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Elevated Expression of IL-23/IL-17 Pathway-Related Mediators Correlates with Exacerbation of Pulmonary Inflammation During Polymicrobial Sepsis¹

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Abstract

Sepsis is a leading cause of death in the United States, claiming more than 215,000 lives every year. A primary condition observed in septic patients is the incidence of acute respiratory distress syndrome (ARDS), which is characterized by the infiltration of neutrophils into the lung. Prior studies have shown differences in pulmonary neutrophil accumulation in C57BL/6J (B6) and A/J mice after endotoxic and septic shock. However, the mechanism by which neutrophils accumulate in the lung after polymicrobial sepsis induced by cecal ligation and puncture (CLP) still remains to be fully elucidated. We show in this study that lung inflammation, characterized by neutrophil infiltration and expression of inflammatory cytokines, was aggravated in B6 as compared to A/J mice and correlated with high expression of p19, the IL-23-specific subunit. Furthermore, LPS stimulation of B6- and A/J-derived macrophages, one of the main producers of IL-23 and IL-12, revealed that B6 mice favored the production of IL-23 whereas A/J-derived macrophages expressed higher levels of IL-12. In addition, expression of IL-17, known to be upregulated by IL-23, was also more elevated in the lung of B6 mice when compared to A/J mice. In contrast, pulmonary expression of IFN- γ was much more pronounced in A/J than in B6 mice, which was most likely a result of a higher production of IL-12. The expression of the IL-17-dependent neutrophil recruitment factors CXCL2 and G-CSF was also higher in B6 mice. Altogether, these results suggest that increased activation of the IL-23/IL-17 pathway has detrimental effects on sepsis-induced lung inflammation, whereas activation of the IL-12/IFN- γ pathway may lead, in contrast, to less pronounced inflammatory events. These two pathways may become possible therapeutic targets for the treatment of sepsis-induced ARDS.

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Keywords

Sepsis; lung inflammation; neutrophil; cytokines; animal model

Introduction

Regulation of the innate immune response is a critical step in the resolution of injury and inflammation. Failure to control this response may result in the activation of secondary conditions, such as systemic inflammatory response syndrome. Indeed, systemic inflammatory response syndrome is a component of sepsis (1). Epidemiological studies have shown that the incidence of sepsis is over 750,000 cases per year in the United States with a mortality rate ranging from 20% to 50% depending on the disease stage (2). In addition, the incidence of sepsis is expected to escalate annually as the population of elderly people as well as immunocompromised and antibiotic-resistant patients continues to rise (2). The high mortality rate associated with sepsis is associated with the incidence of multiple organ dysfunction syndrome (3). Although the etiology of this syndrome remains unclear, it has been proposed that it is the result of an exaggerated or poorly regulated inflammatory response (4). The incidence of sepsis is highly variable among various individuals, which could be explained by the genetic variability in the human population. Indeed, polymorphisms in some genes associated with the inflammatory response have been correlated with changes in the clinical outcome of septic patients (5). The discovery of novel genetic markers that could predict the outcome from sepsis could substantially advance the treatment of this condition since there are currently no reliable diagnostics. However, the identification of a specific locus modifying the response to sepsis is a complicated task requiring linkage or candidate gene analyses, both demanding a large cohort of patients. An alternative approach is to identify these genetic markers using experimental mouse models that mimic the pathophysiological conditions of clinical sepsis (6). In these experimental models, the use of inbred mouse strains and other genetic resources facilitate the mapping of modifier genes for the incidence of sepsis. Previous studies have demonstrated that a higher mortality rate was observed in C57BL/6J (B6) as opposed to A/J mice following *Escherichia Coli* lipopolysaccharide (LPS) challenge (7) and septic shock induced by cecum ligation and puncture (CLP) (8), indicating that the outcome from sepsis is dependent on the genetic background. Moreover, quantitative trait loci analysis of intercrosses between B6 and A/J mice has allowed the identification of several loci containing modifier genes of the inflammatory response to endotoxemia and sepsis (9, 10). The same methodology could certainly be used to identify new markers for the incidence of sepsis. In this regard, IL-23, which has been implicated as a critical pro-inflammatory cytokine in numerous inflammatory diseases, is a potential paradigm for genetic mapping (11). IL-23 belongs to the IL-12 family of pro-inflammatory heterodimeric cytokines that are secreted as a covalently linked complex composed of one unique subunit, p19, and a common p40 subunit shared with the p35 subunit of IL-12 (12). Antigen-presenting cells, such as activated dendritic cells, monocytes, and macrophages (Mφs) are the main sources of IL-23 in response to pathogen-derived stimuli (12). Numerous cell types involved in the innate immune response, often located at the interface between the host and the environment, such as $\gamma\delta$ T cells, NK, and iNKT cells, express the IL-23 receptor and rapidly produce IL-17

(synonymous to IL-17A) following exposure to pathogens and/or IL-23 stimulation (13). The crucial role of the IL-23/IL-17 pathway in the early recruitment of large numbers of neutrophils in mucosal and non-mucosal tissues via several cytokines and chemokines has been well documented (14).

The present study was aimed at investigating the role of the IL-23/IL-17 pathway in lung pathogenesis after sepsis induced by CLP, using two inbred mouse strains, B6 and A/J mice. We found that lung expression of IL-23 and IL-17A after CLP insult was significantly more pronounced in B6 than in A/J mice. Increased expression of IL-17A was accompanied by a higher production of two neutrophil-recruiting factors, CXCL2 and G-CSF, in B6 mice, which correlated with a greater accumulation of neutrophils in the lung. In contrast, A/J mice, which displayed a lower neutrophil infiltration in lung tissues, showed higher IFN- γ production that was most likely the result of higher IL-12 expression.

Materials and Methods

Cecum ligation and puncture

Cecum ligation and puncture was performed as previously described (15) with some modifications. Male C57BL/6J (B6) and A/J mice (8-9 weeks old) were food deprived for 16 h before the procedure. Animals were anesthetized with isoflurane (1.5 to 2.5 MAC), and a 2 cm incision was made in the lower abdominal region and the cecum was exposed under sterile conditions. The distal portion of the cecum was ligated 1.5 cm from the end with a 4-0 silk suture and punctured once with a 16-gauge needle. The cecum was replaced in the peritoneal cavity and squeezed to place a small portion of its contents (bacteria and feces) into the peritoneum. Then, the peritoneal wall and skin were closed with double sutures. Mice were resuscitated with a 1 ml subcutaneous injection of sterile saline (0.9%). As a control, mice were sham operated as described above, except that the cecum was neither ligated nor perforated. Non-operated mice were also used as a second control. After the procedure, mice had access to water and food *ad libitum*. Lungs were perfused with 10 ml of PBS to wash out all remaining blood and harvested at 1, 2, 3, 6, and 20 h after sham or CLP procedure, flash frozen in liquid nitrogen, and then stored at -80°C. These animal protocols have been reviewed and approved by the UCSD Institutional Animal Care and Use Committee according to the NIH guidelines.

Peritoneal macrophage isolation and LPS treatment

Naïve peritoneal M ϕ s (PM ϕ s) were obtained as previously described (16). The peritoneal cavities of B6 and A/J mice were washed with 5 ml of ice-cold RPMI1640 containing antibiotics. Cells were centrifuged for 10 min at 800 rpm and resuspended in RPMI1640 supplemented with 10% FBS and antibiotics. Cells were then plated for 1 h at 37°C, washed twice, and incubated for an additional 16 h. Analysis by flow cytometry showed that over 90% of the cells were M ϕ s (F4/80 positive). PM ϕ s were treated with 100 ng/ml LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, St. Louis, MO) for 3 h at 37°C. Control cells were incubated with PBS alone.

IL-12 and IL-23 enzyme-linked immunosorbent assay (ELISA)

For detection of IL-12 and IL-23 secretion from B6 and A/J PM ϕ s, supernatants from cells incubated for 24 hours with 10 ng/ml LPS or from PBS-treated (control) cells were collected and analyzed by using IL-12p70 and IL-23 sandwich ELISA Ready-SET-Go kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. PM ϕ s from each mouse were divided into two tissue-culture wells, one treated with LPS and one treated with PBS as control. A total of five B6 and five A/J mice were used and each determination was performed in duplicate. Pulmonary levels of IL-12p70 and IL-23 were determined in cell lysates prepared from lung tissues collected 20 hours after CLP or sham procedure from both B6 and A/J animals. Four mice in each group were used and each determination was performed in duplicate. Data are expressed as pg/mL per mg of protein to normalize for the amount of starting material.

Flow cytometry analysis

PM ϕ s were gently scraped, centrifuged and resuspended in FACS staining buffer (FSB, DPBS without Ca²⁺/Mg²⁺ supplemented with 0.5% BSA). PM ϕ s were then incubated with rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA) for 15 m to block Fc γ receptors. In some experiments, PM ϕ s were incubated with APC-conjugated anti-F4/80 antibodies (eBioscience) and biotinylated LPS for 30 m followed by AlexaFluor488-conjugated streptavidin (BD Pharmingen). In other experiments, PM ϕ s were stained for 30 m with a combination of FITC-conjugated anti-CD14 (eBioscience) and APC-conjugated anti-TLR4/MD2 antibodies (eBioscience). PM ϕ s were then washed and resuspended in FSB. Fluorescence was acquired using a BD FACSCanto II flow cytometer and analyzed by FlowJo software (Tree Star, Ashland, OR).

RNA extraction, cDNA isolation, and quantitative real-time PCR (qPCR)

Levels of mRNA were measured by quantitative real-time RT-PCR (qRT-PCR). Lung tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) using an Ultra-turrax T25 (KA, Wilmington, NC). PM ϕ s, treated or not with LPS, were washed twice with PBS, and TRIzol reagent (Invitrogen) was added. RNA was purified according to the manufacturer's protocol and treated with DNase I (DNA-free kit, Ambion, Austin, Tx) to remove any DNA contamination. DNA-free RNA was then reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Newly synthesized cDNA was further diluted and stored at -20°C. The cDNA levels of genes listed in Table 1 were measured by quantitative real-time PCR (qPCR) using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) with the following QuantiTect validated primer sets (Table 1, Qiagen). All PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Melting curve analysis was performed for each primer set to ensure amplification specificity. Corresponding standard curves were added in each PCR reaction. The housekeeping gene GAPDH (Table 1, Qiagen) was used to normalize data to cDNA inputs. All results were expressed as copy numbers of target gene per copy numbers of GAPDH.

Myeloperoxidase assay (MPO)

Myeloperoxidase (MPO) activity was measured according to the method described by Stewart et al (10) with several modifications. Lung tissues were homogenized for 30 s in phosphate buffer (50 mM, pH 7.4). Homogenates were centrifuged at $10,000 \times g$ for 10 m at 4°C, and the resulting pellets were resuspended in phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). Samples were then subjected to three cycles of freezing and thawing after which they were sonicated and centrifuged at $10,000 \times g$ for 5 m at 4°C, and supernatants were stored at -80°C. Supernatants (25 µl) were then mixed with phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide and absorbance at 460 nm was read immediately at regular intervals (15 s) for 5 m. Protein concentration was measured for each sample by using a BCA protein assay, and data (MPO activity) were expressed as change in absorbance per minute and normalized per mg of protein.

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA). Significance was analyzed using a Student's t-test, one-way ANOVA followed by Newman-Keuls multiple comparison test, or two-way ANOVA followed by the Bonferroni multiple comparison test. A p value of < 0.05 was considered statistically significant. Statistical significance for comparison of mortality rates was analyzed by log-rank test.

Results

Survival rate, pulmonary neutrophil recruitment, and inflammatory cytokine expression in CLP-induced sepsis are modulated by the genetic background

Previous studies have demonstrated differences in mortality between B6 and A/J mice after endotoxic and septic shock (7, 8). We first corroborated these prior findings in our CLP procedure (1.5 cm cecum ligation and a single 16G needle puncture). Indeed, a better survival rate was observed in A/J mice (44.8%) as compared with B6 mice (19.2 %) following our CLP conditions ($p = 0.0116$). The median survival rate for A/J mice was 57 h as opposed to 33 h for B6 animals (Figure 1A). Neutrophil infiltration into the lung is a critical event in the development of ARDS in clinical settings as well as in experimental animal models, including CLP (16). Neutrophil infiltration was measured by the MPO assay in PBS perfused lung samples obtained at various time points after CLP or sham operation. The number of neutrophils present in the lung was significantly increased at 2 h, peaking at 3 h, and then declining after 6 h of CLP in both B6 and A/J mice in comparison with sham-operated and non-operated mice (Figure 1B, *upper panel*). However, a higher accumulation of neutrophils was observed in B6 mice at 2, 3, and 6 h than in A/J mice ($p=0.0279$, $p=0.0172$, and $p=0.0153$, respectively). At 20 h after CLP, only B6 mice exhibited a significantly higher level of MPO activity than sham-operated mice ($p=0.0207$; Figure 1B, *upper panel*). The B6-to-A/J MPO ratio, calculated at each time points after CLP, showed that the difference in neutrophil accumulation between these two mouse strains kept increasing in a time-dependent manner (Figure 1B, *lower panel*).

In addition to neutrophil infiltration, we investigated cytokine expression in lung after CLP. Changes in cytokine levels were measured at the mRNA level because this approach allowed us to study the kinetic of the inflammatory process after CLP at very early stages of the process when the concentration of cytokines secreted may be very low (18). Cytokine gene expression after CLP was a very rapid process, occurring within 2 h after the procedure, reaching maximal levels, which were significantly higher than in sham-operated or non-operated mice (Figure 1C) at 3 h (IL-1 β , IL-10, and TNF- α) or 6 h (IL-6) after CLP. IL-1 β and IL-6 mRNA levels were 5- and 24-fold more elevated in B6 mice than in A/J mice ($p = 0.0003$ and $p = 0.0008$, respectively). In contrast, A/J mice showed higher TNF- α levels than B6 animals ($p < 0.0001$). These results are consistent with prior reports on circulating levels of cytokines after CLP in inbred mouse strains (8) demonstrating that the inflammatory response after sepsis was modulated by the genetic background of the experimental subject.

Expression of IL-23, rather than IL-12, is favored in the lung of B6 animals

IL-12 and IL-23 are both heterodimeric cytokines composed of one specific subunit, p35 (IL-12) or p19 (IL-23), combined with a common subunit, p40. Changes in p35, p19, and p40 mRNA levels were examined in lung samples collected at 1, 2, 3, 6, and 20 h after CLP or sham operation obtained from B6 and A/J mice. Basal p19 mRNA levels were 23-fold higher in B6 than in A/J or non-operated mice (Figure 2A, *left panel*, $p < 0.0001$), whereas p35 and p40 mRNA levels were moderately elevated in B6 as opposed to A/J mice (Figure 2A, *middle and right panels*, $p = 0.0126$ and $p = 0.0234$, respectively). During the course of sepsis, expression of p19 increased rapidly, reaching a peak at 2 h after CLP in both B6 and A/J mice and subsequently decreasing toward values similar to sham-operated mice at 6 h for A/J and at 20 h for B6 mice (Figure 2B, *left panel*). The increase of p19 levels observed at 2 h after CLP was more pronounced in B6 (132-fold) than A/J (8-fold) mice in comparison with sham-operated mice ($p = 0.00313$ and $p = 0.002$, respectively), resulting in a 667-fold difference in p19 expression between B6 and A/J mice at this time point. The levels of p35 also increased during the course of sepsis, reaching maximum expression at 2 and 6 h after CLP for B6 and A/J mice, respectively. This increase was followed by a dramatic decrease, reaching values similar to sham-operated mice at 20 h after CLP for both strains (Figure 2B, *middle panel*). At the peak of expression, p35 mRNA levels were 9-fold ($p = 0.0110$) and 6-fold ($p = 0.0025$) more elevated in the CLP group in comparison with sham-operated mice for B6 and A/J mice respectively. Basal p40 mRNA levels increased 2.3-fold in B6 mice as compared with sham-operated mice. In contrast, expression of p40 mRNA was not modified by CLP in A/J animals. Levels of p40 at 3 h after CLP were 6.8-fold higher in B6 than in A/J mice (Figure 2B, *right panel*). To monitor if these differences in p19, p35 and p40 mRNA levels between B6 and A/J animals following CLP-induced sepsis resulted in changes in IL-23 and IL-12 expression in the lungs, ELISA for these two heterodimeric cytokines were conducted at 20 hours after the CLP procedure. Expression of IL-23 was significantly increased in B6 animals after CLP whereas the difference in IL-23 expression between sham-operated and CLP-operated A/J mice did not reach statistical significance (Figure 2C, *left panel*). In contrast, CLP-treated A/J animals expressed significantly more IL-12p70 than their sham-operated counterpart. In B6 animals, although all four CLP-treated animals expressed higher levels of IL-12p70, the difference did not

attain statistical significance using one-way ANOVA. These data, altogether, indicate that the pulmonary production of IL-23 is favored in the lungs of B6 mice following CLP whereas IL-12 expression is more pronounced in A/J animals.

Macrophages from B6 mice produce more IL-23, but less IL-12, than those from A/J animals in response to LPS stimulation

To investigate whether the expression of IL-23 and IL-12 were differentially regulated at the cellular level, naïve PM ϕ s were isolated from B6 and A/J mice and stimulated in culture with LPS. Biotin-conjugated LPS binding and surface expression of CD14 and TLR4 were slightly elevated in unstimulated B6 cells in comparison with A/J-derived PM ϕ s, as detected by flow cytometry (Figure 3A). LPS stimulation resulted in a robust increase in the expression of p19, p35, and p40 (mRNA levels) after 3 h of treatment in both B6- and A/J-derived PM ϕ s (Figure 3B). However, LPS-induced p19 and p40 levels were significantly higher in B6 than in A/J cells (28- and 17-fold, respectively, both $p < 0.0001$). No significant differences for p35 mRNA levels were observed after LPS treatment between PM ϕ s derived from B6 and A/J mice (Figure 3B). In addition, significant amounts of secreted IL-23 and IL-12 (IL-12p70) proteins were measured by ELISA in the supernatants of B6- and A/J-derived PM ϕ s after 24 hours of LPS treatment (Figure 3C). However, higher amounts of IL-23 were secreted from B6-derived PM ϕ s as compared to A/J-derived PM ϕ s (Figure 3C). In contrast, higher accumulation of IL-12 was detected in the supernatants of A/J-derived PM ϕ s (Figure 3C). Altogether these data indicate that the expression of IL-23, rather than IL-12, is favored in macrophages from B6 mice stimulated with LPS, as opposed to A/J animals.

B6 mice showed elevated IL-17A expression while A/J mice displayed higher IFN- γ expression

In vivo and *ex vivo* studies have shown that stimulation of innate immune cells such as $\gamma\delta$ T cells and NK1.1^{neg} NKT with a combination of IL-1 β and IL-23 promote rapid secretion of IL-17A (19, 20). Since the presence of both $\gamma\delta$ T and NKT cells has been reported in mouse pulmonary tissue (21, 22), we next investigated whether the expression of IL-17A was also promoted during sepsis in both mouse strains. IL-17A expression increased at 2 h after CLP in both B6 and A/J mice, reaching a peak at 3 h in comparison with sham-operated mice. IL-17A levels decreased within 20 h after CLP in both strains, reaching similar levels to sham-operated mice (Figure 4A, *left panel*). The expression of 17A was significantly more elevated in B6 mice at 2, 3, and 6 h after CLP than in A/J mice ($p = 0.0438$, $p = 0.0023$, and $p = 0.0423$, respectively). We also investigated the expression of IL-17F, another member of the IL-17 family, which shares structural similarities with IL-17A but seems to exert a different function in the regulation of the inflammatory response (14). The expression of IL-17F was also increased during the development of sepsis, but no significant differences were observed between B6 and A/J mice (Figure 4A, *right panel*). The foregoing data suggest that the higher expression of IL-1 β and IL-23 observed in the lungs of B6 animals after CLP was associated with higher expression of IL-17A. Secretion of IL-12 alone or in combination with IL-1 by antigen-presenting cells, such as M ϕ s and dendritic cells, induce IFN- γ production by various immune cells (12). Since A/J mice displayed a significant increase in IL-12 expression as compared to B6 mice, we postulated that more IFN- γ should,

therefore, be produced in this strain. To test this hypothesis, IFN- γ mRNA levels were measured in lung samples at different time points after CLP and compared between B6 and A/J mice. Indeed, IFN- γ levels peaked at 3 h for B6 and at 6 h for A/J mice after CLP (Figure 4B). The expression of IFN- γ in the lungs of A/J animals at 6 h after CLP was 9.8-fold higher than the equivalent peak levels at 3 h in B6 mice. These results altogether suggest that the elevated expression of p19 subunit observed in B6 mice resulted in higher IL-23 expression, which induced an elevated expression of IL-17A in combination with IL-1 β by innate immune cells. In contrast, the lower expression levels of p19 observed in A/J animals result in more IL-12 expression that, in turn, stimulates higher production of IFN- γ .

Elevated IL-17A expression was associated with higher accumulation of neutrophils in the lungs of B6 mice via upregulation of G-CSF and CXCL2

Several studies have shown that IL-17A stimulates granulopoiesis and neutrophil recruitment by indirect mechanisms involving GM-CSF and G-CSF and several chemokines, including CXCL1 and CXCL2 (23). We assessed the expression of these various factors involved in neutrophil recruitment. Expression of GM-CSF was not affected by CLP, whereas the levels of G-CSF were significantly increased at 2 h, reaching a peak at 6 h after CLP in both mouse strains (Figure 5A). The expression of G-CSF in B6 mice was higher than in A/J mice at 2, 3, 6, and 20 hours of CLP ($p = 0.0116$, $p = 0.0138$, $p = 0.0188$, and $p = 0.0269$, respectively). Expression of both CXCL1 and CXCL2 was increased after CLP treatment (Figure 5B), but only CXCL2 was higher in B6 than in A/J mice. These data suggest that the recruitment of neutrophils following CLP-induced sepsis could be partly mediated through IL-17A-dependent production of G-CSF and CXCL2.

Discussion

Therapy for sepsis, which is a leading cause of death in the United States is still supportive, and mainly includes antibiotic treatment, mechanical ventilation, and fluid resuscitation (2). Targeted therapeutic interventions and reliable biomarkers have been difficult to develop, probably due to the multi-factorial characteristics of this disease. Another layer of complexity in sepsis is related to the contributions of genetic variability of the patient population. Although some genetic polymorphisms have been associated with the incidence of sepsis (6), new and more consistent genetic markers need to be established, which could be used as predictors for the incidence of sepsis. Previous studies have identified modifier genes for the inflammatory response to sepsis using mouse models and genetic crossing (5). In the present study, new paradigms for the identification of potential genetic markers were obtained by comparing the response to sepsis induced by CLP between two inbred mouse strains: B6 and A/J mice.

We observed a rapid infiltration of neutrophils into the lung (2 h of CLP), which was more elevated in B6 mice as opposed to A/J mice, resembling prior observations (7, 8). The higher mortality observed in B6 mice as opposed to A/J mice could be due to this rapid infiltration of neutrophils into the lung, which is consistent with studies demonstrating a reduction in lung severity injury with neutrophil depletion (24). Reduced mortality after

neutrophil depletion was correlated with decreased IL-1 β levels, but not TNF- α in the lung (24), which is similar to our observations between B6 (elevated IL-1 β and IL-6 expression) and A/J mice (elevated TNF- α expression). These differences in pulmonary neutrophil infiltration and cytokine levels between B6 and A/J mice may result in new phenotypes for the mapping of novel modifier genes in the response to sepsis, as previously shown (9).

Since neutrophil infiltration is a key component of ARDS, which is an early event in the development of sepsis and septic shock (17), we investigated the possible mechanism underlying neutrophil migration into the lung following CLP-induced sepsis in B6 and A/J mice. We observed that constitutive expression of IL-23p19 was more elevated in the lungs of B6 mice as compared with A/J mice (23-fold), which was further enhanced after CLP, maintaining the disparity between B6 and A/J mice. Indeed, IL-23p19 levels at the peak of expression (2 h after CLP) were over 650 times more elevated in B6 mice than in A/J mice. These observations open the possibility of correlating elevated IL-23p19 expression with increased neutrophil infiltration into the lung and higher mortality rate, which is consistent with an early report showing reduced mortality in a model of Gram-negative endotoxic shock after neutralization of IL-23p19 by specific antibodies (25). IL-23 is a heterodimeric cytokine composed of two subunits, p19 and p40, the latter is also common for IL12, which is composed of p40 and p35. Thus, we investigated changes in p35 and p40 expression in the lung after CLP. We observed that p35 and p40 expressions were also increased after CLP, but to a lesser extent than p19 in B6 mice, suggesting that IL-23p19 is the main inflammatory component of this cytokine family in the lung after CLP. This assumption was tested in isolated naïve PM ϕ s stimulated with LPS in culture conditions. We also observed significantly higher expression levels of p19 and p40 in B6 stimulated PM ϕ s in comparison with A/J-derived cells. In contrast, no differences in p35 expression were detected in LPS stimulated B6- or A/J-derived PM ϕ s. Since both p19 and p35 compete for the same p40 subunit, production of IL-23, rather than IL-12, was favored in B6 mice as opposed to A/J mice.

Furthermore, we showed that IL-17A expression was increased after CLP in both B6 and A/J mice. Innate immune cells stimulated by IL-1 β and IL-23 such as $\gamma\delta$ T or NKT cells (19, 20) or differentiated effector T helper 17 cells (Th17) (13) are the main producer of IL-17A. The early expression of IL-17A observed in the lungs of B6 and A/J mice after CLP most likely indicates that innate immune cells rather than effector Th17 cells are the main source of IL-17A in this tissue. Indeed, differentiation of naïve T cells into IL-17A-producing Th17 cells required 3 to 5 days following stimulation of antigen-presenting cells by pathogen-associated molecular patterns (13). The importance of IL-17A in the development of CLP-induced sepsis has been addressed in previous studies. However, a clear understanding for the role of this cytokine in the development of sepsis has not been possible due to the contradictory results that were presented. Flierl et al. (26) showed that antibody-mediated neutralization of IL-17A or depletion of IL-17A-producing $\gamma\delta$ T cells improved survival, whereas Freitas et al. (27) demonstrated that deletion of the IL-17 receptor gene led to an increased mortality rate. Herewith, we presented elevated expression of IL-17A in B6 mice that displayed a higher mortality rate after CLP, whereas IL-17A was reduced in A/J mice that are less susceptible to CLP. In support of this assumption, a recent study has demonstrated that anaphylatoxin C5a inhibition increased survival after CLP by diminishing

IL-17A production by $\gamma\delta$ T cells (28). Our results are therefore in good agreement with the report by Flierl et al. (26).

IL-17A has been shown to exert its functions via stimulation of lung stromal cells and the release of several chemokines and colony-stimulating factors including CXCL1, CXCL2, GM-CSF, and G-CSF (29). We found that CXCL1, CXCL2, and G-CSF were highly expressed after CLP in both mouse strains, whereas GM-CSF was either not affected (A/J) or slightly decreased (B6) during sepsis. CXCL2 and G-CSF, but not CXCL1, were significantly more elevated in the high IL-17A-producing B6 mice than in A/J mice. This data suggest that CXCL1 and CXCL2 could be differentially regulated by IL-17A and that the myeloid colony-stimulating factor G-CSF may be a critical factor involved in neutrophil-mediated lung injury. Clinical studies have reported that G-CSF, but not GM-CSF, correlated with neutrophil infiltration, which was significantly higher in ARDS non-survivor versus survivor patients (30). In addition, instillation of G-CSF alone into rat lung has been shown to induce neutrophil recruitment, lung injury, and impaired pulmonary function (31). In fact, measurement of G-CSF levels in blood and tracheal aspirate has been proposed as an early diagnosis of sepsis in neonates and critically ill children (32). Surprisingly, levels of G-CSF remained high throughout the entire twenty hours of the study, whereas all the other cytokines and chemokines tested showed a biphasic expression pattern with an early increase in expression within the first 6 hours and a dramatic decrease thereafter. Since G-CSF is also a powerful anti-apoptotic factor for neutrophils, it is tempting to speculate that this sustained expression of G-CSF prolonged the life of infiltrated neutrophils and, therefore, participated in the neutrophil-mediated lung injury. This conclusion is also supported by previous reports showing that the number of apoptotic neutrophils in the lungs of patient with ARDS is very low and that bronchoalveolar lavage specimens collected at the early stage of the disease display high concentrations of G-CSF, which was capable of inhibiting apoptosis in peripheral blood neutrophils in an *in vitro* stimulation assay (33).

A/J mice, in contrast, displayed much higher expression levels of IFN- γ than B6 mice, which is most likely related to elevated expression of IL-12 by A/J mice and reduced expression of IL-23. A recent study established that during tuberculosis infection, IFN- γ inhibited both neutrophil recruitment and survival, and therefore limited lung inflammation via a mechanism involving IL-17 down-regulation (34). Another study has recently demonstrated that the IL-12/IFN- γ pathway limits neutrophil development and accumulation during inflammation, and, as a result, may protect the host against neutrophil-mediated tissue damage (35). Moreover, IFN- γ has been shown to repress IL-23 expression by increasing IL-23p19 mRNA degradation (36). Conversely, inhibition of IL-12 and IFN- γ production by prostaglandin E2 enhanced IL-17 synthesis and IL-23-mediated neutrophil recruitment in rheumatoid arthritis (37). In a model of CLP after burn injury, IL-12 treatment improved survival in comparison with sham after burn injury control group (38). In severe sepsis induced by CLP, treatment with recombinant IL-12 at 6 h before the procedure reduced mortality rates significantly (39). Our data and previous observations seem to indicate that the IL-12/IFN- γ and IL-23/IL-17 pathways have divergent roles in the host response against infection, with IL-12/IFN- γ providing protection and IL-23/IL-17 being detrimental for the progression of inflammatory diseases. However, several studies

using IL-17A- and IL-23-deficient mice showed that the IL-23/IL-17 axis could also exert a protective role against different pathogens (29).

Conclusions

The study of two inbred mouse strains displaying different outcomes after CLP-induced sepsis has allowed us to propose that increased accumulation of neutrophils in the lung correlates with high levels of IL-23p19 (Figure 6). In addition, innate production of IL-17A, which is induced by a combination of IL-1 β and IL-23, was also increased in the most susceptible mouse strain (Figure 6). In contrast, A/J mice, which were more resistant to sepsis, expressed higher levels of pulmonary IFN- γ than the sensitive B6 mouse strain. We finally showed that the production of several factors involved in IL-17A-dependent neutrophil recruitment and activation, G-CSF and CXCL2, was increased in B6 mice (Figure 6). Altogether, these data indicate that the activation of the IL-12/IFN- γ pathway may improve the resolution of CLP-induced sepsis whereas over-stimulation of the IL-23/IL-17 pathway may have detrimental effects on the progression of the disease. It is possible that modulating these two pathways may be crucial to adequately resolve inflammation after CLP. Therapeutic interventions aimed to restore the balance between IL-23 and IL-12 production may thus prove to be beneficial in the treatment of sepsis.

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Fig 1. Mortality rate, neutrophil infiltration and inflammatory cytokine expression in the lungs of B6 and A/J mice after CLP-induced polymicrobial sepsis

(A) Male B6 and A/J mice (8-9 weeks old) were fasted for 16 h before the procedure. CLP was performed as described under Material and Methods using a 1.5 cm ligated cecum area and 16-gauge needle single perforation. Survival was continuously monitored in B6 (closed squares, $n = 26$) and A/J (open squares, $n = 29$) for 72 h after CLP. Statistical significance was analyzed by log-rank test and * denotes a $p < 0.05$. (B) Male B6 and A/J mice were subjected to CLP or sham operation. A non-operated group was also included as control. At different time points after CLP ($n = 5$) or sham operation ($n = 5$) mice were sacrificed, and lungs were perfused, harvested and snap-frozen. Neutrophil infiltration was then measured by the MPO method. *Upper panel*: pulmonary MPO activities at different time points after CLP or sham procedures were expressed as change in absorbance per minute and normalized per mg of protein. Control animals (non-operated group) are used as time=0. *Lower panel*: Kinetic of B6-to-A/J MPO ratio. Data are presented as average $\pm$ SE ($n = 5$) and statistical analysis was performed by two-way ANOVA followed by the Bonferroni multiple comparison test with * indicating a $p < 0.05$ for B6 after CLP vs. A/J after CLP at each time point. (C) Total RNA was isolated from perfused lung tissues and levels of mRNA were measured by qPCR at 3 hours (IL-1 β , IL-10 and TNF- α) or 6 hours (IL-6) after CLP or sham operation. The mRNA levels were established by comparison with a standard curve and expressed as copy number. Values were normalized to GAPDH mRNA levels. Data are presented as average $\pm$ SE ($n = 5$) and statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls Multiple comparison test with * $p < 0.05$ as indicated on the figure.

Fig. 2. Expression of IL-23 and IL-12 in the lung of B6 and A/J mice following CLP-induced sepsis

Male B6 and A/J mice were subjected to CLP (1.5 cm cecum ligation and 16G needle perforation), sham operated or non-operated controls. At different time points after CLP ($n = 5$) or sham operation ($n = 5$), mice were sacrificed, and perfused lungs were harvested and snap-frozen. Total RNA was then isolated and levels of mRNA for the different IL-23 and IL-12 subunits were measured by qPCR and expressed as copy number. The housekeeping gene GAPDH was used to normalize the data. (A) Basal levels of p19, p35, and p40 in non-operated control mice are represented as bar graphs with filled and open histogram for B6 and A/J mice, respectively. (B) Kinetic of p19, p35 and p40 mRNA levels in lungs harvested at 0 (basal level), 1, 2, 3, 6, and 20 h after CLP or sham operation of B6 and A/J animals. (C) Expression of IL-23 and IL-12 was determined by ELISA in lungs harvested at 20 hours after CLP or sham procedure. ELISA data were normalized with the amount of tissue proteins used in each determination. All results presented in this figure are expressed as average $\pm$ SE ($n = 4$ to 5). For comparison of p19, p35 and p40 basal mRNA levels, significance was analyzed by student's t-test. Statistical analysis of p19, p35 and p40 mRNA levels after CLP and sham operation was performed by two-way ANOVA followed by the Bonferroni multiple comparison test with * indicating a $p < 0.05$ for B6 after CLP vs. A/J after CLP for each time point. Significance for IL-23 and IL-12 protein levels was analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test with # indicating a $p < 0.01$.

Fig. 3. Expression of IL-23 and IL-12 is differentially regulated in LPS-stimulated naïve PMφs isolated from B6 and A/J mice

B6 and A/J mouse peritoneal cavities were washed with 5 ml of ice-cold RPMI1640 containing antibiotics. Peritoneal cells were centrifuged and plated for 1 h at 37°C. Adherent cells (PMφs) were washed twice and were further incubated for 16 h at 37°C. (A) PMφs were gently scraped, centrifuged and incubated with rat anti-mouse CD16/CD32 for 15 min. to block Fcγ receptors. Subsequently, PMφs were either incubated with APC-conjugated anti-F4/80 antibodies and biotinylated LPS for 30 min. followed by AlexaFluor488-conjugated streptavidin (BD Pharmingen) or stained with a combination of FITC-conjugated anti-CD14 and APC-conjugated anti-TLR4/MD2 antibodies. PMφs were then washed and fluorescence was acquired using a BD FACSCanto II flow cytometer. Analysis was performed by FlowJo software and data are presented as either density plots (left panels) or histogram plots (right panels). (B) PMφs were stimulated or not with 100 ng/ml LPS for 3 h. Total RNA was prepared from stimulated and control cells, and p19, p35, and p40 mRNA levels were measured by qPCR. Data were normalized by GAPDH mRNA levels and expressed as average ± SE (n = 5 mice for each strain). Statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls multiple comparison test with * p < 0.05 as indicated on the figure. (C) Expression of IL-23 and IL-12 in the supernatants of PMφs from B6 and A/J mice treated or not for 24 h. with 100 ng/ml LPS was determined by ELISA. Statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls multiple comparison test with # p < 0.01 as indicated on the figure.

Fig. 4. Pulmonary expression of IL-17A is favored in B6 animals, whereas expression of IFN- γ is more pronounced in A/J mice following CLP-induced sepsis

Male B6 and A/J mice were subjected to CLP (1.5 cm cecum ligation and 16G needle perforation), sham operated or non-operated controls. At different time points after CLP ($n = 5$) or sham operation ($n = 5$), mice were sacrificed, and lungs were harvested and snap-frozen. Total RNA was isolated at each time point and reverse-transcribed to cDNA. (A) Levels of mRNA for IL-17A (*left panel*) were measured by qPCR at 1, 2, 3, 6, and 20 h after CLP or sham operation. For IL-17F (*right panel*), mRNA levels were determined at 3 h after CLP or sham operation. (B) Levels of IFN- γ were determined by qPCR at 1, 2, 3, 6, and 20 h after CLP or sham operation. All mRNA levels were established using a standard curve and expressed as copy number. The housekeeping gene GAPDH was used to normalize the data. Results are expressed as average $\pm$ SE ($n = 5$). Comparison between the mRNA levels of IL-17A and IFN- γ in B6 and A/J after CLP at each time point was measured by two-way ANOVA followed by the Bonferroni multiple comparison test with * indicating a $p < 0.05$. Statistical analysis for IL-17F mRNA levels was performed by one-way ANOVA followed by the Newman-Keuls multiple comparison test with * $p < 0.05$ as indicated on the figure.

Fig. 5. Expression of CXC chemokines and colony-stimulating factors in the lung of B6 and A/J mice following CLP

Male B6 and A/J mice were subjected to CLP (1.5 cm cecum ligation and 16G needle perforation), sham operated or non-operated controls. At different time points after CLP ($n = 5$) or sham operation ($n = 5$), mice were sacrificed, and lungs were harvested and snap-frozen. Total RNA was then isolated, reverse-transcribed into cDNA. (A) Levels for GM-CSF (*left panel*) and G-CSF (*right panel*) mRNA were determined by qPCR at 1, 2, 3, 6, and 20 h after CLP or sham operation. (B) Levels of CXCL1 (*left panel*) and CXCL2 (*right panel*) mRNA were determined by qPCR at 1, 2, 3, 6, and 20 h after CLP or sham operation. GAPDH housekeeping gene was used to normalize to cDNA inputs. Data are expressed as average $\pm$ SE ($n = 5$) with * $p < 0.05$ for comparison of B6 after CLP vs. A/J after CLP for each time point as determined by two-way ANOVA followed by the Bonferroni multiple comparison test

Fig. 6. Proposed model for the regulation of pulmonary recruitment of neutrophils between B6 and A/J mice after CLP

We propose that, following CLP injury, the higher neutrophil accumulation observed in the lungs of B6 mice as opposed to A/J animals results from an overstimulation of the IL-23/IL-17A pathway, leading to a higher production of neutrophil-recruiting factors such as G-CSF and CXCL2. In contrast, stimulation of the IL-23/IL-17 pathway following CLP is much reduced in A/J mice, allowing a higher IL-12-mediated IFN- γ production and a limited accumulation of neutrophils. We thus postulate that a fine regulation of these two pathways, namely IL-23/IL-17 and IL-12/IFN- γ , during the development of sepsis is necessary to initially induce a rapid and massive recruitment of neutrophils to the site of infection and to subsequently prevent neutrophil-induced damage of the lung tissue.

Table 1
List of primers used in this study

Genes	NCBI accession	Amplicon size (bp)	Qiagen cat #
IL-1 β	NM 008361	150	QT01048355
IL-6	NM 031168	128	QT00098875
IL-10	NM 010548	103	QT00106169
IL-12a, IL-12p35	NM 008351	90	QT01048334
IL-12b, IL-12p40	NM 008352	97	QT00153643
IL-17A	NM 010552	94	QT00103278
IL-17F	NM 145856	141	QT00144347
IL-23a, IL-23p19	NM 031252	80	QT01663613
IFN- γ	NM 008337	190	QT01038821
TNF- α	NM 013693	112	QT00104006
Colony stimulating factor 2, CSF-2, GM-CSF	NM 009969	115	QT00251286
Colony stimulating factor 3, CSF-3, G-CSF	NM 009971	67	QT00105140
Chemokine (C-X-C motif) ligand 1, CXCL1	NM 008176	93	QT00115647
Chemokine (C-X-C motif) ligand 2, CXCL2	NM 009140	81	QT00113253
Glyceraldehyde-3-phosphate dehydrogenase, GAPDH	NM 008084	144	QT01658692