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Expression of C/EBP β in myeloid progenitors during sepsis promotes immunosuppression

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Abstract

Sepsis-induced myeloid-derived suppressor cells (MDSCs) contribute to immunosuppression associated with sepsis. We reported that the CCAAT enhancer-binding protein C/EBP β activates microRNA (miR)-21 and miR-181b expressions, which induce transcription factor NFI-A to support the generation and expansion of MDSCs in the bone marrow and spleens of septic mice. Here, using a conditional knockout mouse model lacking C/EBP β in the myeloid lineage, we find that without C/EBP β , myeloid progenitor cells could not express miR-21 or miR-181b, and ectopic expression of C/EBP β in the C/EBP β -deficient myeloid progenitors activated the expression of the two miRNAs. Moreover, C/EBP β -reconstituted myeloid cells expressed IL-10 and reduced T cell proliferation and function, similar to control MDSCs that express C/EBP β . Exogenous expression of miR-21 and miR-181b in the C/EBP β -deficient myeloid progenitors from septic mice produced similar results. Notably, NFI-A-dependent transactivation of NF- κ B MDSC generating pathway was reversed in the C/EBP β -deficient myeloid progenitors from septic mice. Together, these results support that decreasing C/EBP β expression prevents MDSC generation and decreases immunosuppression in septic mice, providing a target for sepsis treatment.

Graphical Abstract

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Conflicts of interest

None.

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C/EBP β disrupts myelopoiesis by inducing miR-21 and miR-181b to generate MDSCs in sepsis

Keywords

C/EBP β ; Immunosuppression; MDSC; Sepsis

1. Introduction

The early inflammatory response to sepsis is characterized by a hyperinflammatory reaction that, if not treated early, progresses to a protracted immunosuppressive phase, which alters both innate and adaptive immunity (Hotchkiss *et al.*, 2013b; Liu *et al.*, 2012; Remick, 2007). At the cellular level, sepsis-induced immunosuppression is associated with tolerance of monocytes/macrophages and neutrophils as demonstrated by their decreased responses to bacterial toxins and failure to clear microbial infections (Alves-Filho *et al.*, 2010; Ellaban *et al.*, 2004), depletion of adaptive immune effector cells by apoptosis (Hotchkiss *et al.*, 2013b; Oberholzer *et al.*, 2001; Wesche *et al.*, 2005), and suppression of T cell proliferation and function (Boomer *et al.*, 2011; Guignant *et al.*, 2011).

Our previous studies in a mouse model of polymicrobial sepsis induced by cecal ligation and puncture, which develops into early and late sepsis phases, revealed substantial expansion of myeloid-derived suppressor cells (MDSCs) within the bone marrow and spleen during the late septic phase (Brudecki *et al.*, 2012a). We reported that immature Gr1⁺CD11b⁺ myeloid cell from late septic mice (i.e., MDSCs) produce immunosuppressive mediators, inhibit T cell activation and proliferation, and are unable to differentiate into mature immune cells (McClure *et al.*, 2016; McClure *et al.*, 2014). MicroRNA (miR)-21 and miR-181b inhibit MDSCs differentiation during sepsis by inducing the myeloid cell differentiation-related transcription factor NFI-A (McClure *et al.*, 2016). In vivo inhibition of miR-21 and miR-181b in septic mice decreases repressor MDSCs and improves late sepsis survival (McClure *et al.*, 2014).

The CCAAT enhancer-binding transcription factor C/EBP β supports inflammation-induced proliferation of myeloid cells, where it accelerates myelopoiesis induced by cytokines or infections (Hirai *et al.*, 2006; Marigo *et al.*, 2010). For example, cytokines and growth factors, such as IL-6, G-CSF and GM-CSF, produced during infection or in tumor microenvironment can induce C/EBP β expression (Manz and Boettcher, 2014; Marigo *et al.*, 2010; Zhang *et al.*, 2010). Previous studies have shown that activation of the

transcription factor Stat3 by G-CSF or IL-6 is required for induction of C/EBP β transcription in mouse myeloid progenitors (Hirai *et al.*, 2006; Niehof *et al.*, 2001). In addition, the transcription factor CREB (cAMP-response element-binding protein) is also required for the IL-6 mediated induction of C/EBP β transcription by tethering Stat3 at the C/EBP β promoter (Niehof *et al.*, 2001). In support of the important role of Stat3 in the induction of C/EBP β , recent studies have shown that the phenotype of bone marrow conditional Stat3 knockout mice resembles C/EBP β deficiency since it exhibits impaired accelerated myelopoiesis under the pathophysiological conditions (Hirai *et al.*, 2006; Panopoulos *et al.*, 2006).

We previously reported that C/EBP β in conjunction with Stat3 binds to and activates the promoters of both miR-21 and miR-181b in naive Gr1⁺CD11b⁺ cells (McClure *et al.*, 2017), which are phenotypically similar to MDSCs. We hypothesized that C/EBP β triggers the miR-21 and miR-181b dependent path that generates MDSCs during late sepsis immunosuppression. In this study, we used a mouse model with conditional deletion of *Cebpb* gene in the myeloid lineage to further investigate the mechanism of C/EBP β -induced generation of MDSCs in sepsis. We find that C/EBP β induces miR-21 and miR-181b expression to drive MDSCs during sepsis. Notable, myeloid precursors generated in the C/EBP β conditional knockout mice during sepsis differentiate into competent innate immune cells, supporting that targeting the C/EBP β -mediated pathway may prevent late sepsis immunosuppression.

2. Materials and Methods

2.1. Mice

Generation of BALB/c *Cebpb* conditional, myeloid cell-specific knockout mice have been described previously (McPeak *et al.*, 2017). The *Cebpb*^{flx/flx}; *Lyz2*^{Cre/+} mice, where the expression of the Cre recombinase inactivates the floxed *Cebpb* allele in the myeloid lineage cells, served as our myeloid-specific knockout. The *Cebpb*^{flx/flx}; *Lyz2*^{+/+} mice, which do not express the Cre recombinase and thus the floxed *Cebpb* 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. Male mice, 8–10 weeks old were used. 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

2.2. Induction of sepsis

Polymicrobial sepsis was induced by cecal ligation and puncture (CLP). CLP was performed using a 21-gauge needle and two punctures, and mice were administered antibiotic to generate early/acute and late/chronic septic phases as described previously (Brudecki *et al.*, 2012b). This model creates a prolonged infection with 100% mortality over 4 weeks. To generate late sepsis, mice were subcutaneously administered antibiotic (Imipenem; 25 mg/kg body weight) or an equivalent volume of 0.9% saline. To establish intra-abdominal infection and approximate the clinical situation of early human sepsis where there often is a delay

between the onset of sepsis and the delivery of therapy (Mazuski *et al.*, 2002), injections of Imipenem were given at 8 and 16 hr after CLP, which results in high mortality (~60–70%) during the late/chronic phase (Brudecki *et al.*, 2012b). The presence of early sepsis was confirmed by transient systemic bacteremia and elevated cytokine levels in the first 5 days after CLP. Late/chronic sepsis (after day 5) was confirmed by enhanced peritoneal bacterial overgrowth and reduced circulating proinflammatory cytokines.

2.3. Isolation of Gr1⁺CD11b⁺ cells

Gr1⁺CD11b⁺ cells were isolated from the bone marrow using magnetically assisted cell sorting according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). The bone marrow cells were flushed out of the femurs with RPMI-1640 medium (without serum) under aseptic conditions (Brudecki *et al.*, 2012b). A single cell suspension of the bone marrow was made by pipetting up and down and filtering through a 70-µm 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 antibody (Clone RB6-8C5; eBioscience, San Diego, CA) 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 a MS column. Purified Gr1⁺CD11b⁺ cells were then washed and resuspended in sterile saline. The cell purity was determined by flow cytometry. Typically ~90% Gr1⁺CD11b⁺ cells were obtained by this procedure.

Gr1⁺CD11b⁺ cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Hyclone Laboratories, Logan, UT), and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C and 5% CO₂.

2.4. ELISA

Cytokine production was determined using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) according to the manufacturer's instructions. Each sample was run in duplicate.

2.5. C/EBPβ expression plasmid

Full length mouse *Cebpb* cDNA (transcript variant 1) was cloned in pEZ-M02 expression vector downstream of the CMV promoter, and C/EBPβ protein expression was verified by western blotting. A pReceiver-M02 vector served as a negative control.

2.6. Transfection of C/EBPβ plasmid and miRNA precursors

Plasmid DNA was suspended in HiPerFect reagent (Qiagen, Valencia, CA) (final concentration: 0.5 µg/ml). For miR-21 and miR-181b overexpression, negative control precursor or miR-21 or miR-181b precursor (Ambion) were suspended in a HiPerFect reagent at 50 nM final concentration. Gr1⁺CD11b⁺ cells were transfected using the Gene Pulser MXCell system (Bio-Rad, Hercules, CA). After 24 hr, portions of the cells were removed and either used for RNA isolation and miRNA measurements by PCR or stimulated for 12 hr with 1 µg/ml of LPS, and culture supernatants were used for cytokine

measurements by ELISA. The remainder of the cells was differentiated for 6 days with M-CSF plus rIL-4 and then analyzed by flow cytometry.

For Stat3 knockdown, Gr1⁺CD11b⁺ cells were transfected with pools of Stat3-specific or scrambled (control) siRNAs (Santa Cruz Biotechnology) at a 0.5 μ M final concentration as described above and then incubated for 36 hr.

2.7. miRNA measurement

Expression levels of miR-21 and miR-181b were determined by quantitative real-time PCR (RT-qPCR) using miRNA-enriched RNA and miScript SYBR Green PCR kit with miScript Primer Assays specific to miR-21 and miR-181b according to the manufacturer's instructions (Qiagen). The relative expression was calculated using the 2^{-C_t} cycle threshold method after normalization to the endogenous U6 RNA as an internal control.

2.8. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed to assess *in vivo* DNA-protein interactions at the miR-21 and miR-181b promoters using ChIP-IT Express Enzymatic Shearing kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, Gr1⁺CD11b⁺ cells were harvested from the bone marrow and protein-DNA complexes were cross-linked by fixation in 1% formaldehyde in minimal culture medium for 10 min at room temperature. After washing with cold PBS, cells were lysed in 1x lysis buffer containing protease inhibitor cocktail. Cell lysate was cleared by centrifugation at 5,000 rpm for 10 min at 4°C. The pelleted nuclei were then resuspended in digestion buffer and incubated with the enzymatic shearing cocktail at 37°C for 10 min. The sheared chromatin solution was recovered by centrifugation at 15,000rpm for 10 min at 4°C. Ten microliter of the chromatin solution was reserved as "input" DNA sample. The remaining chromatin solution was immunoprecipitated overnight at 4°C with protein G magnetic beads and 3 μ g of antibody specific to p-Stat3, C/EBP α , C/EBP β , or isotype control antibody (Santa Cruz Biotechnology). The chromatin/antibody complexes captured on the beads were washed three times in ChIP buffer and then eluted by incubation for 15 min in 50 μ l elution buffer. Next, the DNA-protein cross-links were reversed by incubating the eluted chromatin with 50 μ l of reverse cross-linking buffer. The supernatant containing the DNA was then incubated, along with the "input" DNA samples at 95°C for 15 min. After treatment with proteinase K for 1 h at 37°C, the reaction was stopped, and the resulting DNA was extracted and stored at -20°C.

Enrichment of the miR-21 and miR-181b promoter sequences in the ChIPed DNA was measured by quantitative real-time PCR using primer and fluorescently labeled internal probe sequences (Integrated DNA Technologies, Coralville, IA) specific to the miR-21 and miR-181b promoters, as described previously (McClure *et al.*, 2017).

2.9. Flow cytometry

Cells were stained by incubation for 30 min on ice in staining buffer (PBS plus 2% FBS) with the following antibodies: anti-Gr1 conjugated to FITC, anti-CD11b conjugated to phycoerythrin (PE), anti-F4/80 conjugated to allophycocyanin (APC), anti-CD11c

conjugated to PE, anti-MHC II conjugated to FITC, anti-CD4 conjugated to PE (all from eBioscience, San Diego, CA). An appropriate isotype-matched control was used for each antibody. After washing, the samples were analyzed by a FACSCaliber flow cytometer (BD Biosciences, Sparks, MD). About 25,000 events were acquired and analyzed using the CellQuest Pro software (BD Biosciences).

2.10. Gr1⁺CD11b⁺ cell differentiation

Gr1⁺CD11b⁺ cells were cultured for 6 days with complete RPMI-1640 medium in the presence of 10 ng/ml of M-CSF (PeproTech Inc., Rocky Hill, NJ) and 10 ng/ml rIL-4 (eBioscience, San Diego, CA). The cell phenotypes were analyzed by flow cytometry.

2.11. T cell proliferation

Gr1⁺CD11b⁺ cells were cultured with CD4⁺ T cells to determine their effects on T cell proliferation and IFN- γ production. Briefly, spleen CD4⁺ T cells were isolated by positive selection using biotinylated anti-CD4 magnetic beads (Miltenyi). Cells were fluorescently labeled with carboxy-fluoresceindiacetate, succinimidyl ester (CFSE) dye using the Vybrant CFDA SE Cell Tacer Kit (Invitrogen Molecular Probes, Eugene, OR). Cells were incubated for 10 min at room temperature with 10 μ M CFSE dye and then co-cultured (at 1:1 ratio) with Gr1⁺CD11b⁺ cells. T cell proliferation was induced by the stimulation with an anti-CD3 plus an anti-CD28 (R&D Systems, Minneapolis, MN) antibody (1 μ g/ml/each). After 3 days, cells were harvested and CD4⁺ T cell proliferation was determined by the step-wise dilution of CFSE dye in dividing, CD3-gated CD4⁺ cells using flow cytometry. Culture supernatants were collected for the IFN- γ measurement.

2.12. Western blot

Equal amounts of protein extracts were mixed with 5x Laemmli sample buffer, separated by a SDS-10% polyacrylamide gel (Bio-Rad) and subsequently transferred to nitrocellulose membranes (Thermo Scientific, Waltham, MA). After blocking with 5% milk in Tris-buffered saline/Tween-20 for 1 hr at room temperature, membranes were probed overnight at 4°C with the appropriate primary antibodies. After washing, blots were incubated with the appropriate HRP-conjugated secondary antibody (Life Technologies, Grand Island, NY) for 2 hr at room temperature. Proteins were detected with the enhanced chemiluminescence detection system (Thermo Fisher Scientific). The developed bands were visualized using the ChemiDoc XRS Detection System (Bio-Rad) and the images were captured with the Image Lab Software V3.0. Membranes were stripped and re-probed with b-actin antibody (Sigma-Aldrich) as a loading control.

2.13. Statistical analysis

All data are expressed as mean $\pm$ s.d. and were analyzed by Microsoft Excel, V3.0. Differences among groups were analyzed by a two-tailed student's *t* test for two groups and by ANOVA for multiple groups. Statistical significance was designated at *p* < 0.05.

3. Results

3.1. Expression of miR-21 and miR-181b is lost in C/EBP β -deficient Gr1⁺CD11b⁺ cells

Our previous studies showed that myeloid cell-specific inactivation of the *Cebpb* gene expression prevents the Gr1⁺CD11b⁺ MDSCs generation during sepsis and increases survival by 80% (McPeak *et al.*, 2017). Moreover, mice with C/EBP β conditional knockout survive late sepsis and generate Gr1⁺CD11b⁺ immune competent myeloid progenitors (McPeak *et al.*, 2017). C/EBP β induces miR-21 and miR-181b to generate immunosuppressive Gr1⁺CD11b⁺ in the bone marrow of septic mice (McClure *et al.*, 2017). In this study, we hypothesized that genetic deletion *Cebpb* will disrupt the path that leads to Gr1⁺CD11b⁺ MDSC generation. To test this, we first determined miR-21 and miR-181b expressions in sepsis Gr1⁺CD11b⁺ cells isolated from the bone marrow of control and C/EBP β knockout mice. As shown in Fig. 1A, miR-21 and miR-181b expression was induced in early sepsis and further increased in late sepsis cells. However, C/EBP β -deficient Gr1⁺CD11b⁺ cells from septic mice did not express miR-21 and miR-181b similar to cells from sham mice (lower panel).

We also reported that C/EBP α , and not C/EBP β , binds the same consensus motif at miR-21 and miR-181b promoters and is required for generating immune competent Gr1⁺CD11b⁺ cells (McClure *et al.*, 2017), thus disrupting expression of miR-21 and miR-181b. In this study and using Chromatin immunoprecipitation (ChIP) assay, we show that C/EBP β binding at the miR-21 promoter in sepsis but not sham Gr1⁺CD11b⁺ cells from the control mice, which further increased in late sepsis cells (Fig. 1B), concurrent with the miRNA induction. In sepsis Gr1⁺CD11b⁺ cells from the C/EBP β knockout mice, C/EBP α binding simulated cells from sham mice (Fig. 1B, lower panel). These results demonstrate that in the absence of C/EBP β expression, C/EBP α binds to and activates the miR-21 and miR-181b promoters in sepsis Gr1⁺CD11b⁺ cells.

3.2. C/EBP β plays an essential role in limiting Gr1⁺CD11b MDSC differentiation and maturation during sepsis

To confirm the role of C/EBP β in the activation of miR-21 and miR-181b expressions in sepsis Gr1⁺CD11b⁺ cells, we ectopically expressed C/EBP β in the C/EBP β -deficient cells from late septic mice. As shown in Fig. 2A, we detected C/EBP β protein at the miR-21 promoter with its binding pattern, Stat3, similar to that seen in control Gr1⁺CD11b⁺ cells. C/EBP β binding paralleled miR-21 expression (Fig. 2B) and miR-181b expression (data not shown). Of note, C/EBP β attenuated the Gr1⁺CD11b⁺ cells differentiation into macrophages and dendritic cells (Fig. 2C).

We reported that late sepsis Gr1⁺CD11b⁺ cells are immunosuppressive producers of IL-10 (MDSCs) and inhibit T cell proliferation and activation (Brudecki *et al.*, 2012; McClure *et al.*, 2016). Here, septic control mice transfected with empty vector, Gr1⁺CD11b⁺ cells from the C/EBP β conditional knockout mice reciprocally produced more TNF α and less IL-10 and did not suppress CD4⁺ T cell proliferation or IFN γ production (Fig. 3). Moreover, ectopic expression of C/EBP β reprogrammed these cells into the immunosuppressive phenotype (Fig. 3). Together, these results demonstrate that C/EBP β plays a pivotal role in

supporting miR-21 and miR-181b expression in Gr1⁺CD11b⁺ immunosuppressive cells during sepsis.

3.3. C/EBP β primarily generates immunosuppressive Gr1⁺CD11b⁺ MDSCs in sepsis by inducing miR-21 and miR-181b

We next investigated the essential role of miR-21 and miR-181b in generating immunosuppressive Gr1⁺CD11b⁺ cells in sepsis, using C/EBP β -deficient cells without miR-21 and miR-181b expression (Fig. 1A). Transfection of miR-21 and miR-181b precursors elevated both miRNAs to a level even higher than C/EBP β -expressing mice and subsequently attenuated their differentiation into macrophages and dendritic cells (Fig. 4). We then examined Gr1⁺CD11b⁺ cell cytokine production and immunosuppression of CD4⁺ T cells. miR-21 and miR-181b transfection into the sepsis C/EBP β -deficient Gr1⁺CD11b⁺ cells diminished the TNF α levels and increased IL-10 production (Fig. 5A) and repressed CD4⁺ T cell proliferation and activation, as demonstrated by IFN γ production (Fig. 5B). Transfection with precursors of either miRNA alone did not elicit any significant effects (data not shown). These data support that, miR-21 and miR-181b expression is dominant and lies downstream of C/EBP β in generating Gr1⁺CD11b⁺ MDSCs.

3.4. C/EBP β conditional knockout reverses the immunosuppressive Gr1⁺CD11b⁺ MDSC development during sepsis

We previously reported that NFI-A induction by miR-21 and miR-181b drives Gr1⁺CD11b⁺ MDSC development in sepsis (McClure *et al.*, 2016), by a complex pathway involving cyclin-dependent kinase (cdk4) and its inhibitor p21, PTEN, and NF-kB proteins (McClure *et al.*, 2016). Here, we show that NFI-A expression was completely inhibited in the C/EBP β -deficient cells with increased p21 levels, similar to cells from sham mice (Fig. 6A). Furthermore, reversing NFI-A and p21 expression patterns disrupted cdk4 protein complex and inactivated NF-kB (Fig. 6B), a terminal supporter of Gr1⁺CD11b⁺ cell expansion (McClure *et al.*, 2016). Thus, C/EBP β is a key and proximally drives Gr1⁺CD11b⁺ MDSC development in sepsis.

We also examined the p-Stat3 and miRNA levels in the presence or absence of exogenous C/EBP β protein. Both total and phosphorylated (active) Stat3 were expressed in the C/EBP β -deficient cells (data not shown), and ectopic expression of C/EBP β did not affect p-Stat3 levels, and meantime activated the miR-21 and miR-181b expressions (Fig. 7A). To confirm the above results, we ectopically expressed the C/EBP β protein in sepsis C/EBP β -deficient cells with or without Stat3 knockdown. As shown in Fig. 7B, lack of p-Stat3 prevented the miR-21 and miR-181b expression, despite the presence of the C/EBP β protein. These results extend our previous findings of a synergism between p-Stat3 and C/EBP β for supporting miR-21 and miR-181b expressions.

4. Discussion

We previously reported that C/EBP β plays an essential and proximal role in generating immunosuppressive Gr1⁺CD11b⁺ myeloid cells (McClure *et al.*, 2017; McPeak *et al.*, 2017). Taking a genetic approach, we confirm and extend support of the concept that C/EBP β

epigenetically controls miR-21 and miR-181b expression to drive MDSC reprogramming to the repressor phenotype so important in sepsis outcome. Immunosuppressive Gr1⁺CD11b⁺ cells (i.e., MDSCs) secrete IL-10 to repress CD4⁺ T cells and likely innate immune phagocytes during sepsis. Our genetic based study further supports the clinically important concept that MDSC generation bridges repressor responses of innate and adaptive immune during sepsis—thereby promoting uncontrolled infection with many microbes, including fungi (Hotchkiss *et al.*, 2013a; Hotchkiss *et al.*, 2013b). Since late/chronic sepsis survival correlates with resolution of the immunosuppressive state, this study supports C/EBP β targeting as a novel therapy for improving human sepsis outcomes. Most human sepsis deaths occur during the late stage of sepsis (Hotchkiss *et al.*, 2013a; Mira *et al.*, 2017).

Steady-state myelopoiesis maintains innate immunity cells (Ueda *et al.*, 2005). However, sepsis accelerates myelopoiesis. The rapid mobilization of innate immunity cells, especially granulocytes, from the bone marrow in response to the microbial products and damage-associated molecular patterns creates niches in the bone marrow (Manz *et al.*, 2014; Ueda *et al.*, 2005), which under the stress of sepsis preferentially differentiate toward the myeloid lineage and more immature (progenitor) myeloid cells accumulate (Furze and Rankin, 2008; Manz *et al.*, 2014). While this “emergency” myelopoiesis may aim to repopulate the innate immune system, in sepsis the cells do not mature normally. Because MDSCs are intermediate between myeloid progenitors arising from the hematopoietic stem cells and mature myeloid cells, a block in differentiation and maturation may account for MDSC generation (Dilek *et al.*, 2012; Gabrilovich and Nagaraj, 2009; Talmadge and Gabrilovich, 2013). We have identified C/EBP β as a key checkpoint for arresting Gr1⁺CD11b⁺ cells differentiation and maturation and promoting immune repressor cells. The numbers of the myeloid progenitors in the C/EBP β conditional knockout mice are similar to the control, C/EBP β -expressing mice (McPeak *et al.*, 2017), suggesting that C/EBP β is not involved in the steady-state myelopoiesis during sepsis-driven immune suppression.

MiR-21 and miR-181b expression in Gr1⁺CD11b⁺ cells acts distal to C/EBP β during sepsis to arrest differentiation and maturation (McClure *et al.*, 2014). Mechanistically, C/EBP β , jointly with p-Stat3, bind to and activate the miR-21 and miR-181b promoters (McClure *et al.*, 2017). In line with this, expression of miR-21 and miR-181b was inhibited in C/EBP β -deficient cells. In addition, we have previously shown that C/EBP β and p-Stat3 proteins binds the miRNA promoters in a single complex (McClure *et al.*, 2017). Together these data identify an epigenetic cooperative that includes NF-kB/RelB to silence selective genes by forming heterochromatin (El Gazzar *et al.*, 2009; McCall *et al.*, 2010; Chen *et al.*, 2009). Importantly, this epigenetic reprogram is reversible and can be a target for promoting sepsis resolution.

There are potential extensions and implications of this study. Immunosuppression with disrupted development and differentiation of innate and adaptive immunity is a major part of sepsis, but the latest sepsis III 2016 conference report places organ failure as a primary cause of death and dysregulated inflammation and immunity as a major contributor (Singer *et al.*, 2016). It will be important to determine if the C/EBP β pathway, a ubiquitous transcription factor, disrupts organ physiology during sepsis.

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References

1. Alves-Filho JC, Spiller F, Cunha FQ. Neutrophil paralysis in sepsis. *Shock*. 2010; 34(Suppl 1):15–21. [PubMed: 20714263]
2. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD, Kreisel D, Krupnick AS, Srivastava A, Swanson PE, Green JM, Hotchkiss RS. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA*. 2011; 306:2594–2605. [PubMed: 22187279]
3. Brudecki L, Ferguson DA, McCall CE, El Gazzar M. Myeloid-derived suppressor cells evolve during sepsis and can enhance or attenuate the systemic inflammatory response. *Infect Immun*. 2012a; 80:2026–2034. [PubMed: 22451518]
4. Brudecki L, Ferguson DA, Yin D, Lesage GD, McCall CE, El Gazzar M. Hematopoietic stem-progenitor cells restore immunoreactivity and improve survival in late sepsis. *Infect Immun*. 2012b; 80:602–611. [PubMed: 22144495]
5. Chen X, Yoza BK, El Gazzar M, Hu JY, Cousart SL, McCall CE. RelB sustains IkappaBalpha expression during endotoxin tolerance. *Clin Vaccine Immunol*. 2009; 16:104–110. [PubMed: 19020113]
6. Dilek N, Vuillefroy de SR, Blanco G, Vanhove B. Myeloid-derived suppressor cells: mechanisms of action and recent advances in their role in transplant tolerance. *Front Immunol*. 2012; 3:208. [PubMed: 22822406]
7. El Gazzar M, Yoza BK, Chen X, Garcia BA, Young NL, McCall CE. Chromatin-specific remodeling by HMGB1 and linker histone H1 silences proinflammatory genes during endotoxin tolerance. *Mol Cell Biol*. 2009; 29:1959–1971. [PubMed: 19158276]
8. Ellaban E, Bolgos G, Remick D. Selective macrophage suppression during sepsis. *Cell Immunol*. 2004; 231:103–111. [PubMed: 15919375]
9. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008; 125:281–288. [PubMed: 19128361]
10. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009; 9:162–174. [PubMed: 19197294]
11. Guignant C, Lepape A, Huang X, Kherouf H, Denis L, Poitevin F, Malcus C, Cheron A, Allaouchiche B, Gueyffier F, Ayala A, Monneret G, Venet F. Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock patients. *Crit Care*. 2011; 15:R99. [PubMed: 21418617]
12. Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J, Akashi K, Tenen DG. C/EBPbeta is required for ‘emergency’ granulopoiesis. *Nat Immunol*. 2006; 7:732–739. [PubMed: 16751774]
13. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *Lancet Infect Dis*. 2013a; 13:260–268. [PubMed: 23427891]
14. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol*. 2013b; 13:862–874. [PubMed: 24232462]
15. Liu TF, Brown CM, El Gazzar M, McPhail L, Millet P, Rao A, Vachharajani VT, Yoza BK, McCall CE. Fueling the flame: bioenergy couples metabolism and inflammation. *J Leukoc Biol*. 2012; 92:499–507. [PubMed: 22571857]
16. Manz MG, Boettcher S. Emergency granulopoiesis. *Nat Rev Immunol*. 2014; 14:302–314. [PubMed: 24751955]
17. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, Ugel S, Sonda N, Biccato S, Falisi E, Calabrese F, Basso G, Zanollo P, Cozzi E, Mandruzzato S, Bronte V. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity*. 2010; 32:790–802. [PubMed: 20605485]

18. Mazuski JE, Sawyer RG, Nathens AB, DiPiro JT, Schein M, Kudsk KA, Yowler C. The Surgical Infection Society guidelines on antimicrobial therapy for intra-abdominal infections: an executive summary. *Surg Infect (Larchmt)*. 2002; 3:161–173. [PubMed: 12542922]
19. McCall CE, Yoza B, Liu T, El Gazzar M. Gene-specific epigenetic regulation in serious infections with systemic inflammation. *J Innate Immun*. 2010; 2:395–405. [PubMed: 20733328]
20. McClure C, Ali E, Youssef D, Yao ZQ, McCall CE, El Gazzar M. NFI-A disrupts myeloid cell differentiation and maturation in septic mice. *J Leukoc Biol*. 2016; 99:201–211. [PubMed: 26259914]
21. McClure C, Brudecki L, Ferguson DA, Yao ZQ, Moorman JP, McCall CE, El Gazzar M. MicroRNA 21 (miR-21) and miR-181b couple with NFI-A to generate myeloid-derived suppressor cells and promote immunosuppression in late sepsis. *Infect Immun*. 2014; 82:3816–3825. [PubMed: 24980967]
22. McClure C, McPeak MB, Youssef D, Yao ZQ, McCall CE, El Gazzar M. Stat3 and C/EBPbeta synergize to induce miR-21 and miR-181b expression during sepsis. *Immunol Cell Biol*. 2017; 95:42–55. [PubMed: 27430527]
23. McPeak MB, Youssef D, Williams DA, Pritchett CL, Yao ZQ, McCall CE, El Gazzar M. Frontline Science: Myeloid cell-specific deletion of Cebpb decreases sepsis-induced immunosuppression in mice. *J Leukoc Biol*. 2017; 102:191–200. [PubMed: 28476751]
24. Mira JC, Gentile LF, Mathias BJ, Efron PA, Brakenridge SC, Mohr AM, Moore FA, Moldawer LL. Sepsis Pathophysiology, Chronic Critical Illness, and Persistent Inflammation-Immunosuppression and Catabolism Syndrome. *Crit Care Med*. 2017; 45:253–262. [PubMed: 27632674]
25. Niehof M, Streetz K, Rakemann T, Bischoff SC, Manns MP, Horn F, Trautwein C. Interleukin-6-induced tethering of STAT3 to the LAP/C/EBPbeta promoter suggests a new mechanism of transcriptional regulation by STAT3. *J Biol Chem*. 2001; 276:9016–9027. [PubMed: 11114305]
26. Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: understanding the role of innate and acquired immunity. *Shock*. 2001; 16:83–96. [PubMed: 11508871]
27. Panopoulos AD, Zhang L, Snow JW, Jones DM, Smith AM, El Kasmi KC, Liu F, Goldsmith MA, Link DC, Murray PJ, Watowich SS. STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. *Blood*. 2006; 108:3682–3690. [PubMed: 16888100]
28. Remick DG. Pathophysiology of sepsis. *Am J Pathol*. 2007; 170:1435–1444. [PubMed: 17456750]
29. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016; 315:801–810. [PubMed: 26903338]
30. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nat Rev Cancer*. 2013; 13:739–752. [PubMed: 24060865]
31. Ueda Y, Kondo M, Kelsoe G. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J Exp Med*. 2005; 201:1771–1780. [PubMed: 15939792]
32. Wesche DE, Lomas-Neira JL, Perl M, Chung CS, Ayala A. Leukocyte apoptosis and its significance in sepsis and shock. *J Leukoc Biol*. 2005; 78:325–337. [PubMed: 15817707]
33. Zhang H, Nguyen-Jackson H, Panopoulos AD, Li HS, Murray PJ, Watowich SS. STAT3 controls myeloid progenitor growth during emergency granulopoiesis. *Blood*. 2010; 116:2462–2471. [PubMed: 20581311]
34. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. *Proc Natl Acad Sci U S A*. 2011; 108:9184–9189. [PubMed: 21576471]

Highlights

- A mechanism for MDSC expansion during sepsis is proposed.
- The mechanism involves induction of miR-21 and miR-181b by C/EBP β .
- Inhibiting C/EBP β expression can prevent MDSC expansion and sepsis immunosuppression.

Figure 1. Loss of miR-21 and miR-181b expression in C/EBPβ-deficient Gr1⁺CD11b⁺ cells during sepsis

Sepsis was induced by cecal ligation and puncture (CLP) using a 23-gauge needle, and mice were given antibiotics (imipenem) with fluid resuscitation, which produced early (days 1–5) and late (days 6–28) sepsis phases. The Gr1⁺CD11b⁺ cells were isolated from the bone marrow of sham and septic mice using magnetic beads. **(A)** Measurements of miR-21 and miR-181b expression. MiRNA-enriched RNA was isolated, and levels of miR-21 and miR-181b were measured by RT-qPCR using miR-21 and miR-181b specific assay primer sets. Values were normalized to U6 RNA as an internal control. Data are expressed as mean $\pm$ s.d. (* p < 0.05) of 5 mice per group and are presented relative to values from, control sham cells (set at 1-fold). Data are from one of two experiments. *, compare with sham. **(B)** Transcription factor binding to the miRNA promoters. Gr1⁺CD11b⁺ cells were isolated and pooled (n = 3 mice per group) from the bone marrow of sham and septic mice. Cells were fixed in 1% formaldehyde to cross-link protein-DNA complexes. Cells were lysed and the pelleted nuclei were digested with chromatin shearing enzymatic cocktail. The chromatin solution was then immunoprecipitated with antibodies specific to p-Stat3 (Tyr⁷⁰⁵), C/EBPβ, C/EBPα, or IgG isotype control antibody. Next, chromatin cross-links were reversed to recover the protein-bound DNA. The purified DNA was amplified by real-time PCR to measure the level of enrichment of miR-21 and miR-181b sequences in the immunoprecipitated complexes using promoter-specific primer/probe sets. PCR reactions were performed in triplicate. Samples were normalized to the “input” DNA (i.e., DNA isolated before immunoprecipitation) and are presented as fold enrichment relative to the IgG-immunoprecipitated samples (set at 1-fold). Data are expressed as mean $\pm$ s.d. (* p < 0.05) of 5 mice per group and represent one of two experiments. *, compared with IgG. cKO, conditional knockout.

Figure 2. Expression of C/EBPβ in C/EBPβ-deficient Gr1⁺CD11b⁺ cells from late septic mice reactivates miRNA expression and attenuates cell differentiation

Bone marrow Gr1⁺CD11b⁺ cells were isolated from control or C/EBPβ conditional knockout mice during late sepsis. Cells were transfected with a C/EBPβ expression plasmid or an empty vector. After 36 hr, portions of the cells were harvested and used for ChIP assay and PCR. (A) Transcription factor binding to the miR-21 promoter. Enrichment of the miR-21 promoter sequences in the ChIP DNA was measured as described in Fig. 1. Right panel show western blotting of C/EBPβ protein levels after transfection with C/EBPβ. *, compare with IgG. (B) miR-21 expression was determined by real-time PCR as in Fig. 1. (C) The remainder of the cells (2×10⁶) was differentiated for 6 days with M-CSF plus rIL-4 (10 ng/ml/each), and numbers of differentiated cells were determined by flow cytometry. Percentages of differentiated cells (left) and absolute numbers of cells (right) in the culture after 6-day incubation are shown. Data are expressed as mean ± s.d. (*/#*p* < 0.05) of 7 mice per group and represent one of two experiments. *, compared with cells from C/EBPβ control mice; #, compared with cells from C/EBPβ cKO mice and transfected with vector. cKO, conditional knockout.

Figure 3. Expression of C/EBPβ in C/EBPβ-deficient Gr1⁺CD11b⁺ cells from late septic mice switches them from the immunocompetent to the immunosuppressive phenotype
Gr1⁺CD11b⁺ cells were isolated from the bone marrows of control and C/EBPβ conditional knockout mice during late sepsis and transfected with a C/EBPβ expression plasmid or an empty vector for 36 hr in the presence of 10 ng/ml of recombinant mouse IL-10 in order to activate Stat3. (A) C/EBPβ expression inhibits TNFα while inducing IL-10 production in C/EBPβ-deficient cells. Cells (1×10⁶) were cultured with 1 μg/ml LPS for 12 h. Supernatants were collected and levels of TNFα and IL-10 were determined by ELISA. (B) C/EBPβ-deficient cells reconstituted with C/EBPβ suppress T cell proliferation. Gr1⁺CD11b⁺ cells were co-cultured (at 1:1 ratio) with spleen CD4⁺ T cells isolated from normal (naive) mice were labeled with the fluorescent dye CFSE for 10 min at room temperature. The cells were then incubated in the presence of anti-CD3 plus anti-CD28 antibodies (1 μg/ml/each). After 3 days, CD4⁺ T cell proliferation was determined by the step-wise dilution of CFSE dye in dividing CD4⁺ T cells by flow cytometry. Percentages of cell proliferation were calculated as follow: % cell proliferation = 100 x (count from T cell + Gr1⁺CD11b⁺ cell culture/count from T cell culture). Data are expressed as mean ± s.d. (*/# *p* < 0.05) of 6 mice per group and represent one of two experiments. *, compared with cells isolated from control mice; #, compared with cells isolated from C/EBPβ cKO mice and transfected with vector. cKO, conditional knockout.

Figure 4. Expression of miR-21 and miR-181 in C/EBP β -deficient Gr1⁺CD11b⁺ cells from late septic mice attenuates their differentiation capacity

Gr1⁺CD11b⁺ cells were isolated from the bone marrow of control and C/EBP β conditional knockout mice during late sepsis. Cells were transfected with negative control, miR-21 or miR-181b precursor at 50 nM final concentration. (A) Measurement of miR-21 and miR-181b levels. After 24 hr in culture, portion of the cells were used for RNA isolation and miRNA measurement by PCR. The results are presented as fold change relative to cells from control, sham mice (set at 1-fold). (B) Cell differentiation. The remainder of the cells was differentiated for 6 days with M-CSF plus rIL-4, and percentages of macrophage (F4/80⁺CD11b⁺) and dendritic cell (CD11c⁺MHC II⁺) were determined by flow cytometry. Data are expressed as mean $\pm$ s.d. (*/# $p < 0.05$) of 4 mice per group and represent one of three experiments. *, compared with cells isolated from control mice; #, compared with cells isolated from C/EBP β cKO mice and transfected with control precursor. cKO, conditional knockout.

Figure 5. Expression of miR-21 and miR-181 in C/EBPβ-deficient Gr1⁺CD11b⁺ cells from late septic mice switches them into the immunosuppressive phenotype

Gr1⁺CD11b⁺ cells were isolated from the bone marrow of control and C/EBPβ conditional knockout mice during late sepsis. Cells were transfected with negative control, miR-21 or miR-181b precursor (50 nM final concentration) in the presence of 10 ng/ml of recombinant IL-10. **(A)** Cytokine production. Cells (1×10⁶) were cultured with 1 μg/ml LPS for 12 h. Supernatants were collected and levels of TNFα and IL-10 were determined by ELISA. **(B)** Effect on T cell proliferation. Gr1⁺CD11b⁺ cells were co-cultured (at 1:1 ratio) with spleen CD4⁺ T cells that have been isolated from normal (naive) mice and labeled with the fluorescent dye CFSE for 10 min at room temperature. The cells then were incubated in the presence of anti-CD3 plus anti-CD28 antibodies (1 μg/ml/each). After 3 days, CD4⁺ T cell proliferation was determined by the step-wise dilution of CFSE dye in dividing CD4⁺ cells by flow cytometry. Percentages of cell proliferation were calculated as follow: % cell proliferation = 100 x (count from T cell + Gr1⁺CD11b⁺ cell culture/count from T cell culture). Data are expressed as mean ± s.d. (*/#*p* < 0.05) of 5 mice per group and represent one of two experiments. *, compared with cells isolated from control mice; #, compared with cells isolated from C/EBPβ cKO mice and transfected control precursor. cKO, conditional knockout.

Figure 6. C/EBP β conditional knockout leads to differential changes in expression of the proteins involved in Gr1⁺CD11b⁺ cell expansion in sepsis

Gr1⁺CD11b⁺ cells were isolated from the bone marrows of control and C/EBP β conditional knockout mice. Levels of the indicated proteins were determined by western blotting. The results represent one of three experiments. cKO, conditional knockout.

Figure 7. Induction of miR-21 and miR-181b expression ex vivo in C/EBPβ-deficient cells by C/EBPβ and Stat3

Gr1⁺CD11b⁺ cells were isolated from the bone marrow of C/EBPβ conditional knockout mice during late sepsis. Cells were transfected either with a C/EBPβ expression plasmid or an empty vector alone (**A**) or plus Stat3-specific siRNA or control siRNA (**B**). After 36 hr, cells were stimulated with 10 ng/ml of recombinant mouse IL-6 to activate Stat3. Portions of the cells were harvested and levels of C/EBPβ and p-Stat3 were determined by western blotting. Levels of miR-21 and miR-181b were determined by real-time PCR. Data are expressed as mean ± s.d. (**p* < 0.05) of 3 mice per group and represent one of three experiments. The results are presented as fold change relative to cells from control, sham mice (set at 1-fold) as in Fig. 1. *, compared with cells transfected with vector.

Figure 8. A model for sepsis-induced myelopoiesis and generation of MDSCs

During the immune response to sepsis, inflammatory cytokines such as IL-6 and IL-10, and bacterial endotoxins induce expression of C/EBP β , which synergizes with Stat3 to activate the miR-21 and miR-181b promoters in the Gr1⁺CD11b⁺ myeloid progenitors. The miRNAs then induce NFI-A expression, which attenuates the cell differentiation and maturation via decreasing p21, increasing cdk4, and decreasing PTEN protein levels (not shown). These persistently activate the NF-kB and thus accelerate myelopoiesis and expand numbers of Gr1⁺CD11b⁺ cells. During the early phase of sepsis, these cells are immunocompetent like Gr1⁺CD11b⁺ cells generated under steady-state conditions. During the late phase, Gr1⁺CD11b⁺ cells are reprogrammed into the immunosuppressive phenotype, i.e., MDSCs. The myeloid cell-specific knockout of C/EBP β blocks this process during sepsis and thus prevents immunosuppression.