

Frontline Science: Myeloid cell-specific deletion of Cebpb decreases sepsis-induced immunosuppression in mice

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ABSTRACT

Sepsis inflammation accelerates myeloid cell generation to compensate for rapid mobilization of the myeloid progenitors from bone marrow. This inflammation-driven myelopoiesis, however, generates myeloid progenitors with immunosuppressive functions that are unable to differentiate into mature, innate immune cells. The myeloid-derived suppressor cells (MDSCs) expand markedly in the later phases of sepsis, suppress both innate and adaptive immunity, and thus, elevate mortality. Using a murine model with myeloid-restricted deletion of the C/EBP β transcription factor, we show that sepsis-induced generation of MDSCs depends on C/EBP β . C/EBP β myeloid cell-deficient mice did not generate MDSCs or develop immunosuppression and survived sepsis. However, septic mice still generated Gr1⁺CD11b⁺ myeloid progenitors at the steady-state levels similar to the control sham mice, suggesting that C/EBP β is not involved in healthy, steady-state myelopoiesis. C/EBP β -deficient Gr1⁺CD11b⁺ cells generated fewer monocyte- and granulocyte-like colonies than control mice did, indicating reduced proliferation potential, but differentiated normally in response to growth factors. Adoptive transfer of C/EBP β -deficient Gr1⁺CD11b⁺ cells from late septic mice exacerbated inflammation in control mice undergoing early sepsis, confirming they were not immunosuppressive. These results show that C/EBP β directs a switch from proinflammatory to repressor myeloid cells and identifies a novel treatment target. *J. Leukoc. Biol.* 102: 000-000; 2017.

Abbreviations: C/EBP β = CCAAT/enhancer binding protein β , cKO = conditional knockout, CLP = cecal ligation and puncture, M-CSF = macrophage CSF, MDSC = myeloid-derived suppressor cell, MHC II = MHC class II, miRNA/miR = microRNA, M-MDSC = monocytic myeloid-derived suppressor cell, PMN-MDSC = polymorphonuclear myeloid-derived suppressor cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

A hyperinflammatory reaction to infection initiates highly lethal sepsis [1, 2]. This early hyperinflammatory state follows excessive production of proinflammatory cytokines, such as TNF- α and IL-6 [3, 4]. Although this proinflammatory phase predominates during the early sepsis response, recent reports of gene expression in animals and human sepsis provide evidence that an opposing anti-inflammatory response occurs concomitantly and dominates during a profoundly immunorepressed and catabolic state with continued organ dysfunction [5–7]. Sepsis-induced immunosuppression increases production of immunosuppressive soluble mediators, depletion of T cells and dendritic cells by increased apoptosis, immune exhaustion with paralysis of CD8⁺ T cell effector properties, and fosters bacterial growth [8, 9]. Sepsis immunosuppression prolongs the primary microbial infection and increases the risk of opportunistic infections and organ dysfunctions and predicts poor outcome [1, 10, 11]. Earlier attempts to treat human sepsis by reducing the early/acute hyperinflammatory reaction uniformly failed, mainly because of the rapid shift of sepsis immune responses from immune activation to immune suppression [12, 13]. Thus, understanding how sepsis promotes the shift from effector to repressor immune phenotypes is critical for designing a treatment presently unavailable to septic humans.

We [14] and others [15, 16] have reported increased generation and expansion of MDSCs in mice during sepsis. These immature myeloid cells include progenitors and precursors of monocyte, granulocyte, and dendritic cells; express the myeloid differentiation markers Gr1 and CD11b; and are immunosuppressive [17, 18]. MDSCs accumulate in peripheral blood of patients with sepsis [19] and the bone marrow and spleens of septic mice [14, 15]. Our studies in a mouse polymicrobial sepsis model, which uses limited antibiotic treatment to promote a chronic immunosuppressive state, show progressive increases in Gr1⁺CD11b⁺ MDSCs in the late sepsis

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phase because of failure of the Gr1⁺CD11b⁺ myeloid progenitors to differentiate into immunocompetent, innate immune cells [14]. We identified expression of miR-21 and miR-181b as promoting expansion of Gr1⁺CD11b⁺ repressor cells after sepsis onset, and our results also implicated myeloid differentiation transcription factor NFIA, which activates NF- κ B, in attenuating differentiation of the Gr1⁺CD11b⁺ cells [20, 21]. Importantly, in vivo blockade of miR-21 and miR-181b with specific antagomirs prevented MDSC generation [20], whereas ex vivo knockdown of NFIA in Gr1⁺CD11b⁺ cells enhanced their differentiation and maturation [21]. We also showed that C/EBP β induces miR-21 and miR-181 in Gr1⁺CD11b⁺ cells during sepsis [22]. We found that nuclear C/EBP β and Stat3 bind to, and activate, miR-21 and miR-181b promoters in sepsis but not naive, Gr1⁺CD11b⁺ cells [22]. In vitro knockdown of C/EBP β inhibited miR-21 and miR-181b expression in bone marrow Gr1⁺CD11b⁺ cells from septic mice [22]. C/EBP β promotes inflammation-induced myelopoiesis, but its role in sepsis immunosuppression is unknown [23].

Here, we investigated the role of C/EBP β in sepsis-induced generation and expansion of Gr1⁺CD11b⁺ MDSCs, using mice with a conditional deletion of the *Cebpb* allele in the myeloid lineage. We find that C/EBP β -deficient, septic mice were unable to generate MDSCs but still generated healthy Gr1⁺CD11b⁺ cells, which supports sepsis survival in the absence of MDSCs. We conclude that myeloid cell C/EBP β controls the polymicrobial sepsis outcome in mice by promoting immunosuppression.

MATERIALS AND METHODS

Production of BALB/cJ *Cebpb*-floxed mice

The BALB/cJ *Cebpb*-floxed allele was created by gene targeting in BALB/cJ embryonic stem cells (PRX-Balb/cJ 9; Primogenix Biological Laboratories, Laurie, MO, USA) with a gene-targeting vector described by Sterneck et al. [24]. The *Cebpb* targeting event inserted a loxP site 227 bp 5' of the *Cebpb* transcriptional start site and a loxP-flanked neomycin-resistance cassette 228 bp 3' of the *Cebpb* polyadenylation site (Supplemental Fig. 1). Targeted embryonic stem cells were initially identified by long-range PCR with 1 primer outside the vector homology arm and a second primer in the inserted sequence. PCR-positive clones were validated by Southern blot analysis using 5' and 3' probes external to the vector homology arms and with a neomycin internal probe. A single, correctly targeted clone (2B) was electroporated with a Cre expression plasmid, and subclones were screened by PCR for removal of the neomycin cassette and retention of the *Cebpb* coding sequence. Three subclones were injected into C57BL/6 blastocysts to generate chimeras, which were mated to BALB/cJ females for germline transmission of the *Cebpb*-floxed allele.

Production of BALB/cJ *Lyz2*-Cre knock-in mice

The construction of the BALB/cJ-*Lyz2*^{Cre} knock-in allele was similar to the allele described by Clausen et al. [25] and is shown in Supplemental Fig. 2.

Generation of BALB/cJ *Cebpb* cKO mice

The myeloid-specific C/EBP β knockout mice were generated by breeding the above-described *Cebpb*-floxed mice with the *Lyz2*^{Cre} knock-in mice. *Cebpb*^{flox/flox}; *Lyz2*^{Cre/+} mice were crossed to *Cebpb*^{flox/flox}; *Lyz2*^{+/+} mice to generate *Cebpb*^{flox/flox} mice with and without Cre. Genotypes were verified for all mice by PCR. The *Cebpb*^{flox/flox}; *Lyz2*^{Cre/+} mice, in which expression of the Cre recombinase under the control of the *Lyz2* gene promoter inactivates the floxed *Cebpb* allele in the myeloid lineage cells, served as our myeloid-specific knockout. The *Cebpb*^{flox/flox}; *Lyz2*^{+/+} mice, which do not express the Cre recombinase and thus the floxed *Nfia* allele is still expressed in the myeloid lineage cells, served as controls.

The mice were bred and housed in a pathogen-free facility in the Division of Laboratory Animal Resources. All experiments were conducted in accordance with National Institutes of Health guidelines and were approved by the East Tennessee State University Animal Care and Use Committee.

Sepsis

Polymicrobial sepsis was induced by CLP in 8–10-wk-old mice. We used a 21-gauge needle and 2 punctures, followed by antibiotic [imipenem (Merck, White House Station, NJ, USA); 25 mg/kg body weight] administration to generate early/acute and late/chronic septic phases, as described previously [26]. Mice were s.c. administered antibiotic or an equivalent volume of 0.9% saline. This model creates a prolonged infection with 100% mortality over 4 wk. To establish intra-abdominal infection and approximate the clinical situation of early human sepsis, in which there often is a delay between the onset of sepsis and the delivery of therapy [27], injections of imipenem were given at 8 and 16 h after CLP, which resulted in high mortality (~60–70%) during the late/chronic phase [26]. The presence of early sepsis was confirmed by transient, systemic bacteremia and elevated cytokine levels in the first 5 d after CLP. Late/chronic sepsis (after d 5) was confirmed by enhanced peritoneal bacterial overgrowth and reduced circulating levels of the proinflammatory cytokines TNF- α and IL-6.

We used male mice because several clinical and experimental studies have shown that cell-mediated immune responses are depressed in males with sepsis, whereas they are unchanged or enhanced in females [28, 29]. In addition, previous studies using CLP model provided evidence that female mice are more immunologically competent than male mice in surviving this insult [30]. Because MDSCs suppress both innate and adaptive immune responses, we used male mice so we could assess the maximal effect of this immunosuppressive cell population on sepsis outcome.

Bacterial culture

Immediately after mice were euthanized, the peritoneal cavity was lavaged with 5 ml PBS. The lavage was cleared by centrifugation. Blood was collected via cardiac puncture in heparinized tubes. Lavage or blood was plated on trypticase soy agar base (BD Biosciences, Sparks, MD, USA). The plates were incubated for 24 h at 37°C under aerobic conditions. The plates were read by a microbiologist, and the CFUs were determined.

Gr1⁺CD11b⁺ cells

Bone marrow or spleen Gr1⁺CD11b⁺ cells were isolated with MACS according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). The bone marrow cells were flushed from femurs with RBMI-1640 medium (without serum) under aseptic conditions. The spleens were minced in RBMI-1640 medium. A single-cell suspension of the bone marrow or spleen was made by pipetting up and down and filtering through a 70- μ m, nylon strainer, followed by incubation with erythrocyte lysis buffer. After washing, total Gr1⁺CD11b⁺ cells were purified by subjecting the single-cell suspension to positive selection of the Gr1⁺CD11b⁺ cells by incubating with biotin-coupled mouse anti-Gr1 Ab (clone RB6-8C5; eBioscience, San Diego, CA, USA) for 15 min at 4°C. Cells were then incubated with anti-biotin magnetic beads for 20 min at 4°C and subsequently passed over an MS column. The cell purity was determined by flow cytometry. Typically, ~90% Gr1⁺CD11b⁺ cells were obtained by that procedure.

For the adoptive cell transfer, mice were injected via the tail vein with 4×10^6 of Gr1⁺CD11b⁺ cells or an equivalent volume (100 μ l) of saline immediately after induction of sepsis. Mice were followed for 5 d after cell transfer.

Cell culture

Gr1⁺CD11b⁺ cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (all from GE Healthcare Life Sciences, Logan, UT, USA), and 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA) at 37°C and 5% CO₂. In some experiments, Gr1⁺CD11b⁺

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