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Polymerization Sepsis Diminishes Dendritic Cell Numbers and Function Directly Contributing to Impaired Primary CD8 T Cell Responses In Vivo

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Patients surviving acute stages of sepsis often display impaired adaptive-immune responses. Using the cecal ligation and puncture model, we demonstrated that sepsis leads to substantial and long-lasting changes in the naive CD8 T cell repertoire, affecting the capacity of the host to respond to new infections. However, the identity of CD8 T cell–extrinsic factor(s) and mechanism(s) that contribute to impaired CD8 T cell responses after sepsis is unknown. Priming of naive CD8 T cells is critically dependent on the ability of dendritic cells (DCs) to provide Ag, costimulation, and inflammatory signal 3 cytokines; therefore, the sepsis-induced changes in the DC compartment might represent a contributing factor leading to diminished CD8 T cell immunity in septic hosts. In a direct test of this hypothesis, we show that, in addition to numerical decline, sepsis leads to functional impairments in DCs, diminishing their capacity to produce cytokines upon TLR stimulation in vitro or postinfection in vivo. Importantly, we demonstrated a direct link between DC dysfunction and impairments in CD8 T cell immunity after sepsis by directly targeting Ag to DCs. Finally, postsepsis Flt3 ligand treatment increased the number of DCs and improved DC function, including the ability to sense inflammation and produce IL-12, leading to improved primary CD8 T cell responses to newly encountered Ags. Thus, sepsis-induced numerical and functional loss of DCs contributes to the observed defects in CD8 T cell immunity, and therapeutic approaches designed to improve the status of the DC compartment after sepsis might facilitate the recovery of CD8 T cell immunity.

Sepsis is characterized as an injurious immune response resulting from an uncontrolled systemic infection. The global death toll of sepsis is estimated at 5.3 million individuals annually, yet even those surviving the initial septic insult suffer from long-term impairments and chronic immunosuppression characterized by increased susceptibility to new (secondary) infections and reactivation of latent viruses (1–6). Increased T cell apoptosis observed in human patients suggests that defects in T cell–mediated immunity can be an underlying cause, at least in part, of sepsis-induced general immunosuppression (7–9). Using the murine cecal ligation and puncture (CLP) model of sepsis induction, we recently showed that sepsis leads to a numerical loss of naive (Ag-nonexperienced) CD8 T cells and impairs primary CD8 T cell responses to acute and chronic infections (10–13). In addition, polymicrobial sepsis alters Ag-dependent and -independent memory CD8 T cell functions (i.e., provide protection to pathogen rechallenge or perform innate functions, such as the capacity to produce IFN-γ in response to heterologous infections, respectively) (12, 13). Although these observations demonstrated that sepsis leads to sustained impairments in naive (primary) and memory (secondary) CD8 T cell responses, the contribution of the environment, in which CD8 T cells recognize and respond to their cognate Ag, to sepsis-induced immunosuppression is not well defined.

The optimal expansion of CD8 T cells following interaction with cognate Ag during an infection and/or vaccination is reliant on CD8 T cell–extrinsic factors, including Ag/MHC complex (signal 1), costimulatory ligands (signal 2), and signal 3 cytokines (e.g., IL-12 and type I IFNs) (14–17). Dendritic cells (DCs) are professional APCs capable of providing CD8 T cells with Ag, costimulation, and signal 3 inflammatory cytokines that are critical for primary CD8 T cell expansion (18–20). Murine DCs are generally divided into two large subgroups: plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of cDCs (21). pDCs, which express low to moderate levels of
spleen contains three main cDC subtypes: CD4+ cDCs (CD4+ CD8−), CD8+ cDCs (CD4− CD8+), and double-negative (DN) cDCs (CD4− CD8−) (21). CD4+ cDCs, which make up the greatest percentage of cDCs in the spleen, are located in the marginal zones of the spleen and efficiently activate CD4+ T cells (21, 23). CD8+ cDCs are primarily located in the T cell zones of the spleen, express CD205, have the capacity for cross-presentation and induction of CD8 T cell responses (21–23), and are potent producers of IL-12 (23). Therefore, the postsepsis status of DCs, as well as their ability to provide the necessary signals for optimal priming of naive CD8 T cells, could be an extrinsic factor contributing to the observed defect in primary CD8 T cell responses (18, 19, 24).

Sepsis leads to a loss of DCs in the spleen (25) and a reduction in myeloid DCs and pDCs in the blood of septic patients (26). Moreover, DCs from septic patients have diminished HLA-DR expression and decreased capacity to produce proinflammatory cytokines in response to LPS stimulation (26). Importantly, low DC counts in patient blood correlates with increased sepsis severity (27), suggesting that the DC compartment might play an important role during sepsis progression. The importance of DCs in sepsis was also established in experimental models of sepsis, including the murine CLP model that closely mimics the disease course of septic patients (28–30). Studies using CD11c-diphtheria toxin receptor DC knockout transgenic (Tg) mice indicated that mice treated with diphtheria toxin to reduce DC numbers had increased sepsis severity that was partially recovered upon reconstituting the DC compartment with adoptively transferred DCs from nonseptic hosts (31). In the CLP model, loss of DCs occurs in the spleen, peritoneum, bone marrow, and select lymph node (LN) subsets of septic mice (29, 30, 32, 33). Bone marrow–derived DCs from septic mice exhibited increased IL-10 production and impaired Th1 CD4 T cell priming (33). Additional functional studies established that DCs obtained from septic hosts have a diminished capacity to produce IL-12 in vitro in response to TLR ligands (29, 30, 33–35). Decreased IL-12 and increased IL-10 production by DCs isolated from the lungs of septic mice also was observed (35–37). Mechanistically, epigenetic changes in the IL-12 promoter correlate with the diminished capacity of DCs from the septic host to produce sufficient amounts of IL-12 (34).

Because these previous studies demonstrated a reduction in DC number and function following sepsis, restoration of the number and function of DCs after sepsis could help to restore immune function and provide therapeutic benefit. Indeed, the transfer of bone marrow–derived DCs into septic mice reduces inflammation in the lungs, increases Th1 and decreases Th2 cytokine levels, and increases survival (36, 38). Flt3 ligand (Flt3L), a hematopoietic growth factor capable of stimulating DC expansion, improves survival and protects against opportunistic infection when administered before and after burn wound infections in mice (39, 40). Furthermore, Flt3L administration to mice subjected to yersinoinduced peritonitis helped to ameliorate the loss of pulmonary DCs and IL-12 production after yersinoin administration, as well as reduced pulmonary tissue damage and mortality (41).

The observed loss of DC numbers and their diminished capacity to produce IL-12 postsepsis provide support for a lesion in DCs contributing to CD8 T cell dysfunction. However, studies seeking to understand the status of DCs in the context of diminished primary CD8 T cell responses are lacking. In this article, we establish the role of DCs in sepsis-induced CD8 T cell dysfunction by elucidating the contribution of the DC compartment in primary CD8 T cell responses to model pathogens. In addition, we show that therapeutic (e.g., postsepsis Flt3L treatment) approaches designed to increase DC number and function aid in reversing the sepsis-induced lesions in CD8 T cell immunity.

MATERIALS AND METHODS

EFFECT OF POLYMICROBIAL SEPSIS ON DC AND CD8 T CELL IMMUNITY

Materials and Methods

All experimental procedures using mice were approved by the University of Iowa Animal Care and Use Committee under ACRUF protocol number 1312217. The experiments performed in this study were done under strict accordance with the Office of Laboratory Animal Welfare guidelines and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Cell isolation/analysis

Before removal of lymphoid organs and tissues, samples of blood were obtained by retro-orbital puncture. For experiments enumerating DC subsets, spleens were treated with collagenase XI (125 U/ml) and DNase I (10 μg/ml) and shaken at ~450 rpm for 10 min at 30°C. Single-cell suspensions were prepared, and samples were shaken again at ~450 rpm for 10 min at 30°C. Appropriate volumes of EDTA were added to the samples for 5 min at room temperature to inhibit the action of collagenase. Samples were then spun down and resuspended in fresh media. Single-cell suspensions from spleen and LNs were washed before Ab staining.

Cecal ligation and puncture

Polymicrobial sepsis was induced by CLP (11, 12, 43). Briefly, mice were anesthetized, the abdomen was shaved and disinfected, and a midline incision was made. After identification of the cecum, the distal third was ligated with 4-0 silk sutures and punctured once using a 25-gauge needle to extrude a small amount of cecal content. The cecum was returned to the peritoneum was closed via continuous suture, and the skin was glued together using tissue adhesive (3M; Vetbond, St. Paul, MN). Saline (1 ml) was administered s.c. following the procedure for resuscitation. Bupivacaine was given at the incision site, and flunixin meglumine was administered twice for postoperative analgesia. This level of injury was used to create a chronic septic state characterized by the loss of appetite and body weight, ruffled hair, shivering, diarrhea, and/or periorbital edema and with 5–10% mortality. Sham-treated mice underwent the same procedure, excluding CLP.

Abs, peptides, and intracellular stains

Flow cytometry data were acquired using a FACSCanto (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR). For enumerating DCs, an empty channel was used to eliminate autofluorescence contamination. To assay the expression of cell surface proteins, mAbs were incubated at 4°C for 30 min and fixed using Cytofix/Cytoperm Solution (BD Biosciences). Surface molecules were detected with the following mAbs: CD8 (clone 53-6.7; eBioscience), Thy1.1 (HIS51; eBioscience), CD4 (clone GK1.5; eBioscience), CD19 (clone ID3; Tonbo Biosciences), B220 (clone RA3-6B2; eBioscience), CD11c (clone HL3; BD Biosciences), 1A-1E (clone 2G9; BD Biosciences), CD40 (clone 3G1; eBioscience), CD80 (clone 16-10A1; eBioscience), DEC-205 (clone NLDC-145; BioLegend), Ve2 TCR (clone B20.1; eBioscience), Vβ3.1,5.2 (clone MR9-4; BD Biosciences), and appropriate isotype controls. Endogenous OVA-specific CD8 T cell responses were quantified using Kb-OVA tetramers, with the limit of detection (LOD) determined by calculating the

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background OVA+ cells in a naive noninfected mouse without adoptively transferred OT-I cells.

**Ex vivo intracellular stains**

Mice infected with virulent *Listeria* were harvested 14 h postinfection. After obtaining single-cell suspensions from the spleen, cells were incubated for 5 h at 37°C in the presence of Brefeldin A (BD Biosciences) prior to ex vivo intracellular staining for IL-12/IL-23p40 (clone C15.6; BioLegend). Intracellular staining was performed after surface staining and fixation/permeabilization of the cell membrane using Cytofix/Cytoperm solution (BD Biosciences).

**DC enrichment and TLR stimulation**

Single-cell suspensions were produced from pooled groups of two spleens, filtered, and incubated with anti-CD11c mAb-conjugated magnetic MicroBeads (clone N418; Miltenyi Biotec) for 15 min in the dark at 4°C, according to manufacturer’s instructions. Cells were washed and resuspended in cold autoMACS buffer and passed through an LS MACS separation column (Miltenyi Biotec). Columns were washed three times with 3 ml of cold autoMACS buffer, and the positive fraction was collected. Enriched cells were plated at 0.4–1 × 10^6 cells per dish and stimulated for 7 h at 37°C with 1 μg/ml LPS (055:B5; Sigma-Aldrich) or 1 μg/ml CpG (ODN 1826; Integrated DNA Technologies). After 2 h of stimulation, 1 mg/ml of Brefeldin A (BD Biosciences) was added to each dish. After 7 h of incubation, intracellular staining for IL-12(IL-23p40 (clone C15.6; BioLegend) and TNF-α (clone MPG-X22; eBioscience) was performed after surface staining and fixation/permeabilization of the cell membrane using Cytofix/Cytoperm solution (BD Biosciences).

**DEC-205 Ab production and administration**

The anti–DEC-205 hybridoma (NLDC-145) was a gift from Deborah Palliser (Albert Einstein College of Medicine). The anti–DEC-205 mAb was purified with a protein G column prior to peptide conjugation. The peptides used for conjugation were synthesized with an extra cysteine on the C-terminus (GenScript). Sulfo-SMCC (Thermo Fisher Scientific) was used as a linker, allowing for conjugation between the cysteine on the peptide and the amine groups of the mAb. The conjugation was performed according to the manufacturer’s instructions. Conjugated DEC-205 mAb/Ag complex was diluted in sterile PBS to reach its desired concentration (10 μg/ml per mouse) prior to i.v. administration. Immediately following administration of the DEC-205 mAb/Ag complex, Cpfil diluted in sterile PBS (50 μg/mouse) was administered i.p.

**Serum ELISAs**

IL-12 and IFN-γ levels (pg/ml) were detected from serum collected by retro-orbital bleed at 24 h postinfection using a Mouse IL-12 Platinum ELISA (catalog number BM5616) and a Mouse IFN-γ Platinum ELISA (catalog number BMS606; both from Affymetrix). Tests were performed according to the manufacturer’s instructions, and absorbance values (450 nm) were measured and assessed using Gen5 software (BiorTek).

**Flt3L production and administration**

Flt3L-Ig was purified from cell culture supernatants via Protein A affinity chromatography. A total of 100 μg of Flt3L-Ig was administered i.p. on days 1–4 post-CLP.

**Statistical analysis**

Data were analyzed using Prism6 software (GraphPad), and a two-tailed, unpaired Student t test or one-way ANOVA with a confidence interval > 95% was used to determine significance. Data generated as scatter plots or bar graphs are presented as mean ± SEM.

**Results**

**A septic environment contributes to impaired primary CD8 T cell expansion**

Previous work from our laboratory showed that vaccine- or infection-induced memory CD8 T cells present at the time of sepsis induction have diminished per-cell capacity to undergo proliferative expansion upon pathogen rechallenge or exert their innate functions and produce IFN-γ upon heterologous infection compared with memory CD8 T cells from control (sham-treated) mice (12). Interestingly, this decrease in Ag-dependent and -independent functions also occurred in memory CD8 T cells that were adoptively transferred into CLP mice days after the surgery and resolution of the sepsis-induced hyperinflammatory state. These data suggest that the sepsis environment controls, at least in part, the ability of memory CD8 T cells to respond to cognate Ag stimulation and/or sense the inflammatory cues in the environment.

To formally prove that CD8 T cell–extrinsic factors present early after sepsis induction can also control primary CD8 T cell responses, naïve TCR-Tg OVA257-specific Thy 1.1 OT-I CD8 T cells were adoptively transferred into naïve Thy 1.2 B6 mice 2 d after sham or CLP surgery (Fig. 1A). To examine the ability of sensor OT-I CD8 T cells to respond to cognate Ag stimulation, sham- and CLP-treated mice were infected with recombinant *L. monocytogenes*–OVA (Fig. 1A) 3 d after sepsis induction. As shown previously (44), the magnitude of the proliferative expansion of endogenous K^b^-OVA–specific CD8 T cells at the peak of the response (day 7 post–LMOVA) in the PBL and spleen was diminished in CLP mice compared with sham mice (two of four CLP mice had K^b^-OVA–specific CD8 T cells above the LOD, Fig. 1B–D). Importantly, reduced numbers and decreased primary expansion were also observed with the adoptively transferred OT-I CD8 T cells (Fig. 1B, 1E, 1F). Of note, differences in OT-I cell engraftment could account for the differences in *L. monocytogenes*–induced expansion observed in CLP and sham hosts. However, similar seeding of CFSE-labeled splenocytes (transferred 2 d postsurgery) was detected in the spleens of both groups of mice (data not shown), suggesting that the diminished primary CD8 T cell accumulation in CLP-treated hosts was not due to differences in the number of naïve CD8 T cell precursors present at the time of Ag encounter (infection). Thus, these data indicate that sepsis-induced changes in the environment contribute to suboptimal expansion of naïve CD8 T cells upon Ag encounter in vivo, even when the naïve CD8 T cells were not exposed to the inflammatory cytokine milieu that characterizes early sepsis.

**Sepsis leads to a reduction in number and a change in relative composition of the DC compartment**

A reduction in DC number or a deficiency in the capacity of DCs to provide CD8 T cells with the necessary expansion cues could lead to the suboptimal primary CD8 T cell expansion observed in septic mice. To explore how sepsis affects DC numbers, we performed sham or CLP surgery on naïve B6 mice, followed by flow cytometric analysis of the spleen and LNs (Fig. 2A, 2B). Two days after the initial septic insult, there was a nearly 2-fold decline in total splenic cellularity (Fig. 2C). Importantly, cDCs (CD11c^hi^) and pDCs (CD11c^med^B220^hi^CD19^-) were reduced 4- and 2-fold, respectively, in CLP-treated mice compared with their sham counterparts. Interestingly, the varying degrees of numerical loss in the cDC populations in the spleen led to a change in the relative composition of the DC compartment (Fig. 2B, 2C), and cDCs from septic mice have an increased proportion of DN (CD11c^-CD4^-CD8^-) DCs, making them the major subset of the surviving cDCs (Fig. 2D).

A decline in DC numbers was observed in the spleens early after sepsis induction, suggesting that CLP leads to apoptosis of DCs. Alternatively, as a result of the sepsis-induced inflammation, DCs can follow inflammatory cues and be present in substantially higher numbers at locations proximal to the initial insult. To address these possibilities, DC numbers were determined in inguinal LNs (iLNs; nonreactive, distal) and mesenteric LNs (mLNs; reactive, proximal) 2 d after surgery. The total cellularity of iLNs was diminished 5-fold and the number of cDCs (CD11c^hi^) and pDCs (CD11c^med^B220^hi^CD19^-) were recovered 12-fold and decreased in mLNs compared with sham mice (Fig. 2E). However, no significant change was observed in the number of cDCs or the subtype distribution in mLNs that drain the gut mucosa and are located proximal to the site of the initial
The previous experiments clearly demonstrate that sepsis leads to a decline in DC numbers; however, these experiments did not address the maturation status of the surviving DCs. Thus, the expression of costimulatory ligands CD40 and CD80 was assessed on splenic DCs 2 d after sham or CLP surgery (Supplemental Fig. 1A). As a positive control, LPS was administered in a separate group of naive mice (45, 46). Interestingly, although cDCs from CLP mice had increased expression of CD40 and CD80 (as determined by geometric mean fluorescence intensity) compared with sham controls, the increase in CD40 and CD80 expression was significantly lower than on DCs recovered from mice after LPS stimulation (Supplemental Fig. 1B, 1C). Thus, polymicrobial sepsis does not evoke full maturation of DCs in vivo, suggesting that the ability to acquire and process new Ag is not impaired in sepsis-surviving DCs.

**Sepsis diminishes the capacity of DCs to produce IL-12 in vitro and in vivo**

Thus far, our results indicate that sepsis induces a numerical loss of DCs without significantly changing their maturation status. Next, we investigated the functionality of the surviving DCs, specifically their ability to produce inflammatory cytokines upon stimulation. To address this, an enriched CD11c+ population was obtained from sham and CLP hosts 2 d postsurgery. Following enrichment, cells were stimulated with TLR4 or TLR9 ligands (LPS or CpG, respectively), and IL-12p40 and TNF production was assessed (Fig. 3A). Unstimulated DCs from septic mice showed decreased production of IL-12p40 compared with sham mice, as well as decreased production when stimulated by LPS or CpG (Fig. 3B, 3C). In addition to decreased IL-12p40 production, diminished TNF production was observed (Fig. 3C). Therefore, sepsis changes the ability of purified DCs to respond to TLR stimulation in vitro.

In vitro studies reveal important phenomena, yet they often fall short in accurately recapitulating the physiologic environment. To test the capacity of the host (in general) and DCs (in particular) to sense inflammation and provide important signal 3 cytokines (i.e., IL-12) in vivo, sham or CLP B6 mice were challenged with LM 2 d after surgery (Fig. 4A). We and other investigators showed that *L. monocytogenes* has the capacity, in a dose-dependent manner, to induce a vigorous early inflammatory response characterized by the production of IL-12 and IFN-γ (15, 47, 48). Importantly, no difference in serum levels of IL-12 and IFN-γ was detected in sham or CLP mice at the time of *L. monocytogenes* infection (48 h postsurgery), indicating that the CLP-induced hyperinflammatory phase had passed. One day after *L. monocytogenes* infection, sham mice showed a substantial increase (44-fold greater than CLP mice) in serum IL-12 concentration, whereas there was almost no increase in IL-12 production in the CLP-treated mice (Fig. 4B). Similar results were obtained when examining the production of IFN-γ. Thus, these data strongly suggest that CLP-induced immunoparalysis is characterized by the inability of the host to produce proinflammatory cytokines upon secondary, unrelated infection.

To formally confirm that the observed impairment in IL-12 production could be attributed to decreased IL-12 production by DCs in vivo, sham or CLP mice were infected with virulent *L. monocytogenes* 2 d after surgery, and analysis was performed on day 3. Similar to our results from noninfected mice, septic mice had significantly reduced numbers of splenocytes and cDCs compared with sham controls (Fig. 4C). Importantly, the bacterial *L. monocytogenes* burden in the spleen and liver was similar, if not increased, in CLP mice, suggesting that the inability of the CLP host to sense the infection and respond with production of inflammatory cytokines was not due to the decreased bacterial load.

**FIGURE 1.** Septic environment contributes to the impaired primary CD8 T cell expansion. (A) Experimental design. Naive OVA257-specific TCR-Tg OT-I CD8 T cells (Thy1.1; 10^3 per mouse, i.v.) were adoptively transferred into B6 (Thy1.2) mice 2 d postsham or post-CLP surgery. All groups of recipient mice were challenged 1 d later with recombinant virulent *L. monocytogenes* (10^3 CFU per mouse, i.v.). (B) Representative dot plots show the frequency of tetramer-positive (Kb-OVA) CD8 T cells (endogenous = Thy1.2 or TCR-Tg = Thy1.1) of CD8 T cells in PBLs at day 7 postinfection. Naive noninfected mice were used to calculate the LOD for tetramer staining. (C) Total number of endogenous Kb-OVA+ cells in PBLs on day 7 post-*L. monocytogenes* infection. Numbers inside parentheses indicate the number of mice that had detectable (above the LOD) Thy1.2 OVA257-specific CD8 T cell responses. (D) Total number of endogenous CD8 T cells isolated from the spleen on day 7 post-*L. monocytogenes* infection. Total number of OT-I OVA257-specific CD8 T cells isolated from the peripheral blood at days 6 and 7 post-*L. monocytogenes* infection (E) or from the spleen on day 7 post-*L. monocytogenes* infection (F). Data are mean ± SEM of three or four mice per group and are representative of two independent experiments. LOD, LOD as defined in naive noninfected B6 mice. *p ≤ 0.05, Student’s t test.

septic insult (Fig. 2F), suggesting that sepsis did not change the distribution of DCs. Rather, these data suggest that sepsis leads to a significant decline in DC numbers at sites distal to the insult.
in vivo (Fig. 4D) (48). Direct ex vivo intracellular cytokine staining showed that the frequency of cDCs producing IL-12p40 was significantly diminished in CLP mice (Fig. 4E, 4F), suggesting that sepsis changes the per-cell capacity of DCs to respond to inflammatory cues in the environment. This 3.6-fold decrease in the frequency of DCs producing IL-12p40, coupled with a 3-fold decrease in the total number of cDCs recovered from CLP mice, led to an 11-fold decrease in the number of IL-12p40–producing DCs compared with sham mice. Thus, the diminished ability of DCs to produce signal 3 inflammatory cytokines in response to secondary infection suggests a potential role for the DC compartment in the observed suboptimal primary CD8 T cell responses after sepsis.

Reduced number and impaired functionality of DCs early after sepsis induction in outbred hosts

The use of inbred mouse strains, such as B6, provides substantial advantages to the experimentalist (i.e., precise determination of pathogen-specific CD8 T cell responses); however, this genetically homogenous population does not reflect the true genetic diversity observed in the human population. To determine whether the loss of DCs following sepsis could be recapitulated in a genetically heterogeneous population, we performed sham or CLP surgery on outbred Swiss Webster mice (Fig. 5A). Similar to results in inbred B6 mice, a decline in total cellularity, cDC numbers, and changes in the composition of remaining cDC populations in the spleens from outbred Swiss Webster mice were observed after the septic event (Fig. 5B, 5C). Thus, the loss of cDCs and their subtypes in genetically diverse outbred mice 48 h following sepsis indicates that the sensitivity of DCs to sepsis is not limited to inbred mouse strains. Furthermore, in a manner similar to B6 mice, cDCs from outbred CLP mice 2 d after surgery showed increased expression of CD40 and CD80 (as determined by geometric mean fluorescence intensity) compared with outbred sham controls (SF1D-F). cDCs from sham and CLP groups of outbred mice were also probed for their ability to produce IL-12 following L. monocytogenes infection (Fig. 5D). Septic outbred mice showed a significant decline in the number of cDCs and the frequency of cDCs producing IL-12p40 compared with sham mice (Fig. 5E–5G). This decrease in both number and frequency led to a significant decline in IL-12–producing cDCs, a result that was similar to the observed impairment in B6 mice. Thus, IL-12 production from postsepsis cDCs following secondary infection is diminished in inbred and genetically heterogeneous populations.
The spleens of sham and CLP mice were incubated in the presence or absence of TLR agonists (LPS and CpG) for 7 h at 37˚C in the presence of Brefeldin A. IL-12p40 and TNF upon TLR stimulation. Data are mean ± SEM of four mice per group in pooled samples of two spleens. Data are representative of three similar and independent experiments. *p ≤ 0.05, **p ≤ 0.01, Student t test.

**FIGURE 3.** Sepsis diminishes the capacity of DCs to respond to TLR stimulation in vitro. (A) Experimental design. Enriched CD11c⁺ DCs from the spleens of sham and CLP mice were incubated in the presence or absence of TLR agonists (LPS and CpG) for 7 h at 37˚C in the presence of Brefeldin A. (B) Representative graphs show the frequency of CD11c⁺ cells producing IL-12p40 after in vitro incubation. (C) Percentage of CD11c⁺ cells producing IL-12p40 and TNF upon TLR stimulation. Data are mean ± SEM of four mice per group in pooled samples of two spleens. Data are representative of three similar and independent experiments. *p ≤ 0.05, **p ≤ 0.01, Student t test.

Direct targeting of DCs using DEC-205 mAb conjugated to OVA reveals diminished expansion of adoptively transferred OT-I cells

The experiments discussed thus far suggested that the numerical loss of DCs and their functional impairments in producing cytokines upon secondary pathogen exposure after sepsis are T cell–extrinsic factors directly contributing to suboptimal CD8 T cell immunity; however, a direct link has not been provided. To definitively link the postseptic DC lesion with suboptimal CD8 T cell expansion upon exposure to cognate Ag, we made use of an anti–DEC-205 mAb/peptide conjugate system (49). DEC-205 (CD205) is a surface receptor that CD8⁺ cDCs use for receptor-mediated endocytosis of Ag prior to processing and presentation to T cells (50). To determine the extent to which sepsis impacts CD205-expressing DCs, we performed sham and CLP surgery and analyzed DCs from the spleen 2 d later (Fig. 6A). As expected, sepsis leads to a significant decline in the number of CD11c⁺CD8⁻CD205⁺ DCs (Fig. 6B). To directly probe the ability of the remaining CD205⁺ DCs to prime naive Ag-specific CD8 T cells that were not initially influenced by the septic event, we adoptively transferred the same number of naïve OT-I CD8 T cells into sham or CLP mice 2 d postsurgery. A day later, anti–DEC-205 mAb conjugated with the SIINFEKL peptide of OVA protein (DEC-205 mAb/Ag conjugates) in the presence of CpG was administered, and the total number of OT-I CD8 T cells was determined (Fig. 6A). Interestingly, CLP-treated mice had significantly diminished OT-I CD8 T cell expansion in the blood (days 6–7) and spleen (day 7) postconjugate administration compared with sham mice (Fig. 6C–E). Thus, direct targeting of DCs using DEC-205 mAb/Ag conjugates reveals the role of DCs in suboptimal priming of primary CD8 T cell responses after polymicrobial sepsis induction.

Flt3L treatment following sepsis induction reveals diminished expansion and reduced primary CD8 T cell expansion. Reversing the loss of DC number (as well as function) could prove beneficial in the recovery of CD8 T cell expansion upon pathogen encounter and could contribute to a partial recovery in primary CD8 T cell responses.

Flt3L is a hematopoietic growth factor that is capable of increasing DC numbers and could aid in reversing the effects of the DC lesion in septic mice (51). Repeated treatments of Flt3L for consecutive days are needed to expand the DC pool. Our previous studies indicated that sepsis leads to a decline in DC numbers after sepsis induction. To determine how long the decline in DC numbers persists in CLP-treated mice, we performed sham or CLP surgery and analyzed them at 7 d postsepsis induction (Supplemental Fig. 2A). Although the total cellularity of the spleen was not significantly different between sham and CLP mice, a significant reduction in cDCs, including the CD4⁺ and CD8⁺ cDC subsets, was still detected at day 7 postsurgery (Supplemental Fig. 2B). Also, CLP mice had diminished cellularity of the iLNs, as well as significant declines in the number of cDCs (CD11c⁺) and pDCs (Supplemental Fig. 2C). Thus, the loss of DCs persists in the spleen and iLNs for ≥7 d following sepsis induction. We next examined the effect of Flt3L administration on septic mice; Flt3L treatment began 1 d after CLP surgery and continued for four consecutive days prior to analysis (Fig. 7A). Importantly, septic mice treated with Flt3L showed significantly increased numbers of CD11c⁺ cells in the spleen, as well as significantly increased numbers of CD8⁺ cDCs, DN cDCs, and pDCs compared with untreated septic mice (Fig. 7B). Thus, Flt3L treatment following sepsis induction leads to numerical recovery of DC populations in vivo.

To determine whether the administration of Flt3L could aid in the reversal of DC functionality, septic mice were treated with Flt3L prior to infection with virulent LM on day 5, and direct ex vivo analysis of IL-12p40⁺ DCs was performed on day 6 postsurgery (Fig. 8A). After *L. monocytogenes* infection, the number of CD11c⁺ cells, cDC subsets (CD4⁺, CD8⁺, and DN), and pDCs was significantly increased in the spleen of Flt3L-treated mice, indicating that infection with *L. monocytogenes* did not diminish the DC expansion induced by Flt3L administration (Fig. 8B). Similar to previously
discussed experiments, bacterial burden after *L. monocytogenes* infection was comparable (Fig. 8C). Direct ex vivo intracellular cytokine staining revealed that Flt3L treatment significantly (3.2-fold) increased the numbers of IL-12p40–producing CD11chi DCs compared with the PBS-treated CLP group (Fig. 8D).

To further characterize whether Flt3L therapy postsepsis could aid in the reversal of DC functionality and support the recovery of primary CD8 T cell expansion, groups of CLP mice that received Flt3L or control treatments were adoptively transferred with physiological numbers of naive OT-I TCR-Tg or gp33-specific P14 TCR-Tg CD8 T cells (10^3 per mouse) 1 d before DEC-205 mAb/Ag + CpG administration (Fig. 8E) or infection with LM-GP33 (Supplemental Fig. 3A), respectively. Importantly, Flt3L treatment significantly improved the accumulation of primary effector CD8 T cells after direct targeting of DCs with Ag (Fig. 8F) or postinfection with pathogen expressing the Ag (Supplemental Fig. 3B, 3C), suggesting that treatments aimed at recovery of the DC compartment also have the capacity to lead to downstream recovery of primary CD8 T cell responses.

Once more, we used an outbred mouse model to verify that our results seen in a genetically homogenous population could be extrapolated to a genetically diverse one. In a similar experiment as in inbred B6 hosts, septic outbred mice were injected with PBS or treated with Flt3L prior to *L. monocytogenes* infection (Supplemental Fig. 4A). Similar to inbred mice, septic outbred mice treated with Flt3L exhibited significantly increased numbers of cDCs, cDC subsets (CD4+, CD8+, and DN), and pDCs compared with untreated controls (Supplemental Fig. 4B). Ex vivo IL-12p40 staining revealed a significantly increased (8.6-fold) number of IL-12p40–producing CD11c^hi^ cells in Flt3L-treated septic mice compared with untreated septic mice (Supplemental Fig. 4C).

In summary, these data demonstrate that Flt3L administration to septic inbred or outbred mice leads to a significant recovery of DC number and function, including the ability to prime primary CD8 T cell responses.

**Discussion**

Sepsis results from a systemic immune response to an infection that leads to an early hyperinflammatory state, followed by a state of immunosuppression (52). The early hyperinflammatory state, characterized by increased production of proinflammatory and anti-inflammatory cytokines, leads to increased levels of small molecules, such as NO and leukotrienes, which mediate physiological responses capable of producing massive hypotension, shock, and/or cardiovascular collapse that may lead to early septic deaths (53, 54).

The transition from this hyperinflammatory state to immunosuppression is evident in septic patients who show increased levels of lymphocyte apoptosis and often fall victim to secondary bacterial infections and reactivation of latent viruses, such as HSV or CMV (3–5, 7, 55). CLP is an experimental animal model of sepsis that mimics the course of sepsis in human patients; it results in an initial hyperinflammatory stage, followed by a protracted stage of immunosuppression, and it is also characterized by apoptosis of lymphocytes and increased susceptibility to secondary infections (43, 56).
CLP model, we reported a decrease in both the number and function of naive and memory CD8 T cells (10, 12, 57), and rescuing primary and secondary CD8 T cell responses following sepsis could represent an important avenue for future treatment. In this study, we provide evidence of a T cell–extrinsic factor (i.e., DCs), whose decline in both number and signal 3 cytokine production contributes to the observed impairments in primary CD8 T cell responses in septic mice. Additionally, we demonstrate the use of the DC-mobilizing cytokine Flt3L to rescue the lesion of DC numbers in septic mice and contribute to the faster recovery of CD8 T cell immunity in general.

Sepsis-induced apoptosis of T and B cells, as well as loss of DCs, is well characterized in human patients (7, 25, 55, 58). Furthermore, the apoptotic loss of DCs in the spleens of septic mice was shown at 24–48 h postsepsis induction, with significant losses occurring in the CD4+ and CD8+ cDC populations (28, 30). Consistent with these previous reports, our data show that sepsis leads to a significant decline in the CD4+ and CD8+ cDC populations, as well as the pDC population, in the spleen at 2 d postsepsis. In

**FIGURE 5.** Reduced number and impaired functionality of DCs early after sepsis induction in outbred hosts. (A) Experimental design. Sham or CLP surgery was performed on outbred Swiss-Webster mice and analysis performed 2 d postsurgery. (B) Splenic cellularity and total number of DCs in the spleen. The fold difference in the number of cells detected in sham and CLP groups of mice is shown. (C) Representation of DC subsets in the spleen 2 d postsepsis induction. (D) Experimental design. Sham or CLP surgery was performed on outbred Swiss-Webster mice 2 d prior to infection with *L. monocytogenes* (10⁵ CFU per mouse, i.v.). (E) DC numbers 1 d after *L. monocytogenes* infection. The fold difference in the number of CD11c⁺ cells detected in sham and CLP groups of mice is shown. Frequency (F) and total number (G) of splenic CD11c⁺ cells producing IL-12p40 14 h after *L. monocytogenes* infection. The fold difference in the frequency and number of cells producing cytokine in sham and CLP groups of mice is shown. Data are mean ± SEM of four to six mice per group and are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test.

**FIGURE 6.** Sepsis leads to impaired CD8 T cell expansion after direct targeting of Ag to DCs. (A) Experimental design. Naive OVA257-specific TCR-Tg OT-I CD8 T cells (Thy1.1; 10⁶ per mouse, i.v.) were adoptively transferred into B6 (Thy1.2) mice 2 d postsham or post-CLP surgery. All groups of recipient mice were administered anti–DEC-205 Ab/OVA conjugates (10 μg, i.v.) and CpG (50 μg, i.p.) 1 d after adoptive transfer of naive OT-I CD8 T cells (day 3 postsurgery). (B) Total number of CD8⁺ DEC-205⁺ DCs in the spleen. The fold difference in the number of cells detected in sham and CLP groups of mice is shown. Data are mean ± SEM of five or six mice per group and are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test.
contrast with previous investigations, we also demonstrated a significant decline in the DN cDC population; however, this difference is likely due to differences in CLP induction and the time point of analysis because differences in the timing of analysis may accurately demonstrate the loss of a certain cell population while failing to capture the loss of a different cell population. In addition to timing, the site of analysis (e.g., spleen, LNs, bone marrow) influences the variability observed between studies. Our results recapitulate previous studies reporting the loss of DCs following sepsis while providing additional data demonstrating that the sepsis-induced reduction in DCs in outbred mice more closely resembles the genetic heterogeneity found in humans; this indicates that the loss of DCs following sepsis occurs regardless of host genetic background.

The numerical reduction in DCs following sepsis suggests a possible mechanism for impaired CD8 T cell immunity, because a decrease in the number of DCs displaying cognate Ag could lead to decreased primary expansion of CD8 T cells. DCs in secondary lymphoid organs are a dynamic mixture of immature DCs, which are capable of vigorous Ag uptake but have minimal capacity to activate T cells, and mature DCs, which have a high capacity to generate a T cell response due to upregulation of costimulatory ligands but have a diminished capacity to uptake Ag (59). DC expression of costimulatory ligands and their interactions with CD8 T cells (signal 2) are necessary for the expansion of Ag-specific CD8 T cells upon Ag recognition (15). Our results, like those from a previous study, demonstrate that sepsis leads to moderate upregulation of costimulatory ligands in septic mice (30), but the increased expression of costimulatory ligands on DCs from septic mice is still substantially lower than that observed after LPS stimulation (45, 46). Preliminary data also showed that CLP induction does not change the ability of enriched CD11c+ cells in vitro to acquire and process exogenous Ag. Thus, an altered maturation status of DCs does not control the suboptimal

![Figure 8. Functional recovery of DC compartment after Flt3L treatment of septic mice.](http://www.jimmunol.org/)

## FIGURE 8.

**Numerical recovery of DC compartment after Flt3L treatment of septic mice.** (A) Experimental design. Sham or CLP surgery was performed on naive B6 mice 5 d prior to infection with *L. monocytogenes* (1 × 10^5 CFU per mouse, i.v.). Flt3L-Ig (100 μg i.p.) was administered on days 1–4 following surgery and analysis was performed on day 6. (B) Spleenic DC numbers 1 d after *L. monocytogenes* infection (day 6 postsurgery). (C) *L. monocytogenes* titers in the spleen 1 d postinfection. (D) Total number of CD11c^hi^ cells producing IL-12p40 1 d postinfection. The fold difference in the number of cells producing IL-12 in CLP- and CLP+Flt3L-treated groups of mice is shown. (E) Experimental design. CLP surgery was performed on naive B6 mice 5 d prior to adoptive transfer of naive OT-I CD8 T cells (Thy1.1, 10^3 per mouse, i.v.). The Flt3L-treated CLP group was administered Flt3L-Ig (100 μg i.p.) on days 1–4 following surgery. All mice were administered DEC-205 OVA (10 μg, i.v.) and CpG (50 μg, i.p.) 1 d after adoptive transfer of naive OT-I CD8 T cells (day 6 postsurgery). (F) Total number of OT-I CD8 T cells isolated from the spleen 7 d after DEC-205 OVA administration (day 13 postsurgery). Data are mean ± SEM of four to six mice per group and are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test.
priming of naive CD8 T cells in vivo that is observed early after CLP-induced sepsis.

The primary expansion of Ag-specific CD8 T cells is predicated by the encounter of CD8 T cells with their cognate Ag in the presence of costimulation. Furthermore, proinflammatory cytokines, such as IL-12 and IFN-γ, influence the degree to which CD8 T cells expand, survive, and mediate effector function (15, 18–20, 60, 61). CD8 T cells exposed to signal 1 (Ag) and signal 2 (costimulation) in the presence of signal 3 (IL-12) have an increased ability to expand, survive, and kill compared with T cells exposed to signals 1 and 2 alone (18–20). Our study demonstrates that DCs from septic mice have a diminished capacity to produce IL-12 when stimulated with TLR ligands in vitro, a result that was described previously (30, 33, 34). In addition, using secondary infection with L. monocytogenes as an in vivo tool for probing the host’s capacity to produce proinflammatory cytokines, we demonstrated that septic mice have a systemic loss in their capacity to produce the proinflammatory cytokines IL-12 and IFN-γ. This result is consistent with the paradigm of immunoparalysis in septic survivors and demonstrates an in vivo functional impairment in signal 3 production upon infection, linking the significant decline in DC number with the impairment in IL-12 production. Interestingly, a previous study (34) showed that the decline in IL-12 production by septic DCs corresponds with alterations in the methylation patterns of IL-12p35 and IL-12p40 promoters that are suggestive of a repressive modification in chromatin structure associated with diminished gene expression. Furthermore, this study demonstrated that the impairment in IL-12 production by septic DCs, as well as their epigenetic changes, persists for up to 6 wk after sepsis induction (34). Thus, enduring changes at the genomic level might lead to a long-lasting impairment in cytokine production that contributes to persistent dysfunction in CD8 T cell immunity after sepsis. The timing and downstream effects of these genomic-level changes could be an intriguing area of future inquiry.

Addressing the DC lesion after sepsis may serve as an important therapeutic target for reversing some of the sepsis-induced deficits in immunity. To this end, we demonstrated that administration of Flt3L in inbred and outbred septic hosts leads to a significant increase in DCs, as well as IL-12–producing DCs, compared with untreated septic mice. Moreover, this increase in DCs and IL-12–producing DCs contributed to an increase in the number of expanded CD8 T cells in Flt3L-treated mice. This important finding demonstrates the capacity of Flt3L to recover DC number and functionality after sepsis, as well as reveals that targeted therapy of the DC lesion postsepsis may allow reversal of lesions in the immune system, in our case CD8 T cell immunity, in which DCs play a vital role. Therapeutic use of Flt3L was reported in experimental tumor models (62). Daily administration of Flt3L to mice rapidly increases the number of DCs in multiple tissues (51, 63). Importantly, this increase in DCs is maintained only as long as the Flt3L is given, and the organs that display the increased size and cellularity during Flt3L therapy return to normal within 7–10 d of stopping treatment. The potent hematopoietic effects of Flt3L in preclinical models led to its initial testing in clinical trials nearly 20 y ago (18–20). Despite being well tolerated and demonstrating biologic activity, clinical development of Flt3L was suspended. Clinical interest in Flt3L has been renewed, with a recent report examining the efficacy and safety of a recombinant human Flt3L (CDX-301) (71). Based on the long history of testing Flt3L in multiple immunological settings, it is tempting to speculate on its potential benefits in sepsis patients based on the data presented in this article.

In summary, we showed that CLP-induced sepsis leads to numerical and functional reductions in DCs that contribute to the suboptimal CD8 T cell expansion upon Ag encounter. Furthermore, through administration of the DC-mobilizing cytokine Flt3L, we established that the number of DCs may be recovered in septic mice, leading to an increase in the number of IL-12–producing DCs, as well as an increase in primary CD8 T cell expansion. These data highlight the fact that sepsis-induced immune suppression is a multicellular defect in which T cell–intrinsic and -extrinsic numerical and functional deficiencies likely contribute to the increased susceptibility of sepsis survivors to secondary infections.

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References


