the PAMP ligand for TLR4 (Sunil et al., 2007; Abbas et al., 2012). This acute endotoxemia was associated with up-regulation of NGAL expression in the lungs. In a mouse model with a non-functional, mutated TLR4, only low levels of NGAL could be observed (Sunil et al., 2007). This suggests that TLR4 plays a major role in LPS-induced NGAL expression. The low levels of NGAL could be due to activation of signalling pathways by LPS that are not TLR4-dependent (Wong et al., 2000). Sunil et al. speculate that NGAL is up-regulated in this model to contribute to tissue remodelling and repair and to inhibit bacterial growth (Sunil et al., 2007).

In an experimental mouse model of pulmonary tuberculosis infection, NGAL levels were increased as well (Nasioudis et al., 2015). *Mycobacterium tuberculosis* (*M. tuberculosis*) is recognised mainly by TLR2, TLR4 and TLR9 (Bafica et al., 2005; Kleinnijenhuis et al., 2011). NGAL was secreted into the alveolar space by macrophages, but also by epithelial cells. In an *LCN2* knock-out mouse model, increased growth of *M. tuberculosis* was observed in alveolar epithelial cells (Saiga et al., 2008). Another study showed limited growth of *M. tuberculosis* within macrophages in vitro, when NGAL was administered exogenously (Johnson et al., 2010).

The described studies suggest that the major roles of NGAL during inflammatory conditions in the respiratory system include its classical function to limit bacterial growth and its ability to contribute to tissue remodelling and repair.

Gastrointestinal system

An inflammatory condition in the digestive system with high prevalence worldwide is gastritis mediated by *Helicobacter pylori* (*H. pylori*) (Suerbaum et al., 2002). Microbial components of *H. pylori* are recognised by TLR2 and to a minor extent by TLR4 as well (Rad et al., 2009). In the immune response against *H. pylori*, inflammatory mediators are released by epithelial and infiltrating inflammatory cells into the gastric mucosa. These mediators include NF-κB and IL-1β (Alpízar-Alpízar et al., 2009), earlier shown to be involved in the induction of NGAL expression (Iannetti et al., 2008). Alpízar-Alpízar studied NGAL expression during human *H. pylori* induced gastritis and found significantly increased NGAL levels in the gastric mucosa, which supports the antimicrobial function of NGAL in gastrointestinal inflammatory diseases (Alpízar-Alpízar et al., 2009).

Gastritis initiated by *H. pylori* seems to be a risk factor for gastric cancer (Amieva et al., 2016). Therefore, another point of interest comprises increased NGAL levels observed in gastro-intestinal cancers. Although mechanisms explaining the role of NGAL in carcinogenesis remain unknown, it is thought that NGAL might stimulate growth of carcinoma cells by providing the ability to acquire iron. To confirm this speculation, further research on the expression of NGAL in gastric cancer in connection to progression of the disease is required (Alpízar-Alpízar et al., 2009).

Inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD), are also characterised by an overexpression of the *LCN2* gene (Bolignano et al., 2010; Østvik et al., 2013). Østvik et al. found significantly increased NGAL expression in epithelial cells and infiltrating neutrophils in active UC and CD colonic biopsies compared with inactive UC and CD colonic biopsies. The

study indicates that TLR3, which is constitutively expressed on colonic epithelium, has a central role in the NGAL response in IBDs. Expression of TLR3 itself is also enhanced in active UC and CD biopsies (Østvik et al., 2013). This increased TLR3 expression may contribute even more to the over-expression of the *LCN2* gene. As mentioned before, the ligand for TLR3 is viral dsRNA (Abbas et al., 2012). Østvik et al. state that TLR3 senses endogenous mRNA from damaged tissue in the inflammatory intestinal tract as well, since the pathogenesis of IBDs is often mediated by genetics and the innate immune system itself.

Hepatic system

Another organ of interest is the liver, a significant source of NGAL production under certain inflammatory conditions (Nasioudis et al., 2015). Hepatocytes express TLRs and release APPs, including NGAL, in response to infection or tissue injury (Tilg et at., 1997; Broering et al., 2011). Therefore, NGAL production in hepatocytes is possibly mediated by activation of TLRs.

Rapid and sustained NGAL production by injured hepatocytes can be observed in experimental liver injury. Exposure to either carbon tetrachloride (CCl_4) or LPS induces this described liver injury (Borkham-Kamphorst et al., 2013). CCl_4 induces acute liver injury and hepatotoxicity by its reactive metabolites (Boll et al., 2001). Endogenous DAMPs following this liver injury may be recognised by TLRs. Furthermore, LPS is sensed by TLR4 in this liver injury model (Abbas et al., 2012). In an *LCN2* knock-out mouse model of acute liver injury, increased liver damage was observed (Borkham-Kamphorst et al., 2013). NGAL expression was induced by IL-1β and NF-κB activation in both acute and chronic experimental liver injury (Borkham-Kamphorst et al., 2011). In chronic liver injury *LCN2* knock-out models, the amount of fibrosis is slightly increased compared to non-*LCN2* knock-out condition (Borkham-Kamphorst et al., 2013). Borkham-Kamphorst et al. state that increased expression of NGAL is a reliable marker of liver damage and that NGAL has significant hepatoprotective effects in acute liver injury. No significant correlation between NGAL levels and the degree of liver fibrosis was found. However, a positive correlation between NGAL levels and degree of inflammation was confirmed (Borkham-Kamphorst et al., 2013).

Acute endotoxemia in mice, induced by administration of LPS, resulted in significantly increased expression of NGAL. This increase was evident within 4 hours after onset of acute endotoxemia and persisted for 24 to 48 hours. NGAL expression was increased in isolated Kupffer cells, which are residential macrophages in the liver, as well. The increased NGAL expression was to a great extent mediated by TLR4, sensing LPS. In this condition, NGAL contributes to the resolution of the inflammatory process and to the reestablishment of liver homeostasis (Sunil et al., 2007).

Renal system

NGAL might be a new marker for both acute kidney injury (AKI) and chronic kidney disease (CKD), because its expression is heavily increased in the kidney during these conditions (Bolignano et al., 2009; Haase et al., 2009). The expression of NGAL rises a thousand-fold in response to tubular injury. Thereby, NGAL appears rapidly in both urine and serum, which contributes to the fact that NGAL is useful as biomarker for renal failure, such as AKI or CKD (Schmidt-Ott et al., 2007).

NGAL is expressed in the kidney by tubular epithelial cells and in cells of the innate immune system, such as neutrophils (Eller et al., 2013). Neutrophils provide organs with a mobile source of NGAL during inflammation, while the production of NGAL by tubular epithelial cells might be important for local protection against infections (Mårtensson et al., 2014). It was found that tubular epithelial cells express TLR1, TLR2, TLR3, TLR4 and TLR6, which leads to contribution to the activation of immune responses in tubular injury (Anders et al., 2004). This finding contributes to the understanding of NGAL expression by tubular epithelial cells in renal inflammation or

injury, since TLRs recognise their ligands. Next to its antimicrobial effect, it seems that NGAL is an important growth factor that modulates a variety of cellular responses, including proliferation, apoptosis and differentiation in renal tubular cells (Schmidt-Ott et al., 2007).

Eller et al. investigated the functional roles of NGAL in nephrotoxic serum nephritis (NTS). NTS was induced by glomerular basement membrane antibodies in both wildtype and *LCN2* knock-out mice. It was observed that glomerular damage and interstitial leukocyte accumulation were increased in *LCN2* knock-out mice. When NGAL could not be expressed by innate immune cells, decreased apoptosis, but increased necrosis and formation of HMBG-1 and other DAMPs was seen. HMBG-1 is a TLR2 agonist and induces inflammation (Eller et al., 2013). As described, TLR2 activation induces NGAL expression as well. Eller et al. state that NGAL, expressed by innate immune cells, has a protective role in NTS by inducing apoptosis. An important aspect of NGAL-regulated apoptosis is its ability to inhibit inflammation in NTS, by limiting necrosis and thereby formation of HMGB-1 and pro-inflammatory cytokine production via TLR2 signalling.

Another murine model showed that α-intercalated cells, located in the renal collecting ducts and modulating acid-base balance, are capable to detect uropathogenic *E. coli* via TLR4 signalling and actively secrete NGAL in response. This NGAL secretion contributes to bacterial clearance of the urinary tract and mediates inflammatory responses (Paragas et al., 2014; Nasioudis et al., 2015).

Furthermore, Lee et al. indicated that glomerular podocytes can produce NGAL as well. This was observed after signalling LPS and DAMPs in a model of acute glomerular injury (Lee et al., 2012).

Taken together, the described studies show that NGAL is expressed by various cell types in the kidney via TLR signalling in response to exogenous PAMPs or endogenous DAMPs. Major functions of NGAL in the kidney include protection against pathogens and tissue injury, regulation of apoptosis and differentiation of renal tubular cells.

Sepsis

In contrast to the many local inflammatory diseases described in the previous sections, sepsis is a life-threatening condition of systemic inflammation. Usually, a local infection spreads throughout the whole body, resulting in sepsis (Lever et al., 2007). Dysfunction of one or multiple organ system(s) is a common complication in sepsis (Abraham et al., 2007, Zarjou et al., 2011).

It has been shown that NGAL expression is elevated in both serum and specific organs during sepsis. When LPS, the ligand for TLR4, was administered systemically to mice, a significant increase in serum NGAL levels was observed. NGAL expression was markedly increased as well in different organs, such as the liver, lungs and kidneys, in response to LPS (Sunil et al., 2007; Mårtensson et al., 2014; Nasioudis et al., 2015). These elevated NGAL levels might be a compensatory mechanism for the increased amounts of bacteria during sepsis (Martensson et al., 2014).

Sepsis is associated with hypoferremia, also described as an iron deficiency. This condition limits availability of iron to pathogens. Furthermore, iron-mediated oxidative stress is reduced in hypoferremia. These processes are both mediated by NGAL, playing an essential role in iron transport (Srinivasan et al., 2012). Increased LPS-related toxicity, pro-inflammatory gene expression and oxidative stress could be observed in *LCN2* knock-out mice (Nasioudis et al., 2015). *LCN2* knock-out mice were more sensitive to endotoxemia and more organ damage could be observed. In addition, *LCN2* knock-out mice showed delayed LPS-induced hypoferremia, indicating the regulative role of NGAL once more. In conclusion, NGAL protects the host against sepsis by regulating iron availability and transport (Srinivasan et al., 2012).

The kidney is rapidly affected during sepsis, often resulting in AKI. Pathophysiology of

septic AKI is complex and has not been fully elucidated yet (Nasioudis et al., 2015). Serum and urine levels of NGAL rise quickly in septic AKI, as well as local NGAL expression in the kidney (Nasioudis et al., 2015). TLR4 expression in renal tubular epithelium also increases markedly during LPS-induced sepsis in a murine model (El-Achkar et al., 2006). This suggests that locally expressed TLRs and induced NGAL expression could potentially protect the kidney to sepsis.

Discussion and conclusion

This review focused on the relationship between TLR activation and NGAL expression in different organ systems during inflammatory conditions. Many studies indicated an important role for TLR activation, either by exogenous PAMPs or endogenous DAMPs, in the up-regulation and expression of NGAL. A potential signalling pathway of TLR activation towards NGAL expression was highlighted as well. TLR-induced *LCN2* gene expression is mainly mediated by NF-κB activation.

NGAL contributes to an anti-inflammatory response by binding to bacterial siderophores and thus preventing bacterial growth and dissemination through the body. Apoptosis seems to be regulated partly by NGAL, whereas it inhibits necrosis. Stimulation of apoptosis and inhibition of necrosis also contributes to an anti-inflammatory state: DAMPs are not released in apoptosis - in contrast to necrosis - and thus inflammation does not proceed.

It is speculated that NGAL has functions in tissue remodelling and repair, in particular in the respiratory system. NGAL seems to be an important growth factor, modulating cellular responses, in renal tubuli.

Negative effects of NGAL are also reported. For instance, its probable detrimental effects in neuroinflammation, contributing to loss of astrocytes and microglia. On the other hand, this could be described as anti-inflammatory effect as well, causing apoptosis instead of necrosis. The role of NGAL in growth of carcinoma cells has to be further investigated. Whether this NGAL expression relies on activation of TLRs is also questionable.

The described studies implicate a crucial role for activation of TLR2, TLR3 and TLR4 that induce NGAL expression in inflammatory diseases. The functional role of other TLR subtypes in NGAL up-regulation during injury or inflammatory diseases should be further investigated. Further research on recognition of DAMPs leading to NGAL expression in non-infectious inflammatory diseases should be conducted as well.

Next to TLRs, other PRRs might be involved in NGAL expression. No literature on activation of cytosolic PRRs, such as RIG-I-like receptors (RLRs) or Nod-like receptors (NLRs), leading to NGAL expression could be found. However, signalling pathways of activated RLRs or NLRs ultimately lead to NF-κB activation (Martinon et al., 2005; Loo et al., 2011). Therefore, NGAL expression might be induced as well by activation of those other PRRs.

In conclusion, this review confirms that activation of TLRs induces expression of NGAL during inflammatory conditions. NF-κB activation is a crucial factor in TLR-mediated NGAL expression.

Acknowledgements

I would like to thank J. Moser and E.R. Popa for their guidance during this writing process.

References

Abbas, A.K., Lichtman, A.H., Pillai, S. (2012). *Cellular and Molecular Immunology* (7th Edition). Philadelphia: Elsevier Saunders.

Abraham, E., Singer, M. (2007). Mechanisms of Sepsis-Induced Organ Dysfunction. *Critical Care Medicine*, 35(10): 2408-2416.

Alpízar-Alpízar, W., Laerum, O.D., Illemann, M., Ramírez, J.A., Arias, A., Malespín-Bendaña, W., Ramírez, V., Lund, L.R., Borregaard, N., Nielsen, B.S. (2009). Neutrophil Gelatinase-Associated Lipocalin (NGAL/Lcn2) is Upregulated in Gastric Mucosa Infected with *Helicobacter pylori*. *Virchows Archiv*, 455(3): 225-233.

Amieva, M., Peek Jr., R.M. (2016). Pathobiology of *Helicobacter pylori*-Induced Gastric Cancer. *Gastroenterology*, 150: 64-78.

Anders, H.J., Banas, B., Schlöndorff, D. (2004). Signalling Danger: Toll-Like Receptors and their Potential Roles in Kidney Disease. *Journal of the American Society of Nephrology*, 15(4): 854-867.

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Baré, O., Auron, P. E., Calderwood, S. K. (2002). Novel signal transduction pathway utilized by extracellular HSP70 role of Toll-like receptor (TLR) 2 and TLR4. *Journal of Biological Chemistry*, 277(17), 15028-15034.

Bafica, A., Scanga, C.A., Feng, C.G., Leifer, C., Cheever, A., Sher, A. (2005). TLR9 Regulates Th1 Responses and Cooperates with RL2 in Mediating Optimal Resistance to *Mycobacterium tuberculosis*. *Journal of Experimental Medicine*, 280(12): 20961-20967.

Bolignano, D., Lacquaniti, A., Coppolino, G., Donato, V., Campo, S., Fazio, M.R., Nicocia, G., Buemi, M. (2009). Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Progression of Chronic Kidney Disease. *Clinical Journal of the American Society of Nephrology*, 4: 337-344.

Bolignano, D., Torre, A.D., Lacquaniti, A., Costantino, G., Fries, W., Buemi, M. (2010). Neutrophil Gelatinase-Associated Lipocalin Levels in Patients with Crohn Disease Undergoing Treatment with Infliximab. *Journal of Investigative Medicine*, 58(3): 569-571.

Boll, M., Weber, L.W., Becker, E., Stampfl, A. (2001). Mechanism of Carbon Tetrachloride-Induced Hepatotoxicity. Hepatocellular Damage by Reactive Carbon Tetrachloride Metabolites. *Zeitschrift für Naturforschung C*, 56(7-8): 649-659.

Borkham-Kamphorst, E., Drews, F., Weiskirchen, R. (2011). Induction of Lipocalin-2 Expression in Acute and Chronic Experimental Liver Injury Moderated by Pro-Inflammatory Cytokines Interleukin-1β through Nuclear Factor-κB Activation. *Liver International*, 31(5): 656-665.

Borkham-Kamphorst, E., van de Leur, E., Zimmermann, H.W., Karlmark, K.R., Tihaa, L., Haas, U., Tacke, F., Berger, T., Mak, T.W., Weiskirchen, R. (2013). Protective Effects of Lipocalin-2 (LCN2) in Acute Liver Injury Suggest a Novel Function in Liver Homeostasis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1832(5): 660-673.

Broering, R., Lu, M., Schlaak, J.F. (2011). Role of Toll-Like Receptors in Liver Health and Disease. *Clinical Science*, 121(10): 415-426.

Cowland, J.B., Borregaard, N. (1997). Molecular Characterization and Pattern of Tissue Expression of the Gene for Neutrophil Gelatinase-Associated Lipocalin from Humans. *Genomics*, 45: 17-23.

Cowland, J.B., Sørensen, O.E., Sehested, M., Borregaard, N. (2003) Neutrophil Gelatinase-Associated Lipocalin is Up-regulated in Human Epithelial Cells by IL-1 Beta, but not by TNF-alpha. *Journal of Immunology*, 171(12): 6630-6639.

El-Achkar, T.M., Huang, X., Plotkin, Z., Sandoval, R.M., Rhodes, G.J., Dagher, P.C. (2006). Sepsis Induces Changes in the Expression and Distribution of Toll-Like Receptor 4 in the Rat Kidney. *American Journal of Physiology*, 290(5): 1034-1043.

Eller, K., Schroll, A., Banas, M., Kirsh, A.H., Huber, J.M., Nairz, M., Skvortsov, S., Weiss, G., Rosenkranz, A.R., Theurl, I. (2013). Lipocalin-2 Expressed in Innate Immune Cells is an Endogenous Inhibitor of Inflammation in Murine Nephrotoxic Serum Nephritis. *PLOS ONE*, 8(7): 1-15.

Filiano, A.J., Gadani, S.P., Kipnis, J. (2015). Interactions of Innate and Adaptive Immunity in Brain Development and Function. *Brain Research*, 1617: 18-27.

Flo, T.H., Smith, K.D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S., Aderem, A. (2004). Lipocalin 2 Mediates an Innate Immune Response to Bacterial Infection by Sequestrating Iron. *Nature*,432: 917-922.

Flower, D.R. (1996). The Lipocalin Protein Family: Srucure and Function. *Biochemical Journal*, 318: 1-14.

Haase, M., Bellomo, R., Devarajan, P., Schlattmann, P., Haase-Fielitz, A. (2009). Accuracy of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diagnosis and Prognosis in Acute Kidney Injury: A Systematic Review and Meta-Analysis. *American Journal of Kidney Diseases*, 54(6): 1012-1024.

Iannetti, A., Pacifico, F., Acquaviva, R., Lavorgna, A., Crescenzi, E., Vascotto, C., Tell, G., Salzano, A.M., Scaloni, A., Vuttariello, E., Chiappetta, G., Formisano, S., Leonardi, A. (2008). The Neutrophil Gelatinase-Associated Lipocalin (NGAL), a NF-κB-Regulated Gene, Is a Survival Factor for Thyroid Neoplastic Cells. *Proceedings of the National Academy of Sciences of the United States of America*, 105(37), 14058-14063.

Iwamura, C., Nakayama, T. (2008). Toll-Like Receptors in the Respiratory Sytem: Their Roles in Inflammation. *Current Allergy and Asthma Reports*, 8(1): 7-13.

Johnson, E.E., Srikanth, C.V., Sandgren, A., Harrington, L., Trebicka, E., Wang, L., Borregaard, N., Murray, M., Cherayil, B.J. (2010). Siderocalin Inhibits the Intracellular Replication of *Mycobacterium tuberculosis* in Macrophages. *FEMS Immunology and Medical Microbiology*, 58(1): 138-145.

Karlsen, J.R., Borregaard, N., Cowland, J.B. (2010). Induction of Neutrophil Gelatinase-Associated Lipocalin Expression by Co-stimulation with Interleukin-17 and Tumor Necrosis Factor-alpha is Controlled by IkappaB-zeta but Neither by C-EBP-beta Nor C-EBP-delta. *Journal of Biological Chemistry*, 285(19): 14088-14100.

Kawai, T., Akira, S. (2009). The Roles of TLRs, RLRs, NLRs in Pathogen Recognition. *International Immunology*, 21(4): 317-337.

Kleinnijenhuis, J., Oosting, M., Joosten, L.A.B., Netea, M.G., van Crevel, R. (2011). Innate Immune Recog-

nition of *Mycobacterium tuberculosis*. *Clinical and Developmental Immunology*, 2011: 1-12.

Lee, S., Kim, J.H., Seo, J.W., Han, H.S., Lee, W.H., Mori, K., Nakao, K., Barasch, J., Suk, K. (2011). Lipocalin-2 is a Chemokine Inducer in the Central Nervous System: Role of Chemokine Ligands 10 (CXCL10) in Lipocalin-2-Induced Cell Migration. *Journal of Biological Chemistry*, 286: 43855-43870.

Lee, S.J., Borsting, E., Declèves, A.E., Singh, P., Cunard, R. (2012). Podocytes Express IL-6 and Lipocalin 2/Neutrophil Gelatinase-Associated Lipocalin in Lipopolysaccharide-Induced Acute Glomerular Injury. *Nephron Experimental Nephrology*, 121(3-4): 86-96.

Lever, A., Mackenzie, I. (2007). Sepsis: Definition, Epidemiology and Diagnosis. *British Medical Journal*, 335: 879-883.

Loo, Y.M., Gale, M. (2011). Immune Signaling by RIG-I-like Receptors. *Immunity*, 34(5): 680-692.

Mårtensson, J., Bellomo, R. (2014). The Rise and Fall of NGAL in Acute Kidney Injury. *Blood Purification*, 37: 304-310.

Martinon, F., Tschopp, J. (2005). NLRs Join TLRs as Innate Sensors of Pathogens. *TRENDS in Immunology*, 26(8): 447-454.

Nasiousis, D., Witkin, S.S. (2015). Neutrophil Gelatinase-Associated Lipocalin and Innate Immune Responses to Bacterial Infections. *Medical Microbiology and Immunology*, 204: 471-479.

Østvik, A.E., Granlund, A.V.B., Torp, S.H., Flatberg, A., Beisvåg, V., Waldum, H.L., Flo, T.H., Espevik, T., Damås, J.K., Sandvik, A.K. (2013). Expression of Toll-Like Receptor 3 is Enhanced in Active Inflammatory Bowel Disease and Mediates the Excessive Release of Lipocalin 2. *Clinical and Experimental Immunology*, 173: 502-511.

Paragas, N., Qiu, A., Zhang, Q., Samstein, B., Deng, S.X., Schmidt-Ott, K.M., Viltard, M., Yu, W., Forster, C.S., Gong, G., Liu, Y., Kulkarni, R., Mori, K., Kalandadze, A., Ratner, A.J., Devarajan, P., Landry, D.W., d'Agati, V., Lin, C.S., Barasch, J. (2011). The NGAL Reporter Mouse Detects the Response of the Kidney to Injury in Real Time. *Nature Medicine*, 17(2): 216-222.

Paragas. N., Kulkarni, R., Werth, M.,Schmidt-Ott, K.M., Forster, C., Deng, R., Zhang, Q., Singer, E., Klose, A.D., Shen, T.H., Francis, K.P., Ray, S., Vijayakumar, S., Seward, S., Bovino, M.E., Xu. K., Takabe, Y., Amaral, F.E., Mohan, S., Wax, R., Corbin, K., Sanna-Cherchi, S., Mori, K., Johnson, L., Nickolas, T., d'Agati, V., Lin, C.S., Qiu, A., Al-Awqati, Q., Ratner, A.J., Barasch, J. (2014). α-Intercalated Cells Defend the Urinary System from Bacterial Infection. *Journal of Clinical Investigation*, 124(7): 2963-2976.

Rad, R., Ballhorn, W., Voland, P., Eisenächer, K., Mages, J., Rad, L., Ferstl, R., Lang, R., Wagner, H., Schmid, R.M., Bauer, S., Prinz, C., Kirschning, C.J., Krug, A. (2009). Extracellular and Intracellular Pattern Recognition Receptors Cooperate in the Recognition of *Helicobacter pylori*. *Gastroenterology*, 136(7): 2247-2257.

Rathore, K.I., Berard, J.L., Redensek, A., Chierzi, S., Lopez-Vales, R., Santos, M., Akira, S., David, S. (2011). Lipocalin 2 Plays an Immunomodulatory Role and has Detrimental Effects after Spinal Cord Injury. *Journal of Neuroscience*, 31: 13412-13419.

Saiga, H., Nishimura, J., Kuwata, H., Okuyama, M., Matsumoto, S., Sato, S., Matsumoto, M., Akira, S., Yoshikai, Y., Honda, K., Yamamoto, M., Takeda, K. (2008). Lipocalin 2-Dependent Inhibition of Mycobacterial Growth in Alveolar Epithelium. *Journal of Immunology*, 181(12): 8521-8527.

Schmidt-Ott, K.M., Mori, K., Li, J.Y., Kalandadze, A., Cohen, D.J., Devarajan, P., Barasch, J. (2007). Dual Action of Neutrophil Gelatinase-Associated Lipocalin. *Journal of the American Society of Nephrology*, 18(2): 407-413.

Srinivasan, G., Aitken, J.D., Zhang, B., Carvalho, F.A., Chassaing, B., Shashidharamurthy, R., Borregaard, N., Jones, D.P., Gewirtz, A.T., Vijay-Kumar, M. (2012). Lipocalin 2 Deficiency Dysregulates Iron Homeostasis and Exacerbates Endotoxin-Induced Sepsis. *The Journal of Immunology*, 189(4): 1911-1919.

Suerbaum, S., Michetti, P. (2002). *Helicobacter pylori* Infection. *The New England Journal of Medicine*, 347(15): 1175-1186.

Sunil, V.R., Patel, K.J., Nilsen-Hamilton, M., Heck, D.E., Laskin, J.D., Laskin, D.L. (2007). Acute Endotoxemia is Associated with Upregulation of Lipocalin 24p3/Lcn2 in Lung and Liver. *Experimental and Molecular Pathology*, 83: 177-187.

Takeda, K., Akira, S. (2004). TLR Signaling Pathways. *Seminars in immunology*, 16(1): 3-9.

Takeda, K., Akira, S. (2005). Toll-Like Receptors in Innate Immunity. *International Immunology*, 17(1): 1-14.

Thorley, A.J., Tetley, T.D. (2007). Pulmonary Epithelium, Cigarette Smoke, and Chronic Obstructive Pulmonary Disease. *International Journal of Chronic Obstructive Pulmonary Disease*, 2(4): 409-428.

Tilg, H., Dinarello, C.A., Mier, J.W. (1997). IL-6 and APPs: Anti-Inflammatory and Immunosuppressive Mediators. *Immunology Today*, 18: 428-432.

Vabulas, R.M., Ahmad-Nejad, P., da Costa, C., Miethke, T., Kirschning, C.J., Häcker, H., Wagner, H. (2001). Endocytosed HSP60s Use Toll-Like Receptor 2 (TLR2) and TLR4 to Activate the Toll/Interleukin-1 Receptor Signalling Pathway in Innate Immune Cells. Journal of Biological Chemistry, 276(33): 31332-31339.

Warszawska, J.M., Gawish, R., Sharif, O., Sigel, S., Doninger, B., Lakovits, K., Mesteri, I., Nairz, M., Boon, L., Spiel, A., Fuhrmann, V., Strobl, B., Müller, M., Schenk, P., Weiss, G., Knapp, S. (2013). Lipocalin 2 Deactivates Macrophages and Worsens Pneumococ-

cal Pneumonia Outcomes. *Journal of Clinical Investigation*, 123(8): 3363-3372.

Wong, P.M., Chugn, S.W., Sultzer, B.M. (2000). Genes, Receptors, Signals and Responses to Lipopolysaccharide Endotoxin. *Scandinavian Journal of Immunology*, 51: 123-127.

Wu, H., Santoni-Rugiu, E., Ralfiaer, E., Porse, B.T., Moser, C., Høiby, N., Borregaard, N., Cowland, J.B. (2010). Lipocalin 2 is Protective Against *E. Coli* Pneumonia. *Respiratory Research*, 15(11): 96-103.

Zarjou, A., Agarwal, A. (2011). Sepsis and Acute Kidney Injury. *Journal of the American Society of Nephrology*, 22: 999-1006.

Zhao, P., Stephens, J.M. (2013). STAT1, NF-κB and ERKs Play a Role in the Induction of Lipocalin-2 Expression in Adipocytes. *Molecular Metabolism*, 2(3): 161-170.