Sex-Based Differences in Susceptibility to Respiratory and Systemic Pneumococcal Disease in Mice

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Systemic infection with Streptococcus pneumoniae was investigated in male and female mice in models of invasive pneumonia and sepsis. Male mice were found to be more susceptible to infection, exhibiting greater weight loss, marked decrease in body temperature, and a significantly higher mortality rate compared with female mice. For pneumonia, there were significant differences in survival rates. Female mice cleared their lung infections over time, whereas male mice, compared with female mice, had significantly increased numbers of colony-forming units in early stages of infection accompanied by higher levels of neutrophil recruitment in the first 24 hours after infection. Importantly, there were significant increases in proinflammatory cytokine levels during both sepsis and pneumonia in male compared with female mice. These cytokines were indicative of T-helper 1–type responses. The data presented here describe surprising differences in survival rates, neutrophil recruitment, and proinflammatory cytokine levels, indicating a sex-based difference in susceptibility to respiratory and systemic pneumococcal disease.

Streptococcus pneumoniae (pneumococcus) is a major human pathogen responsible for an extensive burden of human disease and death. It is a major cause of otitis media, bacterial meningitis, and septicemia [1–3]. It is also the most common bacterial respiratory pathogen in the United Kingdom, frequently causing community-acquired pneumonia and resulting in mortality rates of >20% for those with concurrent pneumococcal septicemia [4, 5]. Worldwide, the situation is worse, with pneumococcal septicemia as the major cause of infant mortality in developing countries, causing ~25% of all preventable deaths in children under the age of 5 years, with an annual burden of ~1 million infant deaths [6]. Consequently, the investigation of pathophysiology in pneumococcal disease is of basic scientific and clinical importance because the elucidation of these processes could offer new targets for vaccination and therapeutics.

In terms of the proportion of individuals infected and the severity of infection, human males in general have a higher burden of viral, bacterial, fungal, and parasitic diseases than females [7–10]. Differences in human susceptibility to invasive diseases such as pneumococcal pneumonia have been described recently, including pneumococcal pneumonia, in which the incidence is higher in males than in females in all age groups studied [11–14]. Furthermore, it has been reported that males have a significantly higher incidence of pneumococcal septicemia [15–17] and meningitis [18, 19] than females. Interestingly, the sex difference in susceptibility to pulmonary infection is also present in young infants [13]. It has been shown that male infants are more susceptible to infection and sepsis, with greater associated morbidity.
and mortality than female infants [14, 20, 21]. In general, females respond better to vaccination with higher immunoglobulin levels [22–24]. Evidence from other disease studies indicate that sex-specific differences in host immunity, such as differences in neutrophil apoptosis and cytokine secretion patterns, are linked to the presence of female sex hormones [20, 24]. It has been suggested, however, that increased mortality from infectious diseases in males is related to testosterone-induced immunosuppression in postpubertal males [25]. However, currently there is limited understanding of the molecular and cellular processes that lead to either immunosuppression in males or increased immune function in females. What is generally accepted is the fact that sex-based differences are a consequence of genetic differences that are attributable to X-chromosome inactivation, differences in the expression of steroid hormones, and differences in anatomy [9]. Interestingly, lack of knowledge of sex-based differences in drug study design and analysis has led to standardized treatments for both men and women. Consequently, that has led to differences in drug efficacy and side-effect profiles. For example, in the United States recently, 8 of 10 prescription drugs had to be withdrawn from the market specifically because of drug-related health issues in women [26]. Indeed, the underrepresentation of female subjects in studies of clinical disease has resulted in disparity in both the understanding and the treatment of diseases in the individual sexes [9].

Sex differences in susceptibility to disease have been demonstrated in mouse models of infectious and autoimmune diseases and may be related to differences in the expression pattern of immune response genes [24, 27–30]. Indeed, recent data indicate that there is a clear sexual dimorphism after puberty in innate and adaptive immune response genes in C57BL/6 mice, with innate immune response genes being highly up-regulated in postpubertal male mice but not female mice [28]. In contrast, postpubertal female mice preferentially express adaptive immune response genes, and expression of these genes occurs at lower levels in postpubertal male mice [28]. It is also known that female mice produce higher levels of immunoglobulins to a variety of antigens than do male mice [31–33].

Despite such evidence, an in-depth comparison of the host response to infection to a major bacterial human pathogen has not been reported to date. The analysis of sex-based differences in responses to bacterial infection may have a bearing on differential treatment of female and male patients during inflammatory disease. S. pneumoniae is both the main cause of acute community acquired pneumonia and sepsis in children and the elderly. In addition, mouse models for this pathogen are reliable, consistently reproducible, and well described [34–40]. For these reasons, we investigated the hypothesis that sex differentially affects disease severity, pneumococcal survival in vivo, and cytokine production. We tested this hypothesis by using 2 well-defined models of respiratory and systemic infection in mice.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Media**

Wild-type S. pneumoniae serotype 2, strain D39, was obtained from the National Collection of Type Cultures (NCTC 7466). Pneumococci were grown in brain-heart infusion broth or on blood agar base supplemented with 5% (vol/vol) defibrinated horse blood. When required, suspensions were thawed at room temperature.

**Infection of Mice**

All experiments were carried out in male and female C57BL/6 mice using both intranasal and intravenous challenges. Intravenous infection experiments (sepsis model) were done in Siena, Italy, with mice from Charles River (Italia); the intranasal challenge (pneumonia model) experiments were done in Leicester, United Kingdom, with mice obtained from Harlan. Experiments were done in accordance with respective national and institutional guidelines. Mice were inoculated with 1 × 10⁶ or 1 × 10⁷ colony-forming units (CFU) of pneumococci intranasally for the pneumonia model [36, 38]. Mouse survival, disease signs, and bacterial levels in the lungs and/or blood were measured to evaluate pneumococcal virulence [36].

**Sepsis Model**

Intravenous challenge (sepsis model) experiments were performed using 8-week-old male and female mice in groups of 15. Infections were transmitted by administering 100 μL of tryptic soy broth (TSB) containing 4 × 10⁸ CFU of pneumococci directly into the dorsal tail vein of the mice [37, 38]. The control group was inoculated intravenously with 100 μL of TSB. The inoculum dose was confirmed by viable count after plating on blood agar plates. Signs of disease, weight, and rectal temperature (GTH1170 digital thermometer; Greisinger Electronic) were recorded throughout the experiments. At 6, 12, and 24 hours after infection, blood was collected from a facial vein, and 10 μL of each sample was plated onto blood agar. The remaining sample was clotted at room temperature. All serum samples were stored at −80 °C and brought back to room temperature 30 minutes before testing.

**Pneumonia Model**

Ten male and female mice were 8–10 weeks old when infected and weighed; the mice weighed 25–30 g (Harlan). As described elsewhere [36], mice were lightly anesthetized with 2.5% (vol/vol) flourothane (AstraZeneca) over oxygen (1.5–2 L/min), and 50 μL of phosphate-buffered solution (PBS) containing 10⁶ CFU of S. pneumoniae were administered into the nostrils of the mice. For the lower-dose pneumonia model, 50 μL containing 10⁵ CFU of S. pneumoniae was used. The inoculum dose was confirmed by viable count after plating out on blood agar plates after infections. At prechosen time intervals after infection, groups of mice were terminally anesthetized and bronchoalveolar lung (BAL) lavage specimens were collected with
were significant (\(P < .01\)) in both sepsis (\(A\)) and pneumonia (\(B\)) models. Statistical significance was determined by the log–rank test.

Cytokine Assay
A Bio-Plex immunoassay was performed using a Luminex 100 (Bio-Rad) to quantify cytokines. Serum samples (~100 \(\mu\)L) from sepsis experiments were analyzed using the mouse cytokine 23-Plex assay system (Bio-Rad) for interleukin (IL) 1\(\alpha\), IL-1\(\beta\), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN) \(\gamma\), KC, monocyte chemoattractant protein (MCP) 1 (MCAF), macrophage inflammatory protein (MIP) 1\(\alpha\), MIP-1\(\beta\), RANTES, and tumor necrosis factor (TNF) \(\alpha\). BAL lavage samples from pneumonia experiments were analyzed using the 23-Plex assay system and the 9-Plex assay for IL-15, IL-18, basic fibroblast growth factor, LIF, macrophage colony-stimulating factor (M-CSF), monokine induced by IFN-\(\gamma\) (MIG), MIP-2, platelet-derived growth factor BB, and vascular endothelial growth factor. Serum samples and BAL lavage samples were treated, and the assays were performed according the supplier’s instructions (Bio-Rad). Cytokine concentrations were automatically calculated based on standard curve data using Bio-Plex Manager software (version 4.0). Statistical data evaluation was done using a 2-tailed Mann–Whitney test. All cytokine data not included in the article and their statistical analysis are available on request.

Lung Neutrophil Counts
Lungs were removed from preselected groups of mice, and leukocytes were isolated using methods published elsewhere [36]. Briefly, the lungs were homogenized in the presence of 0.5 mg/mL collagenase (Sigma) and 30 \(\mu\)g/mL (87 units) DNase I (Sigma). After homogenization, cells were washed before final resuspension in 5% fetal bovine serum in Roswell Park Memorial Institute 1640 medium. For differential leukocyte analysis, 50 \(\mu\)L of cell suspension was centrifuged onto cyto spin slides (Shandon), stained using Giemsa stain (BDH), and analyzed at \(\times 400\) magnification. At least 200 cells were counted on each slide.

RESULTS

Sex Difference in Pneumococcal Sepsis
To investigate the effect of sex during pneumococcal sepsis, female and male C57BL/6 mice were infected with an intravenous dose of \(5 \times 10^4\) CFU of \(S.\) pneumoniae D39. A marked difference \((P < .01)\) in susceptibility to infection was observed among female and male mice in terms of survival after intravenous challenge (Figure 1A). As shown in Figure 1A, male mice were significantly more susceptible to sepsis, exhibiting 100% mortality by 48 hours after infection \((P < .01)\), whereas female mice exhibited 55% mortality by 72 hours after infection, with the remaining mice surviving the intravenous challenge. Disease signs were also in accordance with the survival data; a significant difference between male and female mice was apparent by 48 hours after infection, with male mice exhibiting significant weight loss \((P < .05)\) and body temperature decrease \((P < .01)\) compared with female mice at equivalent time points (Figure 2A and B).

Cytokine levels in blood of mice infected intravenously were analyzed using a multiplex bead assay (Bio-Plex; Bio-Rad), which permits reliable quantification of multiple cytokines by flow cytometry. Samples were collected at 0, 6, 24, and 48 hours after infection, and the concentrations of 23 cytokines were
analyzed. After intravenous infection, male mice had significantly increased levels of the cytokines IFN-γ, KC, RANTES, IL-6, IL-17A, IL-12(p70), and G-CSF (Figures 3 and 4 and Table 1) compared with female mice at 24 and 48 hours after challenge ($P < .05$). Only IL-5 levels were significantly ($P < .05$) higher in female mice during sepsis at equivalent time points.

**Sex Difference in Pneumococcal Pneumonia**

To investigate the effect of sex during pneumococcal pneumonia, female and male C57BL/6 mice were infected intranasally with $10^6$ CFU *S. pneumoniae* D39. As in the sepsis model, the survival rates in male and female mice were significantly different ($P < .01$), with male mice exhibiting 100% mortality by 56 hours after infection (Figure 1B). Only 20% mortality was observed in female mice by 72 hours after infection, with none of the survivors showing signs of disease having cleared their infections. To determine bacterial kinetics in lung tissue after respiratory infection, time-pointed infection experiments using $10^6$ CFU and a 10-fold lower dose of $10^5$ CFU were performed. In these experiments, lung samples were collected for bacterial counts, leukocyte counts, and cytokine analysis (Figure 5). In the higher-dose lung infection challenge ($10^6$ CFU/mouse), male mice exhibited significantly greater mortality (Figure 1B) than female mice, and a significantly higher bacterial load 6 hours after infection ($P < .01$) (Figure 5A). However, bacterial counts in lungs of both male and female mice were similar at 24 and 48 hours after infection. Male mice exhibited a significant increase in neutrophil influx into their lungs at 6 and 24 hours after infection, compared with female mice ($P < .01$). However, by 48 hours, there was no significant difference between the 2 groups ($P > .05$) (Figure 5B). In the lower-dose lung infection challenge ($10^5$ CFU/mouse), bacterial counts showed that female mice cleared pneumococci from their lungs within 24 hours, whereas male mice had significantly higher

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**Figure 2.** Disease signs in *Streptococcus pneumoniae* sepsis model. C57BL/6 male (continuous line, filled squares) and female (dashed line, open circles) mice were infected intravenously with $1 \times 10^4$ colony-forming units (CFU) of *S. pneumoniae* D39. Disease signs in each mouse were monitored every 24 hours. A, Median rectal temperatures. B, Percentage of initial weight lost.

**Figure 3.** Cytokines at higher concentrations in mice with sepsis than in mice with pneumonia. Groups of male (blue) and female (red) mice were infected either intranasally, in the pneumonia model (open symbols), or intravenously, in the sepsis model (filled symbols). Graphs show mean cytokine concentrations plus standard deviations (sepsis, $n = 15$; pneumonia, $n = 10$) plus standard deviation. Abbreviations: IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.
pneumococcal numbers at 24 and 48 hours after infection (P < .01) (Figure 5C). In contrast to the higher-dose model, neutrophil influx was significantly greater in female mice than in male mice by 6 hours after infection (P < .01). In male mice there was an increased neutrophil influx by 24 hours after infection (Figure 5D).

Cytokine levels in lung lavage samples of intranasally infected mice were analyzed at 0, 6, 24, and 48 hours after infection. No

Table 1. Overview on Cytokines Showing Significant Differences in Concentration Between Male and Female Mice after Pneumococcal Infection

<table>
<thead>
<tr>
<th>Difference</th>
<th>Th1-Type cells</th>
<th>Macrophages and Dendritic cells</th>
<th>Neutrophils</th>
<th>Th2-Type Cells</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher in male mice in sepsis</td>
<td>IL-12(p70), IFN-γ, RANTES</td>
<td>IL-6</td>
<td>IL-17, KC</td>
<td>...</td>
<td>G-CSF</td>
</tr>
<tr>
<td>Higher in female mice in sepsis</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>IL-5</td>
<td>...</td>
</tr>
<tr>
<td>Higher in male mice in pneumonia</td>
<td>IL-18, RANTES, TNF-α, MIP-1α</td>
<td>IL-1β</td>
<td>MIP-2, IL-17, KC</td>
<td>IL-15</td>
<td>IL-1α, MIP-1α, MIP-1β, LIF</td>
</tr>
<tr>
<td>Higher in female mice in pneumonia</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>IL-5</td>
<td>...</td>
</tr>
</tbody>
</table>

Statistical significance at P < .05.

Abbreviations: G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; KC, keratinocyte-derived chemokine; LIF, Leukemia Inhibitory Factor; MIP, macrophage inflammatory protein; Th1, T-helper 1; Th2, T-helper 2; TNF, tumor necrosis factor.

* Cytokines are grouped according to functional categories based on the literature; only those with significant increase at 6 hours after infection are shown (statistical analysis by Mann–Whitney U test).
significant difference was observed in the cytokine patterns in the 2 experiments with different challenge doses; thus, both sets of pneumonia data were pooled for statistical analysis. Male mice exhibited significantly increased levels of TNF-α, RANTES, KC, MIP-2, IL-1β, IL-17A, IL-18, and GM-CSF in lung lavage samples compared with female mice at 24 and 48 hours after infection (Figures 3 and 4). As during sepsis experiments, only the level of IL-5 was significantly (*P* < .05) higher in female mice than in male mice during pneumonia experiments at 48 hours.

The mice tested with this intranasal infection showed early increases in levels of most of the neutrophil- and macrophage-related cytokines assayed (IL-6, IL-1β, MIG, MCP-1, GM-CSF, MIP-2, IL-17A, KC), whereas levels of those related to a T-helper 1 (Th1)–type response increased less or later than in sepsis (Figures 3 and 4). All levels of cytokines related to neutrophil infiltration and chemotaxis were significantly higher in male mice (MIP-2, IL-17A, KC), as were levels of cytokines related to Th1-type response (IL-18, IFN-γ, RANTES, TNF-α, MIP-1α), indicating a higher proportion of septic mice in the male group.

**Cytokine Differences in Male and Female Mice During Sepsis and Pneumonia**

Male mice exhibited significantly increased levels of several cytokines during both sepsis and pneumonia compared with female mice. Increases in IL-6, IL-12(p70), IFN-γ, and G-CSF levels were seen only during sepsis, whereas increases in IL-1β, IL-15, IL-18, TNF-α, MIP-2, and GM-CSF levels were seen only during pneumonia. Increased levels of the cytokine IL-17A and the chemokines RANTES and KC were seen in both sepsis and pneumonia in male mice. During both sepsis and pneumonia,
only IL-5 levels were higher in female than in male mice (Figure 6 and Table 1).

**DISCUSSION**

It has been known for some time that sex is a contributing factor in the incidence and progression of a number of inflammatory diseases of autoimmune origin [41, 42]. There is also evidence to suggest that this is true for the incidence of bacterial disease in humans; several studies have shown that susceptibility to invasive bacterial disease is higher in male than in female humans in all age groups studied [11–14]. The notion that males are more susceptible to bacterial disease is further supported by data from mouse models of bacterial infection and endotoxic shock, where female mice exhibit longer survival than male mice when subjected to severe sepsis [42]. Sex-based differences have also been observed in other mouse models of disease, for example, where *Mycobacterium marinum* infection leads to increased disease severity, higher levels of bacterial burden, and increased mortality rates in male mice compared with female mice [43]. Studies in mice have also provided evidence to suggest that bacterial sepsis in males is associated with increased levels of inflammatory cytokines such as TNF-α, correlating with worse disease prognosis for males [10]. Despite the demonstration of such differences, a detailed analysis of sex-based differences in response to a major human bacterial pathogen has not been performed. Therefore, in this study, we provide clear evidence to show that male C57Bl/6 mice are significantly more susceptible to respiratory infection and sepsis caused by pneumococci, with increased disease severity, significantly higher bacterial burden, and significantly increased mortality rates compared with female mice. To our knowledge this is the first such study for pneumococcal disease. We found that mortality rates in the pneumonia model seem not to correlate directly with the overall bacterial burden in lung. Although there are significantly more CFUs in lungs of male mice at 6 hours after infection than in lungs of female mice, we found that these differences disappear by 24 hours. These findings suggest that the reasons behind male mortality during respiratory infection may have less to do with total bacterial burden overall than with the early, sharp increase in CFUs in lung of male mice, which could act as a trigger for sustained levels of inflammation over time. The overall implication is that male mice exhibit potent immune responses (triggered by early increases in pneumococcal bacterial numbers), which are more vigorous than those in female mice, and that this increased level of response plays a key role in the progression of severe inflammation in lungs and reduced host survival.

As part of our effort to elucidate these differences, we analyzed the levels of 32 cytokines likely to play a role in lung inflammation and sepsis over a 48-hour period. We found that during pneumonia, male mice exhibited a cytokine profile consistent with severe inflammation associated with the recruitment of neutrophils, which are the major effector cells in host pulmonary defense against pneumococcal infection [44–48]. Levels of the proinflammatory cytokines IL-1β, IL-6, and IL-17A; the neutrophil chemoattractants KC and MIP-2; and the activator/proliferator of monocytes, macrophages, and neutrophils (M-CSF, MCP-1, GM-CSF) were all significantly higher in lung samples from male mice at 24 hours after infection than in samples from female mice. By 48 hours after infection, levels of IL-17A, IL-12(p40), and the CD4 T-cell chemoattractant RANTES were significantly higher in male than in female mice. In sepsis, the levels of inflammatory mediators that precipitate the systemic effects associated with sepsis, that is, IL-6, IFN-γ, TNF-α, and IL-12(p70), were significantly higher in male than in female mice.

One reason for this finding could be that because male mice have more pneumococcal CFUs in their lungs in the crucial early stages of infection (the first 6 hours), they may also have higher pneumolysin (PLY) levels in their lungs, which would drive the release of proinflammatory cytokines along with increased recruitment of neutrophils. We have recently shown that the PLY can dramatically amplify the production of proinflammatory cytokines from macrophages and dendritic cells, such as IFN-γ, IL-17A, IL-6, IL-12, TNF-α, and IL-1β (via the activation of the NLRP3 inflammasome) [49]. Another possibility is that male mice may have lower levels of cytokines and regulatory T cells, which may normally control the severity of inflammation and progression of disease.

Indeed, it has been suggested that females are protected from endotoxic shock by producing lower levels of inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) that cause the systemic effects of sepsis and also by increasing the expression of immunosuppressive cytokines (such as IL-10), which down-regulate systemic inflammation [42]. Such a theory is in line with data published elsewhere, which show that female-derived splenic macrophages secrete higher levels of IL-10 than male-derived macrophages after exposure to lipopolysaccharide [50] and that peritoneal macrophages derived from young male mice produce higher levels of proinflammatory cytokines, such as IL-1β and IL-6, when exposed to lipopolysaccharide compared with female-derived macrophages [10, 50]. Further investigation of this sex-based difference in pneumococcal disease will be of interest.

We have shown that potent proinflammatory cytokines are highly elevated in male mice experiencing pneumonia and that these levels correlate with greater neutrophil recruitment into lungs and significantly increased host mortality. Female mice, which do not produce such high levels of proinflammatory cytokines, had a slower neutrophil recruitment rate in the first 24 hours after infection, during the higher-dose challenge experiments and survived their respiratory infection despite exhibiting equivalent numbers of pneumococci in their lungs after 24 hours. It seems that increased host mortality is linked to
higher levels of proinflammatory cytokines and heavily sustained neutrophil recruitment in the early stages after infection, rather than overall pneumococcal numbers in lungs at later stages of infection. This is evidenced by the same pattern of cytokine release, heavy neutrophil infiltration, and increased mortality in male mice infected with a 10-fold lower pneumococcal challenge dose but with a similar 6-hour increase in pneumococcal numbers. Therefore, it seems that male mice respond rapidly and strongly to bacterial infection in the crucial early stages of infection (the first 6 hours). This early potent response is highly proinflammatory and damaging to the host owing to sustained increase in levels of proinflammatory mediators, regardless of consequent increases in bacterial numbers. This observation is true for male mice in models of both respiratory infection and systemic disease.

In sepsis, male mice exhibit high and sustained levels of the classic proinflammatory cytokines associated with bacterial sepsis, that is, TNF-α as well as IL-6, IFN-γ, and IL-12(p70), compared with female mice. Once again, the highly elevated levels of these cytokines correlate with host death in male mice in this model. On the other hand, female mice with sepsis do not express significant levels of these cytokines and survive sepsis. One interesting difference in female compared with male mice is the significantly increased levels of IL-5 at 24–48 hours after infection. IL-5 is a T-helper 2 (Th2)-type cytokine that is important in B cell activation and subsequent production of immunoglobulin M and A. Both antibodies are important in pneumococcal clearance.

We have shown that the male host responds vigorously, but detrimentally, to pneumococcal infection with an early and sustained heavy neutrophil recruitment accompanied by significant increases in proinflammatory cytokines in both respiratory and systemic disease models. This response positively correlates with significantly increased host mortality but does not correlate with clearance of pneumococci from infected lungs. In contrast, female mice exhibit significantly lower levels of all proinflammatory cytokines with delayed neutrophil recruitment into lungs and survive the infection of their lungs and manage to clear pneumococci. Our findings demonstrate that greatly increased levels of proinflammatory cytokines and prolonged recruitment of neutrophils play a key role in the progression of disease and survival in male hosts.

**Notes**

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