Splenic studies: prevention of pneumococcal disease on organisational, clinical and experimental level

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Chapter 8

Enhanced vulnerability for *Streptococcus pneumoniae* sepsis during asplenia is determined by the bacterial capsule


*Immunobiology 2011*
Part III

Abstract

Patients without a spleen are susceptible for overwhelming sepsis with *Streptococcus pneumoniae*. We investigated the relative contribution of the pneumococcal capsule in the reduced host defense after splenectomy.

Sham-operated or splenectomized mice were inoculated with serotype 2 or 4 *S. pneumoniae* (D39, TIGR4) or the isogenic nonencapsulated mutants (D39Δcps, TIGR4Δcps). After splenectomy, intranasal infection with D39 resulted in increased mortality, increased bacterial dissemination and exaggerated systemic inflammation rather then altering inflammation in the lungs. Intravenous infection also resulted in enhanced mortality, bacterial growth and systemic inflammation after splenectomy. In contrast, the spleen did not contribute to host defense during infection with D39Δcps. Similar observations were made for TIGR4: increased bacterial growth and inflammation after intravenous infection with wild-type, but not nonencapsulated bacteria in splenectomized mice.

These results indicate that the capsule of *S. pneumoniae* is indeed responsible for increased vulnerability of asplenic mice to invasive pneumococcal disease.
Introduction

Individuals with asplenia are known to be at risk for post-splenectomy sepsis (PSS), which carries a high mortality of 50-70%. Encapsulated bacteria are thought to be the responsible pathogens for PSS, since *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis* account for more than half of septic episodes. Although other microorganisms are considered to carry some risk as well, all preventive strategies traditionally have focused on encapsulated pathogens.

The importance of the spleen in host defense against encapsulated bacteria has been invigorated by several experimental models. The first in a series of animal studies was published in 1919, wherein rats proved to be more susceptible to bacterial infection after splenectomy than after orchidectomy. Subsequently, animal studies have focused on *S. pneumoniae*, using different routes of administration and different serotypes, all confirming the capacity of this bacterium to cause severe infection after removal of the spleen. The mechanism of increased susceptibility to infection is thought to be related to the splenic efficient innate and adaptive immunological surveillance of the circulation. Its specific role in the removal of encapsulated bacteria is related to marginal zone macrophages, which are able to detect and capture encapsulated bacteria and marginal zone B cells, which respond to capsule polysaccharide antigens by differentiating into IgM-producing memory B cells or antigen presenting cells.

Although the literature collectively describes an established role of encapsulated bacteria in severe infection after splenectomy, to the best of our knowledge the contribution of the bacterial capsule herein has never been studied directly. We sought to dissect the relative contribution of the pneumococcal capsule versus other virulence factors of the pneumococcus (not residing in the capsule) in the diminished resistance of asplenic mice by comparing the host response of splenectomized and sham-operated mice after pulmonary or systemic infection with wild-type (WT) or isogenic mutant *S. pneumoniae* lacking the polysaccharide capsule. We demonstrate for the first time that it is indeed the capsule of *S. pneumoniae* that is responsible for enhanced susceptibility to infection after splenectomy.

Methods

**Animals**

In all experiments, male, age matched, specific pathogen-free C57BL/6 mice (Charles River, Maastricht, Netherlands) were used. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, Netherlands).
Part III

**Bacteria**

*S. pneumoniae* strains used in this study were WT isolates D39 (serotype 2) and TIGR4 (serotype 4) and their isogenic capsule locus (*cps*) deletion mutants D39Δ*cps* and TIGR4Δ*cps*, which were constructed as described previously.  

**Splenectomy**

Mice were given buprenorphine (Temgesic®, Schering-Plough, Amstelveen, Netherlands) 0.075 mg/kg subcutaneously 15 minutes preoperatively, and anesthetized via inhalation of a mixture of O2 (1-2 l/min) and isoflurane 2.0-2.5% (Abbott, Kent, UK). A 1-cm incision was made in the left flank and the spleen was mobilized. In sham-operated (Sham) mice the spleen was replaced. Splenectomy was performed after ligating efferent and afferent vessels with Sofsilk 4-0 (Tyco Healthcare Group, Connecticut) and skin was closed with Vicryl 4-0 (Ethicon, Johnson&Johnson, Belgium). After 8 hours 0.05 mg/kg buprenorphine was administered. Mice were given 2 weeks to recover before administering *S. pneumoniae*.

**Experimental design**

*S. pneumoniae* strains (WT and Δ*cps*) were grown for 3-6 hours to mid-logarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), with yeast extract (0.5%). Bacteria were harvested by centrifugation (4000 rpm) and washed twice in isotonic saline. For induction of pneumonia, bacteria were administered intranasally (total volume 50 μl) under light anaesthesia by inhalation of isoflurane (Abbott, Kent, UK) as described previously. For systemic infection, bacteria were injected intravenously (total volume 200 μl) via the tail vain. Infectious doses were as described in the Results section and table/figure legends. For determining bacterial loads, mice were sacrificed under isoflurane anaesthesia (2%/2L), samples were collected and processed as described. Briefly, lungs and liver were homogenized at 4°C in 5 volumes of isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK). Homogenates and blood were serially diluted 10-fold in isotonic saline, and 50μl volumes were plated onto sheep-agar plates, incubated overnight at 37°C and colony forming units (CFU) were counted. Homogenates were prepared for cytokine measurements in lysis buffer containing 300mM NaCl, 30mM Tris, 2mM MgCl₂,6H₂O, 2mM CaCl₂,2H₂O and 1% Triton X-100 (pH 7.4) with 0.5 ml protease-inhibitor (Roche Complete, 1 tablet in 5 ml demi-water), incubated for 20 minutes (4 °C), centrifuged 10 minutes (3600 rpm) and supernatants were stored at -20 °C until assays were performed.

**Assays**

Lung cytokines and chemokines (TNF-α, keratinocyte chemoattractant (KC/CXCL1), and macrophage inflammatory protein 2 (MIP-2/CXCL2) were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to manufacturers’ instructions.
Plasma TNF-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemoattractant protein-1 (MCP-1/CCL2) were measured using a commercially available cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with manufacturers’ recommendations.

Aspartate aminotransferase (ASAT) (U/L), alanine aminotransferase (ALAT) (U/L) and lactate dehydrogenase (LDH) (U/L) were determined in plasma with a fully automated Roche Modular P800 analyzer (Roche Diagnostics, Basel, Switzerland).

**Histology**

Lungs were fixed in 4% formalin and embedded in paraffin. Five μm sections were stained with hematoxylin and eosin (HE). All slides were analyzed by a pathologist blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis and endothelialitis. Each parameter was graded 0 to 4, with 0 being ‘absent’ and 4 being ‘severe’. Total ‘lung inflammation score’ was expressed as the sum of the scores for each parameter. Granulocyte staining was done using FITC-labeled rat anti-mouse Ly-6 mAb (Pharmingen, San Diego, CA) as described earlier. Ly-6G expression in the lung tissue sections was quantified by digital image analysis. In short, lung sections were scanned using the Olympus Slide system (Olympus, Tokyo, Japan) and TIF images, spanning the full tissue section were generated. In these images Ly6G⁺- and total surface area were measured using Image J (U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij), the amount of Ly6G positivity was expressed as a percentage of the total surface area.

**Statistical analysis**

Statistics were performed with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA. Data are given as means ± SEM or as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. Differences between groups were analyzed using Mann-Whitney U test. For survival analyses, Chi-square tests were performed at different time points for the proportion of survivors. A value of p<0.05 was considered statistically significant.

**Results**

*Splenectomy results in an enhanced mortality after induction of pneumonia with D39 but not with D39Δcps*

First we investigated the impact of splenectomy on the outcome of pneumonia with WT (serotype-2-encapsulated) D39. For this we infected mice intranasally with D39 at a dose known to
be nonlethal to normal WT mice (4 x 10^5 CFU)\textsuperscript{21} two weeks after splenectomy (Splx-mice) or sham surgery (Sham-mice), and followed them for 48 hours (Table 1A). As expected, all Sham-mice survived. In sharp contrast, 6 of 7 Splx-mice died within the 48-hour observation period (P < 0.01 versus sham). Intranasal infection with D39 at a lower dose (4 x 10^4 CFU) resulted in death in 1/8 Splx-mice versus none in Sham-mice.

Pneumonia caused by D39Δcps in doses up to 4x10^7 CFU was not associated with mortality in either Splx-mice or Sham-mice (Table 1B). Of note, in our hands mortality after infection with D39Δcps does not occur beyond 24 hours of infection (data not shown). These data demonstrate that splenectomy renders mice highly susceptible to mortality caused by pneumonia with encapsulated D39.

<table>
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<tr>
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<td>Splx</td>
<td>Sham</td>
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<td>8/8</td>
<td>6/6</td>
<td>7/7</td>
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<tr>
<td>48h post-inoculation</td>
<td>Sham</td>
<td>Splx</td>
<td>Sham</td>
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<tr>
<td>6/6</td>
<td>7/8</td>
<td>6/6</td>
<td>1/7 **</td>
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<th>Dose 4x10^7 CFU</th>
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<td>Splx</td>
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<td>48h post-inoculation</td>
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<td>8/8</td>
<td>8/8</td>
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</table>

Table 1. Mortality after intranasal infection with (encapsulated) S. pneumoniae D39 (A), and (nonencapsulated) D39Δcps (B). (**) P = 0.002, by Chi-square test

*Splenectomy results in enhanced dissemination of D39 but not of D39Δcps after induction of pneumonia*

Next we wished to establish whether the increased mortality of Splx-mice after induction of pneumonia with D39 was associated with an accelerated bacterial growth at the primary site of infection and/or at distant body sites. For this purpose we intranasally infected Splx- and Sham-mice with 8 x 10^5 CFU D39 (i.e. a dose expected to cause mortality in Splx-mice beyond the 24-hour time point) and determined bacterial loads in lungs, blood and liver 24 hours post infection. The numbers of D39 harvested from lungs were similar in Splx- and Sham-mice (Figure 1A). In contrast, D39 loads were much higher in blood (Figure 1B, P < 0.05) and liver (Figure 1C, P < 0.005) of Splx-mice when compared with Sham-mice. To directly assess the contribution of the pneumococcal capsule to the increased dissemination of D39 after induction of pneumonia in
Splex-mice, we intranasally infected Splex- and Sham-mice with D39Δcps (1 x 10^7 CFU). Because in earlier experiments we had established that D39Δcps is cleared rather rapidly (data not shown), we harvested lungs, blood and liver 6 and 24 hours after infection. The numbers of D39Δcps recovered from lungs were similar in Splex- and Sham mice at both time points, and the majority of mice in both groups had cleared D39Δcps from their lungs at 24 hours (Figure 1D). Blood cultures only became positive in 2/8 Splex-mice and 1/8 Sham-mice 6 hours after infection, whereas none of the animals in either group had a positive blood culture at 24 hours. Liver cultures remained sterile at both time points in both groups.

**Figure 1.** Splenectomy results in increased bacterial outgrowth in distant body sites after induction of pneumonia with (encapsulated) *S. pneumoniae* D39 but not with (nonencapsulated) D39Δcps. Bacterial loads in lung, blood and liver in sham operated and splenectomized (Splex) mice 24 hours after intranasal infection with 8x10^6 CFU D39 (A-C), and in lungs of mice 6 and 24 hours after infection with 1x10^7 CFU D39Δcps (D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8-10 mice per group). In panel D, 24 hour time point, the numbers indicate the number of positive cultures. * P < 0.05, ** P < 0.005 versus sham-operated mice, by Mann-Whitney U test.
Splenectomy has no impact on lung inflammation during pneumonia with D39 or D39Δcps

Previous studies have suggested that splenectomy may influence innate immune responses in the lung. To obtain insight into the impact of splenectomy on lung inflammation during pneumococcal pneumonia and the influence of the pneumococcal capsule herein, we performed semi-quantitative analyses of lung histology slides prepared from Sham and Splx-mice 24 hours after intranasal infection with D39 (8 x 10^5 CFU) or 6 hours after infection with D39Δcps (1 x 10^7 CFU). The extent of lung pathology did not differ between Splx- and Sham mice after intranasal infection with either D39 or D39Δcps, as determined by the scoring system outlined in Methods section (Figure 2A-D). In addition, the extent of neutrophil influx into lung tissue did not differ between groups, as reflected by Ly-6G stainings (insets in Figure 2). Furthermore, except for IL-10 (which was modestly higher in Splx-mice), splenectomy was not associated with differences in cytokine or chemokine concentrations in the lung after infection with D39 (Table 2A). Infection with D39Δcps also did not result in differences in lung cytokine/chemokine levels between Splx- and Sham-mice, except for MCP-1, which was slightly higher in the former group (Table 2A).

![Image](image_url)

**Figure 2.** Lung pathology after intranasal infection with D39 and D39Δcps.

Representative slides of lung tissue obtained 24 hours after induction of pneumococcal pneumonia with D39 S. pneumoniae (upper panels; A-Sham and B-Splx) and with D39Δcps S. pneumoniae (lower panels; D-Sham and E-Splx). Haematoxylin and eosin staining, showing levels of inflammation. Magnification 10x. **Insets:** representative slides of lung Ly-6G staining (brown), showing influx of neutrophils.

Findings are quantified by total pathology scores (TLIS) and scores of pulmonary Ly-6G at 24 hours after induction of pneumococcal pneumonia with D39 (C) and D39Δcps (F). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8-10 mice per group); statistics between the groups were computed by Mann-Whitney U test.
<table>
<thead>
<tr>
<th></th>
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<th>D39Δcps</th>
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<tbody>
<tr>
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<tr>
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<td>IL-10</td>
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<td>MCP-1</td>
<td>6166±1626</td>
<td>2143±242</td>
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<td></td>
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<td>3040±277*</td>
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<tr>
<td><strong>B</strong></td>
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<tr>
<td></td>
<td>Plasma</td>
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<tr>
<td>TNF-α</td>
<td>118±70</td>
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<tr>
<td>IL-6</td>
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<td>LDH</td>
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<td>121±20</td>
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**Table 2.** Pulmonary and plasma cytokine concentrations and clinical chemistry after induction of pneumonia with (encapsulated) *S. pneumoniae* D39 or (nonencapsulated) D39Δcps.

Lung (A) and plasma (B) cytokines were determined by CBA, samples were obtained at 24 hours after infection with D39 (8 x 10^5 CFU) and at 6 hours after infection with D39Δcps (1 x 10^8 CFU), all concentrations are in pg/ml. Aspartate aminotransferase (ASAT) (U/L), alanine aminotransferase (ALAT) (U/L), and lactate dehydrogenase (LDH) (U/L) were determined in plasma obtained at 24 hours after infection (for further description see Methods section).

Data are means ± SEM, n= 8-10 mice per group. * indicates P<0.05, ** indicate P<0.005, *** indicate P< 0.001, by Mann-Whitney U test.

*Splenectomy impacts systemic inflammation during pneumonia with D39, but not with D39Δcps.*

The results described above established that splenectomy does not influence bacterial growth or pathology in the lungs during pneumonia, while strongly increasing bacterial loads in distant body sites and mortality after infection with D39. In order to obtain more insight into the cause of death in Splx-mice during pneumonia, we next investigated the extent of systemic inflammation in Sham and Splx-mice after induction of pneumonia with either D39 or D39Δcps. Splenectomy was associated with markedly elevated plasma concentrations of TNF-α, IL-12, MCP-1 and IL-10 after infection with D39, as compared with Sham-operated mice (Table 2 B). Such differences between
Sham and Splx-mice were not observed after intranasal infection with D39Δcps. In addition, Splx-mice displayed evidence of distant organ damage during pneumonia with D39 (but not with D39Δcps), i.e. the plasma levels of ASAT (indicative of hepatocellular injury) and LDH (indicative of cellular injury in general) were higher in Splx- than in Sham mice (Table 2B).

**Splenectomy causes increased mortality in sepsis with D39, but not with D39Δcps.**

The data presented above strongly suggest that splenectomy has a limited effect on local immune defense within the lungs and that the increased lethality of Splx-mice after intranasal infection with D39 is caused mainly by an enhanced growth of pneumococci at distant body sites. In addition, the pneumonia studies with D39Δcps indicated that nonencapsulated pneumococci are not capable of causing systemic infection after induction of respiratory tract infection in either Splx- or Sham-mice. To further study the impact of splenectomy on host defense against *S. pneumoniae* and the role of the pneumococcal capsule herein, we next infected Splx- and Sham mice with D39 or D39Δcps intravenously. In accordance with earlier studies 11, 12, 24, we found that Splx-mice displayed a strongly increased lethality after intravenous injection of D39 (Table 3A). 48 hours after injection, the low dose (2x10^5 CFU) of D39 was lethal to none of the Sham-mice whereas all Splx-mice died within 24 hours. Intravenous infection with a higher dose of D39 (2x10^6 CFU) was lethal to all Splx-mice within 24 hours as opposed to 1 of 4 Sham-mice. In sharp contrast, splenectomy did not influence mortality after intravenous injection of D39Δcps: whereas all mice injected with 4x10^6 CFU D39Δcps survived (Table 3B), all mice injected with 4x10^7 CFU succumbed very rapidly (within one hour) irrespective of the group (Splx or Sham) (Table 3B).

<table>
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<td>4/4</td>
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*Table 3.* Mortality after intravenous infection with (encapsulated) *S. pneumoniae* D39 (A), and (nonencapsulated) D39Δcps (B). (**) P=0.008, by Chi-square test
These data demonstrate that splenectomy renders mice highly susceptible to mortality caused by primary sepsis with encapsulated but not nonencapsulated D39, unless given in an extremely high concentration.

*Splenectomy results in enhanced growth of D39, but not of D39Δcps after intravenous infection*

We next determined the impact of splenectomy on the growth of D39 and D39Δcps in the systemic infection model. For experiments with D39 we chose to euthanize the mice 24 hours after infection with $8 \times 10^3$ CFU, i.e. a dose not expected to cause lethality in either group within 24 hours. As expected $^{9-12}$, Splx-mice had much higher D39 loads than Sham-mice in all body compartments tested (blood: $P<0.01$, lung: $P<0.0001$, and liver: $P=0.0001$; Figure 3A).

For experiments with D39Δcps, we determined bacterial loads at 6 hours after intravenous infection with $2 \times 10^7$ CFU of D39Δcps. At this time point, all mice had cleared D39Δcps from blood and liver, whereas in lungs of 1/9 Sham- and 2/9 Splx-mice there was minor bacterial outgrowth (no significant difference). Therefore, next we chose to euthanize mice at 2 hours after infection, examining 4 different doses of D39Δcps (i.e. $3 \times 10^7$, $10^6$, $10^5$ and $10^4$ CFU). After infection with $3 \times 10^7$ CFU, bacterial loads in blood, lung and liver were not significantly different between Sham and Splx-mice (Figure 3B). After administration of lower inocula of D39Δcps there were no significant differences in lung and liver cultures, and bacteria were cleared from the blood except for 1/8 Splx-mice.
Figure 3. Splx results in enhanced bacterial outgrowth after intravenous infection with (encapsulated) S. pneumoniae D39, but not with (nonencapsulated) D39Δcps.

Bacterial loads of plasma, lung and liver in Sham and splenectomized (Splx) mice 24 hours after intravenous infection with $8 \times 10^8$ CFU D39 (A) and 2 hours after intravenous infection with $3 \times 10^7$ CFU of D39Δcps (B). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group).

** indicate $p<0.01$, *** $p<0.0001$ versus sham-operated mice, by Mann-Whitney U test.

**Splenectomy results in exaggerated systemic inflammation after intravenous infection with D39, but not with D39Δcps**

Next, we determined the extent of systemic inflammation in Sham and Splx-mice after intravenous infection with D39 ($8 \times 10^8$ CFU; at 24 hours) or D39Δcps ($2 \times 10^7$ CFU; at 6 hours). Splenectomy resulted in markedly elevated plasma levels of all cytokines and chemokines measured (Table 4). Similarly, the concentrations of these mediators were also much higher in lungs and livers from Splx-mice, when compared with Sham mice (data not shown). In contrast, after infection with D39Δcps the plasma levels of TNF-α, IL-12, IFN-γ and MCP-1 were lower in Splx-mice. Moreover, Splx-mice demonstrated evidence of distant organ injury after intravenous infection with D39 (but not with D39Δcps), as reflected by much higher plasma levels of ASAT, ALAT and LDH (Table 4). Lung and liver homogenates did not show any differences in concentrations of cytokines or chemokines after infection with D39Δcps (data not shown).
Table 4. Plasma cytokine concentrations and clinical chemistry after intravenous infection with (encapsulated) *S. pneumoniae* D39 or (nonencapsulated) D39Δcps.

Mice were intravenously infected with D39 (8 x 10^7 CFU) or D39Δcps (2 x 10^7 CFU). Plasma samples were obtained at 24 hours after infection with D39 and at 6 hours after infection with D39Δcps. All cyto- and chemokine values are in pg/ml. Aspartate aminotransferase (ASAT) (U/L), alanine aminotransferase (ALAT) (U/L), and lactate dehydrogenase (LDH) (U/L) were determined in plasma (for further description see Methods section). Data are means ± SEM (n=8-9 per group). * indicates P= 0.05, ** indicate P< 0.005, *** indicate P<0.0005 versus sham operated mice, by Mann-Whitney U test.

**Splenectomy also enhances bacterial growth after intravenous infection with TIGR4, but not with TIGR4Δcps**

D39 is a serotype 2 pneumococcus. To increase generality of our findings in the intravenous model, we next assessed the impact of splenectomy on bacterial growth after intravenous infection with a serotype 4 *S. pneumoniae* strain (TIGR4) or the isogenic nonencapsulated mutant strain TIGR4Δcps. In strict accordance with the results obtained with D39, intravenous administration of 10^3 CFU TIGR4 resulted in much higher (4 to 6 orders of magnitude) bacterial loads in blood, lungs and liver in Splx-mice when compared with Sham mice (data not shown). In contrast, both Sham and Splx-mice rapidly cleared TIGR4Δcps (given intravenously at a dose of 3 x 10^5 CFU), with only 2/9 Sham mice with positive blood cultures 6 hours post infection versus none of the Splx mice (not different between groups).
Discussion

Asplenia renders the host at increased risk for pneumococcal pneumonia and sepsis with fatal outcome. The bacterial capsule is considered an important virulence factor of \textit{S. pneumoniae} in general and especially in asplenic patients. The pneumococcus expresses multiple virulence factors that are unrelated to the capsule (reviewed in \cite{25}). In the present study we addressed the question whether virulence factors other than the capsule contribute to the enhanced susceptibility to severe pneumococcal infection in the asplenic host.

\textit{Respiratory tract infection}

We demonstrate that after splenectomy, mice display a strongly impaired host defense and enhanced mortality during respiratory tract infection caused by encapsulated but not by nonencapsulated \textit{S. pneumoniae}. This is caused by an accelerated growth and dissemination to distant body sites in the presence of an unremarkable antibacterial defense in the lungs. During infection with either D39 or D39\textit{Δcps} via the airways, splenectomy had no impact on bacterial growth in the lungs. In addition, lung inflammation accompanying pneumococcal pneumonia was similar in Splx- and Sham-mice, as reflected by histopathology, neutrophil influx and pulmonary cytokine/chemokine concentrations. In literature however, several investigations have pointed to local pulmonary mechanisms contributing to the increased vulnerability to pneumococcal pneumonia after splenectomy. Indeed, it has been suggested that the spleen has the capacity to modulate lung immunity.\cite{22, 23}. Specifically, the experiments showed that alveolar macrophages of Splx-mice displayed diminished bactericidal activity and phagocytosis \textit{ex vivo}, 2 hours after incubation with \textit{S. pneumoniae}. Differences between alveolar macrophages of Sham and Splx-mice after saline pretreatment however were obvious only early after inflammation (at 2 hours).\cite{22, 23}. Our findings do not support diminished alveolar clearance; rather our results strongly suggest that the immune defect during respiratory tract infection by \textit{S. pneumoniae} after splenectomy exclusively resides in the systemic compartment, leaving local pulmonary defenses unaffected.

\textit{Systemic infection}

Our finding that mice subjected to splenectomy have a strongly increased mortality after \textit{S. pneumoniae} sepsis confirms previous studies.\cite{9-13}. Present observation studies show that all Splx mice die within 24 hours after systemic infection with D39, which was associated with impressive differences in bacterial growth in lungs as well as blood and livers of Splx mice, as compared to Sham operated animals. Furthermore, after induction of D39 sepsis, all cytokines (TNF-α, IL-6, IL-10, IL-12, IFN-γ and MCP-1) were significantly increased after Splx as compared to Sham, most likely secondary to the much higher bacterial loads in Splx-mice, which likely provided a more potent stimulus for the induction of cytokine release in the circulation. The capacity of nonencapsulated \textit{S. pneumoniae} to elicit systemic cytokine release was low, most likely due to the
rapid clearance of the bacteria and the absence of the proinflammatory properties of the capsule. Interestingly, cytokine production was even lower during D39Δcps sepsis after splenectomy as compared to sham. Since bacterial loads of D39Δcps were similar in both groups, this probably reflects the contribution of the spleen to the systemic immune response.

The mechanism by which the spleen confers protection against *S. pneumoniae* within the circulation is at least in part by providing an optimal environment for antigen clearance and immunologic surveillance within the circulation. The spleen is especially important for IgM and IgG2 antibody responses to polysaccharide antigens, generated by B cells in the marginal zone. After splenectomy this antibody response is attenuated, resulting in a reduced recognition of carbohydrate neoantigens and a delayed bacterial clearance upon entry in the circulation. Moreover, mouse splenic marginal zone macrophages express a C-type lectin called Specific ICAM-3-grabbing nonintegrin-related 1 (SIGNR1), the murine homolog of the human dendritic cell-specific SIGN (DC-SIGN), which plays an eminent role in capturing encapsulated *S. pneumoniae* from the circulation. The capsule of *S. pneumoniae* is therefore a major virulence factor by virtue of its capacity to impede opsonization by immunoglobulin and complement, and to inhibit phagocytosis. Several other components are necessary for its full virulence, most notably pneumolysin, pneumococcal surface protein A and C, lipoteichoic acid and phosphorylcholine.

We here directly addressed the question whether host defense against other pneumococcal virulence factors also in part relies on an intact spleen. The experiments using isogenic mutant serotype 2 and 4 *S. pneumoniae* lacking a capsule clearly demonstrate that even in the absence of a spleen nonencapsulated pneumococci are rapidly cleared after infection. Although we could not confirm this with our experiments, the liver may at least partially compensate for the missing spleen. Is has been described that 70% of the bacteria entering the bloodstream become entrapped by Kupffer cells and hepatocytes. Conceivably, during infection with a non-virulent nonencapsulated bacterium, hepatic clearance might be sufficient to sterilize the bloodstream independently of the spleen.

Recently, the spleen has been identified as an essential peripheral part of the “cholinergic anti-inflammatory reflex.” In this pathway, enhanced efferent activity of parasympathetic nerve endings results in the release of acetylcholine, which by a specific action on α7 cholinergic receptors on macrophages suppresses proinflammatory cytokine production. Splenectomy disrupts this neural-based inhibitory system, which may have contributed to the strongly exaggerated systemic cytokine response in Splx-mice. However, Splx-mice showed lower plasma levels of TNF-α, IL-12, IFN-γ and MCP-1 upon intravenous infection with D39Δcps, arguing against a significant role for the cholinergic anti-inflammatory pathway via the spleen in this condition.

Of note, infection with D39 resulted in mortality between 24 and 48 hours after infection. Although this time frame seems relatively brief, all mice were from the same genetic background and infected with exactly the same inoculum, resulting in minimal variability. The exact cause of
death cannot be dissected from our data; it is likely, however, that mice died from a combination of severe pneumonia (with high bacterial loads in lungs and an associated local inflammatory response) and systemic inflammation with associated distant organ injury (see tables 2 and 4).

**Conclusion**

Splenectomy results in a strongly increased susceptibility to severe infections caused by *S. pneumoniae*. We here demonstrate that pneumococcal strains that are lacking the capsule but still express other important virulence factors are rapidly cleared even in the absence of the spleen. As such, our data provide solid evidence that indeed the capsule has a prominent role in the enhanced vulnerability to pneumococcal disease in patients after splenectomy or with functional asplenia.
References