Modulation of Dendritic Cell Differentiation in the Bone Marrow Mediates Sustained Immunosuppression after Polymicrobial Sepsis

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Murine polymicrobial sepsis is associated with a sustained reduction of dendritic cell (DC) numbers in lymphoid organs and with a dysfunction of DC that is considered to mediate the chronic susceptibility of post-septic mice to secondary infections. We investigated whether polymicrobial sepsis triggered an altered de novo formation and/or differentiation of DC in the bone marrow. BrdU labeling experiments indicated that polymicrobial sepsis did not affect the formation of splenic DC. DC that differentiated from bone marrow (bone marrow-derived DC [BMDC]) of post-septic mice released enhanced levels of IL-10 but did not show an altered phenotype in comparison with BMDC from sham mice. Adoptive transfer experiments of BMDC into naive mice revealed that BMDC from post-septic mice impaired Th1 priming but not Th cell expansion and suppressed the innate immune defense mechanisms against *Pseudomonas* bacteria in the lung. Accordingly, BMDC from post-septic mice inhibited the release of IFN-γ from NK cells that are critical for the protection against *Pseudomonas*. Additionally, sepsis was associated with a loss of resident DC in the bone marrow. Depletion of resident DC from bone marrow of sham mice led to the differentiation of BMDC that were impaired in Th1 priming similar to BMDC from post-septic mice. Thus, in response to polymicrobial sepsis, DC precursor cells in the bone marrow developed into regulatory DC that impaired Th1 priming and NK cell activity and mediated immunosuppression. The absence of resident DC in the bone marrow after sepsis might have contributed to the modulation of DC differentiation. ***The Journal of Immunology*****, 2011, 186: 977–986.**

The host response to systemic bacterial infection, termed sepsis, is paralleled by the development of immunosuppression that facilitates the expansion of microbes and may lead to multiorgan failure due to persistent or secondary infections (1). Despite advances in modern intensive care medicine, sepsis remains the most frequent cause of death on intensive care units (2).

Conventional dendritic cells (DC) are the most potent APCs due to their high expression of MHC molecules. Microbial agents stimulate DC for an increased expression of costimulatory molecules like CD40 and CD86, a process termed maturation (3). Stimulated DC secrete a distinct pattern of cytokines that is decisive for the type of subsequent Th cell differentiation and, thus, are critical for the link between the innate and the adaptive immune response. The heterodimeric cytokine IL-12 promotes the polarization of naive Th cells toward Th1 that is required for immunity against bacterial infections, whereas IL-10 counteracts Th1 priming but favors the development of regulatory T (Tr) cells (4, 5).

DC in the spleen consist of three subtypes ($CD4^+CD8^-$, $CD4^-CD8^+$, or $CD4^-CD8^-$) are continuously replaced with a $t_{1/2}$ of 2 to 3 d (6). DC originate from precursor cells in the bone marrow and share in part the developmental pathway with the monocyte/macrophage lineage. The mechanisms underlying the lineage development of the different subsets of DC are not completely understood. Diverse progenitor cells and transcription factors are involved in the development of the respective DC lineage in the bone marrow and are decisive for the function of differentiated DC that populate peripheral tissues (reviewed in Refs. 7, 8). Under inflammatory conditions, monocytes may serve as precursors for DC (9). Moreover, the function of finally differentiated DC may be modulated by the local cytokine milieu (e.g., in the presence of IL-10), DC show an impaired capacity to release IL-12 and to induce Th1 priming (10–12).

We have previously shown that during the acute phase of severe sepsis, splenic DC rapidly increase the expression of the costimulatory molecules CD40 and CD86 and simultaneously acquire an impaired capacity to release IL-12 in response to bacterial stimuli like immunostimulatory bacterial oligonucleotides (CpG). Instead, splenic DC from septic mice secrete elevated levels of IL-10 (13). This altered responsiveness of DC from septic mice to bacterial stimuli is shortly termed DC dysfunction.

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; BMDC-CD11c$^-$, BMDC generated from bone marrow after depletion of CD11c$^+$ cells; CLP, cecal ligation and puncture; CLP-BMDC, BMDC generated from bone marrow of CLP mice; CM, culture medium; DC, dendritic cells; pOVA, OVA peptide; Sham-BMDC, BMDC generated from bone marrow of sham mice; Tr, regulatory T; TSB, tryptic soy broth.

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During disease progression, the disappearance of the $CD4^+$ $CD8^-$ subpopulation of splenic DC causes a strong reduction of the total DC number not only in the spleen but also in other lymphoid organs (13, 14). The percentage of DC in the spleen remains reduced for several weeks (15). Whether this DC number diminution is caused by an impaired de novo formation of DC from progenitors in the bone marrow is unknown.

The dysfunction of splenic DC during acute sepsis develops into a chronic dysfunction that is maintained even after resolution of the primary infection and is associated with an impaired capacity of DC to drive an effective Th1 response (15, 16). Previous studies have shown that post-septic mice can be rescued from lethal secondary infections by a treatment with DC from naive mice (16–18). These findings led to the assumption that the enhanced susceptibility of post-septic mice to secondary infections is mediated by the chronic dysfunction of DC. However, the following questions remained unclear: 1) is the long-lasting reduction of the DC numbers in lymphoid organs after sepsis (15) mediated by an defective de novo formation of DC from progenitors in the bone marrow? 2) Does the DC dysfunction originate from an altered differentiation program in the bone marrow or further downstream by the local cytokine milieu in the organ that is populated by newly differentiated DC? 3) Are de novo-differentiated DC from septic mice alone sufficient to mediate immunosuppression? In the current study, we addressed these three questions using the cecal ligation and puncture (CLP), a well-established murine model for human polymicrobial sepsis.

Materials and Methods

Animals

Wild-type BALB/c mice (6–12-wk-old females; Harlan Winkelmann, Borchen, Germany) and DO11.10 mice transgenic for the $OVA_{323–339}$-specific TCR (6–12-wk-old females, originally obtained from The Jackson Laboratory and bred in our own animal facility) were used for the experiments. All mice were housed under specific pathogen-free conditions and had access to standard rodent food and water ad libitum. The animal experiments were performed according to the regulations approved by the local ethic committee.

Cecal ligation and puncture

Polymicrobial sepsis was induced through CLP as described previously (13) with minor modifications. Briefly, mice were anesthetized, and after a midline laparotomy, the cecum was exposed and ligated by 50%. A 27-gauge needle was used to puncture the cecum once, and a small amount of cecum content was extruded. The cecum was then replaced into the abdominal cavity, and the incision was closed with two layers. Sham mice were treated identically except for the ligation and puncture of the gut. All mice were resuscitated by an i.p. injection of 1 ml sterile saline. The mice did not receive any antibiotics. Under these conditions, all CLP mice showed signs of severe illness within 24 h after induction of sepsis, and 20% of these mice died within the first 2 d after the operation but not thereafter. Where indicated, each mouse received 2 mg BrdU (Sigma-Aldrich, Taufkirchen, Germany) in PBS i.p. 2 h before surgery and 1 mg BrdU as daily dose until sacrifice.

Preparation and culture of splenic DC and lymph node cells

The culture medium (CM) was very low endotoxin medium VLE RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% FCS (Biochrom), 10 mM HEPES (Biochrom), 2 mM glutamine, 0.06 mg/ml penicillin, 0.02 mg/ml gentamicin, and 0.05 mM 2-ME (all from Sigma-Aldrich). Medium for the culture of lymph node cells contained 1% mouse serum (PAA Laboratories, Pasching, Austria) instead of FCS.

Single-cell suspensions of spleens were prepared using collagenase digestion as described previously (13). RBCs were lysed through treatment with ammonium chloride. Splenic DC were purified from freshly isolated total spleen cells using CD11c microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and MACS as described previously (13). A part of the isolated cells was stained with fluorescent Ab against CD11c that is characteristic for DC or with an isotype control Ab. The percentage of $CD11c^+$ cells was determined by means of FACS (see below). More than 75% of the cells were positive for CD11c. DC were cultured in the presence of 0.3 ng/ml murine rGM-CSF (R&D Systems, Wiesbaden, Germany) as a survival factor and were either stimulated or not with 5 μg/ml synthetic phosphorothioated 1668 CpG Oligonucleotides (Qiagen, Cologne, Germany) (19). After 18 h, supernatants were harvested. Lymph nodes were meshed through a cell strainer, and single cells were washed.

Generation of bone marrow-derived DC, culture, and T cell activation in vivo

Bone marrow cells were prepared by flushing tibiae and femurs with CM, and RBCs were lysed using ammonium chloride. $CD11c^+$ cells were depleted from bone marrow cells using CD11c microbeads (Miltenyi Biotec) and MACS. Bone marrow-derived DC (BMDC) were generated as described (20). Briefly, bone marrow cells (2×10^6 cells per culture plate) were cultured in CM containing 20 ng/ml GM-CSF. After 7 d days, nonadherent BMDC were harvested. The total activity of the bone marrow per mouse to generate DC was calculated as (number of $CD11c^+$ cells/plate) × (bone marrow cells/mouse)/2.

BMDC were cultured with 5 μg/ml CpG and 100 μg/ml OVA protein (Sigma-Aldrich) for 7 h (termed OVA-loaded BMDC hereafter). Where indicated, FITC-labeled OVA (Molecular Probes, Goettingen, Germany) was used. After 7 h, supernatants were harvested. OVA-loaded BMDC were washed and transferred into fresh culture plates. After additional 20 h culture in the presence or absence of 2.5 μg/ml CD40L (R&D Systems), the supernatants were harvested. All cultures were set up in triplicates.

For T cell activation in vivo, OVA-specific $CD4^+$ T cells were purified from spleens of naive DO11.10 mice using the $CD4^+$ T cell isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. T cells (5×10^6 each) were injected i.v. into the tail veins of naive recipient mice 24 h prior to the s.c. application of 0.5×10^6 OVA-loaded BMDC per hint footpad. Where indicated, 50 μg anti–IL-10 Ab (clone JES5-2A5; BioXCell, West Lebanon, NH) or isotype control Ab (BioXCell) per mouse were injected i.v. 2 h after application of the OVA-loaded BMDC. Seven days later, popliteal lymph node cells were isolated, and triplicate cultures were set up with or without 1 μg/ml OVA peptide (pOVA; aa 323–339, AnaSpec, San Jose, CA) or 100 μg/ml OVA protein for 3 d.

In vivo tracking of BMDC

BMDC were incubated with 0.5 μM CFSE (Molecular Probes) for 12 min at 37°C, washed, and further incubated for 30 min in CM prior to loading with OVA as described above. Cells were washed twice, and 0.5×10^6 CFSE-labeled OVA-loaded BMDC were injected s.c. into each hind footpad of naive recipient mice. Twenty-four hours later, popliteal lymph node cells were isolated.

Flow cytometry

Cells were incubated with total mouse IgG (100 μg/ml; Sigma-Aldrich) and with Abs (from BD Biosciences, Heidelberg, Germany or eBioscience, San Diego, CA) against CD11c, CD11b, CD80, CD86, CD40, CD4, CD8α, MHC class I, MHC class II, CD3ε, Gr-1, CD49b, Ly6G, CD45R/B220, CD45RB, CD69, or $OVA_{323–339}$-specific TCR (Caltag Laboratories, Burlingame, CA) as indicated. For intracellular staining, freshly prepared lymph node cells were incubated for 4–6 h with monensin (0.66 μl/ml; GolgiStop, BD Biosciences). After surface staining of CD11c, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) before addition of Abs against IL-12p40. Appropriate isotype controls were used for all stainings.

Incorporated BrdU was detected using the BrdU flow cytometry detection Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, spleen cells from BrdU-treated mice were stained with Abs against CD11c and CD4 or with CD11c and CD11b (CD11b is only expressed on $CD4^+$ and $CD4^-CD8^-$ DC but not on $CD8^+$ lymphoid DC) and were fixed and permeabilized. After treatment with DNAse I, the BrdU incorporation was detected using anti-BrdU Abs. Isotype control Abs were used to set the threshold for $BrdU^+$ cells. Flow cytometry and analyses were performed using a FACSCalibur and CellQuest Pro software (both from BD Biosciences), respectively. The absolute number of $BrdU^+$ DC per mouse was calculated as: number of spleen cells per mouse × (percent $CD11c^{hi}$ cells among total spleen cells) × (percent $BrdU^+$ cells among gated $CD11c^+$ cells). $BrdU^+$ DC were gated, and the percentage of the DC subtypes was determined according to the CD4 and CD11b expression. The percentage of $CD8^+$ DC was calculated as: 100 − percent $CD11b^+$ cells. The percentage of $CD4^-CD8^-$ DC was calculated as: percent $CD11b^+$ DC − percent $CD4^+$ DC.

Infection with Pseudomonas aeruginosa *and preparation of lung leukocytes*

P. aeruginosa (strain ATCC 27853, American Type Culture Collection) were plated overnight on tryptic soy agar plates at 37°C. After resuspension in tryptic soy broth (TSB), bacteria were grown for 1 h. Bacteria were harvested during the logarithmic growth phase by pelleting and resuspension in fresh TSB. Prior to infection, bacteria were washed twice in RPMI 1640. Mice were intranasally infected with 5×10^8 CFU *P. aeruginosa*. In case of naive mice, *P. aeruginosa* bacteria were applied together with 10^6 BMDC in a total volume of 20 μl. After 24 h, lungs were removed and homogenized in 1 ml PBS. Homogenates were cleared from debris prior to the protein quantification. For the determination of the bacterial load, homogenates were lysed for 10 min with saponin (5 mg/ml) to release intracellular bacteria. Serial dilutions were cultured on TSB agar plates for 18 h, and the colonies were counted. To obtain single-cell suspensions, lungs were perfused with PBS 4 h postinfection and digested using Collagenase P (2 mg/ml, 45 min, 37°C; Roche, Grenzach-Wyhlen, Germany).

ELISA

The content of cytokines was quantified using ELISA for IL-10, IFN-γ, IL-2 (all DuoSet; R&D Systems), IL-6 (BioSource International), or IL-12 (Ready-SET-Go; eBioscience) as recommended by the manufacturers.

Coculture of NK cells and BMDC

For coculture experiments, NK cells were isolated from the spleens of naive mice using the *Pan* NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cell purity was usually >85% as verified by FACS staining. One hundred thousand NK cells were cultured with 1.25×10^4 BMDC in a volume of 150 μl/well (96-well round-bottom plate) medium supplemented with 5 ng/ml rIL-2 (R&D Systems). NK cells and BMDC alone served as controls. The cells were either stimulated or not with UV-irradiated and heat-inactivated (at 65°C for 1 h) *P. aeruginosa* bacteria equivalent to 5×10^6 CFU. After 24 h, the supernatant was harvested for cytokine determination.

Statistics

Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad). Data show the mean and the bar graphs additionally show the SD. Data on lung cytokines were normalized to the mean value of sham mice for each experiment and tested for equal variances prior to further analyses. Differences between sham and CLP groups were tested using the paired or unpaired Student *t* test as indicated in the figure legends. Data obtained from multiple groups were tested using one-way ANOVA followed by Newman-Keuls post test. Data on the bacterial load are presented as scatter plot including the median and were tested using the nonparametric Mann–Whitney *U* test. A p value < 0.05 was considered to be significant.

Results

Number and phenotype of splenic DC during acute infection and recovery

Previous studies on post-septic DC dysfunction used a CLP protocol that induced sepsis in combination with an antibiotic treatment (16–18). Because antibiotics are considered to modulate bone marrow cells (21) and therefore might interfere with our studies on DC development, we set up a CLP protocol that induced a less severe sepsis and that, therefore, was independent from an antibiotic treatment. Twenty-four hours after CLP, a time point that mirrors the acute phase of sepsis, bacteria were present in the peritoneal cavity, spleens, lungs, bone marrow, and blood due to systemic dissemination of the microbes (Supplemental Fig. 1). By day 4 after CLP, no bacteria could be recovered from these compartments, indicating that the infection was contained at that time point (Supplemental Fig. 1). Therefore, this time point of 4 d after CLP was selected to address the post-septic phase.

Next, it had to be confirmed that this model of a less severe sepsis induced DC dysfunction and DC loss similar to the model of severe sepsis as previously reported (13–15, 22). Therefore, the phenotype of DC in the spleen during the acute and post-septic phase was determined by 24 h or 4 d, respectively, after sham or CLP operation. The absolute number of DC in the spleen (gated as $CD11c^{hi}$ cells; Fig. 1*A*) was reduced by 50% in comparison with the DC number in sham mice, both at 24 h and 4 d after CLP (Fig. 1*B*).

During the acute phase of sepsis but not postsepsis, DC expressed higher levels of the costimulatory molecules CD86 and CD40 than the DC isolated from sham mice (Fig. 1*C*). Previous experiments have shown that among several TLR agonists CpG was the most potent agent to induce IL-12 secretion from splenic DC of BALB/c mice (data not shown). Therefore, we used CpG to address the cytokine release from splenic DC. Upon stimulation with CpG, purified splenic DC harvested from mice 4 d after CLP released much higher levels of IL-10 and less IL-12 than the DC extracted from sham mice (Fig. 1*D*) and similarly increased the expression of CD86 and CD40 (Supplemental Fig. 2). The loss of $CD11c^{hi}$ DC in septic mice was mainly due to a diminished subpopulation of $CD4^+$ $CD8^-$ DC at both time points (Fig. 1*E*).

To determine the susceptibility to *P. aeruginosa*-induced pneumonia after the less severe sepsis, mice were infected with *P. aeruginosa* 4 d after sham or CLP operation. CLP mice that were not infected with *P. aeruginosa* served as controls. Twenty-four hours later, the bacterial load in the lungs was quantified. CLP mice contained much more bacteria in the lung after *P. aeruginosa* infection than sham mice (Fig. 1*F*). The lungs of CLP mice that had not been infected with *P. aeruginosa* were free of bacteria (Fig. 1*F*). Thus, a less severe sepsis induces DC dysfunction and an enhanced susceptibility to *P. aeruginosa* as previously observed for severe sepsis.

De novo formation of DC in the spleen

The persistent reduction of the DC number in the spleen of post-septic mice might indicate a defective de novo formation of DC from the hematopoietic compartment during sepsis. To investigate this possibility, mice were treated with BrdU that was incorporated into the DNA during replication, and the content of $BrdU^+$ DC in the spleen, indicating recently divided cells, was determined 4 d after sham or CLP operation. The percentage of $BrdU^+$ cells among gated $CD11c^{hi}$ DC in CLP mice increased 2-fold in comparison with DC from sham mice (Fig. 2*A*). However, with regard to the absolute number of $BrdU^+$ DC per spleen, sham and CLP mice did not differ (Fig. 2*B*). The analyses of the subtype distribution of $BrdU^+$ DC revealed that much less newly generated DC from CLP mice belonged to the $CD4^+$ subtype in comparison with DC from sham mice (Fig. 2*C*). Instead, there was an increased formation of $CD4^-CD8^-$ DC in CLP mice. $CD8^+$ DC were generated to a similar degree in sham and CLP mice (Fig. 2*C*). Thus, sepsis does not inhibit the appearance of newly generated DC in the spleen but rather affects the formation of distinct DC subsets.

Cellular composition of the bone marrow during recovery from sepsis

To investigate whether the less severe sepsis was associated with changes in the bone marrow, the cellular composition of bone marrow 4 d after sham or CLP operation was determined. The bone marrow of CLP mice contained significantly less cells than the bone marrow from sham mice (Fig. 3*A*). The populations of T lymphocytes as well as those of DC (for gating, see Supplemental Fig. 3) were diminished (Fig. 3*B*). The remaining T cells displayed an activated phenotype according to the enhanced expression of the early activation marker CD69 (Fig. 3*C*). The number of NK cells remained unchanged (Fig. 3*B*). Thus, post-septic mice displayed an altered cellular composition of the bone marrow.

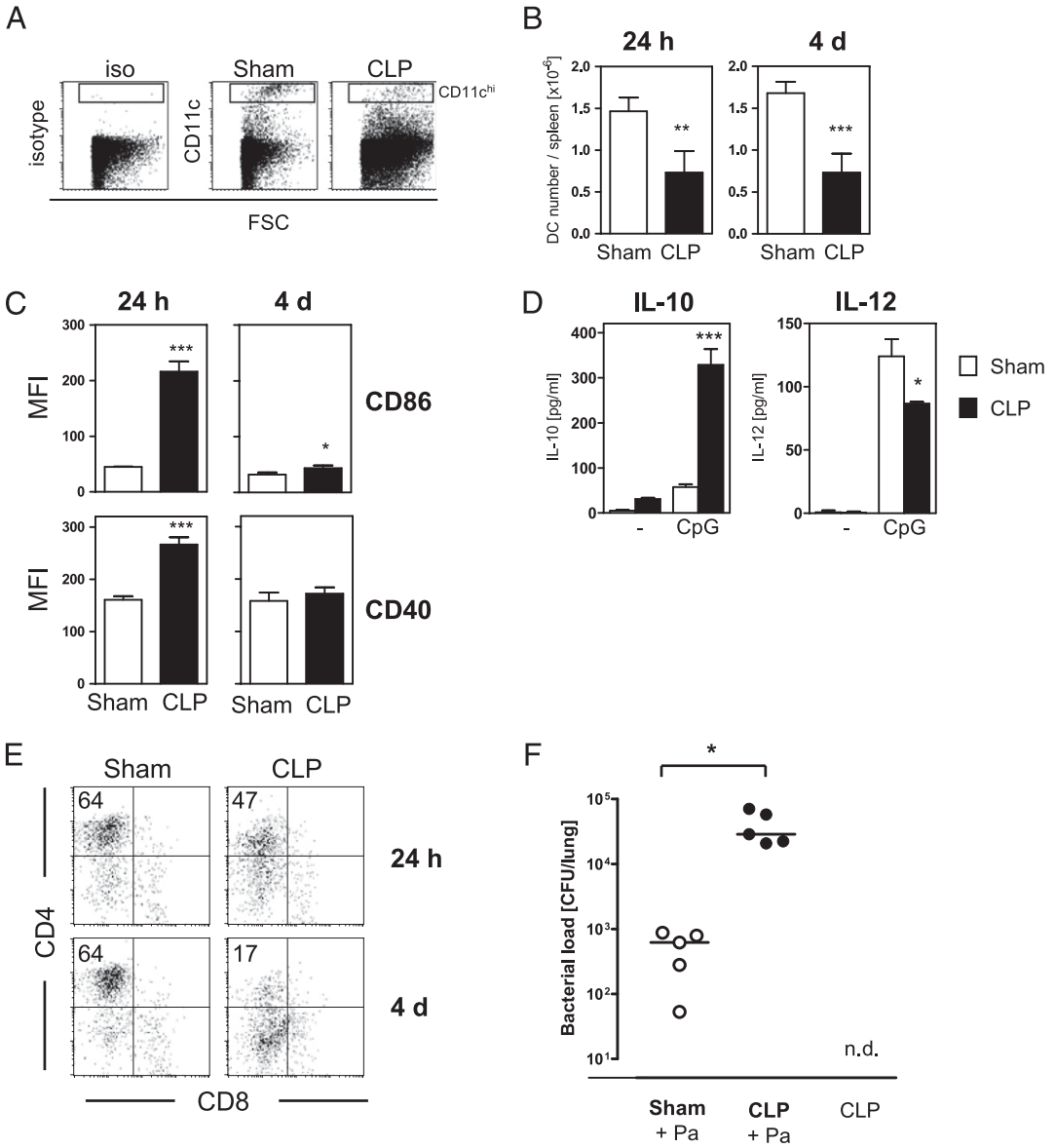

FIGURE 1. DC dysfunction and susceptibility to secondary infections during and after a less severe sepsis. Twenty-four hours or 4 d after sham or CLP operation, total spleen cells were prepared and stained with Abs against CD11c in combination with CD4 and CD8 or with CD40 and CD86 and analyzed by means of flow cytometry. Isotype Abs were used as control. *A*, Gating strategy of conventional $CD11c^{hi}$ DC. *B*, Absolute number of $CD11c^{hi}$ DC per spleen. Data show mean + SD of $n = 3$ to 4 mice per group and are representative of at least three experiments. *C*, Mean fluorescence intensity of CD40 and CD86 expression on $CD11c^{hi}$ DC. Data show mean + SD of $n = 3$ to 4 mice per group and are representative of at least three experiments. *D*, DC purified from total spleen cells were stimulated with or without CpG, and IL-10 and IL-12 were determined in the supernatants. Data show mean + SD from triplicate cultures. Data are representative for two experiments with $n = 3$ to 4 mice per group. *E*, Distribution of $CD11c^{hi}$ DC according to CD4 and CD8 expression. Numbers indicate the percentage of $CD4^+CD8^-$ DC. *F*, Bacterial load in the lung of sham and CLP mice. Four days after sham or CLP operation, the mice were intranasally infected with *P. aeruginosa* (Pa). The bacterial load was determined 24 h later. CLP mice without Pa infection served as control. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ CLP versus sham as tested using an unpaired Student *t* test. n.d., not detected.

Phenotype and function of BMDC from post-septic mice

To analyze DC differentiation from bone marrow of sham and CLP mice under comparable conditions, BMDC were generated from bone marrow cells 4 d after the operation through in vitro culture with GM-CSF that is considered to reflect the differentiation of DC under inflammatory conditions (8). Bone marrow cells from post-septic mice showed an increased proliferative capacity (Fig. 4*A*) and generated a higher number of $CD11c^+$ cells (Fig. 4*B*) in comparison with bone marrow cells from sham mice within 7 d of culture. However, due to the reduced bone marrow cell number in CLP mice, the total activity of the bone marrow per mouse to generate $CD11c^+$ cells was comparable between sham and CLP mice (Fig. 4*C*). BMDC from sham and CLP mice did not differ in their expression of MHC and costimulatory molecules or subtype specific markers, like Gr-1 or CD45RB, and did not express CD4 or CD8 (Fig. 4*D*).

We investigated the cytokine secretion pattern and T cell-stimulatory capacity of BMDC upon stimulation with CpG in analogy to the analyses of splenic DC. BMDC from sham and CLP mice were loaded with OVA and were cultured with or without CpG for 7 h. Thereafter, one part of nonadherent cells was used for T cell activation in vivo (see below). The other part was transferred to fresh culture plates without further stimuli, and the culture was continued in the absence or presence of CD40L to mimic T cell contact in vitro. Uptake of FITC-labeled OVA was comparable

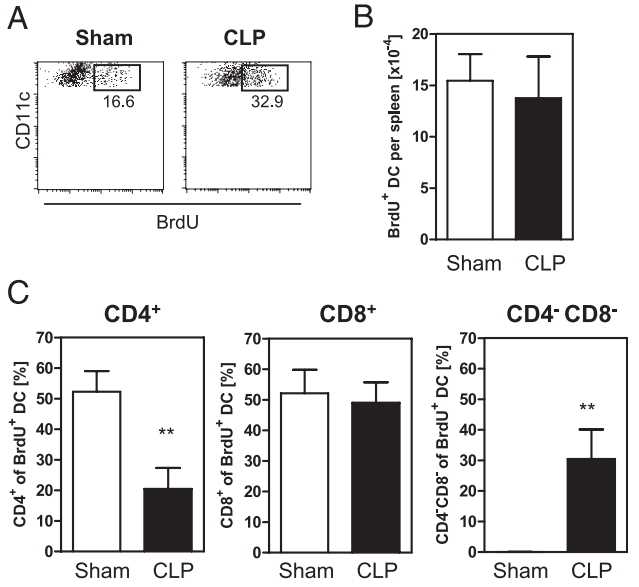

FIGURE 2. De novo formation of DC in the spleen. Mice received BrdU and underwent sham or CLP operation. After 4 d, total spleen cells were prepared and stained against CD11c, BrdU, and CD4 or CD11b. *A*, Numbers indicate the percentage of $BrdU^+$ cells among gated $CD11c^{hi}$ cells. *B*, Total number of $BrdU^+$ $CD11c^{hi}$ DC per spleen. *C*, Percentage of the diverse DC subtypes among newly generated $BrdU^+CD11c^{hi}$ DC in the spleen. $CD8^+$ DC were identified as $CD11b^-$ DC. The percentage of $CD4^-$ $CD8^-$ DC was calculated as the difference of $CD4^+$ DC and $CD11b^-$ DC. Data show mean + SD of $n = 3$ mice per group and are representative for three experiments. Statistical differences were tested using an unpaired Student *t* test. $^{**}p < 0.01$.

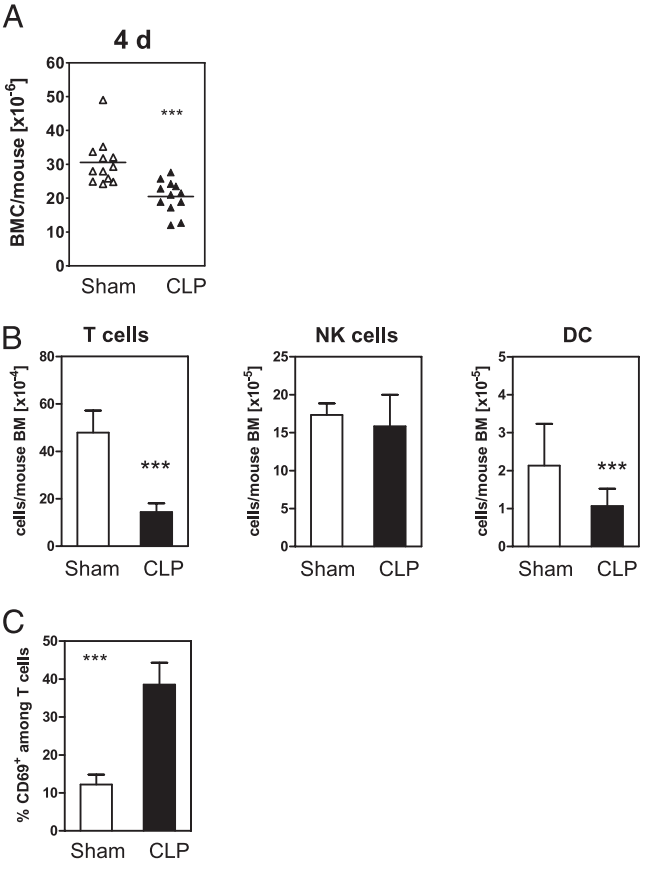

FIGURE 3. Cellular composition of the bone marrow. Four days after sham or CLP operation ($n = 3$ to 4 mice per group), bone marrow cells were prepared, pooled per group, and analyzed flow cytometrically. *A*, Average total bone marrow cell (BMC) number per mouse tibiae and femurs. Horizontal lines indicate the mean values of $n = 12$ experiments. *B*, Absolute number of T cells ($CD3^+CD49b^-CD11c^-$), NK cells ($CD3^-CD11c^-$ $CD49b^+$), and resident DC (MHC class $II^+CD11c^{int/hi}$) in total bone marrow (BM) per mouse tibiae and femurs. Data show mean + SD of $n = 4$ (for T and NK cells) or 8 (for DC) experiments. *C*, Expression of CD69 on T cells. $^{***}p < 0.001$ CLP versus sham as tested using a paired Student *t* test.

between BMDC from sham and CLP mice (Fig. 4*E*). During loading with OVA, CpG-stimulated BMDC generated from bone marrow of CLP mice (CLP-BMDC) released enhanced levels of IL-10 but similar amounts of IL-12 and IL-6 in comparison with BMDC from bone marrow of sham mice (Sham-BMDC; Fig. 4*F*). In the absence of CpG, BMDC from both groups released only low or undetectable levels of these cytokines (data not shown). After transfer to fresh culture plates, the superiority of CLP-BMDC to Sham-BMDC in IL-10 production persisted and was even enhanced upon ligation of CD40 (Fig. 4*G*). The secretion of IL-6 did not differ between BMDC of both groups (Fig. 4*G*). IL-12 levels in supernatants after transfer of BMDC to fresh culture plates were below the detection limit (data not shown). In comparison with other TLR ligands, CpG induced the most striking difference in IL-10 secretion between Sham- and CLP-BMDC (Supplemental Fig. 4).

For the assessment of their Th cell-stimulatory capacity, OVA-loaded BMDC were injected s.c. into the hind footpads of naive mice that had previously received OVA-specific $CD4^+$ Th cells. BMDC were labeled with CFSE to track BMDC after injection and to determine their expression of the IL-12 subunit p40 representative for the IL-12 heterodimer ex vivo. Twenty-four hours after injection, $CFSE^+$ BMDC of either group could be detected in the lymph nodes to a similar extent (Fig. 5*A*, 5*B*). In comparison with $CFSE^+$ BMDC from sham mice, $CFSE^+$ BMDC from CLP mice expressed equally high levels of CD86 (Fig. 5*C*) but less IL-12p40 (Fig. 5*D*).

Seven days after injection of BMDC, the lymph node cells were restimulated with OVA to address the OVA-specific T cell-derived levels of IFN-γ and IL-2 in the supernatants. The lymph node cells contained an equal percentage of OVA-specific $CD4^+$ Th cells irrespective of whether the BMDC originated from sham or from septic mice (Fig. 5*E*). Likewise, the OVA-specific Th cells similarly expressed the T cell activation markers CD25 and CD69 independent from the source of BMDC (data not shown). Lymph node cells from mice that had received CLP-BMDC released strongly reduced levels of the Th1 cytokine IFN-γ upon restimulation with OVA peptide or with OVA protein in comparison with cells from mice that had been treated with Sham-BMDC (Fig. 5*F*). To address the role of IL-10 during BMDC-mediated Th cell priming, anti–IL-10 or isotype control Ab were applied 2 h after application of OVA-loaded BMDC. In comparison with the treatment with the isotype control Ab, the neutralization of IL-10 led to a remarkably increased IFN-γ secretion from restimulated lymph node cells of mice that had received CLP-BMDC (Fig. 5*G*). The secretion of IL-2 from restimulated lymph node cells did not differ between both groups (Fig. 5*F*). Neither Th2 cytokines (IL-4, IL-5, IL-13) nor the Tr cell-associated cytokine IL-10 were detectable in supernatants of the restimulated lymph node cells from either group (data not shown). Thus, CLP-BMDC and Sham-BMDC display a similar phenotype but an altered function as CLP-BMDC strongly release IL-10 and are impaired in Th1 priming.

Transfer of immunosuppression through BMDC from post-septic mice

To investigate whether DC that differentiated from bone marrow of post-septic mice transferred an enhanced risk for *P. aeruginosa* infection, Sham-BMDC or CLP-BMDC were injected intranasally

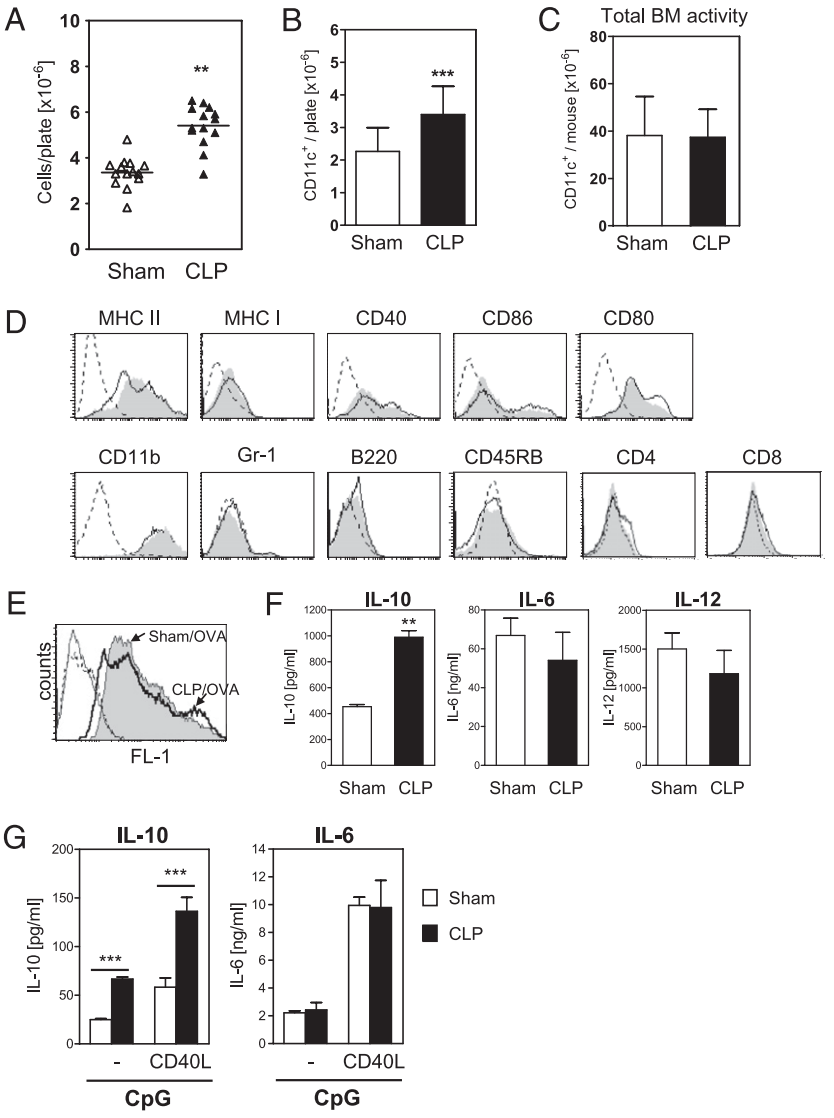

FIGURE 4. Phenotype of BMDC. Four days after sham or CLP operation, bone marrow cells were isolated and cultured for 7 d in the presence of GM-CSF. On day 7, BMDC were harvested. *A*, Total number of cells per culture plate. *B*, Number of $CD11c^+$ cells per culture plate. *C*, Activity of the total bone marrow to generate $CD11c^+$ cells. Data show single values or mean + SD of $n = 14$ experiments with 3 to 4 mice per group. Statistical differences were tested using paired Student *t* test. *D*, Expression of diverse surface markers on gated $CD11c^+$ BMDC from sham (gray shaded area) and CLP (bolt black line) mice. The broken lines show the isotype control stainings from sham cells that were comparable to the isotype stainings of CLP cells. *E*, BMDC were cultured with or without FITC-labeled OVA for 7 h. Cells were stained for CD11c, and the fluorescence intensity in FL-1 representing OVA uptake was determined. The solid gray and the broken black line indicate FL-1 intensity of BMDC from sham and CLP mice, respectively, in the absence of OVA. Arrows indicate histograms of OVA-treated BMDC. *F* and *G*, BMDC were loaded with OVA in the presence of CpG for 7 h as described in *Materials and Methods*. Thereafter, cells were transferred into fresh culture plates, and the culture was proceeded either in the absence or in the presence of CD40L. *F*, Content of IL-10, IL-6, and IL-12 in the supernatants harvested after 7 h. *G*, Content of IL-10 and IL-6 in the supernatants obtained after transfer into fresh plates. Data show mean + SD of triplicate cultures of one experiment that is representative for at least three experiments. Statistical differences were tested using an unpaired Student *t* test. $^{**}p < 0.01$; $^{***}p < 0.001$.

into naive mice along with viable *P. aeruginosa*. Twenty-four hours later, the bacterial load and cytokine levels in the lung were determined. Mice that had received CLP-BMDC contained a higher bacterial load in the lung than Sham-BMDC–treated mice (Fig. 6*A*). The higher bacterial load in the lung after application of CLP-BMDC was associated with a decreased expression of IL-12 and IFN-γ (Fig. 6*B*). The content of TNF-α and IL-10 in the lung did not differ significantly between the two groups (data not shown).

Granulocytes and NK cells play a major role in the defense against *P. aeruginosa* (23, 24). The number of granulocytes and NK cells in the lung of naive mice (for gating, see Fig. 6*C*, 6*F*) was determined 4 h postinfection with *P. aeruginosa* in the presence of Sham-BMDC or CLP-BMDC. The number of $Ly6G^+$ granulocytes clearly increased postinfection independent of the presence of Sham-BMDC (Fig. 6*D*). When the bacteria were applied together with CLP-BMDC, the increase in the number of granulocytes was less prominent (Fig. 6*D*). The expression of CD11b that mirrors the maturation status of granulocytes was reduced in the lung postinfection in comparison with resident granulocytes in the naive lung but did not differ between the BMDC-treated or untreated groups (Fig. 6*E*).

The number of NK cells in the lung of naive mice did not change postinfection with *P. aeruginosa* in the absence or presence of Sham-BMDC or CLP-BMDC (Fig. 6*G*) and thus does not explain the reduced IFN-γ production in the lung that was associated with CLP-BMDC application. Therefore, BMDC were tested for their capacity to modulate the NK cell-derived IFN-γ secretion in vitro. Sham-BMDC or CLP-BMDC were cocultured with naive NK cells and stimulated with UV-irradiated *P. aeruginosa*. NK cells and BMDC alone served as controls. After 24 h, the content of IFN-γ in the supernatant was determined. Sham-BMDC and CLP-BMDC alone did not secrete any IFN-γ (data not shown). No IFN-γ was detected in any culture in the absence of *P. aeruginosa* (Fig. 6*H*). NK cells alone released prominent levels of IFN-γ upon stimulation with *P. aeruginosa* (Fig. 6*H*). However, in the presence of CLP-BMDC, *P. aeruginosa*-stimulated NK cells released less IFN-γ than in the presence of Sham-BMDC (Fig. 6*H*). Taken together, BMDC from post-septic mice transfer enhanced susceptibility to lung infection to naive mice and suppress the IFN-γ production from NK cells.

$CD11c^+$ cells in the bone marrow modulate DC differentiation

The striking alteration of the cellular composition of the bone marrow during sepsis (Fig. 3*B*) might be involved in the diverse DC differentiation in sham and septic mice. We investigated whether DC themselves might modulate the differentiation of new DC in the bone marrow. Therefore, BMDC were generated from bone marrow cells that had been previously depleted from $CD11c^+$

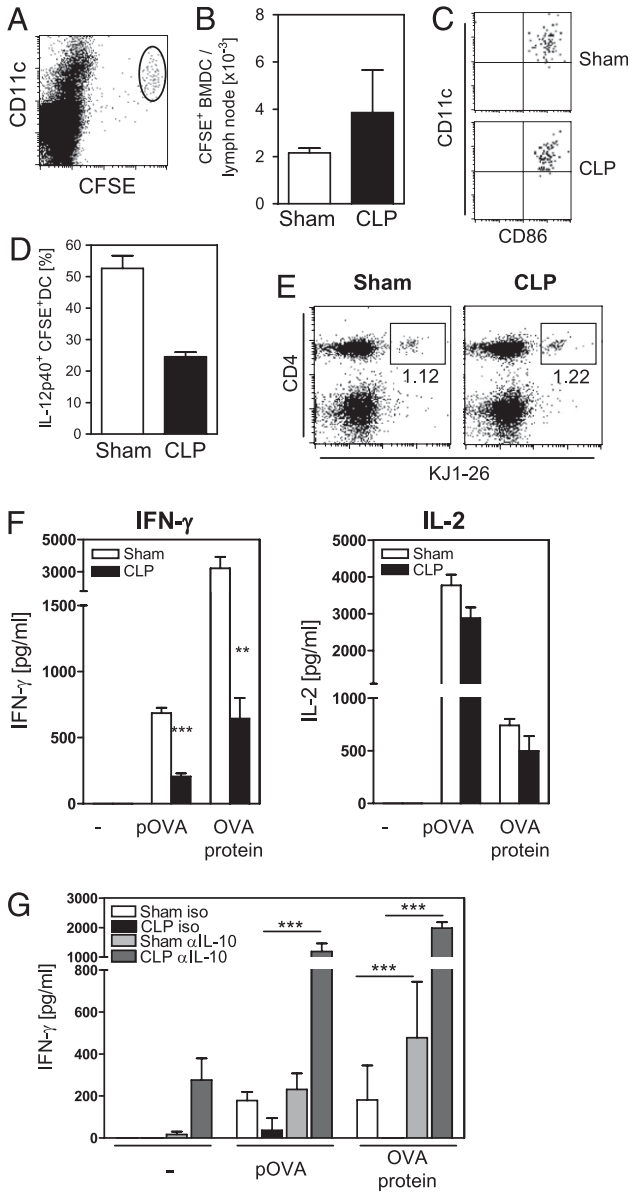

FIGURE 5. Activity of BMDC in Th cell stimulation. Four days after sham or CLP operation, BMDC were generated from bone marrow cells through culture with GM-CSF. *A–C*, On day 7, BMDC were labeled with CFSE, loaded with OVA for 7 h in the presence of CpG, and injected into the hind footpads of naive mice. After 24 h, cells from draining lymph nodes were isolated, pooled, and stained against CD11c. *A*, Representative dot plot of lymph node cells. The region shows $CFSE^+CD11c^+$ cells representing the injected BMDC. *B*, Absolute number of $CFSE^+CD11c^+$ cells in the lymph nodes. Data show mean + range of two experiments with 3 to 4 mice per group. *C*, Lymph node cells were stained against CD11c and CD86. Dot plots show the expression of CD11c and CD86 on gated $CFSE^+$ cells and are representative for two experiments. *D*, Lymph node cells were cultured in the presence of monensin immediately after preparation and were stained against CD11c and intracellular IL-12p40. $CFSE^+CD11c^+$ cells were gated, and the percentage of IL-$12p40^+$ cells was determined. Data show mean + range of two experiments. *E* and *F*, OVA-loaded BMDC were injected into naive mice that had received OVA-specific T cells from DO11.10 mice previously. *E*, After 7 d, lymph node cells were isolated and stained against the OVA-specific TCR (KJ1-26) and CD4. Numbers in the dot plot depict the percentage of $CD4^+KJ1\text{-}26^+$ cells among total lymph node cells. Lymph node cells in the absence of antigenic stimulation contained <0.05% of $KJ1\text{-}26^+$ T cells (not shown). *F*, Lymph node cells were restimulated with OVA protein or pOVA for 3 d, and the content of IFN-γ and IL-2 was determined in the supernatants. Data show mean + SD of triplicate cultures of one experiment that is representative for at least four experiments with similar results. Statistical differences between sham and CLP were tested using an unpaired Student *t* test. *G*, OVA-loaded BMDC and OVA-specific T cells were administered into naive mice as described above. Additionally, all mice received anti–IL-10 or isotype control Ab 2 h after the BMDC injection. The production of IFN-γ was determined in supernatants of restimulated lymph node cells. Statistical differences between the anti–IL-10 Ab- and the corresponding isotype control Ab-treated groups were tested using the ANOVA followed by the Newman-Keuls multiple comparison test. $^{**}p < 0.01$; $^{***}p < 0.001$.

cells (BMDC-$CD11c^-$) (Fig. 7*A*). Prior depletion of $CD11c^+$ cells from bone marrow had no effect on the percentage of differentiated $CD11c^+$MHC class II^+ BMDC on day 7 of culture (Fig. 7*B*).

Upon loading with OVA and stimulation with CpG, BMDC-$CD11c^-$ from sham mice but not from CLP mice secreted more IL-10 than BMDC generated from total bone marrow (Fig. 7*C*). BMDC-$CD11c^-$ from both groups secreted less IL-6 than BMDC generated from the respective total bone marrow (Fig. 7*D*).

OVA-loaded BMDC-$CD11c^-$ from sham and septic mice equally induced the expansion of OVA-specific Th cells in popliteal lymph nodes after s.c. injection into the footpad (data not shown) of naive mice. After the application of BMDC-$CD11c^-$ from sham mice, restimulated lymph node cells released less IFN-γ than lymph node cells that were obtained after application of Sham-BMDC. In contrast, prior depletion of $CD11c^+$ cells from the bone marrow of CLP mice did not further reduce the IFN-γ production of restimulated lymph node cells (Fig. 7*E*). Thus, BMDC generated from CD11c-depleted bone marrow from sham mice displayed an altered cytokine secretion pattern and a reduced extent of Th1 priming.

Discussion

The dysfunction of DC that develops during polymicrobial sepsis is considered to mediate an enhanced susceptibility to secondary infections. To our knowledge, we show for the first time that sepsis modulates the differentiation of DC in the bone marrow. Upon adoptive transfer, DC that had developed from the bone marrow of post-septic mice inhibited Th cell polarization and NK cell function in naive mice. Accordingly, DC from post-septic mice transferred an enhanced susceptibility to *P. aeruginosa* to naive mice. We provide evidence that the altered DC differentiation during sepsis might originate from changes in the content of $CD11c^+$ cells in the bone marrow.

We show in this paper that during sublethal sepsis DC initially underwent maturation (indicated by the upregulation of CD40 and CD86) and acquired long-lasting dysfunction (characterized by the shift of the IL-12/IL-10 balance toward IL-10). In parallel, the $CD4^+CD8^-$ DC subpopulation in the spleen was selectively lost. Moreover, the sublethal sepsis enhanced the risk for secondary infections (Fig. 1*F*) that could be restored through application of naive DC (25).

The mechanisms underlying the sustained reduction of the number of splenic DC in post-septic mice have not been investigated so far. Such mechanisms might include apoptosis of DC as reported for severe sepsis (14, 26) and/or an impaired de novo formation of DC from the bone marrow as known from viral infections (27). We show in this study that in the spleen of post-septic mice, newly generated DC appeared to the same extent as in sham mice (Fig. 2*B*). This observation argues against an impaired de novo formation of DC in post-septic mice but rather indicates that there exists a balance between the appearance of new DC and an ongoing loss of old DC that underwent irreversible maturation during the acute phase of sepsis (Fig. 1*C*). A striking difference between newly generated DC of sham and CLP mice was that

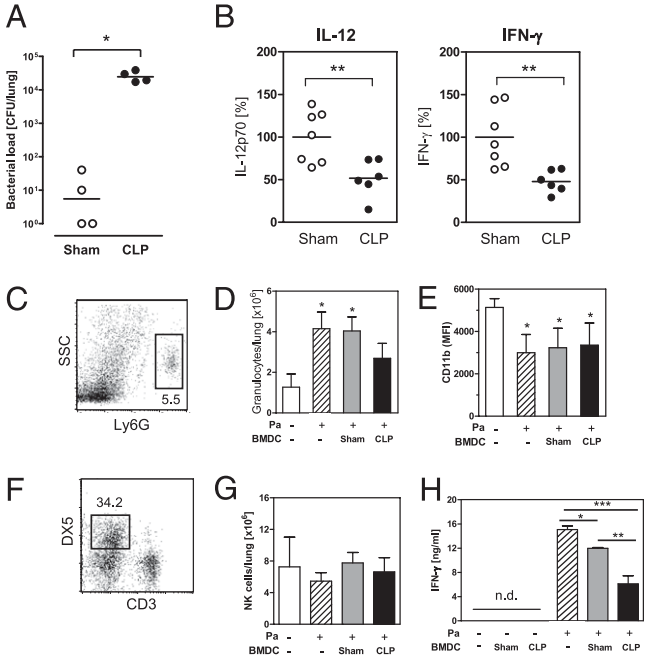

FIGURE 6. Transfer of immunosuppression through CLP-BMDC. *P. aeruginosa* bacteria were applied intranasally into naive mice together with BMDC generated from sham or CLP mice 4 d after the operation. *A* and *B*, After 24 h, lung tissue was homogenized. *A*, Content of bacteria per lung of individual mice from one out of two experiments. Horizontal lines indicate the median value. Significant difference was tested using Mann–Whitney *U* test. *B*, Relative expression of IFN-γ/mg protein and IL-12/mg protein in lung homogenates of individual mice. The mean value of the sham group was used to normalize all values of the corresponding experiment. Data represent pooled data of two experiments with $n = 3$ to 4 per group. Horizontal lines indicate the mean value of normalized data. Significant difference was tested using an unpaired Student *t* test. Absolute values of sham mice ranged between 16 and 47 pg/mg protein for IL-12 and between 13 and 30 pg/mg protein for IFN-γ. *C–G*, Naive mice were intranasally infected with *P. aeruginosa* alone or together with BMDC from sham or CLP mice. Untreated mice served as control. Four hours after the infection, the lung was perfused with PBS, and single cells were prepared, stained for Ly6G, CD11b, CD49b, CD11b, CD3, and analyzed flow cytometrically. *C*, Dot plot showing the gating of $Ly6G^+$ granulocytes. *D*, Absolute number of granulocytes per lung. *E*, Mean fluorescence intensity of CD11b on gated granulocytes. *F*, Dot plot showing the gating of $CD49b^+CD3^-$NK cells. *G*, Absolute number of NK cells per lung. Numbers in the dot plots indicate the percentage of gated cells. Data are representative for two experiments with $n = 3$ to 4 mice per group. Statistical differences were tested using an unpaired Student *t* test. *H*, CLP-BMDC suppress NK cell function. Naive NK cells were cultured alone (hatched bars) or in the presence of Sham-BMDC (gray bars) or CLP-BMDC (black bars) and were either stimulated or not with inactivated *P. aeruginosa* bacteria (Pa). After 24 h, the content of IFN-γ was determined in the supernatants. Statistical differences were tested using the ANOVA. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

~30% of the newly generated DC from CLP mice were $CD4^-$ $CD8^-$ and correspondingly less DC were $CD4^+$ (Fig. 2*C*). The formation of $CD8^+$ DC was not affected during the sublethal sepsis (Fig. 2*C*). Thus, the predominance of the $CD4^-CD8^-$ DC subtype that develops during sepsis is caused by an expansion of this subset and is not only the consequence of a selective loss of the $CD4^+CD8^-$ DC subpopulation (13).

Sepsis did not change the de novo formation of DC as shown in vivo (Fig. 2*B*) and in vitro (Fig. 4*C*) but altered the function of newly differentiated DC. In vitro-generated BMDC from septic mice resembled splenic DC in vivo with regard to impaired Th1 priming (Fig. 5*F*), increased IL-10 production (Fig. 4*F*, 4*G*), and maturation in response to stimulation with CpG (Supplemental Fig. 5). Despite identical culture conditions, isolated bone marrow cells from sham and CLP mice gave rise to BMDC that remarkably differed in function. Therefore, we assume that the sepsis-induced development of DC dysfunction originates in the cells of the hematopoietic compartment. Pène et al. (17) recently reported that BMDC generated from post-septic C57BL/6 mice in comparison with BMDC from sham mice weakly expressed MHC class II and costimulatory molecules and were impaired in IL-12 secretion, whereas the formation of IL-10 remained unchanged. These data are in striking contrast to our findings generated with cells from BALB/c mice as well as from C57BL/6 mice (data not shown) and therefore cannot be explained with the use of different mouse strains. Rather the diversity in the culture conditions during BMDC differentiation between our study that used the original protocol of Lutz et al. (20) and the study from Pène et al. (17) may have led to the contrasting phenotype of the BMDC.

We show in this paper that Ag-loaded CLP-BMDC were suppressed in their capacity to prime a Th1 response when transferred to naive mice. Sham-BMDC and CLP-BMDC did not differ in their phenotype regarding the uptake of Ag (Fig. 4*E*), the expression of MHC class II and of costimulatory molecules (Fig. 4*D*, 5*C*, Supplemental Fig. 5), their migration into the draining lymph nodes (Fig. 5*B*), and the induction of Ag-specific Th cell expansion upon adoptive transfer into naive recipients (Fig. 5*E*). These findings indicate that the failure of CLP-BMDC to induce Th1 priming was not caused by an inadequate expression of molecules that are required for Th cell activation. IL-10 possesses regulatory function (5) and is released by CLP-BMDC at prominent levels especially upon ligation of CD40 that mimics the interaction with T cells (Fig. 4*G*). Unlike splenic DC from septic mice, CLP-BMDC were not suppressed in their capacity to release IL-12 in vitro but showed a reduced expression of IL-12p40 when they appeared in the lymph nodes after adoptive transfer into naive mice (Fig. 5*D*). This observation suggests that CLP-BMDC receive additional signals in the lymphoid organ (e.g., through interaction with other cell types and/or through epigenetic regulation) (15) that might result in a reduced IL-12 release from CLP-BMDC at the site of T cell priming. The neutralization of IL-10 during T cell activation in vivo led to an increased IFN-γ release from OVA-specific T cells especially in mice that had received CLP-BMDC (Fig. 5*G*). This finding indicates the involvement of

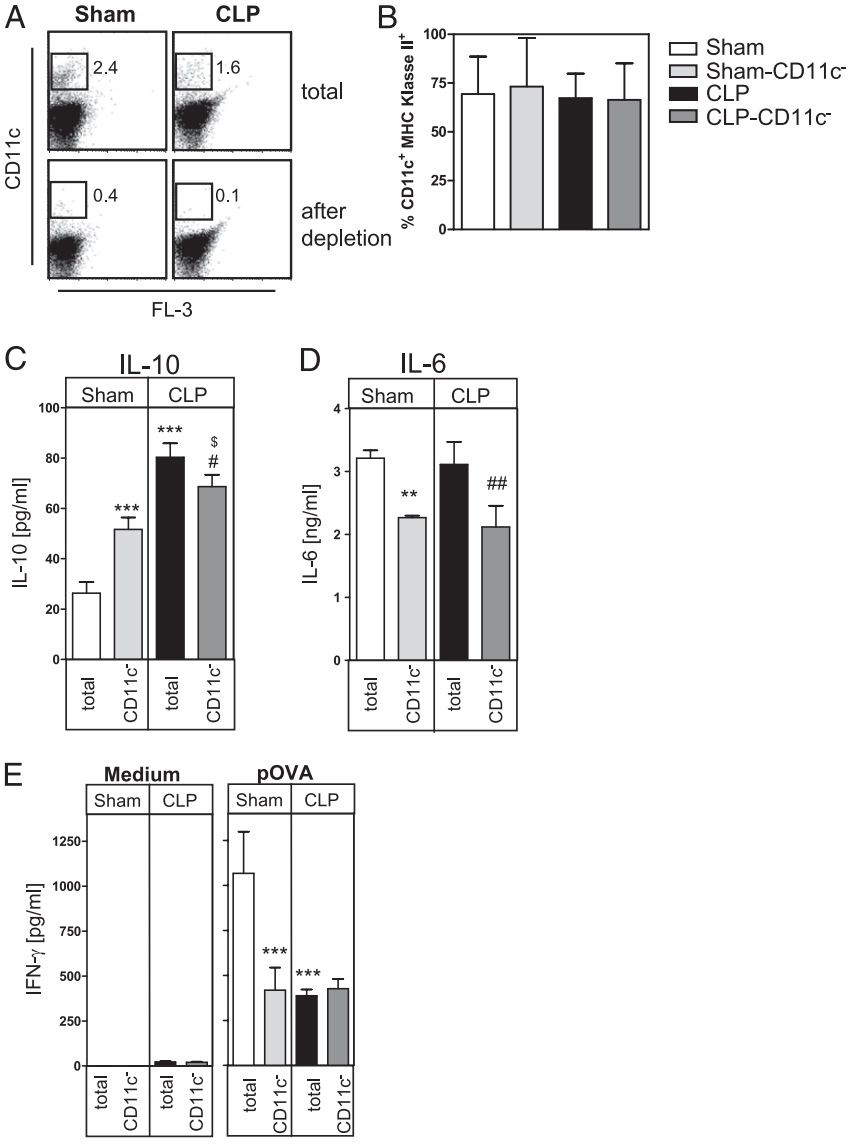

FIGURE 7. Regulatory effect of CD11c$^+$ cells in the bone marrow on the differentiation of BMDC. Four d after sham or CLP operation, bone marrow cells were prepared and one part was depleted from CD11c$^+$ cells. BMDC were generated from total as well as from CD11c-depleted bone marrow cells through culture with GM-CSF. *A*, Bone marrow cells before and after depletion of CD11c$^+$ cells. Numbers indicate the percentage of CD11c$^+$ cells. *B*, Percentage of CD11c$^+$MHC class II$^+$ BMDC on day 7 of culture. *C–E*, BMDC from total and CD11c-depleted bone marrow were loaded with OVA for 7 h in the presence of CpG and transferred into fresh plates. The content of IL-10 (*C*) and IL-6 (*D*) in the supernatants was determined after 20 h. *E*, OVA-loaded BMDC were injected into naive mice that had previously received T cells from DO11.10 mice. After 7 d, lymph node cells were restimulated with pOVA, and the amount of IFN-γ was determined in the supernatants 3 d later. All data show mean + SD of triplicate cultures of one experiment that is representative for three experiments. Statistical differences were tested using the one-way ANOVA followed by the Newman-Keuls post test. $^{**}p < 0.01$; $^{***}p < 0.001$ sham CD11c$^-$ or CLP total bone marrow versus sham total bone marrow; $^{\$}p < 0.05$ sham CD11c$^-$ versus CLP CD11c$^-$ bone marrow; and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ CLP CD11c$^-$ versus CLP total bone marrow.

IL-10 in CLP-BMDC–mediated suppression of Th1 priming. We assume that CLP-BMDC are functional in inducing Th cell activation/proliferation as shown by the expansion of Ag-specific CD4$^+$ Th cells and intact IL-2 secretion (Fig. 5*E*, 5*F*). However, considering the concomitant lack of Th2 cytokines or IL-10 secretion from restimulated Th cells, CLP-BMDC do not provide signals that support the polarization of Th cells toward Th1, Th2, or Tr cells.

Adoptive transfer of CLP-BMDC into naive mice did not only prevent Th1 priming but further had a negative impact on the host response to *P. aeruginosa* infection in the lung (Fig. 6*A*) of naive mice. Previous experiments have shown that the bacterial load in the lungs of naive mice postinfection with *P. aeruginosa* was independent from simultaneously applied Sham-BMDC (data not shown), indicating that Sham-BMDC do not improve the clearance of bacteria through mechanisms that might lack in CLP-BMDC. Granulocytes and NK cells are required for an effective defense against *P. aeruginosa* infection in the lung (23, 24) and, thus, might be target cells of the CLP-BMDC–mediated suppression of the immune response against *P. aeruginosa*. However, the finding that CLP-BMDC did not significantly alter the number or the maturation state of recruited granulocytes in the lung (Fig. 6*D*, 6*E*) argues against a negative effect of CLP-BMDC on granulocyte recruitment.

NK cells represent the major source of IFN-γ in the lung upon *P. aeruginosa* infection (23), and protective immunity against these microbes is associated with the expression of IL-12 and IFN-γ (28, 29). The reduced level of IFN-γ in the lung of naive mice after transfer of CLP-BMDC (Fig. 6*B*) equals the disturbed IFN-γ production in the lung of *P. aeruginosa*-infected post-septic mice (28, 30). This observation points to a role of NK cells in the CLP-BMDC–mediated immunosuppression. Indeed, we found that CLP-BMDC inhibited the *P. aeruginosa*-induced IFN-γ secretion from NK cells (Fig. 6*H*), whereas the number of NK cells in the lung was not affected (Fig. 6*G*). Therefore, we assume that CLP-BMDC prevent the appropriate function of NK cells in the lung and consequently enhance the susceptibility of naive mice to *P. aeruginosa*. Considering the CLP-BMDC–mediated inhibition of Th1 priming and NK cell activation, CLP-BMDC might represent a novel type of regulatory DC (Fig. 4*D*) that does not equal the phenotype of already known CD45RB$^+$MHC class II^{lo}CD11c^{lo} regulatory DC (31–33).

Diverse mediators (e.g., IFN-γ, IFN-α, IL-10) when present during DC differentiation modulate the function and phenotype of developing DC (32, 34, 35). We did not observe any change in DC characteristics when BMDC were differentiated from naive bone marrow in the presence of conditioned media from bone marrow

cells of post-septic mice (unpublished observation). Thus, soluble factors released from bone marrow cells do not seem to be responsible for the altered differentiation of DC in post-septic mice. Rather, the altered cellular composition of the bone marrow after sepsis (Fig. 3*B*) as shown by reduced numbers of T cells and DC might influence the differentiation of DC through so far unknown mechanisms. Although the T lymphocyte number showed a striking reduction, we did not detect any influence of T cells from sham or CLP mice on DC differentiation (data not shown). Instead, we identified a novel function of $CD11c^+$ cells in the bone marrow of sham mice as the presence of these cells during the 7 d culture of bone marrow cells favored the differentiation of BMDC toward a Th1-promoting phenotype. In the absence of $CD11c^+$ cells, bone marrow cells from sham mice gave rise to BMDC that resembled BMDC generated from bone marrow of CLP mice in terms of an increased IL-10 secretion (Fig. 7*C*) and a reduced capacity for Th1 priming (Fig. 7*E*). There exist several $CD11c^+$ cell types in the bone marrow like conventional DC or plasmacytoid DC, and it remains to be elucidated which of these subtypes dictates the developmental program of DC differentiation. DC have been described to promote the formation of megacaryocytes and platelets from bone marrow stem cells through soluble and cell contact-dependent factors (36). The novel function of $CD11c^+$ cells in the bone marrow that we describe in this paper implies that resident DC might control the differentiation of cells of their own lineage in the bone marrow. Consequently, the loss of such $CD11c^+$ cells from bone marrow as it occurs during sepsis might result in the formation of dysfunctional DC.

We conclude that after sepsis, DC with aberrant function are generated in the bone marrow and repopulate lymphoid and nonlymphoid tissues. Due to their regulatory activity, these DC suppress the function of Th cells and NK cells and thereby impair the development of effective immune responses against bacterial infections. The consequences may be persistent infections and/or enhanced susceptibility to secondary infections as observed in patients and rodents after sepsis (1). Thus, normalization of DC differentiation from precursor cells in the bone marrow might be a promising approach to prevent sustained immunosuppression after sepsis.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Benjamim, C. F., C. M. Hogaboam, and S. L. Kunkel. 2004. The chronic consequences of severe sepsis. *J. Leukoc. Biol.* 75: 408–412.
2. Angus, D. C., W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* 29: 1303–1310.
3. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
4. Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of TH1-TH2 development. *Nat. Immunol.* 1: 199–205.
5. Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: the master regulator of immunity to infection. *J. Immunol.* 180: 5771–5777.
6. Kamath, A. T., J. Pooley, M. A. O'Keeffe, D. Vremec, Y. Zhan, A. M. Lew, A. D'Amico, L. Wu, D. F. Tough, and K. Shortman. 2000. The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J. Immunol.* 165: 6762–6770.
7. Wu, L., and Y. J. Liu. 2007. Development of dendritic-cell lineages. *Immunity* 26: 741–750.
8. Naik, S. H. 2008. Demystifying the development of dendritic cell subtypes, a little. *Immunol. Cell Biol.* 86: 439–452.
9. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82.
10. Harizi, H., M. Juzan, V. Pitard, J. F. Moreau, and N. Gualde. 2002. Cyclooxygenase-2-issued prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J. Immuno1.* 68: 2255–2263.
11. De Smedt, T., M. Van Mechelen, G. De Becker, J. Urbain, O. Leo, and M. Moser. 1997. Effect of interleukin-10 on dendritic cell maturation and function. *Eur. J. Immunol.* 27: 1229–1235.
12. Steinbrink, K., M. Wölfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* 159: 4772–4780.
13. Flohé, S. B., H. Agrawal, D. Schmitz, M. Gertz, S. Flohé, and F. U. Schade. 2006. Dendritic cells during polymicrobial sepsis rapidly mature but fail to initiate a protective Th1-type immune response. *J. Leukoc. Biol.* 79: 473–481.
14. Efron, P. A., A. Martins, D. Minnich, K. Tinsley, R. Ungaro, F. R. Bahjat, R. Hotchkiss, M. Clare-Salzler, and L. L. Moldawer. 2004. Characterization of the systemic loss of dendritic cells in murine lymph nodes during polymicrobial sepsis. *J. Immunol.* 173: 3035–3043.
15. Wen, H., Y. Dou, C. M. Hogaboam, and S. L. Kunkel. 2008. Epigenetic regulation of dendritic cell-derived interleukin-12 facilitates immunosuppression after a severe innate immune response. *Blood* 111: 1797–1804.
16. Benjamim, C. F., S. K. Lundy, N. W. Lukacs, C. M. Hogaboam, and S. L. Kunkel. 2005. Reversal of long-term sepsis-induced immunosuppression by dendritic cells. *Blood* 105: 3588–3595.
17. Pène, F., B. Zuber, E. Courtine, C. Rousseau, F. Ouaaz, J. Toubiana, A. Tazi, J. P. Mira, and J. D. Chiche. 2008. Dendritic cells modulate lung response to *Pseudomonas aeruginosa* in a murine model of sepsis-induced immune dysfunction. *J. Immunol.* 181: 8513–8520.
18. Wen, H., C. M. Hogaboam, J. Gauldie, and S. L. Kunkel. 2006. Severe sepsis exacerbates cell-mediated immunity in the lung due to an altered dendritic cell cytokine profile. *Am. J. Pathol.* 168: 1940–1950.
19. Sparwasser, T., T. Miethke, G. Lipford, K. Borschert, H. Häcker, K. Heeg, and H. Wagner. 1997. Bacterial DNA causes septic shock. *Nature* 386: 336–337.
20. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
21. Charak, B. S., R. Louie, B. Malloy, P. Twomey, and A. Mazumder. 1991. The effect of amphotericin B, aztreonam, imipenem and cephalosporins on the bone marrow progenitor cell activity. *J. Antimicrob. Chemother.* 27: 95–104.
22. Flohé, S. B., H. Agrawal, S. Flohé, M. Rani, J. M. Bangen, and F. U. Schade. 2008. Diversity of interferon gamma and granulocyte-macrophage colony-stimulating factor in restoring immune dysfunction of dendritic cells and macrophages during polymicrobial sepsis. *Mol. Med.* 14: 247–256.
23. Wesselkamper, S. C., B. L. Eppert, G. T. Motz, G. W. Lau, D. J. Hassett, and M. T. Borchers. 2008. NKG2D is critical for NK cell activation in host defense against *Pseudomonas aeruginosa* respiratory infection. *J. Immunol.* 181: 5481–5489.
24. Koh, A. Y., G. P. Priebe, C. Ray, N. Van Rooijen, and G. B. Pier. 2009. Inescapable need for neutrophils as mediators of cellular innate immunity to acute *Pseudomonas aeruginosa* pneumonia. *Infect. Immun.* 77: 5300–5310.
25. Pène, F., E. Courtine, F. Ouaaz, B. Zuber, B. Sauneuf, G. Sirgo, C. Rousseau, J. Toubiana, V. Balloy, M. Chignard, et al. 2009. Toll-like receptors 2 and 4 contribute to sepsis-induced depletion of spleen dendritic cells. *Infect. Immun.* 77: 5651–5658.
26. Hotchkiss, R. S., K. W. Tinsley, P. E. Swanson, M. H. Grayson, D. F. Osborne, T. H. Wagner, J. P. Cobb, C. Coopersmith, and I. E. Karl. 2002. Depletion of dendritic cells, but not macrophages, in patients with sepsis. *J. Immunol.* 168: 2493–2500.
27. Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone. 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J. Clin. Invest.* 113: 737–745.
28. Lighvani, S., X. Huang, P. P. Trivedi, R. H. Swanborg, and L. D. Hazlett. 2005. Substance P regulates natural killer cell interferon-gamma production and resistance to *Pseudomonas aeruginosa* infection. *Eur. J. Immunol.* 35: 1567–1575.
29. Moser, C., P. O. Jensen, O. Kobayashi, H. P. Hougen, Z. Song, J. Rygaard, A. Kharazmi, and N. H by. 2002. Improved outcome of chronic *Pseudomonas aeruginosa* lung infection is associated with induction of a Th1-dominated cytokine response. *Clin. Exp. Immunol.* 127: 206–213.
30. Murphey, E. D., C. Y. Lin, R. W. McGuire, T. Toliver-Kinsky, D. N. Herndon, and E. R. Sherwood. 2004. Diminished bacterial clearance is associated with decreased IL-12 and interferon-gamma production but a sustained proinflammatory response in a murine model of post-septic immunosuppression. *Shock* 21: 415–425.
31. Fujita, S., K. Seino, K. Sato, Y. Sato, K. Eizumi, N. Yamashita, M. Taniguchi, and K. Sato. 2006. Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response. *Blood* 107: 3656–3664.
32. Wakkach, A., N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18: 605–617.
33. Wong, K. A., and A. Rodriguez. 2008. *Plasmodium* infection and endotoxic shock induce the expansion of regulatory dendritic cells. *J. Immunol.* 180: 716–726.
34. Wu, X., W. Hou, S. Sun, E. Bi, Y. Wang, M. Shi, J. Zang, C. Dong, and B. Sun. 2006. Novel function of IFN-gamma: negative regulation of dendritic cell migration and T cell priming. *J. Immunol.* 177: 934–943.
35. Carbonneil, C., H. Saidi, V. Donkova-Petrini, and L. Weiss. 2004. Dendritic cells generated in the presence of interferon-alpha stimulate allogeneic CD4+ T-cell proliferation: modulation by autocrine IL-10, enhanced T-cell apoptosis and T regulatory type 1 cells. *Int. Immunol.* 16: 1037–1052.
36. Wang, Q., W. Zhang, G. Ding, L. Sun, G. Chen, and X. Cao. 2001. Dendritic cells support hematopoiesis of bone marrow cells. *Transplantation* 72: 891–899.