Toll-like receptor and associated regulators in pneumonia and sepsis

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Chapter 4

Role of Interleukin-1 receptor like 1 (ST2) in gram-negative and gram-positive sepsis in mice

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Abstract
Background: Interleukin-1 receptor like 1 (ST2) has been implicated as a negative regulator of Toll-like receptor signaling. We here sought to elucidate the role of ST2 in cytokine release and systemic infection caused by two common human sepsis pathogens, *Streptococcus (S.)* pneumoniae (gram-positive) and *Klebsiella (K.)* pneumoniae (gram-negative).

Methods: Whole blood leukocytes and splenocytes were harvested from ST2 deficient (*st2<sup>-/-</sup>) and wild type (WT) mice and stimulated ex vivo with *S. pneumoniae* or *K. pneumoniae*. In addition, *st2<sup>-/-</sup>* and WT mice were infected intravenously with these bacteria and bacterial loads and cytokine levels were measured in blood, spleens and lungs at 6, 24 and 48 hours thereafter.

Results: Unexpectedly, *st2<sup>-/-</sup>* blood leukocytes and splenocytes produced lower levels of cytokines and chemokines than WT cells in response to either pathogen. In contrast, the in vivo role of ST2 during sepsis caused by these bacteria was limited, although at 6 and 24 hours after infection with *S. pneumoniae* bacterial loads were lower in spleens of *st2<sup>-/-</sup>* mice.

Conclusion: ST2 augments rather than inhibits cytokine release by blood leukocytes and splenocytes exposed to *S. pneumoniae* or *K. pneumoniae*, but plays a limited role in host defense during sepsis caused by these pathogens.
Introduction

Toll-like receptors (TLRs) play an eminent role in the induction of an adequate innate immune response to invading pathogens [1, 2]. TLRs are expressed on either the cell surface (TLR1, -2, -4, -5, -6 and -10) or in intracellular compartments (TLR3, -7, -8 and -9), where they recognize conserved motifs from pathogens collectively called pathogen associated molecular patterns. TLRs can also sense so-called damage associated molecular patterns, which are endogenous molecules released by injured cells [3]. TLR signalling is tightly regulated in order to prevent disproportionate inflammation due to unrestrained stimulation of TLRs. Several negative regulators of TLRs have been implicated in this inhibitory mechanism, among which interleukin 1 receptor-like 1 (IL1RL1 also known as ST2) [4, 5]. ST2 is a member of the IL-1 receptor family that is expressed by many hematopoietic cells, Th2 lymphocytes, natural killer (NK) and NKT cells, mast cells, monocytes, dendritic cells and granulocytes. The negative regulatory function of ST2 in TLR signaling is reflected by enhanced cytokine release by st2 deficient (−/−) macrophages upon stimulation with TLR agonists [6]. In addition, ST2 together with IL-1 receptor accessory protein forms the receptor for IL-33 [7].

Sepsis is characterized by an unbalanced response of the host to infection [8, 9]. TLRs are important for defense against bacteria by virtue of their capacity to induce innate immunity; yet, on the other hand, abundant TLR activation during progressive growth of pathogens likely contributes to collateral tissue damage. Along the same line of reasoning, ST2 may serve a dual role during infection. ST2 may impair innate immune responses through inhibition of TLR signaling and/or may attenuate injurious inflammation occurring as a consequence of ongoing TLR activation. Our laboratory recently demonstrated that ST2 strongly impaired host defense during secondary Pseudomonas pneumonia in mice with sublethal polymicrobial sepsis caused by cecal ligation and puncture (CLP); ST2 did not influence the host response during primary Pseudomonas pneumonia (i.e. in previously healthy mice) [10]. In contrast, st2−/− mice showed an increased susceptibility to CLP-induced severe abdominal sepsis as reflected by higher bacterial loads and increased mortality [11]. We here sought to determine the role of ST2 in systemic infection caused by two common humans sepsis pathogens, Streptococcus (S.) pneumoniae (gram-positive) and Klebsiella (K.) pneumoniae (gram-negative).
Methods

Animals
Specific pathogen free 9-11 week old BALB/c mice (wild-type [WT]) were purchased from Charles River (Maastricht, The Netherlands). St2-/- mice [12] backcrossed eight times to a BALB/c background were kindly provided by dr. Andrew N.J. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and bred in the animal facility of the Academic Medical Center in Amsterdam. Age- and sex-matched animals were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Experimental infections
Sepsis was induced by intravenous inoculation of 5x10^5 colony-forming units (CFUs) of Streptococcus pneumoniae serotype 3 (American Type Culture Collection, Rockville, MD; ATCC 6303) or 5x10^5 CFUs of Klebsiella pneumoniae serotype 2 (ATCC 43816) in 200µl normal saline via the tail vein. Mice were sacrificed after 6, 24 or 48 hours of infection. Sample harvesting and processing, including determination of bacterial loads, was done as previously described [13, 14]

Ex vivo stimulation
Heparinized whole blood and splenocytes from naive WT and st2-/- mice were harvested as described [15, 16] and ex vivo stimulated with 5x10^6 or 5x10^7 ml heat killed S. pneumoniae or K. pneumoniae (blood) or in an equivalent of 10 or 100 bacteria to 1 cell (spleen) for 4 or 24 hours at 37 °C.

Assays
In plasma and supernatant tumor necrosis factor (TNF)-α, Interleukin (IL)-6, IL-12p70, IL-10, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 were measured by cytometric bead array multiplex assay (BD Biosciences) in accordance with the manufacturers recommendations. TNF-α, IL-1β, IL-6, IL-33, macrophage inflammatory protein (MIP)-2 and cytokine-induces neutrophil chemo attractant (KC) in spleen and lung homogenates were measured using specific enzyme-linked immunosorbent assays (ELISA) (R&D systems, Abingdon, UK) in accordance with the manufacturer’s recommendations. Plasma aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) were determined with commercially available kits (Sigma-Aldrich, St Louis, MO, USA), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Histopathological analysis
Paraffin-embedded 4 µm lung tissue sections were stained with haematoxylin and eosin (H&E). All slides were coded and scored by a pathologist blinded for experimental
groups for the following parameters: interstitial inflammation, endothelitis, bronchitis, edema, pleuritis, presence of thrombi and percentage of lung surface with pneumonia. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition).

Flow cytometry
Blood cells and splenocytes obtained from WT and st2−/− mice were prepared for flow cytometry as described [15]. Immunostaining for cell surface molecules was performed for 30 min at 4°C, using directly labeled Abs against CD3 (clone 17A2), CD4 (clone RM4-5), CD8 (clone S3-6.7), B220 (clone RA3-6B2), F4/80 (clone 521204 R&D), GR1 (clone RB6-8C5) and NK cells (clone DX5). All Abs were used in concentrations recommended by the manufacturer (BD Pharmingen, Breda, the Netherlands and R&D systems, Abingdon, UK). FACS analysis was done by FACSCalibur (BD Biosciences).

Statistical analysis
Data are expressed as box and whiskers showing the smallest observation, lower quartile, median, upper quartile and largest observation or as means ± SEM. Differences were analyzed by Mann Whitney U test. A value of P < 0.05 was considered statistically significant.

Results
St2 deficiency reduces inflammatory mediator production by whole blood and splenocytes in response to bacteria ex vivo
In order to evaluate a possible role for ST2 in systemic bacterial infection, we first sought to determine the ex vivo cytokine and chemokine response to two common human sepsis pathogens (one gram-positive and one gram-negative) in two relevant body compartments, i.e. blood leukocytes and splenocytes (the spleen being an important organ in protecting the host from bacteremia) [17, 18]. Whole blood and splenocytes from WT and st2−/− mice were harvested and ex vivo stimulated with 2 doses of S. pneumoniae or K. pneumoniae. After 4 or 24 hours cytokines (TNFα, IL-6, IL-10, IFN-γ, IL-12p70, IL-33) and chemokines (MCP-1, KC, MIP-2) were measured in supernatants (Figure 1). Unexpectedly, overall st2−/− blood leukocytes and splenocytes produced lower levels of inflammatory mediators than WT cells in response to either pathogen; differences between genotypes were significant for the vast majority of conditions and mediators. Most notably, the phenotype of enhanced production of inflammatory mediators such as described for st2−/− macrophages [6] was not observed in cultures of whole blood or splenocytes with the single exception of modestly increased release of MIP-2 by st2−/− splenocytes 4 hours post exposure to K. pneumoniae. Stimulation of whole blood or splenocytes with either pathogen did not
induce secretion of IL-33 or IL-12p70. FACS analysis revealed no differences in cellular composition between WT and st2-/- blood and spleen prior to stimulation (data not shown).

**Figure 1:** Impact of ST2 deficiency on cytokine and chemokine release by whole blood leukocytes and splenocytes exposed to S. pneumoniae or K. pneumoniae.

Whole blood and splenocytes were isolated from WT (grey bars) and st2-/- mice (white bars) and stimulated with heat-killed S. pneumoniae or K. pneumoniae for 4 and 24 hours at the indicated concentrations. Data are means ± SE (n = 6). * P < 0.05, ** P < 0.01, versus WT. b.d. = below detection.
St2-/- mice demonstrate a transiently reduced bacterial growth in spleen during gram-positive but not during gram-negative sepsis

Having established that ST has a major impact on the capacity of blood leukocytes and splenocytes to release cytokines and chemokines upon exposure to S. pneumoniae or K. pneumoniae ex vivo, we were interested in the role of ST2 in host defense during primary blood stream infection with these pathogens. Therefore, WT and st2-/- mice were intravenously infected with either S. pneumoniae or K. pneumoniae and euthanized 6, 24 or 48 hours later. To determine the role of ST2 in bacterial dissemination from the circulation and growth in different target organs, we measured bacterial loads in blood, lung and spleen at the time points indicated (Figure 2). During gram-positive sepsis (Figure 2A-C), bacterial burdens did not differ between WT and st2-/- mice in blood or lungs at any time point. However, st2-/- mice did show reduced bacterial loads in their spleens at 6 and 24 hours after infection with S. pneumoniae (both P < 0.05 versus WT mice); at 48 hours this difference was no longer observed. After infection with K. pneumoniae (figure 2D-F) st2-/- mice displayed similar bacterial loads in all body compartments at all time points tested. Notably, the Klebsiella model was associated with mortality in 2/8 WT and 1/8 st2-/- mice at 48 hours post infection.

**Figure 2**: Impact of ST2 deficiency on bacterial growth and dissemination during sepsis caused by S.pneumoniae or K. pneumoniae.

WT (grey whiskers) and st2-/- mice (white whiskers) were given S. pneumoniae (A-C) or K. pneumoniae (D-F) intravenously and bacterial loads in blood, lung and spleen were counted 6, 24 or 48 hours of infection. Data are box and whiskers showing the smallest observation, lower quartile, median, upper quartile and largest observation of 8 mice per group at each time point (except at 48 hours after K. pneumoniae infection: 6 WT vs 7 st2-/- mice; † = dead mouse). * P < 0.05 versus WT.
Influence of ST2 on plasma and tissue levels of cytokines and chemokines during sepsis

To obtain insight into the role of ST2 in the regulation of systemic inflammation during bacterial sepsis in vivo we measured cytokines and a chemokine (TNFα, IL-6, IL-12p70, IL-10, IL-33, IFN-γ and MCP-1) in plasma of mice 6, 24 and 48 hours after intravenous infection with either *S. pneumoniae* or *K. pneumoniae* (Table 1). Although the extent and kinetics of systemic cytokine release clearly differed between the models of gram-positive and gram-negative sepsis, mediator levels did not differ between *st2−/−* and WT mice at any time point with the exception of modestly decreased plasma IFN-γ in *st2−/−* mice 24 hours after infection with *S. pneumoniae* (*P* < 0.05 versus WT mice). IL-10 and IL-33 could not be detected in plasma. To evaluate the role of ST2 in the induction of mediator release in target organs, we measured cytokines (TNFα, IL-1β, IL-6, IL-33) and chemokines (KC, MIP-2) in whole organ homogenates of spleens and lungs. Mediator levels in spleen homogenates did not differ between mouse strains at any time point after infection with either *S. pneumoniae* or *K. pneumoniae* (Table 1). In lung homogenates, *st2−/−* mice demonstrated lower lung levels of IL-6, IL-1β and MIP-2 24 hours after infection with *S. pneumoniae* (all *P* <0.05 versus WT mice); this difference was not found at other time points (Table 1). During *Klebsiella* sepsis no differences between *st2−/−* and WT mice were found with regard to lung cytokine and chemokine levels (Table 1). TNFα lung levels were below detection at all time points both during *S.* and *K. pneumoniae* infection.

Figure 3: Impact of ST2 deficiency on cellular injury and lung pathology during sepsis caused by *S. pneumoniae* or *K. pneumoniae*.

WT (grey whiskers) and *st2−/−* mice (white whiskers) were given *S. pneumoniae* (A–D) or *K. pneumoniae* (E–H) intravenously and plasma levels of ASAT (A, E), ALAT (B, F) and LDH (C, G) were measured and lung histopathology scored according to the scoring system described in the Methods section (D, H). Data are box and whiskers showing the smallest observation, lower quartile, median, upper quartile and largest observation of 8 mice per group at each time point (except at 48 h after *K. pneumoniae* infection: 6 WT vs 7 *st2−/−* mice. **P < 0.01 vs WT, * P < 0.05 versus WT.**
Table I: Glycine and dimethylglycine concentrations after 5% glucose administration.

<table>
<thead>
<tr>
<th>Glycine</th>
<th>Dimeethylglycine</th>
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<td>WT</td>
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ST2 in gram-negative and gram-positive sepsis.
ST2 mice demonstrate modestly increased lung inflammation

To obtain insight into the role of ST2 in organ injury, we measured the plasma levels of ASAT, ALAT (both parameters of hepatocellular injury) and LDH (a parameter for cell injury in general) and semi-quantitatively lung pathology according to the scoring system described in the Methods section. Apart from a minor ALAT increase after 6 hours of K. pneumoniae infection in st2−/− mice, the plasma levels of ASAT, ALAT and LDH were not different between st2−/− and WT mice at any time point after infection with S. pneumoniae or K. pneumoniae (Figure 3). In both sepsis models, mice displayed modest lung inflammation 48 hours after infection, especially in the interstitium (data not shown). St2−/− mice had higher lung pathology scores after both infection with S. pneumoniae or K. pneumoniae (both P < 0.05 versus WT mice; Figure 3).

Discussion

ST2 has been implicated as a negative regulator of TLR signaling capable of inhibiting cytokine release by macrophages upon stimulation of receptors that rely on the common adaptor MyD88 for signal transduction [6]. The present work sought to investigate the role of ST2 in the host response during sepsis caused by two common human pathogens, focusing on the release of cytokines and chemokines by and in relevant body compartments. We here demonstrate that ST2 augments rather than inhibits inflammatory mediator production by blood leukocytes and splenocytes in response to S. pneumoniae and K. pneumoniae ex vivo. Nonetheless, the in vivo role of ST2 during sepsis caused by these bacteria proved to be limited.

Sepsis is a severe systemic syndrome triggered by a disseminating infectious pathogen and characterized by an unbalanced yet rigorous inflammatory response [8, 9]. Upon activation by pathogens, TLRs stimulate the immune system to release pro-inflammatory mediators. MyD88 is a common adaptor for all TLRs (except TLR3) that associates with IL-1R-associated kinases and TNF receptor-associated factor 6, eventually resulting in activation of nuclear factor (NF)-κB [1, 2]. Other receptors that are dependent on MyD88 include those that mediate the cellular effects of IL-1, IL-18 and IL-33 [7, 19]. ST2 is a member of the TLR/IL-1 receptor superfamily that can inhibit NFκB activation through MyD88 dependent receptors by sequestration of MyD88 with another adaptor Mal [6]. As a consequence thereof, st2−/− macrophages were reported to produce increased amounts of pro-inflammatory cytokines in response to IL-1 and TLR ligands [6]. Herein lie two possible mechanisms via which ST2 might influence the development of sepsis and sepsis outcome: impairment of innate immune responses through inhibition of TLR signaling and/or attenuation of injurious inflammation occurring as a consequence of ongoing TLR activation. However, our data suggest a pro-
inflammatory rather than an anti-inflammatory role for ST2 since both st2−/− blood leukocytes and splenocytes produced lower concentrations of cytokines and chemokines in response to either S. pneumoniae or K. pneumoniae ex vivo. Importantly, the earlier study exclusively studied the role of ST2 in thioglycolate induced peritoneal macrophages [6]. This together with our current data suggests a variable role for ST in different tissues and cell types. Of note, as the receptor for IL-33 ST2 elicits pro-inflammatory effects: upon interaction with IL-33 ST2 forms a complex with IL-1R accessory protein, which results in recruitment of MyD88 and activation of NFκB and the production of inflammatory mediators [7]. Thus ST2 can exert opposite functions depending on stimulatory conditions. IL-33 could not be detected in culture supernatants. Therefore, the exact mechanism by which ST2 impacts on cytokine release by blood leukocytes and splenocytes remains to be determined.

Despite the noticeably reduced capacity of st2−/− blood leukocytes and splenocytes to produce cytokines ex vivo, st2−/− mice did not show a clear phenotype when confronted with intravenously administered bacteria: neither plasma cytokine levels nor blood bacterial loads differed between mouse strains. A difference was observed in the spleen 6 and 24 hours after infection with S. pneumoniae: bacterial outgrowth at these time points was lower in st2−/− mice, though the local cytokine response did not differ. The mechanism underlying this finding requires further investigation. Our studies do not provide insight into the impact of ST2 deficiency on survival. In our institution permission for survival studies are only granted in case of clear differences between mouse strains with regard to bacterial growth and inflammation.

Interestingly, in a murine model of polymicrobial sepsis, IL-33 has been shown to attenuate sepsis in vivo in a ST2 dependent manner [20]. IL-33 is mainly an intracellular cytokine that can act as an alarmin, when it is released upon in particular epithelial or endothelial cell damage [5]. An increase in plasma IL-33 has been reported in 50% of septic patients while IL-33 could not be measured in healthy volunteers [20]. In our models of murine sepsis plasma IL-33 levels remained undetectable. IL-33 could be measured in both WT and st2−/− lung and spleen homogenates, especially after 24 hours of S. pneumoniae pneumonia in the lung. Most likely this IL-33 is responsible for the slightly pro-inflammatory function of ST2 observed in the lung at this time, albeit without bactericidal consequences.

In conclusion, we found an unexpected pro-inflammatory role of ST2 in cytokine and chemokine release by blood leukocytes and splenocytes upon exposure to S. pneumoniae or K. pneumoniae ex vivo. ST2 did not impact on the host response during sepsis caused by either one of these pathogens. These data exemplify the complex
nature of innate immunity in general and the receptors involved in particular, revealing opposite functions of ST2 in different experimental conditions.

Reference list

7. Schmitz J, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity, 2005;* 23(5): 479-90.