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IL-10 induces an immune repressor pathway in sepsis by promoting S100A9 nuclear localization and MDSC development

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Abstract

The myeloid-related protein S100A9 reprograms Gr1⁺CD11b⁺ myeloid precursors into myeloid-derived suppressor cells (MDSCs) during murine sepsis. Here, we show that the immunosuppressive cytokine IL-10 supports S100A9 expression and its nuclear localization in MDSCs to function as immune repressors. To support this new concept, we showed that antibody mediated IL-10 blockade in wild-type mice after sepsis induction inhibited MDSC expansion during late sepsis, and that ectopic expression of S100A9 in Gr1⁺CD11b⁺ precursors from S100A9 knockout mice switched them into the MDSC phenotype only in the presence of IL-10. Knockdown of S100A9 in MDSCs from wild-type mice with late sepsis confirmed our findings in the S100A9 knockout mice. We also found that while both IL-6 and IL-10 can activate S100A9 expression in naive Gr1⁺CD11b⁺ cells, only IL-10 can induce S100A9 nuclear localization. These results support that IL-10 drives the molecular path that generates MDSCs and enhances immunosuppression during late sepsis, and inform that targeting this immune repressor path may improve sepsis survival in mice.

Keywords

inflammation; sepsis; innate immunity; MDSCs; S100A9

1. Introduction

Survivors of early sepsis develop a debilitating and lethal chronic critical illness characterized by persistent inflammation and immunosuppression [27]. Dysfunctional innate

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Conflict of interest

The authors declare no conflict of interest.

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immune cells and suppressed adaptive immunity may play a major role in this delayed syndrome [10;30], in which sepsis-induced alterations in immune cells hamper immune homeostasis [1;8], prolong the primary pathogen infection, and increases the risk of opportunistic infections [19]. Mortality rates from chronic sepsis remain high [29].

Myeloid-derived suppressor cells (MDSCs)¹ arise due to aberrant myelopoiesis and possess immunosuppressive and inflammatory properties [12;16], which contribute to persistent inflammation [9]. We previously reported that immature Gr1⁺CD11b⁺ cells, which includes precursors of granulocytes, monocyte and dendritic cells, with immunosuppressive functions (i.e., MDSCs) expand dramatically in a mouse model of polymicrobial sepsis after the early hyperinflammatory phase and often persist during the chronic catabolic sepsis state associated with immunosuppression [3;26]. Others have shown that MDSCs expand and remain elevated in human sepsis [17;22], during persistent inflammation, immunosuppression and increased mortality rate [22].

We recently discovered that the calcium-binding S100A9 protein induces expansion of MDSCs in late septic mice [7]. S100A9 is constitutively expressed and dimerizes with its partner S100A8 in immature myeloid cells, but decreases with differentiation and maturation [31;33]. S100A9 is a known contributor to acute and chronic inflammatory processes [13;15], in part by enhancing phagocyte activation and leukocyte recruitment [13;14]. Most investigations of S100A9 have focused on its extracellular role as a soluble mediator of inflammation [15;32], emphasizing it as an amplifier of inflammation [13].

Our findings that S100A9 protein accumulates in the nucleus in MDSCs during sepsis and that S100A9 knockout mice could not generate MDSCs or develop immunosuppression [7] suggested that S100A9 may contribute to sepsis pathogenesis at the intracellular level. In that study, we showed that S100A9 protein translocated from the cytosol to the nuclear compartment in Gr1⁺CD11b⁺ myeloid progenitors during the late/chronic sepsis and promoted immunosuppression [7]. Importantly, injection of S100A9 into S100A9 knockout mice undergoing sepsis did not affect sepsis response [7], further supporting that intracellular rather than extracellular S100A9 promotes sepsis immunosuppression. In the present study, we used gain-and loss-of-function approach to investigate the path that may dysregulate S100A9 during sepsis. Our results demonstrate that IL-10 induces S100A9 translocation into the nuclear compartment in immature myeloid cells to support their phenotypic switch into MDSCs and thus to act as an immunosuppressive mediator. This novel role of S100A9 import into the nucleus might prove useful in targeting late/chronic sepsis immunosuppression to improve survival.

2. Materials and Methods

2.1. Mice

The C57BL/6N knockout mouse strain used in this study has been described previously [7]. Heterozygous animals were intercrossed to generate homozygous(−/−) mutant animals for the study. The mice were bred and housed in a pathogen-free facility in the Division of Laboratory Animal Resources. Wild-type male C57BL/6N mice, 8–10 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and used as controls. Mice were

acclimated to the new environment before experiments. 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. Sepsis

Sepsis was induced by cecal ligation and puncture (CLP) as described previously [4]. Briefly, a midline abdominal incision was made and the cecum exteriorized, ligated distal to the ileocecal valve, and then punctured twice with a 23-gauge needle. A small amount of feces was extruded into the abdominal cavity. This level of injury creates a prolonged infection with 100% mortality over 4 weeks. Sham-operated mice were treated identically except that the cecum was not ligated or punctured. 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 intraabdominal 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 [23], injections of Imipenem were given at 8 and 16 hr after CLP, which results in high mortality (~70%) during the late/chronic phase [4]. 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. Gr1⁺CD11b⁺ cells

Gr1⁺CD11b⁺ cells were isolated from the bone marrow or spleens using magnetically assisted cell sorting according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). Briefly, the bone marrow cells were flushed out of the femurs with RPMI-1640 medium (without serum) under aseptic conditions. The spleens were minced in RPMI-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 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. The Gr1⁺CD11b⁺ cell purity was typically >90%.

2.4. Cell culture

Gr1⁺CD11b⁺ cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μ g/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.

2.5. Flow cytometry

Gr1⁺CD11b⁺ cells were stained by incubation for 30 min on ice in staining buffer (PBS plus 2% FBS) with the following mouse antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-Gr1, phycoerythrin (PE)-conjugated anti-CD11b, allophycocyanin (APC)-

conjugated anti-F4/80, PE-conjugated anti-CD 11c, FITC-conjugated anti-MHC II. CD4⁺ T cells were stained with PE- conjugated anti-CD4 antibody (all antibodies were from eBioscience, San Diego, CA). An appropriate isotype-matched control was used for each antibody. After washing, the samples were analyzed by an Accuri C6 flow cytometer (BD, Franklin Lakes, NJ).

2.6. S100A9 plasmid

Full length mouse *S100a9* cDNA was cloned in pEZ-M02 expression vector downstream of the CMV promoter. A pReceiver-M02 vector served as a negative control.

2.7. Cell transfection

For S100A9 transfection, S100A9 plasmid DNA or empty vector was suspended in HiPerFect reagent at a 0.5 µg/ml final concentration (Qiagen, Valencia, CA). For S100A9 knockdown, pools of S100A9-specific or scrambled (control) siRNAs were suspended in HiPerFect reagent at a 0.5 µM final concentration. Gr1⁺CD11b⁺ cells were transfected using the Gene Pulser MXCell system (Bio-Rad, Hercules, CA) and then incubated for 36 hr with RPMI-1640 medium.

2.8. 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 of rIL-4 (eBioscience, San Diego, CA). Cells were phenotyped by flow cytometry.

2.9. T cell proliferation

To determine suppressive effects of Gr1⁺CD11b⁺ cells on T cell functions, a co-culture of Gr1⁺CD11b⁺ cells and CD4⁺ T cells were assessed for T cell proliferation and IFN γ production. Briefly, spleen CD4⁺ T cells from naive wild-type were isolated by positive selection using biotinylated anti-CD4 magnetic beads (Myltenyi). Cells were fluorescently labeled with carboxy- fluorosceindiacetate, 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 µM 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 (1µg/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, CD4-gated T cells using flow cytometry. Culture supernatants were collected for the IFN- γ measurement.

2.10. ELISA

Cytokine concentrations in peripheral blood or culture supernatants were determined using specific enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) according to the manufacturer's instructions. Each sample was run in duplicate.

2.11. Protein extracts

Whole cell lysates were prepared by cell lysis in 1x RIPA buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, and 1 mM EDTA (Millipore, Temecula, CA) plus 1x protease inhibitor cocktail.

Cytoplasmic and nuclear proteins were prepared using the NE-PER nuclear and cytoplasmic extraction kit per the manufacturer's instructions (Pierce, Rockford, IL). Briefly, cells were washed in PBS and resuspended in CER1 lysis buffer with protease inhibitor cocktail and incubated on ice for 1 min. CER2 buffer was added and the incubation continued for 5 min. Supernatants (cytoplasmic proteins) were recovered by centrifugation for 5 min at 4°C and 14,000 rpm. The nuclear pellets were resuspended in NER lysis buffer with protease inhibitor cocktail and incubated for 40 min on ice with occasional vortexing. The nuclear proteins were recovered by centrifugation for 10 min at 4°C and 14,000 rpm.

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 β -actin antibody (Sigma-Aldrich) as a loading control.

2.13. Statistical analysis

Data were analyzed by Microsoft Excel, V3.0., and are presented as mean $\pm$ s.d. Differences between two groups were analyzed by a two-tailed student's *t*-test for two groups. Differences among multiple groups were analyzed by one-way ANOVA. Statistical significance is reported for *P*-values < 0.05 .

3. Results

3.1. IL-6 and IL-10 upregulate S100A9 expression in Gr1⁺CD11b⁺ cells.

The proinflammatory cytokine IL-6 and immunosuppressive cytokine IL-10 play a major role in sepsis pathogenesis [25], and have been implicated in the generation and activation of Gr1⁺CD11b⁺ MDSCs under different inflammatory conditions [6;16]. Both IL-6 [20] and IL-10 [18] have been shown to upregulate S100A9 expression in mouse colon epithelial cells and human dendritic cells, respectively. We have shown that S100A9 protein accumulates in MDSCs in late septic mice with immunosuppression and that S100A9 knockout mice could not generate MDSCs [7]. To determine whether IL-6 or IL-10 induces accumulation of S100A9 in sepsis MDSCs, we first measured plasma levels of IL-6 and IL-10 throughout the course of sepsis response in wild-type and S100A9 knockout mice.

Sepsis response in our modified CLP model extends over a 4-week period, with a protracted immunosuppressive phase developing after day 5 of sepsis onset and is associated with a massive expansion of MDSCs in the bone marrow and spleens [3]. As shown in **Fig. 1**, levels of IL-6 increased rapidly in wild-type and S100A9 knockout mice and then decreased dramatically one week after sepsis onset. In contrast, IL-10 levels increased gradually and slightly in both wild-type and knockout mice during the first week after sepsis onset. After day 6, levels of IL-10 further increased until day 28 in wild-type mice, while remaining at very low levels in the S100A9 knockout mice. Given that S100A9 knockout mice cannot generate MDSCs [7], these results indicate that the MDSCs expansion in late septic mice is associated with elevated levels of IL-10.

Gr1⁺CD11b⁺ cells expand in wild-type mice throughout sepsis response, with dramatic increases in late sepsis. However, late sepsis Gr1⁺CD11b⁺ cells are immunosuppressive (i.e., MDSCs) while early sepsis Gr1⁺CD11b⁺ cells are immunocompetent [3]. As shown in **Fig. 2A**, S100A9 expression increased in bone marrow Gr1⁺CD11b⁺ cells throughout sepsis response, with higher levels detected in late sepsis. We next stimulated naive Gr1⁺CD11b⁺ cells, which have same phenotype as MDSCs but are not immunosuppressive [3], with IL-6 or IL-10 and measured S100A9 protein levels in cell lysates. Both cytokines induced S100A9 expression (**Fig. 2B**). In addition, IL-6 and IL-10 also induced Stat3 phosphorylation (**Fig. 2C**), which is known to activate S100A9 expression in mouse myeloid cells [5]. These results demonstrate that IL-6 and IL-10 can induce S100A9 expression in Gr1⁺CD11b⁺ cells during sepsis.

3.2. Blocking IL-10 prevents MDSC expansion during late sepsis.

Because both IL-6 and IL-10 upregulated S100A9 expression (**Fig. 2**), we examined whether blocking their activity in wild-type mice can affect Gr1⁺CD11b⁺ cell expansion. Injection of IL-6 neutralizing antibody at the onset of sepsis significantly decreased Gr1⁺CD11b⁺ cell expansion in early (i.e., day 1–5) septic mice but had no effect on cell expansion during the late sepsis phase (**Fig. 3A**). In contrast, injection of IL-10 neutralizing antibody significantly decreased Gr1⁺CD11b⁺ cell expansion during the late sepsis phase only.

In a reverse experiment, we injected rIL-10 and/or rS100A9 into S100A9 knockout mice following sepsis induction. As in wild-type mice, we observed a significant increase in Gr1⁺CD11b⁺ cell numbers in bone marrow during early sepsis only (**Fig. 3B**). However, injection of rIL-10 and/or S100A9 into knockout mice did not increase Gr1⁺CD11b⁺ cell numbers during the late sepsis response. In addition, injection of rIL-10 or rS100A9 into wild-type mice had no significant effects on Gr1⁺CD11b⁺ cell expansion (**Fig. 3C**). Of note, these mice produce increased amounts of IL-10 (**Fig. 1**) and S100A9 proteins [7]. Together, these results demonstrate that IL-10 plays an essential role in MDSCs expansion in late sepsis and that exogenous/extracellular S100A9 protein has no effect on this process.

3.3. Intracellular S100A9 limits Gr1⁺CD11b⁺ cell differentiation and promotes immunosuppressive function.

Although MDSCs share similar phenotype (i.e., Gr1⁺CD11b⁺) with the Gr1⁺CD11b⁺ cells generated through physiological myelopoiesis, the latter can differentiate *ex vivo* with

growth factors into mature innate immune cells [3]. Gr1⁺CD11b⁺ cells isolated from bone marrow of S100A9 knockout mice during late sepsis are not immunosuppressive [7] and can differentiate into macrophages and dendritic cells when stimulated with macrophage colony-stimulating factor (**Fig. 4** and data not shown). We tested whether expression of S100A9 in Gr1⁺CD11b⁺ cells could affect their phenotype. To this end, we introduced S100A9 expression plasmid into Gr1⁺CD11b⁺ cells isolated from S100A9 knockout mice during the late sepsis phase, cells were then differentiated for 6 days with macrophage colony-stimulating factor. As expected, Gr1⁺CD11b⁺ cells isolated from wild-type mice during the late sepsis phase, and without IL-10 stimulation and S100A9 plasmid transfection, had very limited differentiation capacity compared to cells from sham mice (**Fig. 4**). On the other hand, cells from S100A9 knockout mice with or without IL-10 stimulation exhibited significantly higher differentiation and maturation rates similar to Gr1⁺CD11b⁺ cells from sham mice. Importantly, S100A9 plasmid transfection significantly decreased cell differentiation in the presence of rIL-10 only.

We next found that late sepsis Gr1⁺CD11b⁺ cells isolated from S100A9 knockout and transfected with S100A9 plasmid alone or stimulated with IL-10 alone could not attenuate T cell proliferation or activation in response to CD3/CD28 ligation (**Fig. 5**). In contrast, under these conditions, sepsis Gr1⁺CD11b⁺ cells from wild-type mice significantly attenuated T cell proliferation and activation, which were similar to cells without IL-10 stimulation and S100A9 transfection (**Fig. 5**). Furthermore, S100A9 knockout Gr1⁺CD11b⁺ cells transfected with S100A9 plasmid and stimulated with IL-10 reduced T cell proliferation and IFN γ production significantly (**Fig. 5**). Collectively, the results presented in **Figs. 4 and 5** suggest that intracellular S100A9 can reprogram Gr1⁺CD11b⁺ cells generated during sepsis into MDSCs and that IL-10 drives this regulatory path.

3.4. S100A9 knockdown abolishes the immunosuppressive functions of sepsis MDSCs.

To test whether S100A9 expression in Gr1⁺CD11b⁺ cells is sufficient to reprogram them into the immunosuppressive (MDSC) phenotype, we assessed the effects of S100A9 silencing in late sepsis Gr1⁺CD11b⁺ cells from wild-type mice on their differentiation capacity, as well as their immunosuppressive functions. As shown in **Fig. 6A**, S100A9 silencing significantly increased Gr1⁺CD11b⁺ cell differentiation and maturation into macrophages and dendritic cells, and the presence of IL-10 had no impact. In addition, naive CD4⁺ T cells cultured with Gr1⁺CD11b⁺ cells with S100A9 knockdown exhibited significantly higher proliferation rate and IFN γ production compared with CD4⁺ T cells cultured with control Gr1⁺CD11b⁺ cells (i.e., transfected with control siRNA) (**Fig. 6B and C**). These results suggest that inhibition of S100A9 expression in late sepsis Gr1⁺CD11b⁺ cells is sufficient to promote their maturation and immune competency.

3.5. IL-10 induces nuclear localization of S100A9 in MDSCs.

The results described above suggested that intracellular rather than extracellular S100A9 is responsible for reprogramming late sepsis Gr1⁺CD11b⁺ cells into the MDSC phenotype. Western blot analysis using Gr1⁺CD11b⁺ cells from wild-type mice revealed that S100A9 protein is mainly localized in the cytosol and nuclear compartment, respectively, in early and late sepsis Gr1⁺CD11b⁺ cells (**Fig. 7A**). Because IL-6 or IL-10 could induce S100A9

expression in naive Gr1⁺CD11b⁺ cells (**Fig. 2B**), we asked if these cytokines can also promote S100A9 nuclear localization. The S100A9 protein remained in the cytosol after stimulation of naive Gr1⁺CD11b⁺ cells with IL-6, whereas stimulation with IL-10 induced S100A9 localization to the nucleus (**Fig. 7B**). To confirm that IL-10 was responsible for S100A9 nuclear localization, we transfected naive wild-type Gr1⁺CD11b⁺ cells with S100A9 expression plasmid and stimulated the cells with IL-10. In the absence of IL-10, S100A9 was mainly present in the cytosol, but localized into the nucleus with IL-10 stimulation (**Fig. 7C**). These results suggest that IL-10 induces S100A9 protein accumulation in the nucleus in Gr1⁺CD11b⁺ cells during late sepsis. In addition, we found that IL-10 induced S100A9 protein translocation from the cytosol to the nucleus in MDSCs from early/acute septic patients (**Fig. 7D**).

4. Discussion

The principal new finding of this study is that IL-10 supports both expression and nuclear accumulation of S100A9 in Gr1⁺CD11b⁺ myeloid progenitors to switch them into MDSCs. This implies that IL-10 persistence drives, at least in the chronic catabolic state of late sepsis, immunosuppression. In contrast, our data suggest that increased expression and production of IL-6 is a hallmark of early/acute sepsis [24;28]. We found that the kinetics of IL-6 and IL-10 differential expression mirrored Gr1⁺CD11b⁺ cell expansion. Systemic IL-6 levels gradually increased during early sepsis while IL-10 levels increased during the late sepsis phase. Of note, systemic blockade of IL-10 attenuated Gr1⁺CD11b⁺ cell expansion during early sepsis only, whereas IL-10 blockade inhibited expansion in late sepsis. Notably, we previously showed that Gr1⁺CD11b⁺ cells generated in the bone marrow and spleens of early septic mice are immunocompetent, whereas their counterparts generated in late septic mice are immunosuppressive (i.e., MDSCs) [3]. In *ex vivo* experiments, both IL-6 and IL-10 induced S100A9 expression and Stat3 activation (phosphorylation) in naive Gr1⁺CD11b⁺ cells. We previously reported that the expansion of Gr1⁺CD11b⁺ MDSCs in late sepsis depends on induction of S100A9 [7], whose expression was shown to be induced by p-Stat3 in murine myeloid [5] and epithelial [20] cell lines. Importantly, S100A9 knockout mice can still generate immunocompetent Gr1⁺CD11b⁺ cells during early sepsis [7]. In this study, blockade of IL-6 but not IL-10 inhibited the expansion of early sepsis Gr1⁺CD11b⁺ cells. Moreover, we did not observe expansion of immunosuppressive Gr1⁺CD11b⁺ cells in the S100A9 knockout mice during late sepsis despite the administration of IL-10, yet these mice generated immunocompetent Gr1⁺CD11b⁺ cells during early sepsis. These findings suggest that the S100A9-induced generation and expansion of MDSCs during late sepsis depends on prolonged IL-10 signaling. In support of this, we found that ectopic expression of S100A9 in Gr1⁺CD11b⁺ cells derived from the S100A9 knockout mice during late sepsis blocked their differentiation and induced their immunosuppressive functions, i.e., switched these cells into MDSCs, and this required IL-10 signal. In parallel, siRNA-mediated knockdown of S100A9 in late sepsis MDSCs from wild-type mice restored their differentiation capacity and diminished their immunosuppressive functions. Thus, IL-10 signaling to S100A9 induction during late sepsis is sufficient to reprogram myeloid progenitors into MDSCs.

Although both IL-6 and IL-10 induced S100A9 expression in Gr1⁺CD11b⁺ cells, only IL-10 promoted S100A9 protein translocation to the nucleus. We previously reported that

nuclear localization of S100A9 in myeloid progenitors during late sepsis induces MDSC generation and expansion [7]. The role of IL-10 in the production of S100A9 protein is controversial. A previous study showed that treatment of human monocytes with IL-10 inhibits the release of S100A9 protein [21], which aligns with our new concept. In contrast, another study reported that IL-10 promotes release of S100A9 from human blood monocytes by facilitating S100A9 protein phosphorylation by MAPK p38 [11]. We have shown that S100A9 was phosphorylated and localized in the cytosol in early, but not late, sepsis Gr1⁺CD11b⁺ cells, despite the presence of phosphorylated p38 [7]. Needing further definition and mechanism probing is whether and how IL-6 and IL-10 differentially affect Gr1⁺CD11b⁺ cell generation downstream of Stat3 activation and S100A9 expression.

Stat3 can be both proinflammatory and anti-inflammatory. A recent study found that IL-16 induces proinflammatory response, whereas IL-10 supports anti-inflammatory/tolerogenic response in human dendritic cells, and that IL-6 transiently activates Stat3, whereas IL-10 sustains Stat3 activity [2]. Notably, IL-6 production in mice declined during late sepsis, while IL-10 production continued. We find that Gr1⁺CD11b⁺ cells generated in early sepsis are not immunosuppressive and differentiate into mature cells [3]. Results of this study suggest that Stat3 activation in late sepsis by IL-10 promotes S100A9 expression and nuclear localization, but what retains nuclear S100A9 is unknown.

4.1. Conclusions

This study implicates IL-10 in promoting S100A9 nuclear localization in myeloid cells during late sepsis. This finding is compatible with the notion that IL10 couples to S100A9 to switch the myeloid cells to the immunosuppressive MDSC phenotype, thus supporting chronic sepsis immunosuppression. Finding how this transfer and retention process works at the molecular level could advance precision treatments in murine sepsis.

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Abbreviations

¹MDSC	myeloid derived suppressor cell
CLP	cecal ligation and puncture
IL	interleukin
miR	microRNA
Stat3	signal transducer and activator of transcription 3
siRNA	small interfering RNA

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.Highlights

- The anti-inflammatory cytokine IL-10 enhances sepsis immunosuppression.
- IL-10 modifies S100A9 protein localization.
- IL-10 promotes MDSC development in late sepsis.

Figure 1: IL-6 production is decreased while IL-10 is increased as sepsis progresses from the proinflammatory to the immunosuppressive phase.

Sepsis was induced by cecal ligation and puncture (CLP) using a 23-gauge needle, and mice were given antibiotics with fluid resuscitation. With this injury and treatment, sepsis develops into an early phase (defined as days 1–5 after CLP) and a late phase (day 6 thereafter). Blood was collected from moribund mice that were killed at the indicated times. Plasma was collected and levels of IL-6 and IL-10 were measured by ELISA. Data are means $\pm$ s.d. (* $p < 0.05$) from 3 mice per group/time point. The 0 time point represents baseline values from sham mice. KO, knockout.

Figure 2: Stimulation of naive Gr1⁺CD11b⁺ cells with IL-6 or IL-10 increases S100A9 expression. Gr1⁺CD11b⁺ cells were isolated from bone marrow cells by positive selection and incubated for 12 hr with or without 10 ng/ml of mouse rIL-6 or rIL-10. **(A)** Western blotting of S100A9 in Gr1⁺CD11b⁺ cells from sham and septic mice. **(B and C)** Gr1⁺CD11b⁺ cells from naive mice were stimulated as in *A*. Levels of S100A9, and total and phosphorylated Stat3 proteins were determined by western blotting. The results are representative of three experiments.

Figure 3: The effects of cytokine blockade in wild-type mice, and S100A9 administration into knockout mice on Gr1⁺CD11b⁺ cell expansion during sepsis. Sepsis was induced as described in Fig. 1. (A) Neutralizing IL-10 diminishes Gr1⁺CD11b⁺ cell expansion in late sepsis. Wild-type mice were injected i.p. with 50 µg of anti-IL-6, anti-IL-10 or IgG control antibody on day 0 and day 5 after CLP. (B) Administration of rIL-10 and/or rS100A9 into the S100A9 knockout mice does not increase Gr1⁺CD11b⁺ cell expansion during late sepsis. Mice were injected i.p. with 25 µg of rIL-10 and/or rS100A9 protein after CLP. (C) Administration of rIL-10 or rS100A9 into wild-type mice does not

affect Gr1⁺CD11b⁺ cell expansion during sepsis. Mice were injected with 25 µg of rIL-10 or rS100A9 protein after CLP. Bone marrow cells were harvested at the indicated times, stained with anti-Gr1 and anti-CD11b antibodies and analyzed by flow cytometry. Percentages of the Gr1⁺CD11b⁺ cells gated on Gr1 and CD11b in wild-type (*A*) and S100A9 knockout mice (*B*) are shown. Data are means ± s.d. ([#]/^{*}*p* < 0.05) from 3 mice per group/time point. [#], anti-IL-10 vs. anti-IL-6; ^{*}, anti-IL-10 vs. IgG or anti-IL-6. KO, knockout.

Figure 4: Ectopic expression of S100A9 in late sepsis Gr1⁺CD11b⁺ cells from the S100A9 knockout mice attenuates their differentiation in the presence of IL-10.

Gr1⁺CD11b⁺ cells were selected from bone marrow cells isolated during late sepsis. Cells were transfected with 0.5 µg/ml of the S100A9 expression plasmid or empty vector, cultured for 36 hr. Then, cells were stimulated for 12 hr with 10 ng/ml of rIL-10. Cells were differentiated for 6 days with M-CSF plus rIL-4 (10 ng/ml each). Flow cytometry analysis of the differentiated cells gated on F4/80⁺ CD11b⁺ (macrophage) or CD11c⁺ MHC II⁺ (dendritic cell) staining is shown. Data are means ± s.d. (*/***p* < 0.05) from 5–6 mice per group pooled from two experiments. Note, cells from wild-type mice were not transfected with S100A9

plasmid nor stimulated with IL-10, and are presented here as a negative control. Cells from sham mice are included as a positive control. *, sepsis vs. sham; **, knockout vs. wild-type. KO, knockout.

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Figure 5: Ectopic expression of S100A9 in late sepsis Gr1⁺CD11b⁺ cells from the S100A9 knockout mice switch them into the immunosuppressive phenotype.

Gr1⁺CD11b⁺ cells were selected from bone marrow cells isolated during late sepsis response. Cells were transfected with 0.5 μ g/ml of the S100A9 expression plasmid or empty vector and cultured for 36 hr. Then, cells were stimulated with 10 ng/ml of rIL-10. After 12 hr, the treated Gr1⁺CD11b⁺ cells were cocultured (at 1:1 ratio) with spleen CD4⁺ T cells, which were isolated from naive wild-type mice and labeled with the fluorescent dye CFSE for 10 min at room temperature. The culture was incubated with anti-CD3 plus anti-CD28 antibodies (1

µg/ml each). After 3 days, supernatants and cells were harvested. **(A)** Effect of Gr1⁺CD11b⁺ cells on CD4⁺ T cell proliferation. 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 alone). **(B)** Effect of Gr1⁺CD11b⁺ cells on IFNγ production in activated CD4⁺ T cells. Levels of IFNγ in the culture supernatants were measured by ELISA. Data are means ± s.d. (*/** $p < 0.05$) from 5–6 mice per group pooled from two experiments. Note, cells from wild-type mice were not transfected with S100A9 plasmid nor stimulated with IL-10, and are presented here as a negative control. Cells from sham mice are included as a positive control. *, sepsis vs. sham; **, knockout vs. wild-type. KO, knockout.

Figure 6: Knockdown of S100A9 in late sepsis Gr1⁺CD11b⁺ cells from wild-type mice inhibits their immunosuppressive phenotype.

Gr1⁺CD11b⁺ cells were isolated from bone marrow of wild-type, late septic mice. Cells were transfected with pools of S100A9-specific or control siRNAs and cultured for 36 hr. Then, cells were stimulated for 12 hr with 10 ng/ml of rIL-10. (A) Transfected cells were differentiated for 6 days with M-CSF plus rIL-4 (10 ng/ml each) and percentages of macrophages and dendritic cells were determined by flow cytometry. (B) Transfected cells were co-cultured with spleen CD4⁺ T cells from naive mice, and T cell proliferation and INF- γ production were determined as described in Fig. 5. Data are means $\pm$ s.d. (* p < 0.05)

from 3 mice per group and represent one of three experiments. *, S100A9 siRNA vs. control siRNA. KO, knockout.

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Figure 7: IL-10 induces translocation of S100A9 into the nucleus in Grl⁺CD11b⁺ cells.

(A) Localization of S100A9 protein in Grl⁺CD11b⁺ cells during sepsis. Grl⁺CD11b⁺ cells were isolated from bone marrow of septic wild-type mice, and levels of S100A9 in the cytosol and nucleus were determined by western blotting. (B) Grl⁺CD11b⁺ cells were isolated from bone marrow of naive wild-type mice and cultured for 12 hr with or without 10 ng/ml of mouse rIL-6 or rIL-10. (C) Naive Grl⁺CD11b⁺ cells from wild-type mice were transfected with 0.5 µg/ml of the S100A9 expression plasmid and cultured for 36 hr. Then, cells were treated as in B. (D) IL-10 induces nuclear localization of S100A9 protein in MDSCs from patients with early/acute sepsis. MDSCs (CD33⁺CD11b⁺HLA-DR⁻) were positively selected from PBMCs using magnetic beads and cultured for 12 hr with 10 ng/ml of rIL-10. Levels of cytosolic and nuclear S100A9 proteins were determined by western blotting. The results are representative of three western blots from two independent experiments.