Dendritic Cells Modulate Lung Response to *Pseudomonas aeruginosa* in a Murine Model of Sepsis-Induced Immune Dysfunction[1]

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Host infection by pathogens triggers an innate immune response leading to a systemic inflammatory response, often followed by an immune dysfunction which can favor the emergence of secondary infections. Dendritic cells (DCs) link innate and adaptive immunity and may be centrally involved in the regulation of sepsis-induced immune dysfunction. We assessed the contribution of DCs to lung defense in a murine model of sublethal polymicrobial sepsis (cecal ligature and puncture, CLP). In this model, bone marrow-derived DCs (BMDCs) retained an immature phenotype, associated with decreased capacity of IL-12p70 release and impaired priming of T cell lymphocytes. Eight days after CLP surgery, we induced a secondary pulmonary infection through intratracheal instillation of 5×10^6 CFUs of *Pseudomonas aeruginosa*. Whereas all sham-operated mice survived, 80% of post-CLP mice died after secondary pneumonia. Post-CLP mice exhibited marked lung damage with early recruitment of neutrophils, cytokine imbalance with decreased IL-12p70 production, and increased IL-10 release, but no defective bacterial lung clearance, while systemic bacterial dissemination was almost constant. Concomitant intrapulmonary administration of exogenous BMDCs into post-CLP mice challenged with *P. aeruginosa* dramatically improved survival. BMDCs did not improve bacterial lung clearance, but delayed neutrophil recruitment, strongly attenuated the early peak of TNF-α and restored an adequate Il-12p70/IL-10 balance in post-CLP mice. Thus, adoptive transfer of BMDCs reversed sepsis-induced immune dysfunction in a relevant model of secondary *P. aeruginosa* pneumonia. Unexpectedly, the mechanism of action of BMDCs did not involve enhanced antibacterial activity, but occurred by dampening the pulmonary inflammatory response. *The Journal of Immunology,* **2008, 181: 8513–8520.**

The immune response to microbial pathogens relies on both innate and adaptive components activated in a sequential and coordinated manner. Following pathogen recognition, the innate immune system initiates a cascade of molecular events that leads to the activation of macrophages and monocytes, and to the regulated production of inflammatory cytokines, chemokines, and other costimulatory molecules in an attempt to kill invading microorganisms. Normally, immune and neuroendocrine systems tightly control this local inflammatory process through the coordinated release of anti-inflammatory mediators. Dysfunction of local control mechanisms can lead to septic shock and damage of host tissue, but also accounts for a postinfective immunosuppressive state evidenced by the development of anergy, the frequent inability to clear the primary infection and an increased propensity to acquire secondary infections.

Sepsis-induced immune dysfunction comprise various immune defects, including a shift in the Th-cell pattern to a predominantly Th-2 response with decreased production of proinflammatory cytokines (TNF-α, IL-12) (1, 2) and increased production of IL-10 (3), a deactivation of monocytes with reduced expression of HLA-DR (4, 5) and apoptosis of T and B cell lymphocytes (6, 7), whereas suppressor T cell lymphocytes are relatively increased (8). Mechanisms that underlie the development of postinfective immune dysfunction are unclear but may involve quantitative and/or functional defects of dendritic cells (DCs),[4] the main APCs. DCs play an important role in the initiation and integration of both innate and adaptive immune responses induced by microbial infection. They particularly initiate and orient adaptive immune responses, depending upon their patterns of cytokine secretion (9). Several lines of evidence indicate an important role of DCs in the pathophysiology of sepsis-induced immune dysfunction. Selective depletion of DCs in secondary lymphoid organs was reported in lethal murine models of polymicrobial sepsis (10, 11) and in patients who died from sepsis (12). An early decrease in circulating DCs was similarly reported in patients with septic shock (13). Furthermore, sepsis reprograms spleen DCs toward decreased production of the immunostimulatory cytokine IL-12 and increased release of IL-10 (14, 15).

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[4] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow DC; CLP, cecal ligature and puncture; MHCII, MHC class II; BAL, bronchoalveolar lavage; MPO, myeloperoxydase; flt3L, fms-like tyrosine kinase-3 ligand.

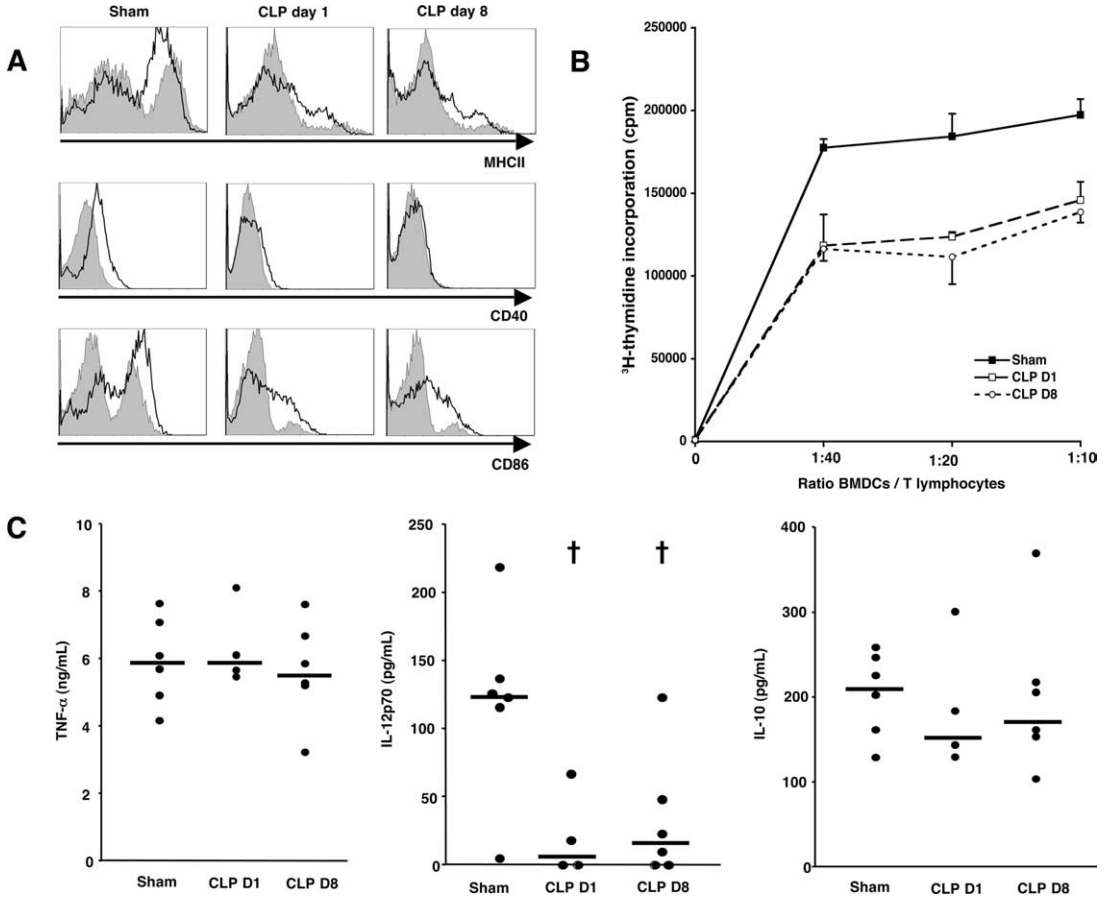

FIGURE 1. Functional abnormalities of BMDCs. Bone marrow cells were obtained from mice that underwent sham-operation or CLP 1 and 8 days before. Cells cultured for 6 days in the presence of GM-CSF were stimulated with LPS (1 μg/ml) for 24 h. Cells were assessed for maturation, priming of T cell lymphocytes, and production of cytokines. *A*, Representative flow cytometry histograms of basal (gray histogram) and LPS-induced (black line) membrane expression of MHCII and costimulatory molecules CD40 and CD86. The FACS analysis shown is representative of five independents experiments. *B*, Allogeneic mixed lymphocytic reaction. BMDCs were cocultured for 72 h with allogeneic T cell lymphocytes obtained from BALB/c mice (ratio 1:40, 1:20, and 1:10 in triplicate). Proliferation of T cell lymphocytes was assessed by measuring the incorporation of [^{3}H]thymidine. The curve shown is representative of three independent experiments. *C*, Quantification of TNF-α, IL-12p70, and IL-10 in cell culture supernatants. †, $p < 0.05$.

Although immune cellular defects consistent with sepsis-induced immune dysfunction have been extensively described in lethal models, how they affect systemic and local immune responses to secondary infections in nonlethal models remains unclear. Up to now, this question has been addressed using models of secondary i.v. infections (16, 17) or models of concomitant pulmonary infection immediately after polymicrobial sepsis (18–20). Although these studies yielded important insights into the host systemic response to infection, they do not establish the contribution of DCs to the pathophysiology of nosocomial infection. We hypothesized that DC abnormalities contribute to postinfective dampening of immune function that may durably alter the lung defense mechanisms despite recovery of primary infection and thereby increase susceptibility to a second infectious insult. To test this hypothesis, we established a relevant murine model of sublethal polymicrobial sepsis followed by late-onset *Pseudomonas aeruginosa* pneumonia in mice that survived the primary injury. We assessed the functions of bone marrow-derived DCs (BMDCs) in this model of sublethal sepsis to investigate whether functional abnormalities of DCs contribute to postinfective immune dysfunction. We report that polymicrobial sepsis durably affects the main functions of BMDCs and increases susceptibility to late-onset secondary *P. aeruginosa* pneumonia. Concomitant tracheal instillation of exogenous BMDCs dampened the local inflammatory response and decreased mortality from this secondary infectious challenge, highlighting the contribution of DCs to mount an efficient lung immune response.

Materials and Methods

Mice

C57BL/6J female mice, 8–12 wk old, were used in all experiments. T cell lymphocytes for allogeneic MLR were obtained from BALB/c mice (8–12 wk old). All animals were purchased from Charles River Laboratories and were maintained in the pathogen-free animal facility of the Cochin Institute. Experiments were conducted in accordance with Cochin Institute guidelines in compliance with European animal welfare regulation.

Model of polymicrobial sepsis

We used a model of cecal ligature and puncture (CLP) as previously described (21). In brief, mice were anesthetized by an i.p. injection of ketamine and xylazine. After a midline incision (<1 cm), the cecum was exposed, ligatured at its external third, and punctured through and through with a 21-gauge needle. Incision was sutured in layers and animals were resuscitated with an i.p. injection of 1 ml of saline. Controls were sham-operated mice undergoing laparotomy with only exposition of cecum without CLP. Six hours following surgery and then every 12 h during 3 days, mice received an i.p. injection of antibiotics (Imipenem cilastatine, Tienam, Merck Sharp and Dohme, 25 mg/kg in 0.5 ml of saline).

Generation of BMDC

Bone marrow cells were flushed from femurs and tibias. After RBC lysis, cells were seeded in 24-well-plates at the concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 5% FCS, 10 mM HEPES, 10 mM

glutamin, 1% penicillin, and streptomycin, in the presence of supernatant (4% v/v) from J558 cells transduced with murine GM-CSF. Cells were cultured for 6 days with medium replacement at day 2, 4, and 6. To assess maturation and cytokine production capabilities, BMDCs were then stimulated for 24 h with LPS 1 μg/ml (*Escherichia coli* K12 ultra-pure LPS, Invivogen). Cells were used for FACS labeling and cell culture supernatants were collected and stored at −80°C for the purpose of cytokine level determination. BMDCs used for intratracheal administration were similarly prepared but not stimulated with LPS.

Flow cytometry analysis

Fluorescent Abs (CD11c, MHC class II (MHCII), CD40, and CD86) and Fc-blocker Ab (anti-CD16/CD32) were purchased from BD Biosciences. Cell suspensions (2×10^5 cells) were first incubated with Fc-blocker Ab for 5 min, and then stained with fluorescent Abs for 30 min at 4°C. Analysis of 10,000 events gated on viable cells was performed on a FACSCalibur (BD Biosciences).

Allogeneic MLR

T cell lymphocytes were isolated from lymph nodes of BALB/c mice using a T cell negative isolation kit (Dynal Biotech), which provides a highly purified CD3-positive fraction (98% of cells). Two $\times 10^5$ T cell lymphocytes were cultured for 72 h in round-bottom 96-well plates in the presence of previously irradiated (3000 rad) BMDCs (ratios 40:1, 20:1, and 10:1 in triplicate). Proliferation of T cell lymphocytes was assessed by measuring the incorporation of [^{3}H]thymidine after a 6-h pulse with 1μCi per well.

Induction of secondary Pseudomonas aeruginosa *pneumonia*

To create a relevant model of secondary pneumonia, we used the *P. aeruginosa* strain PAO1, which notably expresses various virulence factors such as flagellin and type III secretion system. In brief, *P. aeruginosa* was incubated for 12 h in tryptic soy broth at 37°C in a rotating shaker. Bacteria were washed and diluted in PBS, and the desired concentration was adjusted by spectrophotometry (absorbance at 600 nm) according to a reference curve. Bacterial concentration was systematically verified by quantitative culture of the inoculum. For intratracheal instillation of mice, short-duration anesthesia was induced by isoflurane inhalation and a gavage canula was inserted into the trachea. The intratracheal position was verified by respiratory oscillations of a droplet inside a 1-ml syringe. The desired quantity of bacteria was instilled in a volume of 50 μl.

Histological analysis

Whole lungs were successively fixed in 4% paraformaldehyde during 36 h, dehydrated in successive baths with increasing ethanol concentration (30, 50, and 70%), embedded in paraffin and cut into 5-μm sections. The sections were stained with H&E for tissue examination.

Assessment of the pulmonary response

To evaluate the pulmonary response to infection 4 and 24 h after PAO1 instillation, mice were euthanized by pentobarbital i.p. injection. Trachea was exposed and a bronchoalveolar lavage (BAL) of 2 ml of sterile PBS was performed. BAL was subjected to serial 10-fold dilutions and cultured for 24 h in tryptic soy agar to quantify the number of CFUs. The total number of inflammatory cells per BAL was quantified by trypan blue exclusion, and cytospin slides were stained with H&E to determine the respective percentages of macrophages and neutrophils. BAL fluid was stored (−80°C) for quantification of cytokines, protein level, and myeloperoxydase (MPO) activity. BAL protein level was quantified using enzymatic assay (BCA protein assay; Pierce). To determine MPO activity, BAL fluid was incubated with 0-dianisidine dihydrochloride in Hanks' medium supplemented with 0.004% H_2O_2. The reaction was stopped by adding 1% NaN_3 and MPO activity was determined by spectrophotometry (absorbance at 450 nm).

Assessment of bacteremic dissemination

Bacteremic dissemination of *P. aeruginosa* was indirectly assessed through quantitative spleen bacterial cultures on selective medium. Spleen was removed and mechanically homogenized under sterile conditions. Spleen homogenates were subjected to serial 10-fold dilutions and cultured for 48 h in tryptic soy agar supplemented with 100 mg/L amoxycillin-clavulanic acid (to avoid growth of nonpseudomonas bacteria). Bacteria were quantified and controlled to confirm positive oxidase activity.

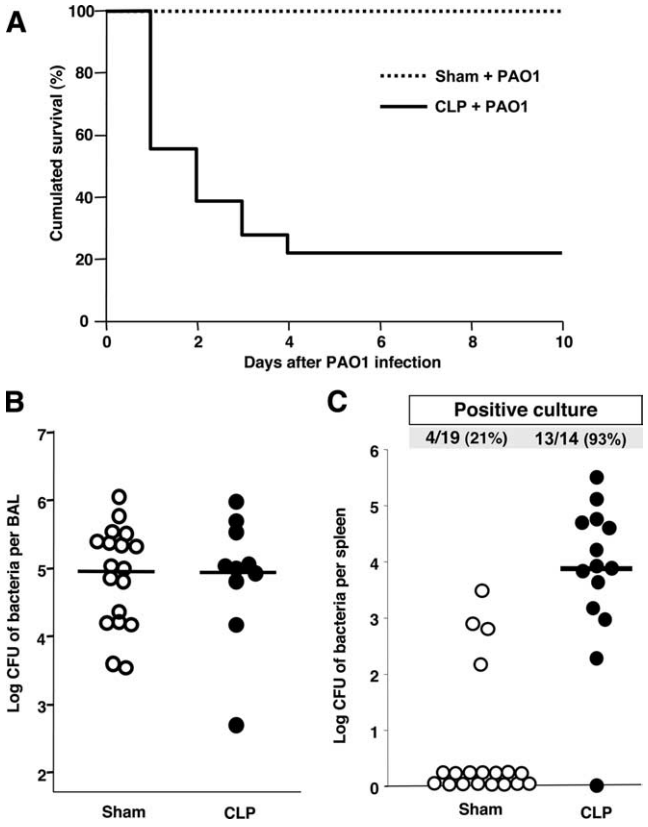

FIGURE 2. Post-CLP mice are highly susceptible to secondary *P. aeruginosa* pneumonia. *A*, Eight days after sham operation or CLP surgery, mice were subjected to intratracheal instillation of 5×10^6 CFUs of *P. aeruginosa* (serotype PAO1). Survival of sham-operated (dotted line, $n = 10$) or post-CLP (continuous line, $n = 18$) mice was monitored for 10 days after intratracheal challenge (log-rank test $p < 0.01$). *B*, Twenty-four hours after intrapulmonary *P. aeruginosa* challenge, mice were euthanized and subjected to BAL for the purpose of quantitative bacterial culture. *C*, Twenty-four hours after intrapulmonary *P. aeruginosa* challenge, mice were euthanized and bacteremic dissemination of *P. aeruginosa* was indirectly assessed through quantitative spleen bacterial cultures. Spleen homogenates were cultured in selective medium to avoid growth of non-pseudomonal bacteria. Bacteria were quantified and controlled to confirm positive oxidase activity.

Determination of cytokines levels in cell culture supernatants and BAL fluid

TNF-α, IL-12p70, and IL-10 concentrations were quantified in cell culture supernatants and in BAL fluid using ELISA according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Survival curves were analyzed using the Kaplan-Meier method and compared using the log-rank test. Continuous variables were expressed as median and interquartile range, displayed using boxplots or scatterplots, and were compared using the Mann-Whitney *U* test. Categorical variables were compared using the Fisher exact test. p values <0.05 indicated statistically significant differences.

Results

Sublethal polymicrobial sepsis induces sustained functional defects of BMDCs

To create a sublethal model of polymicrobial sepsis, CLP was performed using a 21-gauge needle in C57BL/6J female mice. A short antibiotic course resulted in a 70% survival rate while all untreated septic mice died within the first week following CLP

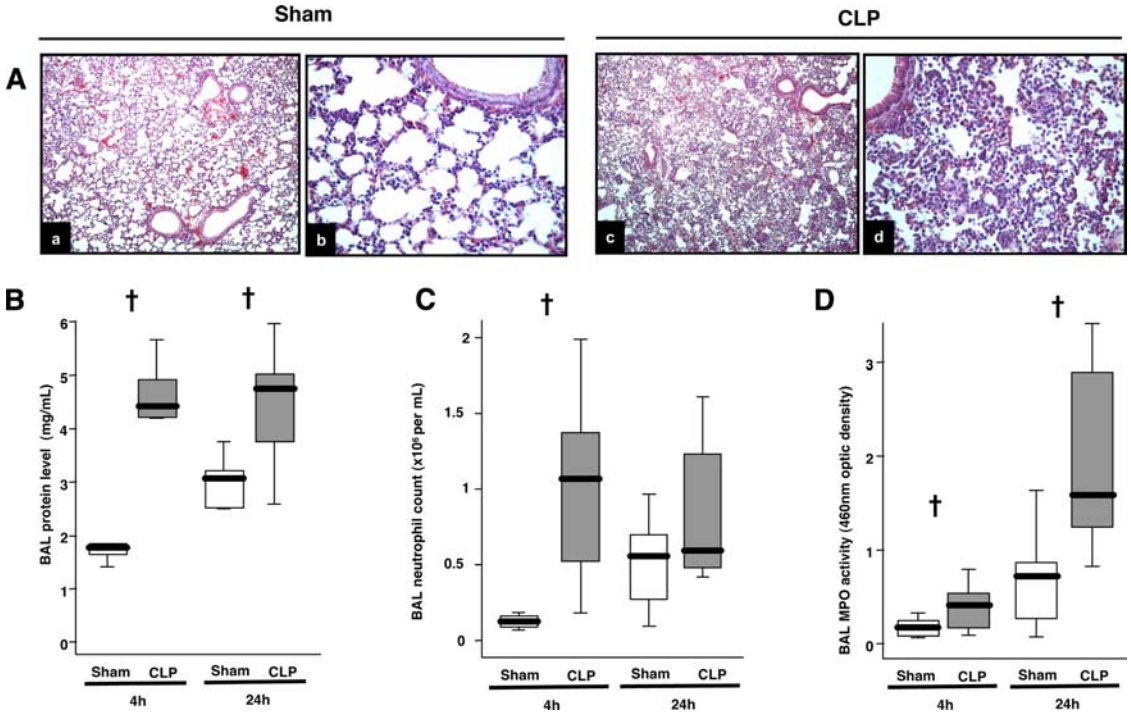

FIGURE 3. Secondary *P. aeruginosa* pneumonia induces lung damage in post-CLP mice. Lung histology from *P. aeruginosa*-infected sham-operated and post-CLP mice (*A*). Lung sections with H&E stain from mice 4 h after intratracheal inoculation of 5×10^6 CFUs of *P. aeruginosa* (serotype PAO1): sham-operated mice at a magnification of ×50 (*a*) and ×200 (*b*), and post-CLP mice at a magnification of ×50 (*c*) and ×200 (*d*). Four and twenty-four hours after intratracheal *P. aeruginosa* challenge, sham-operated and post-CLP mice were subjected to BAL for the purpose of determination of protein level (*B*), neutrophil counts (*C*), and quantification of MPO activity (*D*). Data are represented as boxplots ($n = 8$–15 animals per group and per time-point). †, $p < 0.05$.

(supplemental Fig. S1).[5] To investigate the potential impact of sepsis on the function of DCs, we generated BMDCs from bone marrow precursors collected on day 1 or 8 after CLP. Sepsis did not affect differentiation of BMDCs as confirmed by CD11c expression (>75% of positive cells) and typical morphology (data not shown). However, BMDCs derived from septic mice at the early (day 1) or late (day 8) phase of sepsis retained an immature phenotype with a low basal expression of MHCII and costimulatory molecules CD40 and CD86 (Fig. 1*A*). Furthermore, stimulation with LPS, a TLR4 agonist commonly used to induce DC maturation, failed to restore the membrane expression of MHCII, CD40, and CD86 (Fig. 1*A*). As altered surface expression of MHCII and costimulatory molecules may compromise the interactions of DCs with T lymphocytes, we performed allogeneic mixed lymphocyte reactions to test whether BMDCs derived from septic mice retained the ability to induce proliferation of T lymphocytes. Consistently, BMDCs derived from mice 1 or 8 days after CLP exhibited impaired ability to prime T lymphocytes (Fig. 1*B*). To further investigate the function of BMDCs, we also measured cytokine release after LPS stimulation. Whereas the production of TNF-α and IL-10 was not affected, sepsis significantly impaired the production of bioactive IL-12p70 (Fig. 1*C*).

Post-CLP mice are highly susceptible to secondary P. aeruginosa *pneumonia*

To investigate the impact of sublethal polymicrobial sepsis on long-term lung defense, we created a relevant model of secondary late-onset *P. aeruginosa* pneumonia. For this purpose, we infected mice that had survived and recovered from polymicrobial sepsis 8 days after CLP with various bacterial loads of the *P. aeruginosa* serotype PAO1. Following intratracheal instillation of 5×10^6 CFUs of *P. aeruginosa*, all sham-operated mice survived while 80% of post-CLP mice died within 96 h (Fig. 2*A*), suggesting that sublethal polymicrobial sepsis conferred long-term susceptibility to a secondary pulmonary infectious insult. To test whether sepsis induced defective lung clearance of the pathogen, we performed quantitative bacterial cultures of BAL fluid collected from sham-operated and post-CLP mice. Twenty four hours after tracheal inoculation with *P. aeruginosa*, BAL bacterial counts did not differ between CLP- and sham-operated animals, suggesting that defective bacterial clearance does not account for increased susceptibility to secondary pneumonia (Fig. 2*B*). In contrast, the incidence of bacteremia assessed by positive spleen bacterial cultures was significantly different between groups: quantitative spleen cultures were positive in 93% of post-CLP mice as compared with 21% in sham-operated mice ($p < 0.01$), and bacterial loads tended to be higher in the spleen of post-CLP mice as compared with sham-operated animals with positive spleen cultures (Fig. 2*C*).

P. aeruginosa *pneumonia induces severe lung damage and impairs the IL-12/IL-10 balance in post-CLP mice*

To characterize the lung response to *P. aeruginosa* secondary pneumonia, we assessed morphological changes and determined protein level, neutrophil recruitment, MPO activity, and levels of three representative cytokines (TNF-α, IL-12p70 and IL-10) in the BAL fluid. No lung inflammation was present before instillation of *P. aeruginosa*, as shown by baseline protein levels, cell counts and MPO activity in the BAL fluids of post-CLP and sham-operated mice (Supplemental Figure S2). Instillation of *P. aeruginosa* resulted in severe lung injury and disruption of the alveolo-capillary membrane, as shown by the presence of large foci of alveolar consolidation with neutrophil infiltrates on lung histological

[5] The online version of this article contains supplementary material.

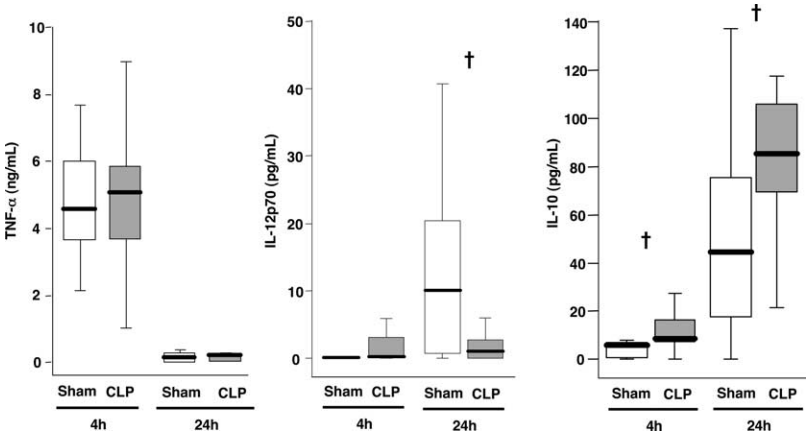

FIGURE 4. Post-CLP mice display imbalanced IL-12p70/IL-10 release in the lung following *P. aeruginosa* pneumonia. Four and twenty-four hours after intratracheal *P. aeruginosa* challenge, mice were euthanized and subjected to BAL for quantification of TNF-α, IL-12p70, and IL-10. Data are represented as boxplots (n = 8–15 animals per group and per time point). †, $p < 0.05$.

sections (Fig. 3*A*). We also found increased BAL protein levels in post-CLP animals (Fig. 3*B*). Quantification of inflammatory cells in the BAL fluid revealed early lung recruitment of neutrophils in post-CLP mice, whereas neutrophil counts did not differ 24 h after pneumonia between sham-operated and post-CLP mice (Fig. 3*C*). MPO activity was also increased in post-CLP mice as compared with sham-operated mice (Fig. 3*D*). To investigate the local host response to secondary pneumonia, we measured BAL concentrations of cytokines. BAL levels of TNF-α peaked early (4 h after inoculation) and decreased to basal levels after 24 h in both sham-operated and post-CLP mice (Fig. 4, *left panel*). Conversely, IL-12p70 levels were significantly lower in the BAL fluid of post-CLP animals as compared with sham-operated mice, whereas IL-10 release was significantly augmented in post-CLP mice (Fig. 4, *middle* and *right panels*), resulting in an IL-12/IL-10 imbalance after pneumonia in post-CLP mice. No differences were noted for BAL levels of IL-17 and KC (supplemental Fig. S3), while IFN-γ was mostly undetectable. Although plasma levels of proinflammatory cytokines (IL-12p70, IL-6, TNF-α) did not differ between sham-operated and post-CLP mice, plasma levels of IL-10 tended to be higher in post-CLP animals (supplemental Fig. S4).

Administration of BMDCs improves survival to secondary P. aeruginosa *pneumonia in post-CLP mice*

We hypothesized that sepsis-induced functional abnormalities of DCs might contribute to impaired lung defense mechanisms against secondary *P. aeruginosa* pneumonia and to the IL-12/IL-10 imbalance observed in our model. To test this hypothesis, post-CLP mice (day-8 survivors) concomitantly received intratracheal instillation of *P. aeruginosa* and BMDCs obtained from nonseptic mice. Tracheal instillation of BMDCs improved the survival rate of post-CLP mice to 70% (Fig. 5*A*), as compared with a 20% survival rate in mice not treated with BMDCs ($p < 0.01$). Interestingly, administration of BMDCs derived from CLP mice did not improve survival and rather worsened outcome in this model of secondary pneumonia (Fig. 5*A*). Altogether, these results suggest that intact DC functions are required to mount an efficient lung response to a secondary infectious insult.

Administration of BMDCs attenuates lung inflammation and restores the IL-12/IL-10 balance in secondary P. aeruginosa *pneumonia*

As sepsis-induced susceptibility to secondary *P. aeruginosa* pneumonia was primarily related to severe lung inflammation rather than inefficient bacterial clearance, we investigated the effect of tracheal instillation of BMDCs on bacterial growth and lung inflammation in post-CLP mice. Whereas instillation of BMDCs did not significantly improve early bacterial lung clearance (Fig. 5*B*), it was associated with decreased systemic bacterial dissemination as compared with untreated post-CLP mice (53 vs 93%, $p < 0.05$) (data not shown).

Tracheal instillation of BMDCs significantly reduced the extent of lung damage as assessed by lung histology (Fig. 6*A*) and BAL protein level (Fig. 6*B*), suggesting that these cells prevented fatal lung inflammation following a *P. aeruginosa* challenge. Consistent with this hypothesis, tracheal instillation of BMDCs also delayed neutrophil recruitment (Fig. 6*C*) and significantly reduced MPO activity in the BAL fluid (Fig. 6*D*), suggesting that BMDCs modulate neutrophils activation.

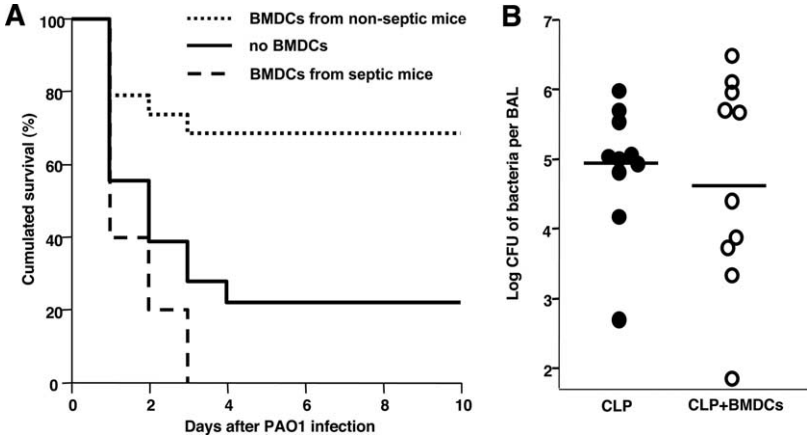

FIGURE 5. Administration of BMDCs reverses susceptibility to secondary *P. aeruginosa* pneumonia in post-CLP mice. *A*, Concomitant to *P. aeruginosa* infection, post-CLP mice were intratracheally administered with 10^6 BMDCs obtained from either nonseptic (dotted line, n = 19) or septic mice (dashed line, n = 5). Continuous line represents survival of post-CLP mice that did not receive BMDCs (n = 18). Survival was monitored for 10 days after intratracheal challenge. Log-rank test $p < 0.05$. *B*, Twenty-four hours after intrapulmonary *P. aeruginosa* infection, post-CLP mice that received intratracheal intillation of BMDCs were subjected to BAL for the purpose of quantitative bacterial culture. Results were compared with those of post-CLP mice with secondary pneumonia not treated with BMDCs (n = 10 animals per group).

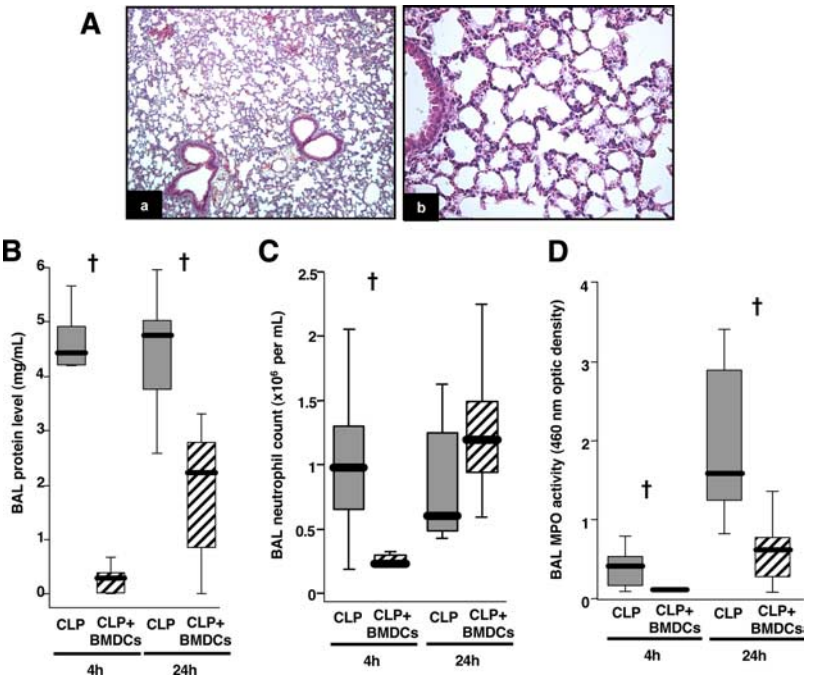

FIGURE 6. Administration of BMDCs dampens pulmonary inflammation in secondary *P. aeruginosa* pneumonia. Lung histology from *P. aeruginosa*-infected post-CLP mice transferred with BMDCs (*A*). Lung sections with H&E stain from mice 4 h after concomitant intratracheal instillation of 5×10^6 CFUs of *P. aeruginosa* (serotype PAO1) and 10^6 BMDCs: original magnification ×50 (*a*) and ×200 (*b*). Four and twenty-four hours after intrapulmonary *P. aeruginosa* challenge, post-CLP mice that were instilled with BMDCs were subjected to BAL for determination of protein level (*B*), neutrophils counts (*C*), and quantification of MPO activity (*D*). Results were compared with those of post-CLP mice with secondary pneumonia not treated with BMDCs. Data are represented as boxplots ($n = 8$–15 animals per group and per time-point). †, $p < 0.05$.

Finally, we investigated the effect of tracheal instillation of BMDCs on cytokine release. BMDCs attenuated the early peak of TNF-α (Fig. 7, *left panel*) and restored a positive IL-12/IL-10 balance. Indeed, IL-12p70 level was transiently but significantly increased at 4 h and returned to basal values at 24 h (Fig. 7, *middle panel*). Conversely, IL-10 production dramatically decreased in the early and late phases of secondary pneumonia (Fig. 7, *right panel*), demonstrating that local instillation of BMDCs can modulate in vivo the IL-12/IL-10 balance.

Discussion

The role of sepsis-induced immune dysfunction in the acquisition of nosocomial infections is unclear. In clinical conditions, analysis of the primary determinants of secondary infections is confounded by underlying comorbidities, care-associated factors as well as incomplete recovery from primary infection. To mimic nosocomial *P. aeruginosa* pneumonia, a frequent clinical situation (22), we established a relevant murine model of sublethal polymicrobial sepsis followed by late-onset *P. aeruginosa* pneumonia. The main results of our investigations can be summarized as follows: 1) post-CLP mice are highly susceptible to secondary *P. aeruginosa* pneumonia after recovery of polymicrobial sepsis; 2) response to secondary infection is characterized by marked lung damage, imbalanced IL-12/IL-10 release and increased systemic bacterial dissemination; 3) whereas polymicrobial sepsis induces sustained functional abnormalities of BMDCs, intratracheal administration of exogenous BMDCs from nonseptic mice improves survival to secondary *P. aeruginosa* pneumonia, dampens pulmonary inflammatory response and restores the IL-12/IL-10 balance.

Several authors have reported impaired lung immunity in animal models of extra-pulmonary infection. In most of these studies, pulmonary bacterial aggression was performed in animals at the early phase (24 h) of sepsis with almost concomitant primary and secondary infections, thereby precluding a precise assessment of their respective contributions to the host response (18, 19). However, timing of the second infectious challenge in respect to the advent or recovery of transient alveolar macrophage deactivation may significantly influence the efficiency of the innate immune response and impact the clinical phenotype (23). In these models, increased susceptibility to early (24 h) *P. aeruginosa* pneumonia was associated with defective bacterial clearance (18, 19). Negative regulation of the LPS/TLR4 signaling pathway by up-regulation of the IL1-receptor-associated-kinase-M likely contributes to the suppression of alveolar macrophage functions in this setting (20). In contrast with the previous models of dual aggressions, we did not observe any impairment of bacterial clearance in our model of

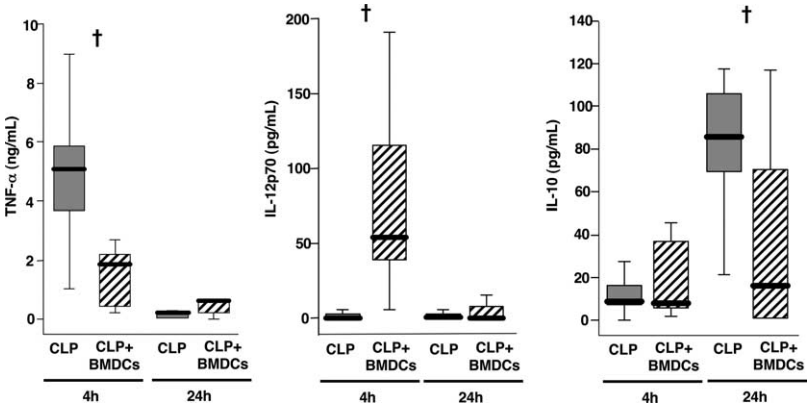

FIGURE 7. Administration of BMDCs attenuates the release of TNF-α and restores the IL-12p70/IL-10 lung balance in secondary *P. aeruginosa* pneumonia. Four and 24 h after intrapulmonary *P. aeruginosa* challenge, post-CLP mice that were administered with BMDCs were subjected to BAL for the purpose of quantification of TNF-α, IL-12p70, and IL-10. Results were compared with those of post-CLP mice with secondary pneumonia not transferred with BMDCs. Data are represented as boxplots ($n = 8$–15 animals per group and per time point). †, $p < 0.05$.

late-onset *P. aeruginosa* secondary pneumonia, suggesting that functional abnormalities of DCs in post-CLP animals do not impede the local antibacterial response against secondary infection. Other models focused on pathogens that are not relevant to the pathogenesis of nosocomial pneumonia to investigate the impact of sepsis on Th-1 and Th-2 immune responses. Hence, mice that survive to CLP have increased susceptibility to tracheal instillation of *Aspergillus fumigatus* and exhibit a dominant Th-2 lung cytokine pattern and defective Th-1 immune response (24). In contrast, postseptic mice challenged with *Schistosoma mansoni* eggs develop larger granulomas in the lung through an enhanced Th-2 cytokine profile (25). In our model, the cytokine response to *P. aeruginosa* instillation in postseptic animals was consistent with a preferential Th-2 immune response to the secondary insult.

In our model, production of IL-12p70 was impaired while IL-10 production was increased in the BAL throughout the early 24 h following pneumonia in post-CLP animals. The role of IL-12 and IL-10 in the antibacterial immune response has been highlighted in different models of Gram-negative pneumonia. Overexpression of IL-12 or neutralization of IL-10 improved bacterial clearance and survival in a model of *Klebsiella* pneumonia (26, 27). In a CLP-model of sepsis-induced immunosuppression, early *P. aeruginosa* pneumonia resulted in enhanced lung IL-10 production and impaired bacterial clearance despite increased neutrophil recruitment (18). In a similar model, LPS-stimulated alveolar macrophages from septic mice displayed a marked impairment in phagocytic activity and produced smaller amounts of IL-12 and TNF-α than alveolar macrophages obtained from sham-operated animals (23). Inhibition of IL-10 activity in vivo reversed the deactivation of alveolar macrophages (23) and improved both pathogen clearance and survival (18), suggesting that IL-10 is a major mediator of sepsis-induced impairment of lung immunity. In our model, bacterial counts did not differ in the BAL fluid from sham-operated and post-CLP mice despite increased local production of IL-10, while we observed important differences in systemic dissemination of infection. Through the release of inflammatory mediators, reactive oxygen species and proteases, activated neutrophils present in the BAL of post-CLP mice may have promoted the epithelial damage that allowed bacteremic dissemination of infection. Hence, as observed in other models of *P. aeruginosa* pneumonia that documented plasma leakage of inflammatory cytokines from the infected lung, loss of compartmentalization due to alveolar epithelial injury was more likely to account for the high rate of bacteremia in post-CLP animals (28).

With their unique ability to link innate and adaptive immunity, DCs may be centrally involved in the development of sepsis-induced immune dysfunction. Commonly used in studies of DC biology and for immunotherapy, BMDCs share common properties and functions with conventional DCs. In our model, BMDCs obtained from septic mice 1 and 8 days after CLP displayed an immature phenotype characterized by low expression of MHCII, CD40, CD86, and decreased IL-12 production. As a consequence, the ability of BMDCs to induce proliferation of allogeneic T cell lymphocytes was consistently decreased. Interestingly, functional abnormalities of BMDCs persisted despite a 7-day period of ex vivo differentiation, suggesting that sepsis induces molecular changes that durably alter the maturation of these cells. Recent data indicate that cytokines such as IL-10 and IL-6, or stimulation of TLRs by pathogen-associated molecular patterns such as LPS, interfere with differentiation and maturation of BMDCs through the modulation of NF-κB and STAT signaling pathways (29–31). During polymicrobial sepsis, sustained stimulation of multiple TLRs or cytokine release might impede the differentiation of BMDCs and compromise the functional interactions between DCs, T lymphocytes and other effector cells that contribute to mount an efficient lung defense.

Whether functional abnormalities of DCs contributed to immune dysfunction and altered lung antimicrobial immunity in human sepsis is unclear. In patients with septic shock, blood monocytes, the cells used to generate human DCs when cultured with GM-CSF and IL-4, interestingly exhibit anergy that closely resembles functional abnormalities of BMDCs evidenced in our study, with impaired production of proinflammatory cytokines (4, 32) and decreased expression of HLA-DR and CD86 (33, 34). Furthermore, the magnitude and the persistence of MHCII reduction correlated with increased susceptibility to infections and subsequent mortality (5, 35). Although the specific role of DCs in pulmonary host defense has not been firmly established in humans, recent experimental studies highlighted the contribution of DCs against primary or secondary lung infection. Hence, a major role of DCs in the organization of a potent Th-1 pulmonary response has been suggested in a model of primary *Klebsiella pneumoniae* pulmonary infection (36). In our study, the restoration of a positive IL-12p70/IL-10 balance after tracheal instillation of exogenous BMDCs suggests that these cells favored a potent pulmonary Th-1 response, which is required to eradicate intracellular pathogens or fungi. Accordingly, administration of BMDCs improves the clearance of the pathogen and survival of postseptic mice challenged with *Aspergillus fumigatus* (24). In contrast, although we observed improved survival with decreased lung damage and inflammation after instillation of BMDCs, bacterial counts in the BAL fluid were similar in all experimental groups. Our data demonstrate that DCs play a minor role in clearance of the extracellular bacteria *P. aeruginosa*, but rather modulate the extent of the inflammatory response and the severity of lung injury. Altogether, these results suggest that functional abnormalities of APCs increase the susceptibility to severe secondary infections, and support the hypothesis that BMDCs are appealing candidates for immunotherapy of sepsis-induced immune dysfunction.

The beneficial role of exogenous DCs in mounting efficient immune response against a secondary pulmonary insult suggests that restoration or enhancement of DCs functions in vivo might be an appealing therapeutic strategy toward bacterial superinfections. IFN-γ has a number of effects on APCs, including up-regulation of MHCII expression and increased production of IL-12, leukocyte recruitment at sites of infection and activation of antimicrobial effector functions. However, administration of IFN-γ to post-CLP mice at the time of an i.v. *P. aeruginosa* challenge did not improve neither survival nor bacterial clearance but sustained proinflammatory response (16). Fms-like tyrosine kinase-3 ligand (flt3L) is an hematopoietic growth factor that stimulates differentiation, mobilization and functions of DCs. Interestingly, either pretreatment with flt3L or adoptive transfer of spleen DCs obtained from flt3L-treated mice, but not from untreated mice, conferred resistance to *P. aeruginosa* burn wound infection (37). In contrast, flt3L pretreatment unexpectedly elicited vasculitis, increased lung permeability, sustained neutrophilic alveolitis, reduced bacterial clearance, and finally increased mortality in a model of pneumococcal pneumonia (38). Altogether, these results suggest that the regulation of immune responses is highly complex and that targeting a single pathway through cytokine administration in vivo deserves caution.

In summary, polymicrobial sepsis increased long-term susceptibility to secondary *P. aeruginosa* pneumonia through persistent functional abnormalities of BMDCs. The response to secondary infection was characterized by marked lung damage and imbalanced IL-12/IL-10 release that can be attenuated by tracheal instillation of exogenous BMDCs. The molecular mechanisms that

contribute to the development of functional abnormalities of DCs remain to be elucidated.

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