Immunotolerance during bacterial pneumonia and sepsis
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Chapter 8

Sepsis-induced suppression of lung host defense is mediated by ST2

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Abstract

Patients with sepsis display - after surviving the initial hyperinflammatory phase - features consistent with immunosuppression, which renders the host susceptible to nosocomial infections, in particular bacterial pneumonia. ST2 is a negative regulator of Toll-like receptor signalling implicated in endotoxin tolerance. We here sought to determine the role of ST2 in modulating host defense in the lung during sepsis, using a murine model of cecal ligation and puncture (CLP)-induced sepsis followed by a secondary infection with *Pseudomonas* (*P.* aeruginosa) via the airways. Therefore CLP or sham surgery was performed on BALB/c wild-type (WT) and ST2 knock out (−/−) mice, and 24 hours later animals were challenged with $10^8$ live *P. aeruginosa*. CLP mice demonstrated impaired clearance of *Pseudomonas* from their lungs and reduced pulmonary levels of tumor necrosis factor (TNF)-α and interleukin-6 as compared with sham mice. Following CLP, ST2−/− mice with secondary pneumonia displayed a strongly improved survival and a better bacterial clearance when compared with WT mice, which was accompanied by enhanced lung inflammation. CLP did not influence the responsiveness of alveolar macrophages toward *P. aeruginosa* ex vivo irrespective of the st2 genotype. In contrast, CLP resulted in a reduced capacity of WT CD4+ and CD8+ T-lymphocytes to produce interferon-γ and TNF-α, an immune suppressive effect that was not seen in ST2−/− mice. These findings indicate that ST2 contributes to the immune compromised state during sepsis and the ensuing disturbed homeostasis of lung host defense.
Introduction

Sepsis is a major health problem which afflicts approximately 750,000 patients in the United States each year and is associated with mortality rates of 20–50%\(^1\). Patients who survive the initial phase of sepsis display features of immunosuppression, which is considered to contribute to the vulnerability of septic patients to nosocomial infections, in particular bacterial pneumonia\(^2,5\). Indeed, animal models have revealed that sepsis leads to an attenuated antibacterial lung host defense and enhanced susceptibility to secondary pneumonia\(^6-11\). Although the underlying mechanisms of immunosuppression are not fully elucidated, anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth-factor (TGF)-β, and deregulated apoptosis of lymphocytes have been implicated as important causative factors\(^3,12,13\).

Additionally, inhibitors of Toll-like receptors (TLRs) - responsible for early recognition of invading pathogens\(^14\) - such as MyD88 short, A20, interleukin-1 receptor-associated kinase (IRAK)-M and ST2 are thought to play a role in the immunosuppression in septic patients\(^8,15-17\). Recently, IRAK-M was found to be upregulated in alveolar macrophages of mice with abdominal sepsis and absence of IRAK-M led to an improved survival and bacterial clearance from the lungs in septic mice exposed to *Pseudomonas (P.)* aeruginosa via the airways\(^8\).

The *st2* gene produces a transmembrane protein (ST2L) and a soluble secreted protein (sST2). ST2 is expressed in several cells including Th2 cells\(^18\), mast cells\(^19\), eosinophils\(^20\) and macrophages\(^21\) and is linked to important Th2 effector functions\(^22-25\). ST2L serves an important negative regulatory function in TLR signaling, as reflected by enhanced cytokine release by ST2 deficient macrophages upon stimulation with TLR agonists such as lipopolysaccharide (LPS)\(^16\). Of importance, mice lacking ST2L are unable to develop endotoxin tolerance after a sublethal priming dose of LPS, suggesting that ST2L may contribute to sepsis induced immunosuppression\(^16\).

Considering that ST2 has been implicated to play an important role in regulating the immune response during severe inflammation, we here sought to investigate the role of ST2 in host defense in the lung during sepsis using the well-established model of cecal ligation and puncture (CLP)-induced sepsis followed by a secondary intrapulmonary challenge with *P. aeruginosa*\(^6,9\).

Materials and Methods

Mice

Pathogen-free 8- to 10-wk-old wild-type (WT) BALB/c mice were purchased from Jackson Laboratory (www.jax.org). ST2 knockout (\(^{-/-}\)) mice\(^24\) were backcrossed eight times to a BALB/c background. Age- and sex-matched animals were used in all
experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Design

CLP and sham surgery were performed as previously described\textsuperscript{26,27}. Details of the CLP procedure, designed to cause <10% mortality, are provided in the on-line supplement. Pneumonia was induced 24 hours after CLP or sham surgery using \textit{P. aeruginosa} (strain PA01; 1 x 10\textsuperscript{8} colony forming units [CFU]/ml) as described\textsuperscript{28,29}. For one experiment \textit{P. aeruginosa} were heat-killed by incubation at 70°C for 15 minutes. Sample harvesting and processing and determinations of bacterial loads were done as described\textsuperscript{28,29}. In a separate study mice were followed for up to 10 days and survival was monitored at least every 12 hours (N=15 per group).

Measurements and assays

Plasma tumor necrosis factor (TNF)-\textalpha, Interleukin (IL)-6 and IL-10 levels were determined using a cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). In lung homogenates, TNF-\textalpha, IL-1\beta, IL-6, IL-4, IL-10, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 levels were measured by ELISA (R&D Systems, Abingdon, UK). Aspartate aminotransferase (ASAT) and lactate dehydrogenase (LDH) were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions.

Pathology

Paraffin lung sections were stained with hematoxilin and eosin as described\textsuperscript{30}, and scored from 0 (absent) to 4 (severe) for the following parameters: necrosis and/or formation of abscess, interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis and percentage of the lung surface demonstrating confluent (diffuse) inflammatory infiltrate by a pathologist blinded for groups. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 32.

Intracellular staining of \textit{ex vivo}-stimulated splenocytes

Splenocytes were obtained 24 hours after CLP or sham surgery by crushing spleens through a 40-\textmu m cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described\textsuperscript{31}. Splenocytes (1 x 10\textsuperscript{6}) were stimulated with phorbol 12-myristate 13-acetate (PMA) 100 ng/ml and Ca ionomycin 4 \textmu g/ml (Sigma) at 37°C and 5% CO\textsubscript{2} in 96-well plates. After 1 hour golgiplug (BD Biosciences) was added to accumulate intracellular cytokines. After another 5 hours of incubation plates were placed at 4°C overnight.
Cells were washed, incubated 10 min at 4°C with anti-mouse CD16/32 mAb to block FcRs, and surface stained with CD3 (PE), CD4 (APC) and CD8 (PerCP)(all BD Pharmingen, San Diego, CA) for 20 min at 4°C. Then the cells were fixed/permeabilized (Cytofix/Cytoperm Plus; BD Biosciences) and stained with PE-conjugated anti-IFN-γ and FITC-conjugated anti-TNF-α mAbs (BD Pharmingen) for 30 min at room temperature. The cells were then washed (Perm/Wash; BD Biosciences), fixed in 1% paraformaldehyde in PBS, and stored at 4°C until flow cytometry analysis. For each experiment, unstained and all single-color controls were processed to allow proper compensation.

Stimulation of alveolar macrophages

Twenty-four hours after CLP or sham surgery, alveolar macrophages were harvested from WT and ST2−/− mice by bronchoalveolar lavage (N=8 per strain) as described30. Cells were resuspended in RPMI 1640 containing 2 mM L-glutamine, penicillin, streptomycin and 10% fetal calf serum in a final concentration of 1x10⁴ cells/100 μl. Cells were then cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2 hours and washed with RPMI 1640 to remove non-adherent cells. Adherent monolayer cells were stimulated with either heat-killed P. aeruginosa (equivalent of 0.2 x 10⁸ CFU/ml) or LPS derived from P. aeruginosa (25 μg/ml; Sigma) for 20 hours at 37°C and 5% CO2. Thereafter supernatants were harvested and assayed for TNF-α and IL-6.

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. For survival analysis, Kaplan-Meier analysis followed by log-rank test was performed. Analyses were performed using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). Values of P<0.05 were considered statistically significant.

Results

CLP results in impaired innate immunity in the lung in WT BALB/c mice

Previous studies, using C57BL/6, CD-1 and ND-4 mice, have documented that CLP-induced sepsis results in an impaired immune response in the lung upon secondary infection with P. aeruginosa6-11. To confirm that CLP causes a similar attenuated pulmonary response in BALB/c mice, WT BALB/c mice underwent either CLP or sham operation and were intranasally infected with P. aeruginosa 24 hours thereafter. 24 hours after induction of pneumonia, CLP mice had significantly higher Pseudomonas loads in their lungs compared with sham mice (P<0.0005; Figure 8.1A). In addition,
CLP mice with *Pseudomonas* pneumonia displayed decreased levels of TNF-α and IL-6 in their lung homogenates compared with sham mice with pneumonia (P<0.005; Figure 8.1B and 8.1C). These data are in accordance with earlier investigations and show that CLP inhibits the innate immune response in the lungs resulting in an impaired antibacterial defense⁶⁻¹¹.

**Figure 8.1** Effect of cecal ligation and puncture (CLP)-induced sepsis on the innate immune response in the lung following intranasal infection with *P. aeruginosa*.

Mice underwent either sham (open bars) or CLP (solid bars) surgery. Twenty-four hours later, *P. aeruginosa* (PA; 10⁸ CFUs/ml) was administered intranasally. Mice were sacrificed 24 hours after induction of pneumonia (N=8 per group). PA colonies in the lung (A) were determined (***P<0.0005). Levels of TNF-α (B) and IL-6 (C) were measured in lung homogenates (**P<0.005). Data are means ± SE.

ST2⁻/⁻ mice are protected from lethality by secondary pneumonia with *P. aeruginosa*

To investigate the role of ST2 in host defense against *Pseudomonas* pneumonia in septic mice, WT and ST2⁻/⁻ mice underwent CLP and were infected with *P. aeruginosa* 24 hours later (T=0). ST2⁻/⁻ mice showed a markedly increased survival following secondary pulmonary infection with *P. aeruginosa*, as compared to WT mice (P<0.005; Figure 8.2A). Indeed, 24 hours after induction of pneumonia only 2/15 ST2⁻/⁻ mice had died versus 5/15 WT mice; at 48 hours 4/15 ST2⁻/⁻ mice and 11/15 WT mice had died.
Overall survival was 11/15 (73%) among ST2−/− mice versus 2/15 (13%) among WT mice. To obtain insight into the impact of ST2 deficiency on antibacterial defense, a separate experiment was performed in which ST2−/− and WT mice were killed 24 hours after induction of post-CLP *Pseudomonas* pneumonia. ST2−/− mice harbored >20-fold fewer *Pseudomonas* CFU in their lungs when compared with WT mice (P<0.001; Figure 8.2B). In addition, ST2−/− mice demonstrated reduced *Pseudomonas* loads in their peripheral blood (P<0.005; Figure 8.2C). Of note, these results underestimate the true difference between ST2−/− and WT mice, since 6/15 WT and only 1/14 ST2−/− mice had died at the predetermined time point of sacrifice, confirming the protection against mortality conferred by ST2 deficiency. Of note, CLP per se (i.e. without subsequent pneumonia) was associated with <10% mortality in both groups, whereas infection with *Pseudomonas* via the airways without preceding CLP did not cause lethality (data not shown).

**Figure 8.2** Protection of ST2−/− mice from lethality by secondary pneumonia with *P. aeruginosa*. Wildtype (WT) and ST2 knock out (−/−) mice underwent CLP and were inoculated with *P. aeruginosa* (PA; 10^8 CFUs/ml) at T=0 (24 hours after CLP). Survival was observed in WT (open squares) and ST2−/− (solid squares) mice (N=15 per group; **P<0.005) (A). In another experiment WT (N=15) and ST2−/− (N=14) mice were infected in order to be sacrificed 24 hours after induction of pneumonia. PA colonies were determined in the lung (B) and in blood (C) (***P<0.005). Six WT mice and 1 ST2−/− mouse had died at the predetermined time point of sacrifice 24 hours after induction of PA pneumonia. Data are means ± SE.
**ST2<sup>-/-</sup>** mice display increased lung inflammation during secondary pneumonia

ST2 has been implicated as a negative regulator of TLR signaling<sup>16</sup>. Since TLRs contribute to the induction of lung inflammation during primary *Pseudomonas* pneumonia<sup>32,33</sup>, we examined the impact of ST2 deficiency on lung histopathology. First we established that 24 hours after CLP (i.e. before infection with *Pseudomonas*) lungs of ST2<sup>-/-</sup> and WT mice did not show obvious signs of pulmonary inflammation (data not shown). At 24 hours after induction of pneumonia, the lungs of WT mice showed interstitial inflammation along with endothelialitis, bronchitis, edema, and pleuritis (Figure 8.3A). Importantly, lung inflammation was exaggerated in ST2<sup>-/-</sup> mice (Figure 8.3B), which was also reflected by higher pathology scores as determined by the semi-quantitative scoring system described in Materials and Methods (<i>P</i>&lt;0.05; Figure 8.3C).

![Figure 8.3](image)

**Figure 8.3** Increased lung inflammation in ST2<sup>-/-</sup> mice during secondary pneumonia. Wildtype (WT; N=15) and ST2 knock out (<sup>/-</sup>; N=14) mice underwent CLP, which was followed by induction of pneumonia by *P. aeruginosa* (PA; 10⁸ CFUs/ml) 24 hours later (T=0). Representative H&E stainings of lung tissue 24 hours after inoculation with *P. aeruginosa* in WT (A) and ST2<sup>-/-</sup> (B) mice. Original magnification, x200. Graphic representation of the degree of lung inflammation (C), determined according to the scoring system described in Materials and Methods (*P*<0.05). Six WT mice and 1 ST2<sup>-/-</sup> mouse had died at the predetermined time point of sacrifice 24 hours after induction of PA pneumonia. Data in panel C are means ± SE.
Impact of ST2 deficiency on lung and plasma cytokine levels

Next we determined the influence of ST2 deficiency on the induction of proinflammatory (TNF-α, IL-1β, IL-6), anti-inflammatory cytokines (IL-4, IL-10) and chemokines (KC and MIP-2). At 24 hours after CLP (i.e. before the induction of pneumonia), the concentrations of these mediators were low and not significantly different in lungs and plasma of ST2−/− and WT mice (data not shown). At 24 hours after pulmonary infection with *Pseudomonas*, ST2−/− mice displayed lower levels of both pro- and anti-inflammatory cytokines as well as chemokines in their lungs relative to WT mice, although for TNF-α, KC and MIP-2 the difference between groups did not reach statistical significance (Table 8.1). In addition, ST2−/− mice had lower plasma concentrations of TNF-α, IL-6 (P=0.07) and IL-10 at this time point (Table 8.1). These findings were counterintuitive in light of the increased lung inflammation in ST2−/− mice (Figure 8.3) and considering that ST2 has been shown to inhibit cytokine release upon activation of TLRs. We argued that ST2−/− mice had lower cytokine concentrations as a consequence of their lower bacterial loads. To test this possibility, we intranasally inoculated ST2−/− and WT mice with heat-killed *Pseudomonas* 24 hours after CLP and measured cytokine levels in lungs 6 hours later (Table 8.2). Although overall ST2−/− mice displayed higher cytokine and chemokine levels in their lungs as compared to WT mice, the differences between groups were modest and statistically significant only for IL-6. (P<0.05), whereas IL-1β levels even tended to be lower in ST2−/− mice. Together these data suggest that ST2 is not a major negative regulator of *Pseudomonas* induced cytokine production in the pulmonary compartment.

<table>
<thead>
<tr>
<th>Table 8.1</th>
<th>Cytokine release during post-CLP Pseudomonas pneumonia.</th>
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<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4877 ± 719</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5183 ± 486</td>
</tr>
<tr>
<td>IL-6</td>
<td>3949 ± 1119</td>
</tr>
<tr>
<td>IL-4</td>
<td>92 ± 11</td>
</tr>
<tr>
<td>IL-10</td>
<td>177 ± 38</td>
</tr>
<tr>
<td>KC</td>
<td>38876 ± 10313</td>
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<tr>
<td>MIP-2</td>
<td>13853 ± 2991</td>
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Cytokine levels (pg/ml) measured in lung homogenate and plasma 24 hours after intranasal infection with *P. aeruginosa* in CLP-induced sepsis. Data are mean ± SE in pg/ml. *P<0.05; **P<0.01 (vs. WT mice).

ST2−/− mice demonstrate reduced distant organ damage during secondary pneumonia

ST2−/− mice had a markedly improved survival in spite of enhanced lung inflammation after induction of pneumonia. The reduced plasma cytokine levels in ST2−/− mice suggested that these animals had an attenuated systemic inflammatory response
syndrome, possibly as a consequence of a diminished dissemination of *Pseudomonas* from the lungs. To obtain support for this hypothesis we performed clinical chemistry on plasma samples harvested 24 hours after pulmonary infection with *Pseudomonas*. Indeed, relative to WT mice, ST2−/− mice had lower levels of ASAT (indicative for hepatocellular injury) and LDH (indicative for cellular injury in general) in their circulation (Figure 8.4; both P<0.05).

Table 8.2  Lung cytokine release upon intranasal inoculation of heat- killed *P. aeruginosa* after CLP.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ST2−/−</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>2351 ± 137</td>
<td>2918 ± 266</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2943 ± 550</td>
<td>2232 ± 221</td>
</tr>
<tr>
<td>IL-6</td>
<td>598 ± 97</td>
<td>1104 ± 192*</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
</tr>
<tr>
<td>KC</td>
<td>32601 ± 1959</td>
<td>38698 ± 6132</td>
</tr>
<tr>
<td>MIP-2</td>
<td>6292 ± 383</td>
<td>7925 ± 1280</td>
</tr>
</tbody>
</table>

Wildtype (WT) and ST2 knock out (−/−) mice underwent CLP, which was followed by intranasal inoculation of HKPA 24 hours later (N=9 per strain). Mice were sacrificed 6 hours after inoculation of HKPA and cytokine levels (pg/ml) were determined in lung homogenates. Data are mean ± SE in pg/ml. *P<0.05; **, P<0.01 (vs WT mice).

Figure 8.4  Reduced distant organ damage during secondary pneumonia in ST2−/− mice.

Wildtype (WT; N=15) and ST2 knock out (−/−; N=14) mice underwent CLP, which was followed by induction of *P. aeruginosa* pneumonia (PA; 10⁸ CFUs/ml) 24 hours later (T=0). Mice were sacrificed 24 hours after pulmonary infection. ASAT and LDH levels were determined in plasma (*P<0.05). Data are means ± SE.

CLP does not influence the responsiveness of ST2−/− and WT alveolar macrophages toward *Pseudomonas*

ST2 has been implicated in the development of LPS tolerance16, a phenomenon characterized by a reduced capacity of immune cells to release proinflammatory cytokines45. A recent study has suggested that alveolar macrophages become tolerant to LPS stimulation 24 hours after CLP, as reflected by a reduced capacity to release TNF-α8. Other investigations, however, have shown that alveolar macrophages, obtained from the lung during a systemic inflammatory response induced by sepsis or
intravenous LPS, do not display tolerance and may even be primed for ex vivo rechallenge. To investigate the impact of CLP on the capacity of alveolar macrophages to respond to _Pseudomonas_ and the role of ST2 herein, we subjected ST2⁻/⁻ and WT mice to CLP or sham surgery and harvested alveolar macrophages 24 hours later. These cells were then stimulated ex vivo with either _P. aeruginosa_ LPS or heat-killed _P. aeruginosa_ for 20 hours (Figure 8.5). When compared with sham surgery, CLP did not significantly impact on the capacity of alveolar macrophages to release TNF-α or IL-6 upon stimulation with either heat-killed _Pseudomonas_ or LPS derived from this bacterium. In addition, ST2 deficiency did not influence stimulated TNF-α or IL-6 release by alveolar macrophages in either sham or CLP mice. These data suggest that CLP does not impact on the responsiveness of alveolar macrophages toward _P. aeruginosa_ and that ST2 does not play an important role in the regulation of proinflammatory cytokine release by these cells.

**Figure 8.5** CLP does not influence TNF-α or IL-6 release by ex vivo stimulated alveolar macrophages.
Wildtype (WT) and ST2 knock out (⁻/⁻) mice were sacrificed 24 hours after sham or CLP (N=8 per group). Alveolar macrophages were harvested 24 hours thereafter. These cells (1 x 10⁶) were then stimulated ex vivo with either _P. aeruginosa_ LPS (25 μg/ml) or heat-killed (HK) _P. aeruginosa_ (equivalent of 0.2 x 10⁸ CFU/ml) for 20 hours, after which TNF-α (A, B) and IL-6 (C, D) levels were determined in supernatants. Data are means ± SE.
ST2 deficiency reverses CLP-induced decreased TNF-α and IFN-γ production by lymphocytes

Experimental models have shown that endotoxin tolerance (or inflammation-induced immunosuppression) is associated with diminished production of the type 1 cytokine IFN-γ by T-lymphocytes. Importantly, a very recent investigation demonstrated a diminished capacity of splenocytes harvested from mice subjected to CLP to produce IFN-γ upon incubation with a T-lymphocytes agonist, which could be linked to the increased lethality in these animals upon (secondary) lung infection with *P. aeruginosa*. Considering that ST2 is expressed by CD4+ and CD8+ T-lymphocytes, we performed experiments to obtain insight in the impact of ST2 deficiency on IFN-γ and TNF-α production by these cell types. Therefore, ST2-/- and WT mice were subjected to CLP or sham surgery; 24 hours after the surgical procedure, splenocytes were harvested and stimulated *ex vivo* with PMA/ionomycin for 6 hours, after which the percentage of IFN-γ and TNF-α producing CD4+ and CD8+ T-lymphocytes was determined by intracellular flow cytometry. Upon PMA/ionomycin stimulation both CD4+ and CD8+ cells from CLP WT mice showed decreased production of IFN-γ and TNF-α compared to cells from sham WT mice, suggesting that CLP induced immunosuppression with regard to the capacity of T-lymphocytes to produce type 1 cytokines (Figure 8.6A-D). Relative to CD8+ cells from sham WT mice, CD8+ lymphocytes from sham ST2-/- mice demonstrated an increased capacity to produce IFN-γ and TNF-α upon stimulation (both P<0.001); these differences between sham ST2-/- and sham WT mice were not found for CD4+ cells. Importantly, ST2-/- mice did not show a reduction in IFN-γ and TNF-α production by CD4+ and CD8+ lymphocytes after CLP, suggesting that the presence of ST2 was essential for the immunosuppression observed in WT mice (Figure 8.6A-D).

ST2 deficiency does not impact on host defense during primary *Pseudomonas* pneumonia

Previously, ST2-/- mice have been found to have an unaltered response to a primary LPS challenge, whereas their reaction to a second LPS challenge is markedly attenuated. We were therefore interested to determine the role of ST2 in host defense against primary respiratory tract infection caused by *Pseudomonas*, i.e. in pneumonia not preceded by CLP. Thus, healthy ST2-/- and WT mice were intranasally infected with *P. aeruginosa* and bacterial loads and the associated inflammation were determined 24 hours later (Figure 8.7). ST2-/- and WT mice had similar *Pseudomonas* loads in their lungs at this time point (Figure 7A). In addition, lung TNF-α, IL-6, KC and MIP-2 concentrations did not differ between groups (Figure 8.7B and C, and data not shown). Finally, lung inflammation, as determined by histopathology, was not different between ST2-/- and WT mice (Figures 8.7D-F).
Sepsis-induced suppression of lung host defense is mediated by ST2

Figure 8.6  IFN-γ and TNF-α production by CD4+ and CD8+ T-lymphocytes of ST2−/− mice after CLP.

Wildtype (WT) and ST2 knock out (−/−) mice were sacrificed 24 hours after sham or CLP (N=8 per group). Splenocytes of sham / CLP in WT (dashed-open / open bars) and ST2−/− (dashed-grey / grey bars) mice were ex vivo stimulated with PMA/ionomycin for 6 hours and cells were stained with intracellular IFN-γ and TNF-α and extracellular CD4, CD8 and CD3. The percentage of medium control of IFN-γ and TNF-α producing CD4+ (A, B) and CD8+ T-lymphocytes (C, D) was determined by flow cytometry (*P<0.05, **P<0.005, ***P<0.0001). Data are means ± SE.
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Figure 8.7  ST2 deficiency does not impact on host defense during primary Pseudomonas pneumonia. Wildtype (WT; N=8) and ST2 knock out (−/−; N=8) mice were intranasally inoculated with P. Aeruginosa (PA; 10⁸ CFUs/ml). Mice were sacrificed 24 hours thereafter and PA colonies were determined in the lung (A). TNF-α (B) and IL-6 (C) levels were determined in lung homogenates. Representative H&E stainings of lung tissue 24 hours after inoculation with P. aeruginosa in WT (D) and ST2 −/− (E) mice. Original magnification, x200. Graphic representation of the degree of lung inflammation (F), determined according to the scoring system described in Materials and Methods. Data in panels A, B, C and F are means ± SE.

Discussion

Sepsis remains a major challenge for clinicians. Recent insights demonstrate that the host response to sepsis involves a prolonged state of immunosuppression, which is considered to contribute to the susceptibility of septic patients to subsequent nosocomial infections. Septic patients are especially vulnerable to nosocomial pneumonia, with P. aeruginosa as a major causative pathogen. At present, the molecular mechanisms underlying the clinical problem of immunosuppression resulting in an inadequate lung host response in sepsis are poorly understood. A recent study has focused the attention on IRAK-M, a negative regulator of TLR signaling. In light of the possible attribution of other negative TLR regulators to immunosuppression and the established role of ST2 in endotoxin tolerance, we were interested to investigate the role of ST2 in sepsis-induced suppression of host defense in the lung. We here report that absence of ST2 protects mice against lethality of secondary pneumonia with P. aeruginosa during CLP-induced sepsis.
CLP is generally considered a clinically relevant model of sepsis. Previously others have demonstrated an impaired antibacterial host response in the lung during CLP-induced sepsis syndrome, which was accompanied with higher mortality rates, impaired bacterial clearance and shifts from pro- to anti-inflammatory cytokine release patterns in the lung. Since our ST2-/- mice are backcrossed to a BALB/c genetic background, we first confirmed the immunosuppressive phenotype and the enhanced susceptibility to secondary pneumonia in BALB/c WT mice, revealing a similarly suppressed lung host defense and attenuated proinflammatory cytokine response during secondary P. aeruginosa pneumonia as earlier reported for C57BL/6, CD-1 and ND-4 mice. The increased susceptibility to Pseudomonas pneumonia after CLP resembles the clinical scenario, where P. aeruginosa is essentially nonpathogenic in the immunocompetent host but a common cause of respiratory tract infection in critically ill patients. In this respect it is important to note that previously healthy mice clear P. aeruginosa from their lungs after infection with bacterial doses that cause mortality after induction of pneumonia following CLP.

The present study identifies ST2 as a negative regulator of inflammation during secondary (post-CLP) Pseudomonas pneumonia. Indeed, although ST2-/- mice had much lower bacterial loads in their lungs 24 hours after induction of pneumonia, they concurrently displayed enhanced lung inflammation as indicated by semi-quantitative pathology scores. At this time point, ST2-/- mice had lower pulmonary cytokine levels when compared with WT mice. We argued that cytokine levels responded more acutely than histopathology to a declining bacterial load and that reduced cytokine concentrations in ST2-/- mice 24 hours after Pseudomonas infection were the consequence of the lower Pseudomonas burden providing a less potent proinflammatory stimulus. To eliminate differences in bacterial loads and to study the early cytokine response to P. aeruginosa infection of the airways, we administered heat-killed bacteria to ST2-/- and WT mice and measured cytokine levels 6 hours later. These experiments revealed modestly (if at all) elevated levels of cytokines and chemokines in the pulmonary compartment of ST2-/- mice, suggesting that ST2 is not a major negative regulator of Pseudomonas induced cytokine production in the lung.

Next, we sought to investigate which cell-type is involved in the CLP-induced immunosuppression in the lung as seen in WT mice when compared to ST2-/- mice. Previous investigations have shown a decrease in production of type 1 cytokines IFN-γ (by T-lymphocytes) and TNF-α (by monocytes/macrophages) during endotoxin tolerance or inflammation-induced immunosuppression. In mice with sublethal abdominal sepsis caused by CLP splenocytes displayed a diminished capacity to release IFN-γ upon incubation with anti-CD3/CD28 (a potent T-lymphocytes agonist). Of considerable interest, treatment of post-CLP mice with a neutralizing anti-IFN-γ antibody prior to induction of secondary Pseudomonas pneumonia strongly enhanced mortality, suggesting that IFN-γ is essential for survival in this “two-hit” model. Since...
ST2 is expressed by CD4+ and CD8+ T-lymphocytes, which are important sources for IFN-γ, the current study investigated the role of ST2 on IFN-γ (and TNF-α) production by these cells. We here show that T-lymphocytes were less capable of producing IFN-γ and TNF-α in mice subjected to CLP 24 hours earlier (i.e. at the time of induction of Pseudomonas pneumonia). Importantly, T-lymphocytes production of these type 1 cytokines was unaltered in septic ST2−/− mice compared to their sham controls, suggesting that the presence of ST2 on CD4+ and CD8+ T-lymphocytes is essential in the immunosuppression observed in WT animals. These data, together with the recently published investigation on the protective role of IFN-γ in post-CLP Pseudomonas pneumonia, suggest that in this model ST2 at least in part impairs host defense by inhibiting IFN-γ production by T-lymphocytes. Notably, this effect of ST2 cannot be examined by measuring IFN-γ levels in vivo, since these remain very low in either plasma or lungs (and data not shown). Our findings corroborate earlier studies describing a role of ST2 in the regulation of effective Th2 responses.

Moreover, administration of the ST2 ligand IL-33 to mice resulted in production of Th2-associated cytokines and an imbalance in the Th1/Th2 ratio. We did not find a role for ST2 in the responsiveness of alveolar macrophages toward Pseudomonas. Indeed, ST2−/− macrophages responded similarly to P. aeruginosa LPS and heat-killed P. aeruginosa, both after CLP and sham surgery. In addition, the capacity of alveolar macrophages from septic mice to produce TNF-α or IL-6 was not significantly decreased when compared with the ability of macrophages from sham operated mice to secrete these cytokines. These data contrast with an earlier report, showing diminished LPS-induced TNF-α production by alveolar macrophages obtained from mice after CLP surgery. Although a clear explanation for this discrepancy is lacking, differences between this and our study include differences in mouse strains (C57BL/6 versus BALB/c), the age range of the animals (6-8 versus 8-10 weeks) and the stimulus (LPS from Escherichia coli versus Pseudomonas LPS and heat-killed P. aeruginosa). The current results argue against an immunotolerant phenotype of alveolar macrophages after CLP and are in line with previous investigations indicating that alveolar macrophages, obtained from the lung of animals or humans with systemic inflammation induced by sepsis or intravenous LPS, do not display tolerance and may even be primed for ex vivo rechallenge with LPS.

A prior study showed ST2 only mitigated the host inflammatory response to a second LPS challenge, while not influencing the reaction to a primary LPS challenge. This prompted us to investigate whether the clear role of ST2 in secondary pneumonia could be reproduced in primary respiratory tract infection caused by Pseudomonas. ST2 did not influence the innate immune response during primary Pseudomonas pneumonia, emphasizing the importance of ST2 during a second “hit” such as nosocomial pneumonia. Clearly, host defense mechanisms operative during primary Pseudomonas pneumonia are markedly different from those active during secondary
respiratory tract infection by this opportunistic pathogen. In this respect it is important to note that IFN-γ plays a protective role during post-CLP *Pseudomonas* pneumonia\textsuperscript{10}, whereas this cytokine impairs host defense in previously healthy mice infected with *P. aeruginosa* via the airways\textsuperscript{48}.

In conclusion, we here established that ST2 is an important factor in the enhanced susceptibility for secondary *Pseudomonas* pneumonia in mice with sublethal polymicrobial abdominal sepsis, possibly by inhibiting type 1 cytokine production by T-lymphocytes.
References

Sepsis-induced suppression of lung host defense is mediated by ST2


Sepsis-induced suppression of lung host defense is mediated by ST2

Online Data supplement

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Cecal Ligation and Puncture (CLP)

CLP was performed as previously described\(^1\)-\(^2\) with minor modifications. Mice were given buprenorfin (Temgesic\(^{\text{®}}\), Schering-Plough, Amstelveen, The Netherlands) 0.075 mg/kg subcutaneously 30 minutes preoperatively. All mice were anesthetized via inhalation of a mixture of O\(_2\) (1-2 l/min) and isoflurane 2.0-2.5% (Burtons, Kent, United Kingdom). During all operations mice were kept on a heating pad at 37°C (Animed, Barneveld, the Netherlands). Under sterile conditions, a 1 cm midline incision was first made on the anterior abdomen and the cecum was exposed. The distal 1 cm of the cecum was ligated (without causing bowel obstruction) with a 4-0 silk suture (Ethicon, Johnson&Johnson, St-Stevens-Woluwe, Belgium) and punctured through-and-through with a 26-gauge needle (Becton Dickinson, Drogheda, Louth, Ireland). A small amount of stool was extruded to ensure wound patency. The cecum was then replaced in the peritoneal cavity and the abdomen was closed in two layers with a running suture using Sofsilk 6-0 (Ethicon). In our hands, ligation of 1 cm of the cecum and puncture with this needle results in a marked septic response and death in 0-10% of animals. Sham-operated (control) animals underwent identical laparatomy, the cecum was exposed but not ligated or punctured and was then replaced in the peritoneal cavity. All mice were administered 1 ml of sterile saline subcutaneously for fluid resuscitation post-operatively and 500 µl saline twice a day thereafter, containing 0.05 mg/kg buprenorfin.
References
