Toll-like receptor and associated regulators in pneumonia and sepsis

Blok, D.C.

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Chapter 9
Summary, general discussion & conclusion
Summary

Humanity struggles against micro-organism invasion on a daily basis. Survival hinges on the ability to maintain host balance in a sometimes hostile environment. When homeostasis is disrupted not only virulent pathogens challenge host survival but otherwise harmless bacteria can cause invasive infection as well. Innate immunity is part of the first line of defense when it comes to preventing infection, signaling a perceived health threat and eliminating microbes before they can cause harm. Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) are vital in those processes [1-3]. The downside of a rigorous inflammatory response elicited by host defense is detrimental collateral tissue damage and, as a consequence thereof (provided damage is extensive enough) dysfunction of affected organs. To regulate immune responses and thus limit unnecessary tissue destruction a crucial role is reserved for inhibitors of inflammation like negative regulators of TLR signaling [4]. This thesis aims to elucidate the role of such negative regulators and associated signaling molecules in pneumonia and sepsis caused by common human pathogens.

Chapter 1 comprises a general introduction and a thesis outline. The disease burden of infections, particularly pneumonia and sepsis, and the importance of advancing our understanding of pathogen evoked host responses are discussed. Innate pulmonary host defense mechanisms and the role of TLRs and TLR inhibitors therein are introduced. In addition, the differential functions of ST2 are considered. The following chapters (2-4) study the role of two specific negative regulators of TLR signaling in the aforementioned disease states. In chapter 2 the role of single immunoglobulin interleukin-1 receptor related molecule (SIGIRR) during pneumococcal pneumonia and sepsis is investigated using murine models of airway and intravenous infection with Streptococcus (S.) pneumoniae respectively. SIGIRR deficiency resulted in delayed mortality and lower bacterial burdens during S. pneumoniae pneumonia. Diminished bacterial outgrowth was accompanied by enhanced lung pathology scores in the early stages of the infection, while pulmonary cytokine and chemokine levels or neutrophil influx were not affected. Notably, SIGIRR deficient neutrophils and alveolar macrophages displayed an enhanced capacity to phagocytose S. pneumonia, which at least in part may explain the improved antibacterial defense of SIGIRR deficient mice in vivo. Chapter 3 demonstrates the diversity in negative regulator function, since ST2 deficiency in the same murine model of pneumococcal pneumonia did not result in diminished bacterial outgrowth as SIGIRR deficiency did. However, during secondary S. pneumoniae pneumonia following influenza A infection (a common clinical scenario), ST2 deficient mice demonstrated modestly higher pulmonary bacterial burdens with an unaltered local inflammatory response. During influenza A infection, prior to secondary infection with pneumococci, ST2 deficient mice cleared the virus as effective as wild-type mice. ST2 deficient mice tended to have a stronger pulmonary inflammatory response upon infection with influenza, especially 14 days after infection, as reflected
Summary & Discussion

Chapter 4 examines the role of ST2 in cytokine release induced by S. pneumoniae and Klebsiella (K.) pneumoniae in whole blood and splenocyte cultures ex vivo and, after intravenous infection, in intact mice in vivo. Unexpectedly, ST2 deficiency resulted in reduced rather than enhanced cytokine release by whole blood leukocytes and splenocytes incubated with either one of the aforementioned bacteria. ST2 did not impact on systemic cytokine release or induction of cytokines in spleens after intravenous infection with S. pneumoniae or K. pneumoniae. The results of chapters 3 and 4 taken together suggest that ST2 plays a limited role in experimental infection caused by three common human pathogens.

Chapter 5 addresses the effect of ST2 stimulation by intravenous administration of its ligand IL-33 to mice prior to infection with K. pneumoniae via the airways. IL-33 treatment strongly improved host defense in a ST2 dependent manner, as reflected by delayed mortality and reduced bacterial outgrowth in IL-33 wild-type but not ST2 deficient mice. Of note, ST2 deficient mice treated with vehicle showed similar bacterial loads and survival as wild-type mice, further indicating that ST2 has an insignificant role in host defense during bacterial infection, thereby confirming the findings in chapters 3 and 4. The beneficial effect of IL-33 was dependent on the presence of type 2 innate lymphoid cells (ILC2s), as demonstrated by a series of experiments involving IL-33 treatment of mice deficient of T and B cells (RAG2 deficient mice), mice deficient of T, B and ILCs (RAG2 plus IL-2 receptor common gamma chain deficient mice) and mice depleted of NK cells; whereas the former mouse strain could be protected by IL-33, the latter mouse strain did not respond. In addition, IL-33 treatment caused high lung levels of the ILC2 cytokines IL-5 and IL-13, and ILC2s purified from the lungs produced high levels of these type 2 cytokines in response to IL-33. The effect of IL-33 did not require IL-5 or IL-13 (shown by experiments using anti-IL-5 antibody treatment and IL-13 deficient mice) or natural killer cells (using antibody mediated depletion of this cell type). Although IL-33 administration caused enhanced influx of neutrophils into the lungs, the protective effect of IL-33 did not require the presence of neutrophils, as demonstrated in studies in which neutrophils were depleted prior to IL-33 treatment and Klebsiella infection. These results identify stimulation of ILC2s as a potential therapeutic approach in the treatment of severe bacterial infections.

Chapter 6 concentrates on the two adaptor molecules responsible for TLR signal transductions upon interaction with bacteria: myeloid differentiation primary response gene (MyD88) and TIR domain-containing adapter-inducing IFN-β (TRIF). We showed that mice deficient for either adaptor molecule have a markedly impaired host defense during K. pneumoniae induced pneumosepsis. To determine the role of MyD88 and TRIF in parenchymal and myeloid cells in this infection, we created bone marrow chimeras with MyD88 or TRIF deficiency in either (radioresistant) resident or (radiosensitive) hematopoietic cells. While MyD88 in both hematopoietic and resident cells contributed by modest elevations in lung cytokine, chemokine and myeloperoxidase levels.
to antibacterial defense and survival, TRIF deficiency in hematopoietic cells alone was enough to result in a hypersusceptible phenotype. Of interest, MyD88 in resident cells and TRIF in hematopoietic cells contributed to cell injury during late-stage infection. As such, these results suggest that mortality in MyD88 or TRIF deficient mice likely occurred as a consequence of excessive bacterial growth and nicely illustrate the double-edged sword character of innate immune activation via MyD88- and TRIF-dependent signaling.

The last two chapters concentrate on human studies. The first, described in chapter 7, focusses on inhibitory molecules of innate immunity in pulmonary tuberculosis and reports soluble (s)ST2 as a potential biomarker for tuberculosis disease activity. In this chapter expression of specific inhibitory genes was measured in peripheral blood mononuclear cells (PBMCs) and alveolar macrophages obtained from patients with active pulmonary tuberculosis in Chittagong, Bangladesh. Patients with tuberculosis had increased expression of IL-1 receptor associated kinase-M (IRAK-M), Toll interacting protein (TOLLIP) and suppressor of cytokine signaling (SOCS)-3 in PBMCs when compared with local healthy control subjects. In contrast, the expression of SIGIRR, A20 and MAP kinase phosphatase (MKP)-1 was not altered during tuberculosis. Although ST2 mRNA levels in PBMCs from tuberculosis patients were not changed, ST2 protein expression was enhanced at the surface of CD4 and CD8 positive lymphocytes. These data provide evidence that activation of innate immunity in circulating cells during tuberculosis is kept in check by negative regulators. Alveolar macrophage mRNA expression of negative TLR regulators did not differ between the infected and contralateral lung side. sST2 plasma levels were elevated in patients with tuberculosis and correlated with established tuberculosis biomarkers, most strongly with soluble interleukin-2 receptor subunit α and IL-8, suggesting that sST2 could be a useful marker for tuberculosis disease activity. To further investigate the value of sST2 as a biomarker in lung infection, sST2 plasma levels were measured in patients with community-acquired pneumonia caused by either Influenza A (H1N1), Q-fever or S. pneumoniae in chapter 8. While all patients exhibited elevated sST2 levels, patients infected with S. pneumoniae, especially those suffering from bacteremia, displayed the highest levels. Moreover, patients with the highest pneumonia severity scores presented with the highest ST2 levels. sST2 plasma concentrations correlated with the concentrations of the established biomarkers C-reactive protein, IL-6, IL-8 and IL-10. These results further indicate that sST2 could be of use as a biomarker reflective of infectious disease severity.
Discussion

Inflammation is a tool used by the immune system to combat (pathogenic) microorganisms and prevent infectious disease. Unfortunately, rigorous inflammation generates tissue damage and dysfunction. Hence, in pursuance of both adequate infection/pathogen control and limited inflammatory host damage, tight regulation of immune responses is needed. TLRs are pattern recognition receptors responsible for the recognition of pathogen- and damage-associated molecular patterns and the initiation of an immune response [1-3]. Negative regulators of TLR signaling, like SIGIRR and ST2, are able to inhibit these pathways and thus limit inflammation [4-6]. Finding the right balance between pro- and anti-inflammatory stimuli is essential to generate a sufficient response followed by a swift return to homeostasis.

The ferocity of inflammation induced by specific pathogens and the magnitude up to which inflammation is beneficial for infection control differs between microorganisms. For example, the common respiratory pathogen used in one of the murine pneumonia models in this thesis, *S. pneumoniae*, is not easily recognized by the immune system due to its thick polysaccharide capsule [7]. Enhanced inflammatory signaling might therefore improve host defense towards this relatively 'invisible' pathogen. As demonstrated in chapter 2, SIGIRR deficiency indeed led to a clear host benefit in this model, accompanied by enhanced inflammation. In contrast, increased inflammation due to SIGIRR deficiency in *Pseudomonas aeruginosa* induced pneumonia (primarily infecting immunocompromised hosts) and *Mycobacterium tuberculosis* (chronic) lung infection, was detrimental for the host [8, 9]. Dampening the enhanced inflammatory response in SIGIRR deficient mice by blocking IL-1R and IL-1β/TNF-α signaling respectively, significantly improved the outcome of both *P. aeruginosa* and *M. tuberculosis* infections. Hence, these data suggest that the effect of manipulating immune responses by eliminating negative TLR regulation is pathogen specific.

Unlike SIGIRR deficiency, removing ST2 driven negative regulation of inflammation from host defense during pneumococcal pneumosepsis did not result in a host benefit, as demonstrated using ST2 deficient mice. Differential effects of different inhibitory TLR regulators in similar disease settings are not unexpected. As described in chapter 7 negative regulation of TLR signaling occurs at different levels in the signaling cascade and individual regulators may have disparate additional functions. SIGIRR and ST2 are both transmembrane, cell-surface associated negative regulators. SIGIRR is constitutively and ubiquitously expressed (in particular in the kidney, liver, digestive tract, lymphoid organs and lung (i.e. bronchial epithelium, endothelium and leukocytes) [8]) and interferes with the association of adaptor molecules to activated TLRs and IL-1R family members [5]. It has been shown that pathogen induced activation of cells results in down-regulated expression of SIGIRR and stable overexpression of SIGIRR resulted in
diminished NF-κB mediated cytokine production [10]. Membrane bound ST2 on the other hand is primarily expressed by hematopoietic cells (granulocytes, monocytes, macrophages, mast cells, dendritic cells, Th2 lymphocytes, NK and NKT cells); it resides intracellularly in resting cells and surfaces on the outer cell membrane after stimulation [11]. ST2 most likely sequesters MyD88, resulting in inhibition of TLR and IL-1R driven MyD88 dependent pathways once ST2 is overexpressed and enhanced cytokine release from ST2 deficient macrophages in response to TLR activation [6]. In addition, ST2 functions as the IL-33 receptor, thus facilitating a pro-inflammatory immune response [12]. IL-33 expression has been established in many cell types and tissues [13]. It has been proposed as an alarmin since it is released from damaged epithelium and endothelial cells (necrosis) and stressed fibroblasts (actively secreted under mechanical strain) [12, 13]. Furthermore, macrophages and NKT cells have been shown to produce IL-33 in response to LPS and influenza virus respectively [14, 15]. In vivo, ST2 has been reported to play an essential role in the development of endotoxin tolerance [6] and the development of an immune-compromised state (increased susceptibility to P. aeruginosa pneumonia following non-lethal peritonitis) [16]. Nonetheless, the research presented in chapter 3 indicates a minor if any role for ST2 in both primary and secondary S. pneumoniae lung infection. Perhaps in the latter disease settings the balance between ST2 facilitated TLR signaling inhibition and IL-33 mediated pro-inflammatory signaling is different from that in for example secondary Pseudomonas pneumonia. Together these results suggest that the role of distinct TLR inhibitors in different infections depend on the cellular distribution of the TLR regulator, its effect on specific signaling pathways, the pathogen and the premorbid condition of the host.

In contrast to the proposed inhibitory effect of ST2 on pro-inflammatory PAMP signaling, as shown by isolated peritoneal macrophages [6], in experiments described in chapter 4 we detected lower cytokine levels produced by ST2 deficient whole blood leukocytes and splenocytes stimulated with bacteria. Although ST2 might enhance pro-inflammatory cytokine release upon activation by IL-33, this cytokine was not detected in culture supernatants. As such, the mechanism by which ST2 deficiency resulted in attenuated pathogen-induced cytokine release should be the focus of future investigations.

While the studies described in chapters 3 and 4 point to a limited role for endogenous IL-33 in host defense during infections caused by S. pneumoniae or K. pneumoniae, in chapter 5 we demonstrate that the administration of recombinant IL-33 strongly improves antibacterial defense during Klebsiella airway infection. This research was prompted by an earlier investigation showing a protective effect of recombinant IL-33 in a model of polymicrobial abdominal sepsis [17]. Herein IL-33 facilitated the recruitment of neutrophils into the peritoneal cavity, which was considered essential for its protective effect. However, while we also found enhanced neutrophil influx to the
primary site of infection (i.e. the lungs), IL-33 remained effective in reducing bacterial burdens in neutrophil depleted mice. Rather, we provide evidence that a recently discovered group of innate lymphoid cells, ILC2s, were at least in part responsible for the beneficial effect of recombinant IL-33. ILC2s per se, like endogenous IL-33, did not play an eminent role in host defense, as illustrated by the fact that mice lacking ILCs were not impaired in their capacity to limit bacterial growth and dissemination after infection with *Klebsiella* via the airways. The findings of chapter 5 provide leads to future research on the role of ILCs in bacterial infection, and the possibility that strategies targeting ILCs may be of use as adjunctive therapies in sepsis. The possible value of IL-33 treatment for sepsis was underlined by our finding that postponed administration of IL-33, 3 hours after infection with *Klebsiella*, was still capable of lowering bacterial loads.

The measurement of biomarkers might assist physicians in clinical decision making. Several biomarkers have been suggested for tuberculosis and pneumonia [18-20]. sST2 has been reported elevated in patient plasma in various disease states [21-24]. During tuberculosis and community-acquired pneumonia sST2 levels were found elevated as well (chapter 7 and 8 respectively). Moreover sST2 levels correlated with disease severity and “established” biomarkers for these infections. Whether sST2 is a valuable predictor of infectious disease severity and whether its measurement could have therapeutic consequences needs to be investigated further. Perhaps, eventually, it could help to predict or monitor the success or failure of treatment in specific situations.

While SIGIRR and ST2 (studied in chapters 2-4) are considered negative TLR regulators, in chapter 6 we engaged in experiments seeking to establish the role of the (signaling enhancing) TLR adaptors MyD88 and TRIF in gram-negative sepsis. Blocking inflammatory signaling by removing one of two TLR adaptors during *Klebsiella* induced pneumosepsis resulted in dramatically diminished survival accompanied by high bacterial burdens. MyD88 is the adaptor for all TLRs except TLR3, while TRIF mediates signaling via TLR3 and TLR4. As such, these adaptors affect the function of multiple TLRs, just like SIGIRR and ST2 can influence signaling of many TLRs (albeit in an opposite way). Different from the studies described in chapters 3 and 4, in chapter 6 we attempted to dissect the cell-specific roles of MyD88 and TRIF by generating chimeric mice with altered TLR adaptor expression in either resident (most notably epithelial and endothelial) cells or hematopoietic cells (most notably neutrophils and monocytes/macrophages). The phenotype of MyD88 and TRIF deficient mice tuned out to be stronger (hypersusceptible) than that of ST2 deficient (no phenotype) in *Klebsiella* pneumonia (chapter 5), which may indicate that during a gradually evolving infection, such as produced in the *Klebsiella* airway infection model used in this thesis, adequate
triggering of TLRs is more important for a balanced host response than inhibition thereof (at least by ST2). Thus far, the role of SIGIRR in Klebsiella infection has not been studied.

Concluding

A balanced immune response consists of a swift pro-inflammatory reaction upon encountering a pathogen followed by a response that on the one hand eliminates the threat and on the other hand limits excessive inflammation and guides repair mechanisms. Regulators tweak distinct signal transduction processes and thereby shape the overall innate immune response. Manipulation of these regulators might tip the balance towards a more pro- or anti-inflammatory state. The net benefit of this manipulation depends on the regulator and its cellular expression, the causative pathogen and type of infection, and the condition of the host. In some infections (temporary) inhibition of inflammation can be advantageous, while in other the same intervention can be detrimental for the host. Therefore, before considering implementation of interventions targeting immune regulators in infectious disease (e.g. pneumosepsis) one has to have a clear understanding of the individual patient’s disease state, the disease setting and the causative pathogen. In the end, a successful immune response (i.e. upholding a state of health) is characterized by maintaining balance and returning to tissue homeostasis as soon as possible. While immune modulation could expedite this process, additional research is needed to provide the detailed knowledge required for use in clinical practice.
Reference list