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Development, Expansion and Role of Myeloid-Derived Suppressor Cells in Post-Sepsis Immune Suppression

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Development, Expansion and Role of Myeloid-Derived Suppressor Cells in Post-Sepsis Immune Suppression

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ABSTRACT

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Myeloid-derived suppressor cells (MDSCs) numbers increase significantly in sepsis and are associated with high mortality rates. These myeloid cell precursors promote immunosuppression, especially in the late (post sepsis) stage. However, the mechanisms that underlie MDSC expansion and programming are not completely understood. To investigate these mechanisms, we used a cecal ligation and puncture (CLP) mouse model of polymicrobial sepsis that progresses from an early/acute proinflammatory phase to a late/chronic immunosuppressive phase. Previous studies in our laboratory showed that microRNA (miR)-21 and miR-181b elevate levels of the transcription factor nuclear factor 1 (NFI-A) that promotes MDSC expansion. We report here that miR-21 and miR-181b regulate NFI-A expression via a post-transcriptional regulatory mechanism by recruiting RNA-binding proteins HuR and Ago1 to stabilize NFI-A mRNA, thus increasing its protein levels. Studies in our laboratory also showed that inflammatory mediator S100A9 accumulates in the nucleus in Gr1+CD11b+ myeloid precursors in the later phases of sepsis and is necessary for their expansion and programming into immunosuppressive MDSCs. We demonstrate here that nuclear S100A9 associates with specific transcription factors that activate miR-21 and miR-181b expressions. In our final manuscript, we uncover another layer of the mechanisms of MDSC expansion and programming. We found that long non-coding RNA (lncRNA) Hotairm1 binds to and recruits S100A9 to the

nucleus to program Gr1+CD11b+ myeloid precursors into MDSCs in the later phases of sepsis. Together, our results reveal three regulatory layers involving NFI-A, S100A9 and Hotairm1 in the pathway leading to MDSCs development in sepsis and suggest that therapeutically targeting these molecular switches might improve sepsis survival.

DEDICATION

I dedicate this work to those who have sacrificed everything for me and have nurtured me to become who I am today, my father Dr. Faisal Alkhateeb and my mother Faten Jaradat. I can never thank them enough for their constant wisdom, encouragement and support. I pray that they are always blessed with health and happiness for the years to come.

This work is also dedicated to my wonderful siblings that were by my side, through it all, Thabit, Hassan, Hiba and Kenan.

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I am grateful for all the countless bounties that God has blessed me with, to pursue and complete my PhD journey. I hope this project contributes to the overall human well-being and alleviates sepsis detrimental outcomes.

I thank my advisor, Dr. Mohamed Elgazzar for allowing me to become an independent researcher and preparing me for a variety of career options. His feedback, support, and willingness to help, whenever I needed it, throughout my PhD years is much appreciated.

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To all the faculty and staff members that were supportive of my growth during the last five years at ETSU, thank you from the bottom of my heart. Special thanks to Dr. Agrawal, Dr. Ordway, Dr. Bradshaw, Dr. Pritchett, Dr. R.Brown, Dr. Roane, Dr. Kilaru, Dr. T.Jones and Mr. Ellis who have left a positive long-lasting effect on my personal development.

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CHAPTER 1

INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al. 2016). Despite advances in understanding sepsis pathophysiology, deaths due to sepsis remain very high (Fleischmann et al. 2016). Sepsis accounts for more than five million deaths annually worldwide (Fleischmann et al. 2016). Roughly, 30 million people in the world are diagnosed with sepsis every year, and this number has been increasing at an alarming rate, exceeding stroke and myocardial infarction (Fleischmann et al. 2016; Seymour et al. 2016; Walkey et al. 2015). It also remains the most common cause of death among critically ill patients where in-hospital mortality is about 25% (Fleischmann et al. 2016). Sepsis is a very devastating healthcare problem, making it the most expensive conditions to treat in U.S. hospitals (Finfer and Machado 2016). Current treatment for septic patients includes only antimicrobials and supportive care. While advances in sepsis care and diagnosis reduced the number of deaths during the early/acute phase, it did not improve the overall mortality rate (Cohen et al. 2012; Vught et al. 2016). Recent studies have shown that a significant number of patients who survive the early sepsis phase rapidly progress to develop a late, post-sepsis phase characterized by protracted immunosuppression and chronic inflammation (Hotchkiss et al. 2013). Currently, there is no approved drug for the treatment of sepsis and thus there is a need for developing molecular-based treatments that can target late, post-sepsis immunosuppression (Patil et al. 2016).

Sepsis Pathophysiology

Sepsis has been known since at least 1000 BC when Ibn Sina, the father of medicine and well-known philosopher, described sepsis as a putrefaction of blood and tissues with fever (Majno 1991). In the latest definition of sepsis, described in the 2016 Sepsis-3 guidelines, sepsis syndrome was redefined as being a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al. 2016). During the last decade, significant improvement has been made in understanding sepsis pathophysiology and the immunological dysfunctions that occur during sepsis (Delano and Ward 2016; Deutschman and Tracey 2014). Sepsis is a diverse syndrome, with various pathways that may be dysregulated during the host response to infection (Steinhagen et al. 2020). For many years, sepsis was considered to solely be composed of the host mounting an initial hyper-inflammatory reaction to infection (Hotchkiss et al. 2013; Poll et al. 2017). Later, pre-clinical and clinical studies suggested that sepsis clinical presentation was not that simple, and the initial inflammatory phase was trailed by an anti-inflammatory or immunosuppressive phase, as well as an inability to restore immune homeostasis (Boomer et al. 2013). In the past decade, it has been shown that both the pro-inflammatory and anti-inflammatory phases in sepsis may occur simultaneously at fluctuating magnitudes and are not biphasic (Delano and Ward 2016; Hotchkiss and Opal 2010; Hotchkiss et al. 2013) (Figure 1.1). Thus, changes that may be either beneficial or harmful occur during the sepsis spectrum, with diverse levels of inflammation and immunosuppression occurring simultaneously, which may contribute to sepsis pathogenesis and dysfunctions of the innate and adaptive immune responses.

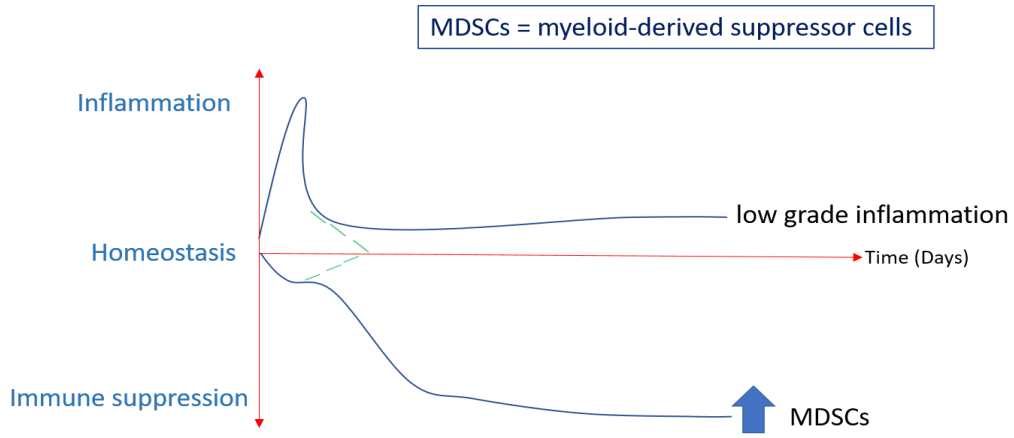

Figure 1.1: Sepsis pathophysiological phenotype shift. Adapted from Hotchkiss et al. 2013; *Nat Rev Immunol*.

Sepsis-Associated Immunosuppression

Post-sepsis immunosuppression may be deleterious to critically ill patients because it increases the risk of viral infections, development of secondary nosocomial infections by opportunistic pathogens, and subsequent multi-organ dysfunctions and death (Delano and Ward 2016; Fleischmann et al. 2016; Gentile et al. 2012; Monneret et al. 2011; Otto et al. 2011). Interestingly, more than 70% of sepsis death occurs after the first three days of sepsis onset and up to several weeks with a one- year mortality rate in discharged patients (Delano and Ward 2016; Fleischmann et al. 2016; Otto et al. 2011). Therefore, targeting the long-term immune perturbations in sepsis that contribute to sepsis-induced immunosuppression may help improve patient survival.

Sepsis-induced immunosuppression may occur due to the dysregulation of several molecular and cellular processes that may worsen sepsis outcomes (Patil et al. 2016). One of these hallmarks, contributing to immune dysregulation, is the excessive release of anti-inflammatory mediators such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-

β), reactive oxygen species (ROS), nitric oxide (NO) and arginase-1 (Arg-1) by immune cells (Gabrilovich and Nagaraj 2009; Peranzoni et al. 2010). Other vital immune alterations that occur after early/acute sepsis include a diminished ability of the immune cells to properly respond to pathogens and to clear infections due to enhanced apoptosis of immune effector cells, suppression of T cell functions, and increases in immunoregulatory cells such as T regulatory cells (Tregs) (Daker et al. 2015; Kulkarni et al. 2018; Mira et al. 2017; Schrijver et al. 2019). Importantly, recent studies have shown that defective myelopoiesis, along with the expansion of myeloid-derived suppressor cells (MDSCs), aggravate this immunosuppressive stage and worsen sepsis patient outcome (Brudecki et al. 2012; Schrijver et al. 2019).

Myeloid-Derived Suppressor Cells

The functional activity of MDSCs became more apparent around half a century ago in a leukocytosis response that was generated in tumor cells and helped sustain tumor growth (Schrijver et al. 2019; Talmadge and Gabrilovich 2013). What led to this favorable pro-tumor environment was also suspected of being responsible for the expansion of cells of myeloid origin with immunosuppressive activity (Talmadge and Gabrilovich 2013). These cells expressed diminished levels of conventional markers for mature myeloid and lymphoid cells (Schrijver et al. 2019). In the recent decades, MDSCs have been identified with several names such as natural suppressor cells, null cells, immature myeloid cells, suppressor macrophages, or myeloid suppressor cells (Murphey et al. 2004; Schrijver et al. 2019). In 2007, “myeloid-derived suppressor cells” was used as a global term to help decrease the confusion in the literature (Gabrilovich et al. 2007).

MDSCs are a heterogeneous population of inducible immature myeloid cells that arise as intermediates between myeloid progenitors and mature myeloid cell populations, generated from self-renewing hematopoietic stem cells (HSCs) (Cuenca et al. 2010; Gabrilovich et al. 2007; Schrijver et al. 2019) (Figure 1.2). Thus, these cells are progenitors and precursors of monocytes, granulocytes, and dendritic cells (Gabrilovich and Nagaraj 2009; Peranzoni et al. 2010). In healthy and homeostatic conditions, immature myeloid cells are present at very low numbers and maintained by a steady-state myelopoiesis (Gabrilovich 2017). However, these cells may dramatically expand, or increase in numbers, under several acute and chronic inflammatory conditions, during natural aging, or with psychological stress (Alves et al. 2018; Mundy-Bosse et al. 2011; Verschoor et al. 2013). Some of the immunological stress conditions where myelopoiesis leads to expansion of these MDSCs include cancer, autoimmune disease, trauma, burns and sepsis (Brudecki et al. 2012; Cuenca et al. 2010; Ostrand-Rosenberg and Sinha 2009; Sica and Massarotti 2017). Other than their vast expansion under chronic inflammatory conditions, these cells possess major regulatory roles due to their immunosuppressive functions and are therefore designated as myeloid-derived suppressor cells (MDSCs) (Bronte et al. 2016; Gabrilovich and Nagaraj 2009).

Sepsis rapidly mobilizes innate immune cells (Cuenca et al. 2010; Delano et al. 2007) and may deplete myeloid cell reserves needed to resist uncontrolled infection (Cuenca et al. 2010; Kelly-Scumpia et al. 2010; Scumpia et al. 2010). During this time, normal immune-competent myeloid precursor cells expand or increase to make up for the lost innate immune cells. However, under a sepsis inflammatory environment, these cells are programmed into MDSCs (Cuenca et al. 2010; Mathias et al. 2017; Ostrand-Rosenberg and Fenselau, 2018). Previous studies in this laboratory (Brudecki et al. 2012) and others (Delano et al. 2007; Derive et al.

2012) reported sustained expansion of Gr1+CD11b+ MDSCs in a model of murine cecal ligation and puncture (CLP) designed to simulate post-acute sepsis. This model causes immunosuppression during the late sepsis phase concomitant with sustained expansion and accumulation of Gr1+CD11b+ MDSCs. Also, using this model, Gr1+Cd11b+ cells increase in early sepsis, but what differentiates these cells from MDSCs in late sepsis is that they increase at lower magnitude than late sepsis, are not immunosuppressive and differentiate normally into mature myeloid cells. Subsequently, persistent increases in MDSCs in a subset of sepsis patients contributed to a chronic post-sepsis syndrome (Mathias et al. 2017; Mira et al. 2017) evidenced by increased nosocomial infections and organ dysfunction (Cuenca et al. 2010; Mathias et al. 2017). This post-acute sepsis (>14 d after hospitalization) increases morbidity and mortality (Hotchkiss et al. 2013; Mira et al. 2017; Patil et al. 2016). However, its molecular underpinnings are unclear.

My projects focus on the molecular mechanisms that underlie the expansion of myeloid precursors and their subsequent programming into MDSCs during sepsis.

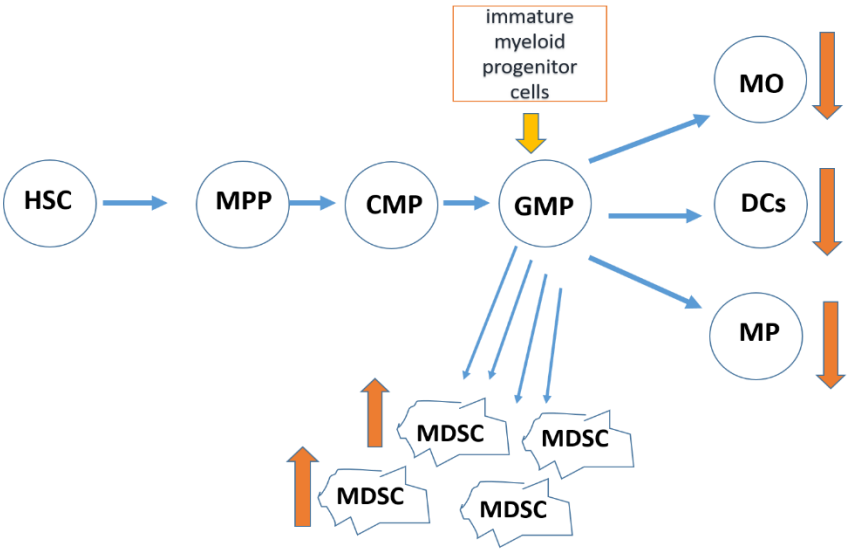

Figure 1.2: MDSC generation and accumulation. In the bone marrow, hematopoietic stem cells (HSCs) generate multipotent progenitors (MPP), common myeloid progenitors (CMP), and granulocyte-macrophage progenitors (GMP) that further differentiate into normal/immune-competent innate immunity cells. However, under various chronic inflammatory conditions such as in cancer and sepsis, myeloid precursors at the GMP stage accumulate in great numbers and do not continue their normal differentiation and maturation pathway. Instead, these acquire immunosuppressive properties and thus are called myeloid-derived suppressor cells (MDSCs). MO, monocytes; DCs, dendritic cells; MP, macrophages. Adapted from Veglia et al, 2018; *Nat Immunol*

MDSC Phenotypes

Murine MDSCs are divided into two main subtypes, polymorphonuclear (PMN- MDSCs) and mononuclear/monocytic (M-MDSCs). This identification is based on their morphological and phenotypical similarities with PMNs and monocytes, and their relative expression of the myeloid cell surface markers CD11b, Ly6G, and Ly6C (Movahedi et al. 2008). The functional difference between the two types of MDSCs in sepsis is their mechanism of immunosuppression. M-MDSCs mediate their action by inducible nitric oxide synthase (iNOS) and produces nitric oxide (NO) while PMN-MDSCs rely on reactive oxygen species (ROS), arginase 1 (Arg1), and interferon-g (IFN-g) production for their immunosuppressive functions (Peranzoni et al. 2010). In our late septic mice model, immunosuppressive MSDCs are identified as undifferentiated

Gr1+ CD11b+ cells, and the Gr1/granulocyte receptor-1 antigen consists of Ly-6G and Ly-6C epitopes (Talmadge and Gabrilovich 2013). Researchers have hypothesized that these MDSCs have the potential to differentiate and mature, but under the chronic septic low-grade inflammation environment, they cannot continue their normal differentiation pathway (Brudecki et al. 2012). Instead, these cells become "trapped" in an immature phenotype and acquire immunosuppressive properties (Figure 1.2) (Cuenca et al. 2010). Studies in our laboratory and others have shown that in healthy or early/acute septic mice, similar Gr1+ CD11b+ cells exist but do not possess immunosuppressive phenotype and can differentiate normally into mature myeloid cells (Brudecki et al. 2012; Gabrilovich and Nagaraj 2009; Kusmartsev and Gabrilovich 2003). In humans, MDSCs are also classified into PMN-MDSCs and M-MDSCs, but because Gr1 is not expressed on human myeloid cells, they are identified by their relative expressions of CD11b, CD14, CD33, CD15 and HLA-DR markers (Bronte et al. 2016; Pawelec et al. 2019).

MDSCs in Sepsis

During sepsis, MDSCs play a dual role depending on the disease stage or progression (Consonni et al. 2019). Like much of the varied host response to inflammation, MDSC expansion can be protective or harmful to the host (Sander et al. 2010). During the early/acute hyperinflammatory stage of sepsis, which is characterized by elevated levels of proinflammatory cytokines such as TNF-alpha and IL-6, MDSCs may be beneficial by suppressing the systemic inflammatory response or "cytokine storm," thus protecting the host from opportunistic infectious insults and decreasing chances of multi-organ dysfunction (Brudecki et al. 2012; Sander et al. 2010; Schrijver et al. 2019). On the other hand, if sepsis is not treated early to restore immune homeostasis, persistent expansion of MDSC occurs and leads to late/chronic sepsis immunosuppression (Mira et al. 2017; Venet and Monneret 2017). Within this context,

MDSCs are harmful because they amplify the anti-inflammatory and persistent immunosuppressive state by producing immunosuppressive mediators and inhibiting effector T cell functions, thus increasing late (post sepsis) mortality. Thus, mediators that promote MDSC expansion and immunosuppressive functions are important targets for improving chronic sepsis outcomes (Consonni et al. 2019).

MDSC Immunosuppressive Functions

Recent findings suggest MDSCs may exaggerate the IL-10–dependent immune suppression and induce Treg cell development. MDSCs accumulation in late sepsis has been linked to T-cell suppression (Cuenca et al. 2010; Hoechst et al. 2008). These cells possess potent immunosuppressive effects via secretion of anti-inflammatory mediators such as IL-10, TGFβ, ROS, NO, and Arg1 (Gabrilovich and Nagaraj 2009; Peranzoni et al. 2010). These mediators may contribute to dampening the immune response if other immune cells also release them. Importantly, reprogramming of myeloid progenitors into immunosuppressive MDSCs in the bone marrow during the late/chronic stage of sepsis may be due to multi-factorial steps (Velten et al. 2017). Also, why these cells only expand in late sepsis with an inability to further differentiate into mature myeloid cells remains unclear. Interestingly, unlike late sepsis, myeloid progenitors that are generated during the early/acute stage of sepsis are not immunosuppressive and retain the ability to differentiate into mature myeloid cells. Several clinical studies in sepsis patients have correlated the high levels of MDSCs in the blood with a worsened clinical condition, occurrences of nosocomial infections, and an increased risk of death (Schrijver et al. 2019). Thus, understanding the mechanisms that underlie MDSC expansion in late sepsis can help in mitigating their detrimental immunosuppressive effects to improve patient outcomes.

Expansion of Gr1+CD11b+ Myeloid Precursors in Sepsis

Role of MiR-21 and MiR-181b

Recent studies in our laboratory have shed light on the mechanisms of MDSC development and expansion during murine sepsis. MDSCs isolated from the bone marrow of mice with polymicrobial sepsis, that underwent cecal ligation and puncture (CLP) and mimic human general peritonitis, showed a differential microRNA (miRNA) expression profile (Laslo et al. 2008; McClure et al. 2014; Oconnell et al. 2011; Tsitsiou and Lindsay 2009). In particular, levels of miR-21 and miR-181b were increased and sustained in the bone marrow and spleens throughout sepsis (McClure et al. 2014). Notably, these two miRNAs have been implicated in myeloid cell development and differentiation (Brudecki et al. 2012; McClure et al. 2014). Later studies showed that in vivo blockade of miR-21 and miR-181b reduced MDSC expansion and late sepsis immunosuppression, enhanced Gr1+CD11b+ myeloid cell differentiation and maturation, and improved survival by 74% (McClure et al. 2014, 2016).

Expression of MiR-21 and MiR-181b in Sepsis

The CCAAT/enhancer-binding protein C/EBPb is a transcription factor that promotes MDSC expansion in the spleens and bone marrow in septic mice via upregulating miR-21 and miR-181b, which increase NFI-A expression (Dai et al. 2017; Minner et al. 2018). Recent studies show that C/EBPb binds to and activates the miR-21 and miR-181b promoters in MDSCs (McClure et al. 2017). On the other hand, loss of C/EBPb allows C/EBPa, which is required for myeloid cell differentiation under normal conditions, to bind to miR-21 and miR-181b promoters and inhibit their expression, allowing normal Gr1+CD11b+ myeloid cell differentiation (Marigo et al. 2010; McPeak et al. 2017). The switch between the binding affinity of C/EBPb and

C/EBPa to miR-21 and miR-181b promoters in Gr1+CD11b+ cells is dependent on retinoblastoma (Rb) protein phosphorylation state (McClure et al. 2017).

Recent studies have shown that C/EBPb and Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylation are required for "emergency" myelopoiesis and MDSC expansion in mice with sepsis (Zhang et al. 2010). Our lab studied this pathway further and concluded that both phosphorylated STAT3 and nuclear C/EBPb act in a synergistic manner to induce miR-21 and miR-181b expressions, leading to NFI-A upregulation and MDSC expansion (McClure et al. 2017). Notably, conditional knockout of C/EBPb, in the myeloid compartment in mouse, attenuated MDSC expansion during sepsis, enhanced normal Gr1+CD11b+ cell differentiation and maturation, diminished late/chronic sepsis immunosuppression, and improved survival (McPeak et al. 2017). Marigo et al. also found that knockout of C/EBPb had the same effect in terms of decreasing MDSC expansion and myeloid cell differentiation in tumor-bearing mice (Marigo et al. 2010).

Effect of MiR-21 and MiR-181b on NFI-A Expression

Mechanistic studies in this laboratory revealed that both miR-21 and miR-181b promote MDSC expansion in sepsis by increasing the expression of the myeloid-related transcription factor NFI-A, which is a member of the nuclear factor I family and has been implicated in myeloid cell differentiation (McClure et al. 2014). Elevated levels of NFI-A maintain myeloid precursors in an undifferentiated state, and thus increases the number of immature myeloid cells (Dai et al. 2017; McClure et al. 2014; Mei et al. 2015). Knockdown of miR-21 and miR-181b in MDSCs reduces NFI-A and allows their differentiation into mature innate immune cells (McClure et al. 2014). Importantly, mice with conditional NFI-A knockout in the myeloid

compartment have diminished MDSCs, do not develop immunosuppression, and have better sepsis outcomes (McClure et al. 2014).

In patients with lung cancer, NFI-A expression was associated with the anti-inflammatory response of MDSCs due to elevated Arg1 levels, while knockdown of NFI-A in MDSCs enhanced anti-tumor immune response and decreased immunosuppression (Tian et al. 2015). Other miRNAs have been shown to regulate NFIA expression under different conditions (Glasgow et al. 2013; Starnes et al. 2009). In leukemia cells, decreases in miR-223 expression resulted in NFI-A upregulation, which inhibited granulocytic and monocytic differentiation (Glasgow et al. 2013). In contrast, miR-136 has been shown to enhance granulocyte differentiation via downregulating NFI-A (Mei et al. 2015; Starnes et al. 2009).

Programming of Gr1+CD11b+ Myeloid Precursors Into MDSCs in Sepsis

Role of S100A9

S100 calcium-binding proteins, S100A8 and S100A9, are expressed in various myeloid cells including monocytes and neutrophils and are predominately cytosolic proteins under normal conditions (Vogl et al. 2018). Under various acute and chronic inflammatory conditions, their expressions increase dramatically, and they function as damage-associated molecular patterns (DAMPs) or alarmins in myeloid immune cells (Bianchi 2007). S100A9 plays a prominent role in sepsis pathogenesis (Vogl et al. 2007). In hospitalized patients diagnosed with sepsis, sustained high level of S100A9 mRNA was a prognostic factor for nosocomial infections (Fontaine et al. 2011). Likewise, immunosuppressed sepsis patients that had an increased chance of acquiring opportunistic infections expressed high S100A9 mRNA levels (Fontaine et al.

2011). In contrast, patients who recovered from sepsis displayed lower levels of S100A8 mRNA and protein (Payen et al. 2008).

In mice with polymicrobial sepsis, IL-10 was shown to induce high S100A9 expression levels that correlated with sustained MDSC expansion during the late sepsis stage (Dai et al. 2017). Although both IL-6 and IL-10 induced S100A9 expression in Gr1+CD11b+ cells (i.e., MDSCs), only IL-10 promoted S100A9 protein translocation from the cytosol to the nucleus (Bah et al. 2018). Mechanistically, translocation of S100A9 into the nucleus increased miR-21 and miR-181b expressions through a feedback loop involving STAT3 and C/EBPb, leading to NFI-A upregulation and MDSC expansion (Dai et al. 2017; El Gazzar 2015). Importantly, *S100a9* gene ablation decreased MDSC accumulation, promoted normal myeloid differentiation, and improved survival in late septic mice (Dai et al. 2017; El Gazzar 2015). Also, in these knockout mice, expression of miR-21 and miR-181b decreased in the late sepsis Gr1+CD11b+ cells. These myeloid precursors were generated at baseline levels, as found in wild-type mice, and had no immunosuppressive functions.

The secretion of S100A9 protein from Gr1+CD11b+ cells occurs under the proinflammatory environment of early sepsis but later decreases during the anti-inflammatory/immunosuppressive sepsis stage (Dai et al. 2017). During this late stage response, S100A9 protein accumulates in the nucleus and contributes to late sepsis death in mice by inducing MDSCs expansion and immunosuppression (Dai et al. 2017). While some studies have reported that extracellular/secreted S100A8 and S100A9 proteins induce proinflammatory responses by enhancing phagocyte activation, other studies have shown that intracellular S100A8 and S100A9 proteins may play an immune-regulatory role in myeloid cell differentiation (Chen et al. 2013; Cheng et al. 2008; El Gazzar 2015). S100A8 and S100A9 are constitutively

expressed in myeloid cells but their expression generally decreases in differentiated cells (Cheng et al. 2008). This may partially explain the high levels of S100A9 in late sepsis MDSCs (Dai et al. 2017). Recent studies have shown that S100A9 plays an anti-inflammatory role in cancer, LPS-induced endotoxemia, kidney transplantation, and autoimmune myocarditis (Ikemoto et al. 2007; Yang et al. 2018). Specifically, in cancer patients, intracellular S100A9 enhanced anti-inflammatory responses that correlated with the accumulation of MDSCs (Sade-Feldman et al. 2013; Zheng et al. 2015). This mechanism was mediated by STAT3 binding to S100A9 promoters, upregulating S100A9 expression, and preventing differentiation of immature myeloid cells (Chen et al. 2013; Cheng et al. 2008). However, mice deficient in S100A9 possessed anti-tumor effects. Studies in our laboratory suggest that S100A9 is required for MDSC expansion and immunosuppressive function during the late stage of sepsis but has no major impact on early/acute sepsis proinflammatory responses (Cheng et al. 2008).

Role of Long Non-Coding RNAs

Recent studies have shown that some long non-coding RNAs (lncRNAs) regulate immune cell development and differentiation (Tian et al. 2018). LncRNAs are non-protein-coding RNA transcripts of more than 200 nucleotides (Bhan et al. 2017). Although lncRNAs have been studied in many diseases during the past decade, researchers have not investigated them extensively in sepsis (Dey et al. 2014). Most research that analyzed lncRNA in sepsis were mainly focused on the hyperinflammatory stage, or early/acute sepsis (Fan et al. 2015; Lin et al. 2015; Wu et al. 2016). However, the effect they may have on MDSC development and immunosuppressive function in late sepsis may be a breaking factor. LncRNAs may have a critical function on modulating gene transcription, specifically when present in the nuclear

compartment of cells and thus may affect epigenetic reprogramming of immune cells (Anfossi et al. 2018; Tian et al. 2018).

Targeting S100A9 by Hotairm1

Around a decade ago, a new lncRNA, Hotairm1 was discovered. Hotairm1 stands for HOXA transcript antisense RNA myeloid-specific 1 and is located between the human myeloid HOXA1 and HOXA2 genes (Zhang et al. 2010). Hotairm1 was found to be expressed at the highest level during the later stages of monocyte differentiation (Tanzer et al. 2005). Thus, it may play a critical role in myeloid cell maturation (Wan et al. 2016). Another study showed that Hotairm1 hindered the differentiation of myeloid cells via binding to miR-3960 and decreasing its expression, which led to the inhibition of myeloid cell maturation (Tian et al. 2018; Xin et al. 2017). Further, the immunosuppressive mediator TGF-β increased Hotairm1 expression (Xin et al. 2017). These findings indicate an inverse relationship between Hotairm1 and myeloid cell differentiation.

Cancer research revealed an association between lncRNAs and MDSCs (Montoya et al. 2019). In lung cancer, a recent study reported that Hotairm1 affects the development and immunosuppressive function of MDSCs (Montoya et al. 2019; Tian et al. 2018). Hotairm1 downregulated the expansion of immunosuppressive MDSCs and enhanced antitumor responses (Tian et al. 2018). Another study showed that Hotairm1 plays a tumor-suppressive role in colorectal cancer where its expression is decreased in colorectal cancer tissue and plasma, but this study did not describe its effect on MDSCs (Wan et al. 2016). The finding that Hotairm1 diminishes MDSCs under certain tumor conditions was linked to elevated expression of HOXA1, that enhanced the antitumor immune response (Montoya et al. 2019). Notably, the effect of Hotairm1 on the development of MDSCs in sepsis is unknown.

Hypotheses and Specific Aims

Sepsis rapidly shifts from early/acute hyperinflammation to a late/protracted immunosuppressed state characterized by high morbidity and mortality (Boomer et al. 2013; Hotchkiss et al. 2013; Poll et al. 2017). Previous studies in our lab have shown that MDSCs significantly expand in the later phases of sepsis and promote immunosuppression (Brudecki et al. 2012). Also, previous studies in the lab have shown that the transcription factor NFI-A is induced during sepsis and that NFI-A knockout in mice reduces Gr+CD11b+ cell generation during sepsis (McClure et al. 2014). We hypothesize that NFI-A expression is upregulated at the post-transcriptional level by miR-21 and miR-181b. Importantly, knockout of the inflammatory mediator S100A9 reduces MDSCs in septic mice and improves mortality (Dai et al. 2017). Preliminary data suggested that lncRNA Hotairm1 binds to S100A9 in Gr+CD11b+ cells. We hypothesize that Hotairm1 targets S100A9 to the nucleus in Gr+CD11b+ cells during late sepsis to switch them into MDSCs.

There are no mechanistic studies that explain why/how immature Gr+CD11b+ cells expand and become MDSCs in late sepsis. The goal of my project is to investigate the molecular pathways that contribute to Gr+CD11b+ cell expansion and MDSC development in sepsis. This project includes three aims. Aim 1 investigates the mechanism of NFI-A upregulation in sepsis. This work is described in a manuscript in Chapter 2, which has been accepted for publication. (Bah et al. 2020). Aim 2 elucidates the link between S100A9 expression and MDSC development in sepsis. This work is described in a manuscript in Chapter 3 and has been published (Alkhateeb et al. 2019). Aim 3 investigates how Hotairm1 switches the proinflammatory Gr+CD11b+ cells into MDSCs in late sepsis. This work is described in a

manuscript in Chapter 4, which is currently under review (Alkhateeb et al. 2020; J. of Innate Immunity).

CHAPTER 2

HUR PROMOTES MIRNA-MEDIATED UPREGULATION OF NFI-A PROTEIN EXPRESSION IN MDSCS DURING MURINE SEPSIS

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Keywords: MDSC, Sepsis, miRNA, NFI-A

Abstract

Myeloid-derived suppressor cells (MDSCs) contribute to high mortality rates during sepsis, but how sepsis induces MDSCs is unclear. Previously we reported that microRNA (miR)-21 and miR-181b reprogram MDSCs in septic mice by increasing levels of DNA binding transcription factor, nuclear factor 1 (NFI-A). Here, we provide evidence that miR-21 and miR-181b stabilize NFI-A mRNA and increase NFI-A protein levels by recruiting RNA-binding proteins HuR and Ago1 to its 3' untranslated region (3'UTR). We also find that the NFI-A GU-rich element (GRE)-binding protein CUGBP1 counters miR-21 and miR-181b dependent NFI-A mRNA stabilization and decreases protein production by replacing 3'UTR bound Ago1 with Ago2. We confirmed the miR-21 and miR-181b dependent reprogramming pathway in MDSCs transfected with a luciferase reporter construct containing an NFI-A 3'UTR fragment with point mutations in the miRNA binding sites. These results suggest that targeting NFI-A in MDSCs during sepsis may enhance resistance to uncontrolled infection.

1. Introduction

Sepsis rapidly mobilizes innate immune cells (Cuenca *et al.*, 2011;Delano *et al.*, 2007) and may deplete myeloid cell reserves needed to resist uncontrolled infection (Cuenca *et al.*, 2011;Kelly-Scumpia *et al.*, 2010;Scumpia *et al.*, 2010). During this time, normal immune competent myeloid precursors cells deviate to myeloid-derived suppressor cells (MDSC), which suppress T cell functions as a contributing cause of disrupted innate and adaptive immune responses (Cuenca *et al.*, 2011;Mathias *et al.*, 2017;Ostrand-Rosenberg and Fenselau, 2018). We (Brudecki *et al.*, 2012a) and others (Delano *et al.*, 2007;Derive *et al.*, 2012) reported sustained expansion of $Gr1^+CD11b^+$ MDSCs in a model of murine cecal ligation and puncture (CLP) designed to simulate post-acute sepsis. Subsequently, persistent increases in MDSCs in a subset of sepsis patients contributed to a chronic post-sepsis syndrome (Mathias *et al.*, 2017;Mira *et al.*, 2017a) evidenced by increased nosocomial infections and organ dysfunction (Cuenca *et al.*, 2011;Mathias *et al.*, 2017). This post-acute sepsis (>14 d after hospitalization) increases morbidity and mortality (Hotchkiss *et al.*, 2013;Patil *et al.*, 2016;Mira *et al.*, 2017b). However, its molecular underpinnings are unclear.

Growth factors and inflammatory mediators drive MDSC expansion under a variety of acute and chronic conditions, including infection and inflammation (Cuenca *et al.*, 2011;Ostrand-Rosenberg and Fenselau, 2018). To better understand MDSCs as contributors to sepsis outcome, we developed a mouse model of polymicrobial sepsis that reprogrammed persistent inflammation and immunosuppression (Brudecki *et al.*, 2012a). We discovered that the CCAAT-box-binding transcription factor NFI-A, which attenuates normal myeloid cell differentiation and maturation (Fazi *et al.*, 2005;Rosa *et al.*, 2007;Zardo *et al.*, 2012), expanded $Gr1^+CD11b^+$ MDSCs in bone marrow and spleens during sepsis (McPeak *et al.*, 2017). Ex vivo knockdown of

NFI-A in $Gr1^{+}CD11b^{+}$ MDSCs obtained from septic mice restores their differentiation and maturation to macrophage and dendritic cells (McClure *et al.*, 2016). Conditional NFI-A knockout in the myeloid cell compartment attenuates MDSC expansion and immunosuppression and improves sepsis survival (McPeak *et al.*, 2017). We then found that microRNA (miR)-21 and miR-181b regulate NFI-A expression in $Gr1^{+}CD11b^{+}$ cells during sepsis (McClure *et al.*, 2014;McClure *et al.*, 2016). MiRNAs post-transcriptionally bind complementary sequences within the 3' untranslated region (3'UTR) of the target mRNAs (Baltimore *et al.*, 2008;O'Connell *et al.*, 2010), which assembles the repressor complex, RNA-induced silencing complex or RISC. RISC decays or represses target mRNA to downregulate protein synthesis (Baltimore *et al.*, 2008;Kim *et al.*, 2009).

In the present study, we enlighten how miR-21 and miR-181b upregulate NFI-A protein expression and MDSC development during sepsis. We found that the RNA-binding proteins HuR and CUGBP1 compete for NFI-A 3'UTR binding and either stabilize or destabilize NFI-A mRNA, and that miR-21 and miR-181b coupling to HuR increases NFI-A mRNA stability and protein levels.

2. Materials and Methods

2.1. Mice

Male BALB/c mice (8-10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in a pathogen-free temperature-controlled room and were acclimated to the new environment for a week before surgery. 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.

We used male mice, because several clinical and experimental studies reported that cell-mediated immune responses are depressed in males with sepsis, and are unchanged or enhanced in females (Angele *et al.*, 2000;De *et al.*, 2005). Because MDSCs suppress both innate and adaptive immune responses, we exclusively used male mice in order to maximize their immunosuppressive effects during sepsis.

2.2. Sepsis model

Polymicrobial sepsis from cecal ligation and puncture (CLP) modeled MDSCs development, as described previously (Brudecki *et al.*, 2012b). Briefly, a midline abdominal incision was made, and the cecum was 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. Mice received (i.p.) 1 ml lactated Ringers plus 5% dextrose for fluid resuscitation. To establish intra-abdominal infection and approximate the clinical condition of human sepsis (Mazuski *et al.*, 2002) and delay in MDSC development, mice were subcutaneously administered antibiotic (imipenem; 25 mg/kg body weight) in saline (0.9% sodium chloride) at 8 and 16 hr after CLP. This results in high mortality (~60-70%) during a post-acute sepsis phenotype (Brudecki *et al.*, 2012b). Survival was followed for 28 days. Mice moribund during acute sepsis (defined as the first 5 d after CLP) or later/chronic sepsis (post 6 d) (Brudecki *et al.*, 2012b) were euthanized and analyzed. A corresponding number of mice from the control/sham group were also analyzed at the same time point.

2.3. $Gr1^+CD11b^+$ cell isolation

$Gr1^+CD11b^+$ cells were isolated from the bone marrow by positive selection using magnetic beads (Miltenyi Biotech, Auburn, CA). Briefly, the bone marrow was flushed out of the femurs with RPMI-1640 medium (without serum) under aseptic conditions. A single cell suspension was made by pipetting up and down and filtering through a 70-μm nylon strainer, followed by incubation with erythrocyte lysis buffer and washing. The cell suspension was subjected to positive selection of the $Gr1^+$ cells by incubating with biotin-conjugated mouse anti-Gr1 antibody (Clone RB6-8C5; eBioscience, San Diego, CA) for 15 min at 4ºC. Cells were then incubated with anti-biotin magnetic beads for 20 min at 4ºC and subsequently passed over a MS column. The cell population was more than 90% $Gr1^+CD11b^+$ as determined by flow cytometry. $Gr1^+CD11b^+$ 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 37ºC and 5% CO_2.

2.4. Cell transfection

For gene expression knockdown, pools of siRNAs specific to Ago1 (Cat #sc-44647), Ago2 (Cat #44659), HuR (Cat #sc-35620), CUGBP1 (Cat #38252), or scrambled (control) siRNAs (Cat #37007) (Santa Cruz Biotechnology) were suspended in HiPerFect reagent (Qiagen, Valencia, CA) at a 0.5 μg/ml final concentration. For miR-21 and miR-181b knockdown, mirVana miRNA inhibitors against miR-21 and miR-181b (Cat #4464084), or negative control inhibitor (Cat #4464077) (Thermo Fisher Scientific, Waltham, MA) were suspended in a HiPerFect reagent at 50 nM final concentration. The cells were incubated for 36 hr with RPMI-1640 medium.

2.5. RNA immunoprecipitation

$Gr1^+CD11b^+$ cells were subjected to formaldehyde cross-linking, to preserve native RNA-protein complexes. Briefly, the cells (~$10x10^6$) were washed with warm PBS and incubated with 0.2% formaldehyde (in PBS) for 10 min at room temperature. The cells were washed in cold PBS, and whole cell lysate was prepared according to published methods (Pillai *et al.*, 2005) with minor modifications, which replaced digitonin with 0.5% NP-40 and 0.5% deoxycholate, and increased incubation time to 30 min. Briefly, cross-linked lysates were incubated for 30 min on ice in lysis buffer containing: 250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5% NP-40, 0.5% deoxycholate, 30 U/ml RNase inhibitor, and 1x protease inhibitor cocktail (Cat #87786; Thermo Fisher Scientific, Waltham, MA). After DNase I treatment for 10 min at 37C, lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4˚C.

Immunoprecipitation was performed as described previously (Pillai *et al.*, 2005), except that the incubation with antibody was performed overnight . Briefly, cell lysates were pre-cleared by incubation with pre-blocked protein A/G-agarose beads for 1 h at 4˚C. The beads were pre-blocked by incubation for 1 h with 100 μg/ml of BSA, and then washed with buffer C (250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 30 U/ml RNase inhibitor, and 1x protease inhibitor cocktail). Cell lysate (900 μl) was added to 100 μl of pre-blocked beads that were coated with 10 μl antibody against Ago2 (clone #4G8; Wako, Richmod, VA), Ago1 (Cat #sc-376696), HuR (Cat #sc-5261), AUF1 (Cat #sc-166577), TTP (Cat #sc-374305), CUGBP1 (Cat #sc-56649), or IgG control antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were rotated overnight at 4˚C, and the beads were centrifuged and washed three times with buffer C. Aliquots of beads-bound protein complexes were saved for protein analysis by western blot, and the remainder was subjected to RNA isolation using TRIzol reagent

for mRNA immunoprecipitation or miRNeasy Mini Kit for miRNA immunoprecipitation. This RNA was used for mRNA and miRNA analysis by PCR.

2.6. Western blots

Whole cell extracts or immunoprecipitated protein complexes were resolved by electrophoresis using SDS-10% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Thermo Fisher Scientific, Waltham, MA). Membranes were blocked with 5% milk in Tris-buffered saline/Tween-20 for 1 hr at room temperature, and then probed overnight at 4ºC with pan-specific anti-NFI (Cat #sc-74444), anti-Ago1 (Cat #sc-376696), anti-HuR (Cat #sc-5261), anti-CUGBP1 (Cat #sc-56649) (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Ago2 (clone #4G8; Wako, Richmod, VA) antibody. After washing, blots were incubated with the appropriate HRP-conjugated secondary antibody for 2 hr at room temperature. Proteins were detected with the enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA), the bands were visualized using the ChemiDoc XRS System (Bio-Rad), and the images were captured with the Image Lab Software V3.0. Membranes were stripped and reprobed with β-actin antibody (Santa Cruz) as a loading control.

2.7. Real-time PCR

Real-time PCR (RT-qPCR) was performed to determine total mRNA levels of NFI-A, as well as its levels in the immunoprecipitated RNA-protein complexes. The RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), reverse transcribed and amplified using SYBR Green RT-PCR kit and QuantiTect Primer Assays specific to NFI-A (Cat #QT00121023; Qiagen, Germantown, MD) or primers that amplify the 3'UTR (Forward, 5'-

ACACTTTGCTCCTCACCTAAC-3'; Reverse, 5'- CACACAGGACACTCAAGACTAC-3') (Integrated DNA Technologies, Coralville, IA). The expression level was calculated using the $2^{-\Delta\Delta Ct}$ cycle threshold. Level of GAPDH (Primer Assay Cat #QT01658692; Qiagen) was used to normalize sample input.

For miR-21 and miR-181b measurements, RNA was isolated as described above. The miRNA levels were determined by RT-qPCR using miScript SYBR Green PCR kit and miScript Primer Assays specific to miR-21 and miR-181b (Qiagen; Cat # MS00011487 and MS00032368). Level of U6 RNA (Qiagen; Cat #MS00033740) was used to normalize sample input.

2.8. Luciferase reporter assay

Our earlier analysis of NFI-A 3'UTR (McClure *et al.*, 2016) for potential miRNA binding sites using limited databases at the time (miRanda; DIANA; TargetScan), did not show any complementary miRNA matching sequences with high binding affinity. However, our most recent bioinformatic screen, using a more refined and expanded miRNA search database (miRSystem, which combines 7 target prediction programs) revealed complementary binding sites for miR-21 and miR-181b, with near-perfect match in the NFI-A mRNA 3'UTR (see **Figure 2.7A**).

An NFI-A 3'UTR fragment (4.906 kb) that contains the miRNA binding sites (**Supplementary Figure 1- Appendix A**) and a GU-rich element (GRE) was cloned in the pEZX-MT06 dual firefly and *Renilla* reporter vector downstream of firefly luciferase (**Figure 2.7A**). Late sepsis $Gr1^{+}CD11b^{+}$ cells were isolated from the bone marrow by positive selection and transfected (~2 x 10^6 cells) with 0.5 μg of luciferase plasmid (GeneCopoeia, Rockville, MD)

using the HiPerFect transfection reagent per the manufacturer's instructions (Qiagen, Valencia, CA). After 48 hr, the cells were harvested, and firefly and *Renilla* luciferase activities were determined with the dual luciferase reporter assay system (Promega, Madison, WI). The pEZX-MT06 empty vector, in which the *Renilla* luciferase gene is controlled by the CMV promoter and an SV40 early enhancer promoter controls the firefly luciferase gene, served as a control for maximum firefly luciferase gene activity. Firefly luciferase values were normalized to *Renilla* luciferase activity.

2.9. Statistical analysis

Results from at least three experiments were analyzed by Microsoft Excel, V3.0 and values were expressed as mean ± s.d. Differences among groups were analyzed by a two-tailed student's *t*-test for two groups and by a one-way ANOVA for multiple groups. *p*-values < 0.05 are shown in the figures.

3. Results

3.1. MiR-21 and miR-181b post-transcriptionally upregulate NFI-A expression.

We first assessed temporal changes in NFI-A mRNA and protein levels in $Gr1^+CD11b^+$ MDSCs isolated from septic mice at times supported by our previous report of the post-acute sepsis model (Brudecki *et al.*, 2012a). **Figure 2.1** shows that parallel increases in NFI-A mRNA and proteins levels align with changes in miR-21 and miR-181b expression. We then showed that combined knockdown of miR-21 and miR-181b in MDSCs from late septic mice significantly reduced both NFI-A mRNA and protein levels (**Figures 2.2A and B**). NFI-A mRNA levels decreased in the presence of transcription inhibitor actinomycin D, resulting in a mRNA half-life

of approximately 2 hr (**Figure 2.2C**). Knockdown of miR-21 and miR-181b decreased NFI-A transcripts close to levels observed after the actinomycin D treatment.

Although NFI-A expression in $Gr1^+CD11b^+$ MDSCs is induced throughout sepsis, we focused our investigation on late sepsis because late sepsis $Gr1^+CD11b^+$ MDSCs promote immunosuppression and chronic sepsis (Brudecki *et al.*, 2012a), which are the focus of our studies.

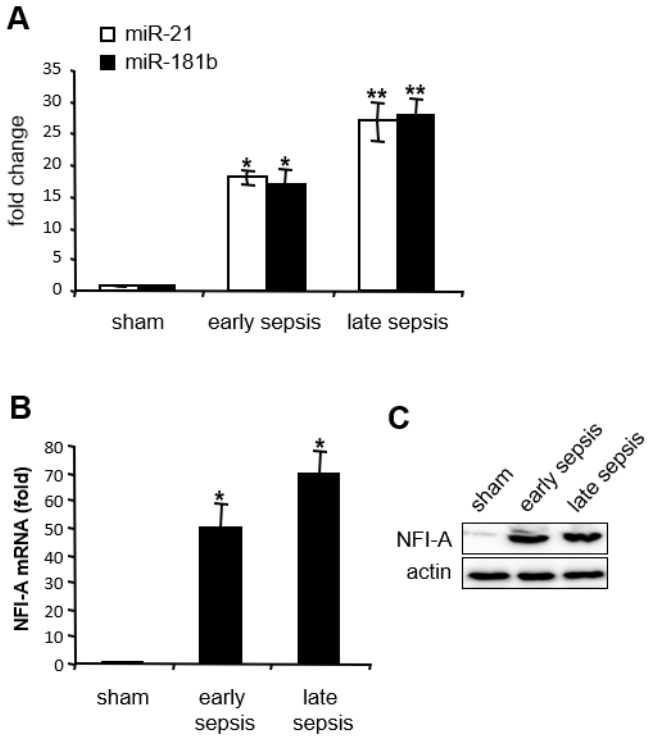

Figure 2.1: Correlation of NFI-A, miR-21 and miR-181b expressions in $Gr1^+CD11b^+$ myeloid cells during sepsis. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of sham and septic mice by positive selection. **(A)** Total RNA was isolated using RNeasy kit, and levels of miR-21 and miR-181b were determined by RT-qPCR using miScript SYBR Green PCR kit and assay primers specific to miR-21 and miR-181b. The miRNA expression was normalized to U6 RNA as an internal control. **(B)** NFI-A mRNA levels determined by RT-qPCR were normalized to GAPDH mRNA. Data in *A* and *B* are means ± SD of three experiments (n = 6-9 mice per group) and are presented relative to sham (1-fold). *$p < 0.05$ vs. sham; **$p < 0.05$ vs. early sepsis. (**C**) Levels of NFI-A proteins in whole cell lysates were determined by western blot using anti-NFI antibody. The results are representative of three experiments.

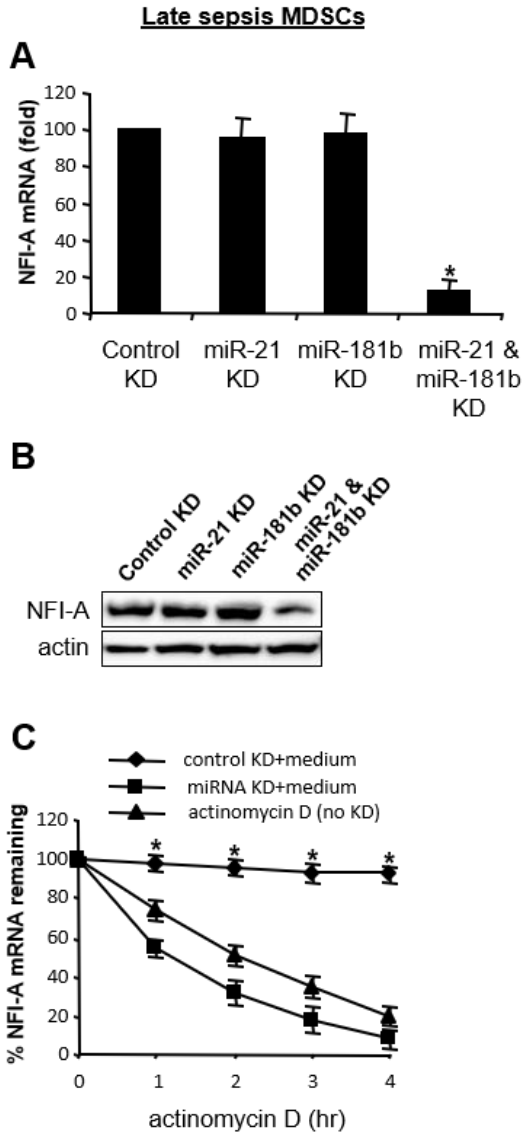

Figure 2.2: Effects of deleting miR-21 and miR-181b on NFI-A mRNA decay in sepsis Gr1$^+$CD11b$^+$ cells. (A) To assess NFI-A mRNA, Gr1+CD11b+ cells isolated from the bone marrow of late septic mice were transfected with miR-21 and/or miR-181b inhibitor, or negative controls, and cultured for 36 hr. RNA was quantified by RT-qPCR and normalized to GAPDH mRNA. Data are means ± SD of three experiments (n = 6-7 mice per group) and are presented relative to control KD. *$p < 0.05$. **(B)** To assess NFI-A protein levels, whole cell lysates were probed by western blot using anti-NFI antibody. The results are representative of two experiments. **(C)** To determine NFI-A mRNA half-life, Gr1+CD11b+ cells were incubated for the indicated times with or without 5 mcg/ml actinomycin D to stop mRNA transcription. NFI-A mRNA was assessed by RT-qPCR as in A. Data are means ± SD from 4 cultures and are presented relative to control KD. *$p < 0.05$ vs. miRNA KD + medium or actinomycin D. KD, knockdown.

3.2. RNA-binding proteins Ago1 and HuR accumulate at NFI-A 3'UTR.

MiRNAs control gene expression post-transcriptionally by binding to complementary sequences within the target mRNA 3' untranslated region (3'UTR) (Filipowicz *et al.*, 2008;Keene, 2007). Since miRNAs act in conjunction with RNA-binding proteins (RBPs) (Barreau *et al.*, 2005;Fabian *et al.*, 2010;Filipowicz *et al.*, 2008), we investigated how miR-21 and miR-181b might stabilize NFI-A mRNA in MDSCs during sepsis. We first performed RNA immunoprecipitation assays to identify RBPs that may target NFI-A 3'UTR. Several RBPs are commonly involved in post-transcriptional gene regulation, including Ago1, Ago2, AUF1, HuR, CUGBP1 and TTP (Barreau *et al.*, 2005;Fabian *et al.*, 2010;Keene, 2007).

Real-time PCR analysis of the immunoprecipitated RNA- using primers specific to the NFI-A 3'UTR- identified significantly higher levels of NFI-A mRNA only in Ago1- and HuR-immunoprecipitated protein complexes compared with the IgG-immunoprecipitated samples (**Figure 2.3A**). To test whether miR-21 and miR-181b recruit RNA binding proteins (RBP) to NFI-A 3'UTR, we performed immunoprecipitation after miR-21 and miR-181b knockdown. **Figure 2.3B** shows that Ago1 and HuR levels decreased concurrent with increased Ago2 and CUGBP1 bindings. Ago1 or HuR knockdown decreased total NFI-A mRNA and protein (**Figures 2.3C and D**), and Ago2 and CUGBP1 replaced Ago1 and HuR proteins when we depleted miR-21 and miR-181b. Changes in Ago1 and HuR did not affect each other's gene expression (**Figure 2.3E).** Ago1/HuR and CUGBP1/Ago2 exchange also occurred in MDSCs during early sepsis (**Supplementary Figure 2- Appendix A**).

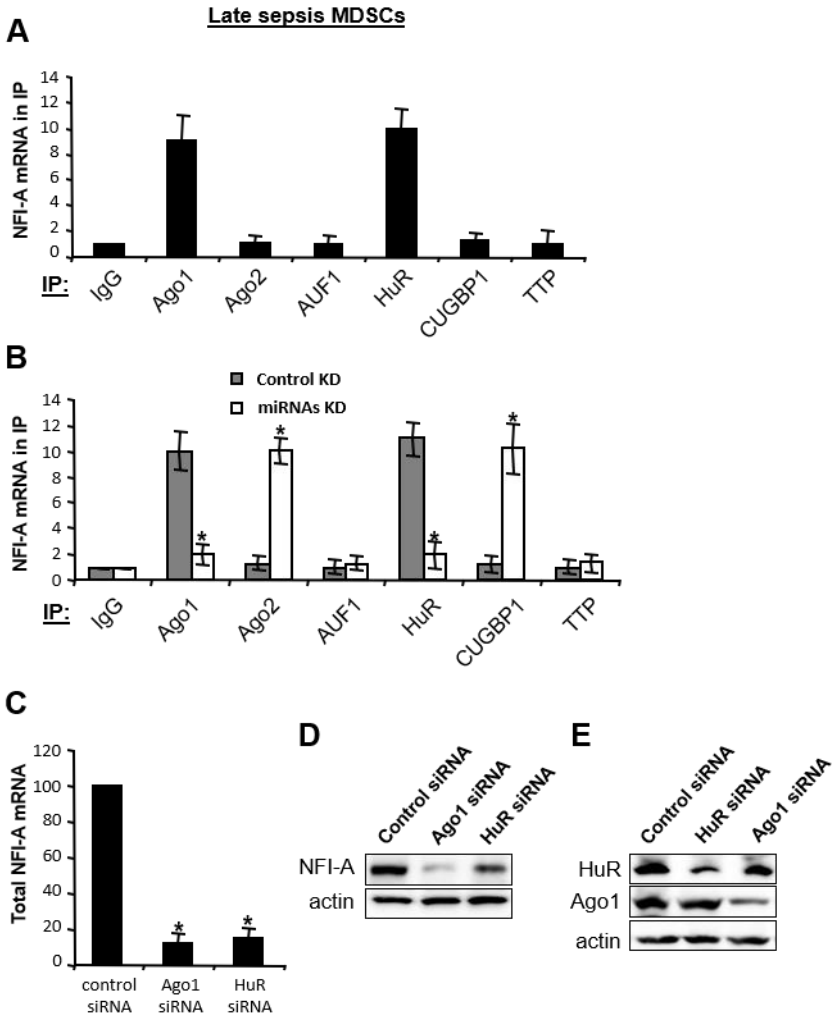

Figure 2.3: Binding of RNA regulatory proteins to NFI-A mRNA in sepsis $Gr1^+CD11b^+$ cells. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of late septic mice. **(A)** Ago1 and HuR bind to NFI-A mRNA. Whole cell lysates were immunoprecipitated using A/G-agarose beads coated with antibody against Ago1, Ago2, HuR, AUF1, TTP, CUGBP1 or IgG isotype control antibody. RNA was extracted from the immunoprecipitates (IP) and analyzed by RT-qPCR for the presence of NFI-A mRNA using primers that amplify the 3'UTR sequence. Values were normalized to GAPDH mRNA. **(B)** To assess Ago1 and HuR bindings to NFI-A mRNA in the absence of miR-21 and miR-181b, the $Gr1^+CD11b^+$ cells were transfected with miR-21 and miR-181b inhibitors or negative control inhibitors, and cultured for 36 hr. Cell lysates were prepared, immunoprecipitated, and NFI-A mRNA was measured as in *A*. Data are means ± SD of three experiments (n = 5-6 mice per group) and are presented relative to IgG IP (1-fold). *$p < 0.05$ vs. control KD. **(C)** NFI-A mRNA and protein levels after Ago1 or HuR knockdown. The $Gr1^+CD11b^+$ cells were transfected with Ago1-, HuR-specific or control siRNAs and cultured for 36 hr. Total RNA was isolated using TRIzol reagent, and levels of cellular NFI-A mRNA were measured by RT-qPCR and normalized to GAPDH mRNA. Data are means ± SD of three experiments (n = 6-8 mice per group) and are presented relative to control siRNA (100%). *$p < 0.05$ vs. control siRNA. **(D)** Levels of NFI-A protein were determined by western blot using anti-NFI antibody. **(E)** Ago1 and HuR do not affect each other expression. Levels of Ago1 and HuR proteins were determined by western blot. The results are representative of two experiments. KD, knockdown.

3.3. HuR loads miR-21 and miR-181b on the NFI-A 3'UTR.

Because knockdown of miR-21 and miR-181b decreased HuR and Ago1 protein bindings at the NFI-A 3'UTR (**Figure 2.3B**), we examined the kinetics of the miRNA interactions with HuR and Ago1 by measuring their association with Ago1 and HuR, using RNA immunoprecipitation assay. **Figures 2.4A and B** show that miR-21 and miR-181b enrich in HuR or in Ago1 immunoprecipitates. Then, we tested whether HuR and/or Ago1 facilitates miR-21 and miR-181b loading on NFI-A 3'UTR using HuR or Ago1 knockdown. MiR-21 and miR-181b associated with HuR before and after Ago1 knockdown, but their levels decreased significantly in Ago1 immunoprecipitated protein complexes after HuR knockdown (**Figures 2.4C and D**). Total cellular miR-21 and miR-181b levels did not change (**Figure 2.4E**), and HuR co-immunoprecipitated with Ago1 (**Figure 2.4F**).

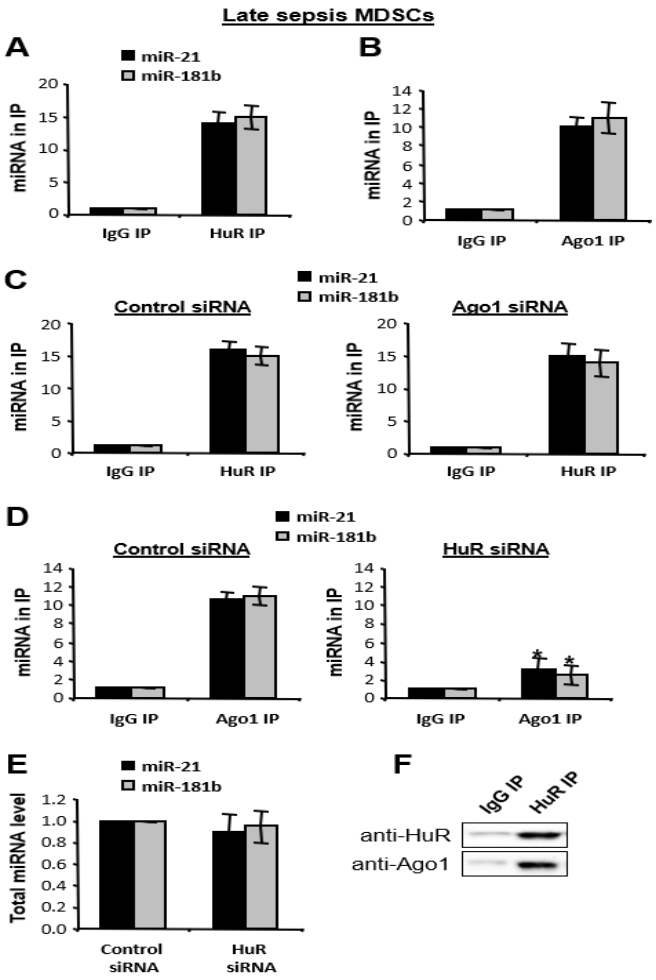

Figure 2.4: HuR effect on the miR-21 and miR-181b bindings with Ago1 protein complex. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of late septic mice. **(A-B)** HuR and Ago1 proteins bind miR-21 and miR-181b. Cell lysates were immunoprecipitated using A/G-agarose beads coated with antibody against HuR, Ago1 or IgG isotype control antibody. RNA was extracted from the immunoprecipitates (IP) using miRNeasy kit, and levels of miR-21 and miR-181b were determined by RT-qPCR using miScript SYBR Green PCR kit assay primers specific to miR-21 and miR-181b. Values were normalized to U6 RNA. **(C)** Ago1 is not involved in HuR binding to miR-21 and miR-181b. The $Gr1^+CD11b^+$ cells were transfected with Ago1-specific or control siRNAs and cultured for 36 hr. Cell lysates were immunoprecipitated with HuR antibody, and levels of miR-21 and miR-181b were determined as in *A*. **(D)** HuR promotes Ago1 binding of miR-21 and miR-181b. The $Gr1^+CD11b^+$ cells were transfected with HuR-specific or control siRNAs and cultured for 36 hr. Cell lysates were immunoprecipitated with Ago1-specific or IgG control antibody. Levels of miR-21 and miR-181b in the immunoprecipitated protein complexes were determined by RT-qPCR as in *A*. Data in *A*, *B*, *C* and *D* are means ± SD of three experiments (n = 5-7 mice per group) and presented relative to the IgG IP sample (set at 1-fold). IP, immunoprecipitation. **(E)** HuR does not affect total cellular miR-21 and miR-181b levels. The $Gr1^+CD11b^+$ cells were transfected with HuR-specific or control siRNAs and cultured for 36 hr. Total RNA was isolated RNeasy kit, and levels of miR-21 and miR-181b were determined by RT-qPCR as in *A*. Data are means ± SD of two experiments (n = 5 mice per group) and are presented relative to control siRNA (1-fold). **(F)** HuR forms a protein complex with Ago1. $Gr1^+CD11b^+$ cell lysates were immunoprecipitated with HuR or IgG control antibody and immunoblotted with HuR or Ago1 antibody. The results are representative of two experiments.

3.4. CUGBP1 destabilizes NFI-A mRNA.

We then assessed whether CUGBP1 associates with NFI-A mRNA 3'UTR and miR-21 and miR-181b after HuR knockdown in MDSCs. **Figure 2.5A** shows that HuR knockdown facilitated CUGBP1 binding to NFI-A 3'UTR and decreased NFI-A protein levels. Meantime, miR-21 and miR-181b were not detected in the CUGBP1 immunoprecipitated protein complexes, either before or after HuR knockdown (**Figure 2.5B**), indicating that miR-21 and miR-181b do not bind to CUGBP1 protein. In addition, co-immunoprecipitation revealed that CUGBP1 and Ago2 form a protein complex only in the absence of HuR (**Figure 2.5C**). We next examined whether miR-21 and miR-181b can bind to Ago2 protein directly. **Figure 2.5D** shows no miRNAs in Ago2 immunoprecipitates, either in the presence or absence of CUGBP1. In addition, levels of cellular NFI-A mRNA increased significantly after CUGBP1 knockdown (**Figure 2.5E**).

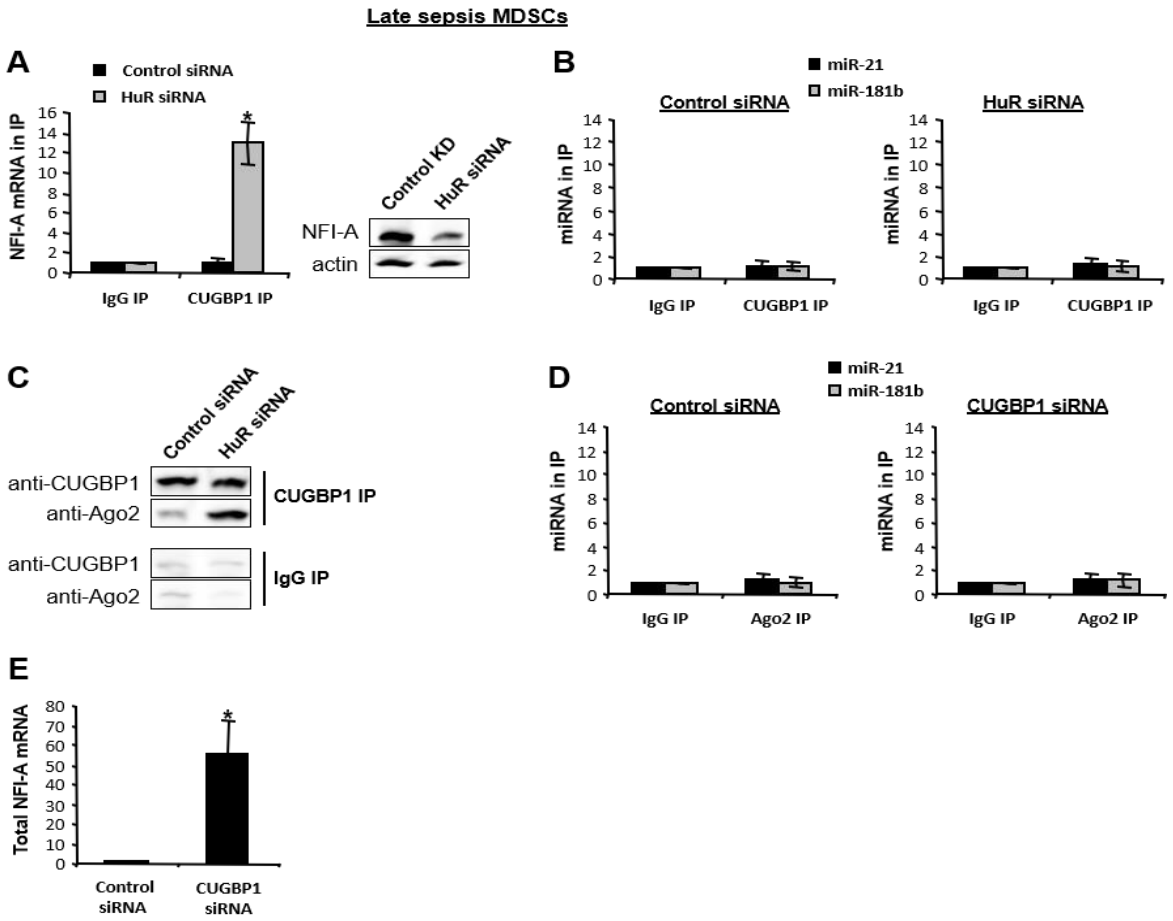

Figure 2.5: CUGBP1 protein binding to NFI-A mRNA in the absence of HuR. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of late septic mice. **(A)** CUGBP1 binds NFI-A mRNA after HuR knockdown. The cells were transfected with HuR-specific or control siRNAs and cultured for 36 hr. Cell lysates were immunoprecipitated with antibody against CUGBP1 or IgG control. RNA was extracted from the immunoprecipitates (IP) using TRIzol reagent and levels of NFI-A mRNA in the IP were measured by RT-qPCR using primers that amplify the 3`UTR sequence. Values were normalized to GAPDH mRNA. NFI-A protein levels in whole cell lysate were determined by western blot using anti-NFI antibody. **(B)** CUGBP1 does not bind to miR-21 and miR-181b. RNA was extracted from CUGBP1 immunoprecipitates using miRNeasy kit, and levels of miR-21 and miR-181b were measured by RT-qPCR using miScript assay primers. Values were normalized to U6 RNA. **(C)** CUGBP1 forms a protein complex with Ago2 in the absence of HuR. The $Gr1^+CD11b^+$ cells were transfected with HuR siRNAs as in *A*. Cell lysates were immunoprecipitated with CUGBP1 or IgG control antibody and immunoblotted with CUGBP1 or Ago2 antibody. The results are representative of three experiments. **(D)** MiR-21 and miR-181b do not bind Ago2. The $Gr1^+CD11b^+$ cells were transfected with CUGBP1-specific, or control siRNAs and cultured for 36 hr. Cell lysates were immunoprecipitated with Ago2 or IgG control antibody. Levels of miR-21 and miR-181b in the IP was determined as in *B*. (**E**) Knockdown of CUGBP1 increases NFI-A mRNA stability in the absence of miR-21 and miR-181b. The $Gr1^+CD11b^+$ cells were simultaneously transfected with miR-21 and miR-181b inhibitors as well as CUGBP1-specific or control siRNAs, and cultured for 36 hr. Total RNA was isolated, and levels of cellular NFI-A mRNA were measured by RT-qPCR using primers that amplify the 3`UTR sequence and normalized to GAPDH mRNA. Data are means ± SD of 6 mice per group and are presented relative to control siRNA (1-fold). Data in *A*, *B* and *D* are means ± SD of three independent experiments (n = 6-7 mice per group) and are presented relative to the IgG IP sample (1-fold). *$p < 0.05$ vs. control siRNA. IP, immunoprecipitation.

3.5. CUGBP1 binds NFI-A 3'UTR directly.

We determined whether CUGBP1 and Ago2 formed a protein complex. We found CUGBP1 and Ago2 formed a protein complex only after the miRNA knockdown (**Figure 2.6A**), which also bound to the NFI-A 3'UTR (**Figure 2.6B**) and paralleled a decrease in NFI-A protein levels (right panel). We then examined whether CUGBP1 and Ago2 bind to NFI-A 3'UTR after combined knockdown of the miRNAs, CUGBP1 and Ago2. The results showed unbound Ago2 in the absence of CUGBP1 (**Figure 2.6C**). In contrast, CUGBP1 binding was not affected by Ago2 knockdown (**Figure 2.6D**) and NFI-A protein levels remained the same with either knockdown.

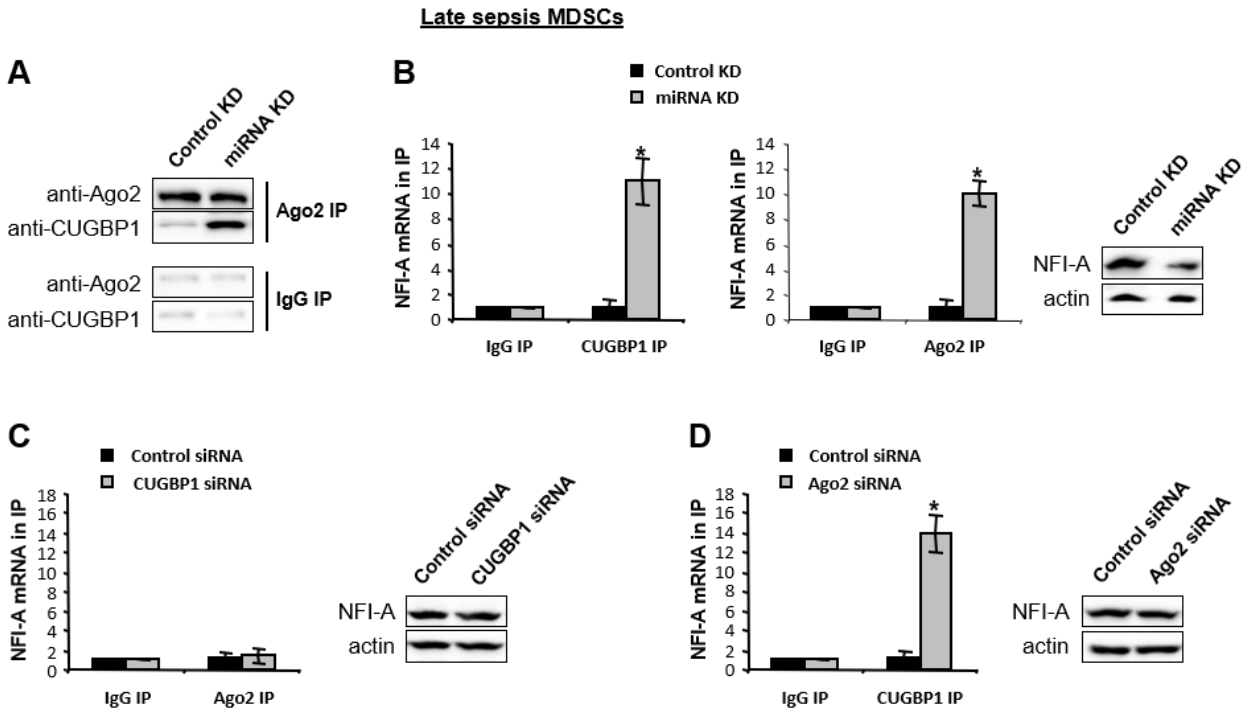

Figure 2.6: CUGBP1 interactions with and recruitment of Ago2 to NFI-A mRNA. $Gr1^{+}CD11b^{+}$ cells were isolated from the bone marrow of late septic mice, transfected with miR-21 and miR-181b inhibitors or negative control inhibitors, and cultured for 36 hr. (**A**) CUGBP1 associates with Ago2 in the absence of the miRNAs. Cell lysates were prepared and immunoprecipitated with CUGBP1 or IgG control antibody and immunoblotted with CUGBP1 or Ago2 antibody. The results are representative of two experiments. (**B**) To assess CUGBP1 or Ago2 binding to NFI-A mRNA, the cell lysates were immunoprecipitated with CUGBP1, Ago2 or IgG isotype control antibody. RNA was extracted from the immunoprecipitates (IP) using TRIzol reagent, and levels of NFI-A mRNA were measured by RT-qPCR using primers that amplify the 3`UTR sequence. Values were normalized to GAPDH mRNA. Data are means ± SD of three experiments (n = 4-7 mice per group). *$p < 0.05$ vs. control KD. **(C-D)** CUGBP1 recruits Ago2 to NFI-A 3'UTR. The $Gr1^{+}CD11b^{+}$ cells were simultaneously transfected with miR-21 and miR-181b inhibitors as well as CUGBP1-, Ago2-specific or control siRNAs, and cultured for 36 hr. Cell lysates were immunoprecipitated, and levels of NFI-A mRNA in the IPs were measured as in *B.* Data are means ± SD of three independent experiments (n = 6-8 mice per group) and are presented relative to the IgG IP sample (1-fold). *$p < 0.05$ vs. control siRNA. Right panels in *B*, *C* and *D* show NFI-A protein levels in whole cell lysates as determined by western blot using anti-NFI antibody. The results are representative of three experiments. IP, immunoprecipitation. KD, knockdown.

3.6. MiR-21 and miR-181b upregulate reporter mRNA expression.

We used a NFI-A 3'UTR reporter construct to assess the effect of miR-21 and miR-181b binding sites. A 3'UTR fragment (4.906 kb) that contains the miRNA binding sites and a GU-rich element (GRE) was cloned in a dual firefly and *Renilla* reporter vector downstream of firefly luciferase (**Figures 2.7 A and B**). **Figure 2.7C** shows that the 3'UTR fragment containing the native miR-21 and miR-181b binding sites along with the GRE element only slightly increased the luciferase reporter expression compared with a control reporter without the NFI-A 3'UTR. In contrast, point mutations in the miR-21 and miR-181b sites decreased luciferase expression and deleting the GRE sequence while retaining native miRNA binding sites restored the luciferase gene expression.

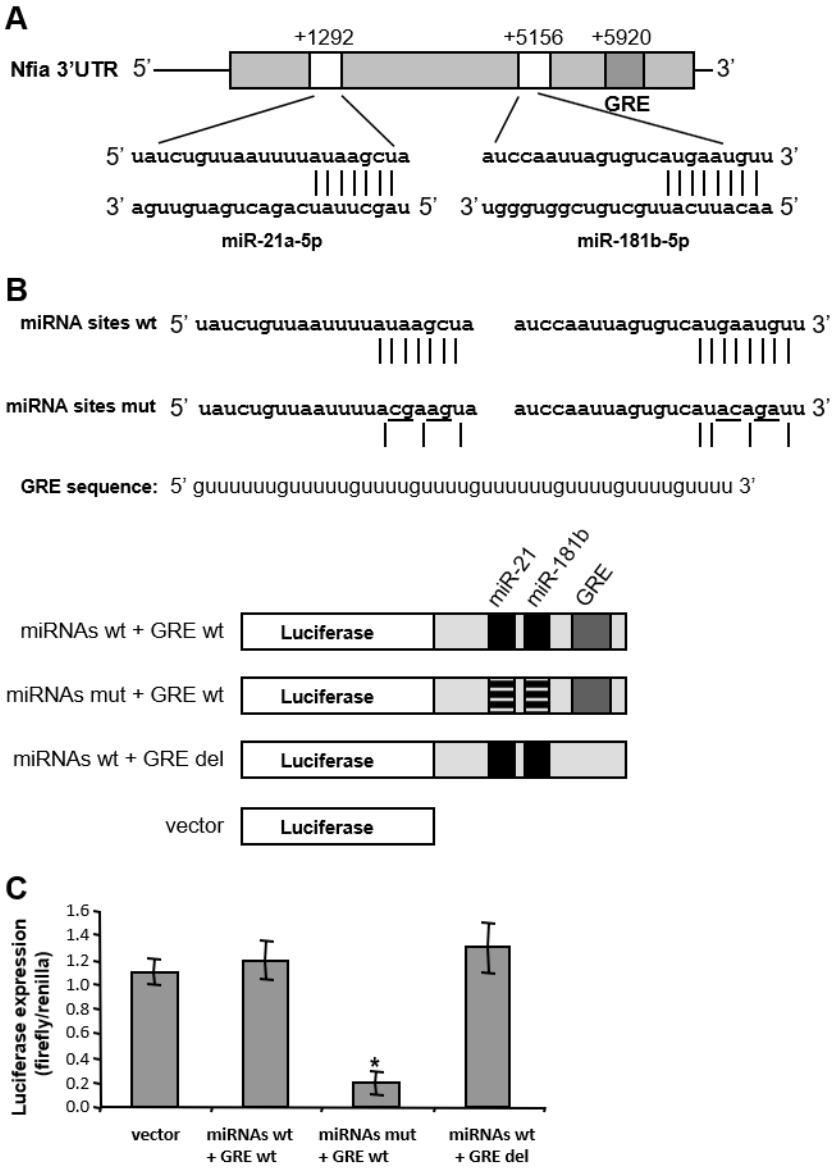

Figure 2.7: MiR-21 and miR-181b effect on reporter mRNA in sepsis Gr1$^+$CD11b$^+$ cells. (**A**) A schematic representation of the NFI-A 3'UTR (7.641 kb), which spans the region from +1634 to +9274 of the mRNA sequence with locations of miR-21 and miR-181b binding sites and GRE element. (**B**) Sequences of the miR-21 and miR-181b as well as the GRE are shown. Mutations introduced in the miRNA binding sites are underlined. A 4.906 kb fragment (from +1168 to +6074) of NFI-A 3'UTR containing the miRNA binding sites and the GRE element was cloned in the pEZX-MT06 dual firefly and *Renilla* reporter vector downstream of firefly luciferase. Diagrams of the luciferase constructs with the miRNAs and GRE locations are shown. GRE, GU-rich element; wt, wild-type; mut, mutant; del, deleted. (**C**) Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of late septic mice. The cells were transfected with indicated luciferase constructs. After 36 hr, cells were harvested for the measurement of firefly and *Renilla* luciferase activities. A luciferase vector without the NFI-A 3'UTR insertion served as a positive control. Firefly luciferase values were normalized to *Renilla* luciferase activity and are presented as a firefly/*Renilla* ratio. Data are expressed as means ± SD of three experiments (n = 4-6 mice per group). *$p < 0.05$. Wt, wild-type; mut, mutant.

4. Discussion

The major finding of this study is that miR-21 and miR-181b promote MDSC development in murine sepsis in part by a post-transcriptional regulation of NFI-A. The post-transcriptional process involves HuR and Ago1 binding to NFI-A 3'UTR. RNA-binding protein CUGBP1 binding to NFI-A 3'UTR in the absence of HuR destabilizes NFI-A mRNA concomitant with exchanging Ago2 and Ago1. The data add a pivotal 3'UTR post-transcriptional mechanism previously unreported to transcriptional and epigenetic control over MDSC development during sepsis (Hollen *et al.*, 2019). The emerging mechanisms that control MDSC development and circulation during murine sepsis, if confirmed in human studies, may inform molecular targeting of human MDSCs.

The inflammatory environment of sepsis caused by accumulation of $Gr1^{+}CD11b^{+}$ MDSCs mutes inflammatory autotoxicity, but concomitantly limits the host clearance of the unresolved original or new infection (Brudecki *et al.*, 2012a;Cuenca *et al.*, 2011;Mathias *et al.*, 2017). Our previous data indicated that NFI-A transcription factor maintains an undifferentiated state (McClure *et al.*, 2016) and promotes MDSCs during sepsis (McPeak *et al.*, 2017) by mechanisms dependent on increased miR-21 and miR-181b expression, but more precise molecular mapping remained unclear. One possibility is control over mRNA coupling at 3'UTR to protein expression. The NFI-A 3'UTR contains consensus sequences complementary to miR-21 and miR-181b "seed" regions. In this study we showed that miR-21 and miR-181b assemble HuR and Ago1 RBPs at the NFI-A 3'UTR. While some studies support that miRNAs downregulate target mRNA expression by inducing mRNA decay or translation repression (Chekulaeva and Filipowicz, 2009;Filipowicz, 2005), other stress that miRNA can upregulate target mRNA expression by increasing mRNA stability (Del *et al.*, 2016;Mortensen *et al.*,

2011;Prislei *et al.*, 2013;Vasudevan *et al.*, 2007). In this study, HuR stabilized NFI-A mRNA in sepsis MDSCs and complexed with Ago1 at NFI-A 3'UTR. Ago1 knockdown, a known mRNA regulatory protein in mammalian cells (Ambros, 2004;Filipowicz, 2005), did not alter miRNAs association with HuR, which post-transcriptionally stabilizes mRNAs of many genes (Abdelmohsen and Gorospe, 2010;Lebedeva *et al.*, 2011;Simone and Keene, 2013). Although the exact mechanism by which HuR promotes target mRNA stability is not completely understood, some studies suggest that HuR competes with other RBPs that promote mRNA degradation (Abdelmohsen and Gorospe, 2010). If so, HuR might be part of a post-transcriptional axis that contributes to sepsis homeostasis dysregulation of inflammatory mediators (El Gazzar, 2014;McCall *et al.*, 2011).

In support of the axis concept, this study showed that the CUG-binding protein CUGBP1, unlike HuR, destabilizes NFI-A mRNA whereas HuR stabilizes NFI-A mRNA. CUGBP1 binds to GU-rich sequences in target mRNAs to promote rapid decay (Rattenbacher *et al.*, 2010;Vlasova and Bohjanen, 2008). The NFI-A 3'UTR contains a 45-nt GU-rich sequence (with 8 GU-rich elements) downstream of miR-181b binding site. In this study, CUGBP1 bound to Ago2 at NFI-A 3'UTR. Ago2 catalyzes endoribonucleolytic cleavage of target mRNA, leading to its degradation (Chen *et al.*, 2009;Meister *et al.*, 2004). We suggest that miR-21 and miR-181b may promote NFI-A expression post-transcriptionally by decreasing mRNA destabilization by CUGBP1. The implication of this study is whether similar switch contributes to the immune resistance to immune tolerance phenotypes of human sepsis.

In summary, this study reveals a newly described post-transcriptional concept for myeloid cell differentiation to the MDSC phenotype, in which the HuR and CUGBP1 axis informs miR-21 and miR-181b control over NFI-A protein support of MDSC development.

Mechanistically, HuR stabilizes NFI-A mRNA to increase protein, and CUGBP1 destabilizes NFI-A mRNA to decrease protein. This unrecognized pivot may inform druggable targets and improve understanding of sustained MDSC expansion during the post-sepsis syndrome of persistent inflammation and catabolism (Gentile *et al.*, 2012;Mathias *et al.*, 2017).

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Conflicts of interest

None.

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CHAPTER 3

S100A9 MAINTAINS MYELOID-DERIVED SUPPRESSOR CELLS IN CHRONIC SEPSIS BY INDUCING MIR-21 AND MIR-181B

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Keywords: Sepsis, Immune suppression, MDSC, S100A9

Abstract

Myeloid-derived suppressor cells (MDSC) expand during sepsis, suppress both innate and adaptive immunity, and promote chronic immunosuppression, which characterizes the late/chronic phase of sepsis. We previously reported that the transcription factors Stat3 and C/EBPβ synergize to induce the expression of microRNA (miR)-21 and miR-181b to promote MDSC expansion in a mouse model of polymicrobial sepsis that progresses from an early/acute proinflammatory phase to a late/chronic immunosuppressive stage. We also showed that $Gr1^+CD11b^+$ cells, the precursors of MDSCs, from mice genetically deficient in the inflammatory protein S100A9 lack miR-21 or miR-181b in late sepsis, and are not immunosuppressive. In the present study, we show that S100A9 induces miR-21 and miR-181b during the late sepsis phase. We find that S100A9 associates with and stabilizes the Stat3-C/EBPβ protein complex that activates the miRNA promoters. Reconstituting $Gr1^+CD11b^+$ cells from S100A9 knockout mice with late sepsis with S100A9 protein restores the Stat3-C/EBPβ protein complex and miRNA expressions, and switches the $Gr1^+CD11b^+$ cells into the immunosuppressive, MDSC phenotype. Importantly, we find that this process requires IL-10 mediated signaling, which induces S100A9 translocation from the cytosol to the nucleus. These results demonstrate that S100A9 promotes MDSC expansion and immunosuppression in late/chronic sepsis by inducing the expression of miR-21 and miR-181b.

Introduction

Myeloid-derived suppressor cells (MDSC) are characterized by the immature myeloid phenotype $Gr1^+CD11b^+$ in mouse and by their immunosuppressive functions, as they suppress both innate and adaptive immunity (Cuenca *et al.*, 2011; Gabrilovich and Nagaraj, 2009; Kong *et al.*, 2013). Generation and accumulation of MDSCs are observed under a variety of pathological conditions where inflammation or infection is a common theme (Dai *et al.*, 2015; Ostrand-Rosenberg and Fenselau, 2018; Veglia *et al.*, 2018). We previously reported expansion and accumulation of MDSCs in the bone marrow and spleens in mouse with polymicrobial sepsis, which progresses from an early/acute proinflammatory phase to a late/chronic stage characterized by protracted immunosuppression (Brudecki *et al.*, 2012), and is associated with elevated mortality and morbidity in animal and humans with sepsis (Delano et al., 2007; Efron et al., 2018; Hotchkiss *et al.*, 2013a; Patil *et al.*, 2016). While $Gr1^+CD11b^+$ cells (precursors of MDSCs) expand and accumulate throughout sepsis, early sepsis $Gr1^+CD11b^+$ cells produce proinflammatory mediators and promote inflammation, whereas their counterparts in late sepsis are immunosuppressive (i.e., functional MDSCs) as they produce immunosuppressive mediators such as IL-10 and arginase 1 (Brudecki *et al.*, 2012; McPeak *et al.*, 2017a).

The mechanisms underlying MDSC expansion remain unclear, but different mediators, including inflammatory cytokines and growth factors, have been thought to drive MDSC expansion under various inflammatory conditions (Condamine and Gabrilovich, 2011; Kong *et al.*, 2013; Ostrand-Rosenberg *et al.*, 2018). In the context of sepsis, we showed that expression of microRNA (miR)-21 and miR-181b is induced after sepsis initiation, where they couple with the myeloid differentiation-related transcription factor NFI-A to drive $Gr1^+CD11b^+$ cell expansion in early and late sepsis (McClure *et al.*, 2016; McClure *et al.*, 2014). The expression of these

miRNAs is induced by the synergistic effect of Stat3 and C/EBPβ, which bind to and activate the miR-21 and miR-181b promoters after sepsis initiation (McClure *et al.*, 2017).

Recently, we reported that mice genetically deficient in the calcium-binding S100A9 protein do not generate MDSCs during the late sepsis phase; however, they still make proinflammatory $Gr1^+CD11b^+$ cells during early sepsis (Dai *et al.*, 2017). Interestingly, expression of miR-21 and miR-181b is lost only in late sepsis $Gr1^+CD11b^+$ cells from the S100A9 deficient mice, and these cells are generated at normal/baseline levels and are not immunosuppressive (Dai *et al.*, 2017). These studies suggested that S100A9 may play a role in the induction of miR-21 and miR-181b expressions and MDSC generation during the late, immunosuppressive phase of sepsis. How S100A9 differentially regulates miR-21 and miR-181b expressions in late/chronic sepsis is unknown.

S100A9 protein is expressed and secreted by various cell types, including phagocytes (Ehrchen *et al.*, 2009; Foell and Roth, 2004a; Vogl *et al.*, 2012), and is induced by many stimuli, including inflammatory mediators and bacterial products (Foell *et al.*, 2004b; Goyette and Geczy, 2011; Vogl *et al.*, 2007). S100A9 is constitutively expressed in immature myeloid cells, including $Gr1^+CD11b^+$ cells, but its expression decreases with differentiation and maturation (Roth *et al.*, 1993; Vogl *et al.*, 2012). Most studies on S100A9 have focused on its role as a proinflammatory, soluble mediator that amplifies inflammation and infection via immune cell activation and recruitment (Ehrchen *et al.*, 2009; Foell *et al.*, 2004; Goyette *et al.*, 2011). Importantly, our studies indicated that S100A9 might play a role in sepsis pathogenesis at the molecular level, by promoting MDSC generation in late sepsis. S100A9 protein translocates from the cytosol to the nucleus in MDSCs during late/chronic sepsis only (Dai *et al.*, 2017). In the present study, we investigated the molecular mechanism by which S100A9 induces miR-21

and miR-181 expressions, and MDSC generation during murine chronic sepsis. We find that nuclear S100A9 acts as a transcription cofactor to facilitate miR-21 and miR-181b induction by Stat3 and C/EBPβ during the late phase of sepsis. The results support that molecular targeting of S100A9 to inhibit MDSC expansion and sepsis-associated immunosuppression might improve sepsis survival.

Materials and Methods

Mice

The C57BL/6N S100a9 knockout mice used for this study have been described previously (Dai *et al.*, 2017). Homozygous(-/-) mice were bred and housed in a pathogen-free facility in the Division of Laboratory Animal Resources. Wild-type C57BL/6N mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used as controls. Male mice, 8-10 weeks old were used in this study and were acclimated to the new environment for a week before surgery. All experiments followed the National Institutes of Health guidelines the East Tennessee State University Animal Care and Use Committee approved the study.

Sepsis

Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) as described (Brudecki *et al.*, 2012). A midline abdominal incision was made and the cecum was ligated distal to the ileocecal valve, and punctured twice with a 23-gauge needle. A small amount of feces into the abdominal cavity. The abdominal wall and skin were sutured in layers with 3-0 silk. Sham-operated mice were treated identically except that the cecum was neither ligated nor punctured. Mice received (i.p.) 1 ml lactated Ringers plus 5% dextrose for fluid resuscitation. To establish

intra-abdominal infection and approximate the clinical condition of early human sepsis where there is a delay between the onset of sepsis and the delivery of therapy (Mazuski *et al.*, 2002), mice were subcutaneously administered antibiotic (Imipenem; 25 mg/kg body weight) or an equivalent volume of 0.9% saline at 8 and 16 hr after CLP. These levels of injury and manipulation create prolonged infections with high mortality (~60-70%) during the late/chronic phase (Brudecki *et al.*, 2012). We followed survival 28 days. We euthanized mice moribund within 5 days after CLP(early sepsis) and after that (Brudecki *et al.*, 2012). We euthanized a corresponding number of healthy appearing mice from the control group to provide control samples.

$Gr1^+CD11b^+$ cell isolation

Bone marrow $Gr1^+CD11b^+$ cells were isolated using magnetic beads according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). Briefly, the bone marrow was flushed out of the femurs with RPMI-1640 medium (without serum) under aseptic conditions. A single cell suspension was made by pipetting up and down and filtering through a 70-μm nylon strainer, followed by incubation with erythrocyte lysis buffer and washing. The cell suspension was subjected to a positive selection of the $Gr1^+$ cells by incubating with biotin-coupled mouse anti-Gr1 antibody (Clone RB6-8C5; eBioscience, San Diego, CA) for 15 min at 4ºC. Cells were then incubated with anti-biotin magnetic beads for 20 min at 4ºC and subsequently passed over an MS column. The cell purity was more than 90% as determined by flow cytometry.

$Gr1^+CD11b^+$ 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% CO_2. In some experiments, cells were stimulated for 6 hr with recombinant murine IL-10.

S100A9 expression construct and transfection

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.

For $Gr1^+CD11b^+$ cell, S100A9 plasmid DNA or empty vector was suspended in HiPerFect reagent at a 0.5 μg/ml final concentration (Qiagen, Valencia, CA). Cells were transfected using the Gene Pulser MXCell system (Bio-Rad, Hercules, CA) and then stimulated with or without recombinant murine IL-6 or IL-10 (PeproTech; Rocky Hill, NJ).

Preparation of protein extracts

Whole cell extract was 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. After 30 min on ice, cell lysate was cleared by centrifugation for 5 min at 4ºC and 14,000 rpm. Protein concentrations were determined by Bradford assay (Bio-Rad), and aliquots were kept at -20ºC.

Cytoplasmic and nuclear proteins were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) per the manufacturer's instructions. Immediately after harvesting, 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 removed by centrifugation for 5 min at 4ºC 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 4ºC and 14,000 rpm.

Co-immunoprecipitation

Immunoprecipitation (IP) of Stat3 or S100A9 proteins was performed to assess protein complex formations in $Gr1^{+}CD11b^{+}$ cells during sepsis. Briefly, whole-cell extracts were pre-cleared by incubation with pre-blocked protein G-agarose beads for one h at 4˚C. The beads were pre-blocked by incubation for one h with 100 μg/ml of BSA. Next, the beads were washed with buffer C (250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 30 U/ml RNase inhibitor, and 1x protease inhibitor cocktail). Cell extract was centifuged at 2,000 rpm for 5 min, and supernatant (900 μl) was added to 100 μl of the pre-blocked beads coated with 10 μl antibody against pStat3 (phospho tyrosine^{705}), S100A9, or IgG control antibody. After rotating overnight at 4˚C, the beads were centrifuged and washed three times with buffer C. Aliquots of bound protein complexes were analyzed by western blotting as described below.

Western blots

Equal amounts of protein extracts or co-immunoprecipitated protein complexes were mixed with 5x Laemmeli sample buffer, separated by SDS-10% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Thermo Fisher Scientific, Waltham, MA). Membranes were blocked with 5% milk in Tris-buffered saline/Tween-20 for 1 hr at room temperature, and then probed overnight at 4ºC with the following primary antibodies: anti-pStat3

tyrosine 705 (sc-8059), anti-C/EBPβ (sc-7962) (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Rabbit S100A9 (ab75478; Abcam, Cambridge, MA). After washing, blots were incubated with the appropriate HRP-conjugated secondary antibody for 2 hr at room temperature. Proteins were detected with the enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA). The protein bands were visualized using the ChemiDoc XRS System (Bio-Rad), and the images were captured with the Image Lab Software V3.0. Membranes were stripped and reprobed with β-actin or nucleoporin antibody (Santa Cruz Biotechnology) as a loading control.

Chromatin immunoprecipitation (ChIP)

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 and fixed in 1% formaldehyde in minimal culture medium for 10 min at room temperature, to cross-link protein-DNA complexes. After washing, cells were lysed in 1x lysis buffer containing protease inhibitor cocktail. The 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 15,000 rpm for 10 min at 4°C. Ten microliters of the chromatin solution were reserved as an "input" sample. The remaining chromatin solution was immunoprecipitated overnight at 4ºC with protein G magnetic beads and 3 μg of antibody specific to pStat3, C/EBPβ (Santa Cruz Biotechnology), S100A9 (Abcam, Cambridge, MA), or isotype control antibody (Santa Cruz Biotechnology). The chromatin/antibody complexes were washed three times in ChIP buffer and then eluted by incubation for 15 min in 50 μl elution buffer. Next, the DNA-

protein cross-links were reversed by incubation with 50 μl 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 37ºC, the reaction was stopped, and the resulting DNA was extracted and stored at -20°C.

Quantitative real-time PCR was used to measure the enrichment of miR-21 and miR-181b promoter sequences in the ChIPed DNA 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).

miRNA measurement

Quantitative real-time qPCR was used to determine levels of miR-21 and miR-181b in $Gr1^+CD11b^+$ cells. miRNA-enriched RNA was isolated and measured using miScript SYBR Green PCR kit with miScript Primer Assays specific to miR-21 and miR-181b according to the manufacturer's protocol (Qiagen). The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ cycle threshold method after normalization to the endogenous U6 RNA as an internal control.

$CD4^+$ T cells suppression assay

To determine the suppressive effects of $Gr1^+CD11b^+$ cells on T cell functions, we tested $Gr1^+CD11b^+$ $CD4^+$ T cells for proliferation and IFNγ production. Splenocytes from naive wild-type were used to purify $CD4^+$ T cells by positive selection using biotinylated anti-CD4 magnetic beads (Myltenyi). Purified cells were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) dye using the Vybrant CFDA SE Cell Tracer Kit (Invitrogen Molecular Probes,

Eugene, OR). Cells were incubated for 10 min at room temperature with 10 μM CFSE dye. Labeled cells were co-cultured (1:1 ratio) with $Gr1^+CD11b^+$ cells. We induced T cell proliferation by the stimulation with an anti-CD3 antibody plus an anti-CD28 antibody (1 μg/ml/each). After three days, cells $CD4^+$ T cell proliferation was determined by stepwise dilution of CFSE dye in dividing, CD3-gated $CD4^+$ T cells using flow cytometry. Culture supernatants were collected for the IFN-γ measurement by ELISA.

Statistical analysis

Data were analyzed with Microsoft Excel, V3.0., and expressed as mean ± s.d. Differences between 2 groups were determined by use of unpaired student's *t*-test. One-way analysis of variance was used to analyze the data with more than two groups. Statistical significance is reported for *p*-values < 0.05.

Results

S100A9 knockout disrupts pStat3 and C/EBPβ bindings at miR-21 and miR-181b promoters

Expression of miR-21 and miR-181b is induced in $Gr1^+CD11b^+$ cells after sepsis initiation via pStat3 and C/EBPβ mediated activation of their promoters during the early and late sepsis phases (McClure *et al.*, 2017). We recently discovered that genetic ablation of S100A9 prevents expression of miR-21 and miR-181b only during the late sepsis phase, despite elevated levels of pStat3 and C/EBPβ (Dai *et al.*, 2017).

To determine the differential effect of S100A9 protein on the miRNA expression, we compared pStat3 and C/EBPβ occupancy at the miRNA promoters in early and late sepsis

$Gr1^+CD11b^+$ cells from wild-type and S100A9 knockout mice using chromatin immunoprecipitation assay. As shown in **Figure 3.1**, both pStat3 and C/EBPβ proteins were detected at the miR-21 promoter in early sepsis $Gr1^+CD11b^+$ cells from the wild-type and knockout mice. In late sepsis, however, binding of pStat3 and C/EBPβ occurred in $Gr1^+CD11b^+$ cells from wild-type mice, but was abolished in cells from the S100A9 knockout mice. Similar binding patterns of pStat3 and C/EBPβ were detected at the miR-181b promoter (**Supplementary Figure 3 – Appendix B**). These results suggest that S100A9 supports the expression of miR-21 and miR-181b in late sepsis only.

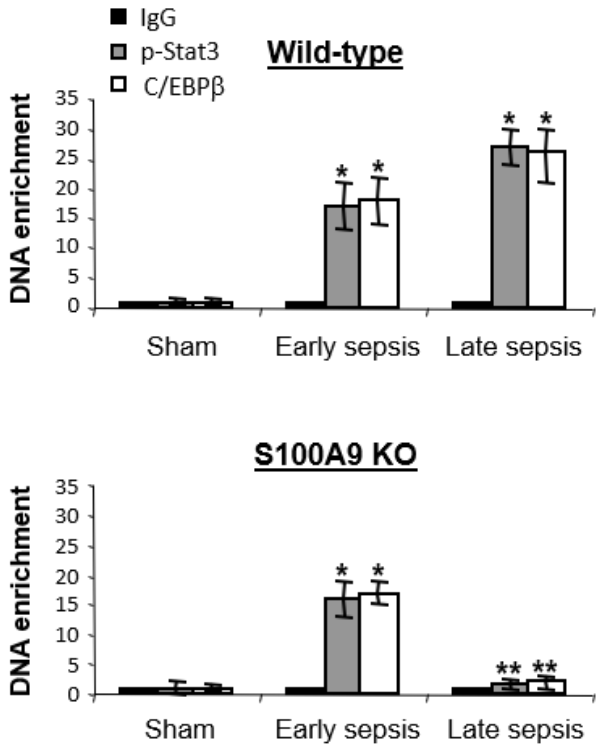

Figure 3.1: Lack of S100A9 expression prevents assembly of the pStat3-C/EBPβ protein complex at the miR-21 promoter in $Gr1^+CD11b^+$ cells in late sepsis. Detection of transcription factor binding at the miRNA promoters. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of sham and septic mice (n = 3-4 mice per group). Formaldehyde-fixed cells were lysed, and the pelleted nuclei were digested with chromatin shearing enzymatic cocktail. The chromatin was immunoprecipitated with antibodies specific to pStat3 (Tyr^{705}), C/EBPβ, or IgG isotype control antibody. Next, chromatin cross-links were reversed to recover the protein-bound DNA. The purified DNA was amplified by quantitative real-time PCR to measure the level of enrichment of miR-21 and miR-181b promoter DNAs in chromatin, using promoter-specific primer/probe sets. PCR reactions were performed in triplicate. Samples values were normalized to the "input" DNA (DNA isolated before immunoprecipitation) and are presented as fold enrichment relative to the IgG-immunoprecipitated samples (set at 1-fold). Data are mean ± s.d. (n = 3-4 mice per group). *$p < 0.05$ vs. sham; **$p < 0.05$ vs. early sepsis. The results are representative of three independent experiments. KO, knockout.

S100A9 is required for assembly of the pStat3-C/EBPβ protein complex

PStat3 and C/EBPβ bind at the miR-21 and miR-181b promoters in $Gr1^{+}CD11b^{+}$ cells as one protein complex (McClure *et al.,* 2017). Western blotting confirmed high levels of S100A9 along with pStat3 and C/EBPβ in early and late sepsis cells (**Figure 3.2A**). To determine whether S100A9 is required for the pStat3-C/EBPβ complex assembly, we performed co-immunoprecipitation using Stat3 and S100A9 antibodies. Western blotting of Stat3 immunoprecipitates detected pStat3-C/EBPβ protein complex in early and late sepsis $Gr1^{+}CD11b^{+}$ cells from the wild-type mice, whereas S100A9 immunoprecipitation showed that S100A9 associated with pStat3-C/EBPβ complex in late sepsis cells only (**Figures 3.2B and C**). Notably, lack of S100A9 protein expression resulted in inhibition of the pStat3-C/EBPβ complex formation in late sepsis $Gr1^{+}CD11b^{+}$ cells only (**Figure 3.2D**). These results demonstrate that S100A9 promotes formation of pStat3-C/EBPβ protein complex in $Gr1^{+}CD11b^{+}$ cells in late sepsis, but has no effect during early sepsis response.

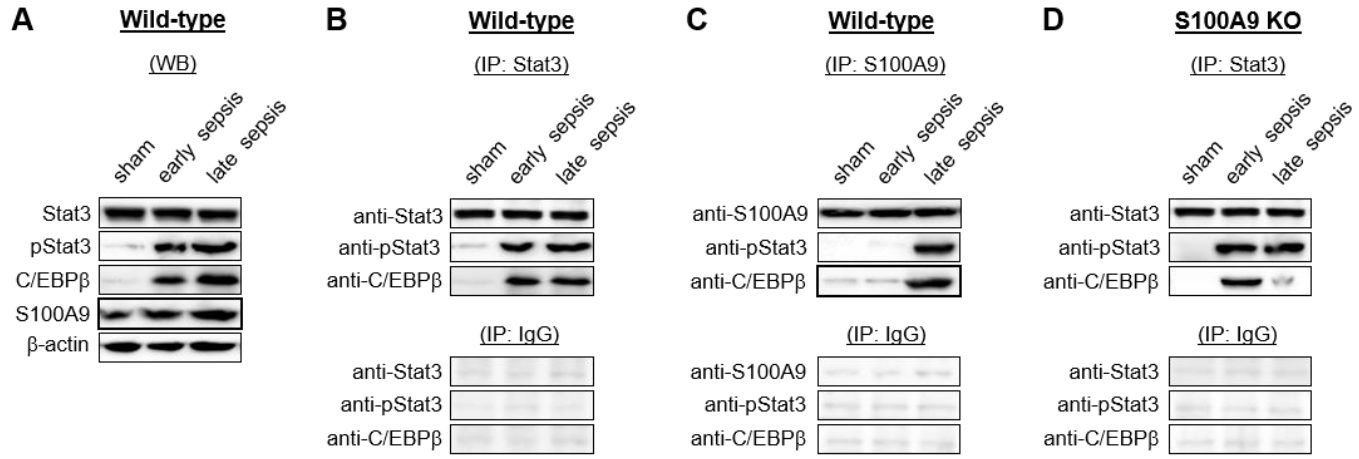

Figure 3.2: The S100A9 forms a protein complex with pStat3 and C/EBPβ proteins in late sepsis. $Gr1^{+}CD11b^{+}$ cells were isolated from the bone marrow of sham and septic mice. (**A**) Levels of pStat3, C/EBPβ, and S100A9 proteins were determined by western blot. (**B-D**) Co-immunoprecipitation analysis of pStat3(Tyr^{705}), C/EBPβ and S100A9 protein interactions. $Gr1^{+}CD11b^{+}$ cell lysates were prepared and immunoprecipitated with pStat3, S100A9, or IgG isotype control antibody using protein G-agarose beads. The immunoprecipitated protein complexes were resolved on denaturing polyacrylamide gel and then immunoblotted with specific antibody against Stat3, pStat3, C/EBPβ, or S100A9. To obtain enough cell lysates for immunoprecipitation, cells were pooled from 2-3 mice per group. The results are representative of three independent experiments. KO, knockout.

Reconstituting late sepsis $Gr1^+CD11b^+$ cells from the S100A9 knockout mice with S100A9 restores the pStat3-C/EBPβ protein complex binding at the miRNA promoters.

We investigated whether S100A9 is sufficient to induce the pStat3-C/EBPβ complex assembly and binding at the miR-21 and miR-181b promoters. To do this, we introduced S100A9 expression plasmid into $Gr1^+CD11b^+$ cells from the S100A9 knockout mice with late sepsis, where formation of the pStat3-C/EBPβ protein complex is disrupted (**Figure 3.2D**). The cells were stimulated with the immunosuppressive IL-10 cytokine because our previous studies showed that IL-10 induced Stat3 phosphorylation, as well as S100A9 translocation to the nucleus (Bah *et al.*, 2018). IL-10 is produced by many myeloid cells during inflammation and infection (Hotchkiss *et al.*, 2013b; Shubin *et al.*, 2011). Notably, IL-10 production is increased as sepsis progresses to the immunosuppressive stage (Bah *et al.*, 2018), and late sepsis MDSCs produce high levels of IL-10 upon ex vivo stimulation with bacterial LPS (McPeak *et al.*, 2017a). Western blotting confirmed S100A9 expression after the transfection with the S100A9 plasmid (**Figure 3.3A**). S100A9 immunoprecipitation assay showed that S100A9 formed a protein complex with pStat3 and C/EBPβ in the presence of IL-10, and these results were similar to what we observed in late sepsis $Gr1^+CD11b^+$ cells from wild-type mice (**Figure 3.3B**). Importantly, PCR analysis of the immunoprecipitated chromatin revealed simultaneous bindings of pStat3, C/EBPβ and S100A9 at the miR-21 promoter (**Figure 3.3C**) and the miR-181b promoter (data not shown). These results indicate that pStat3-C/EBPβ protein complex assembly and binding to the miR-21 and miR-181b promoters in $Gr1^+CD11b^+$ cells during late sepsis requires S100A9.

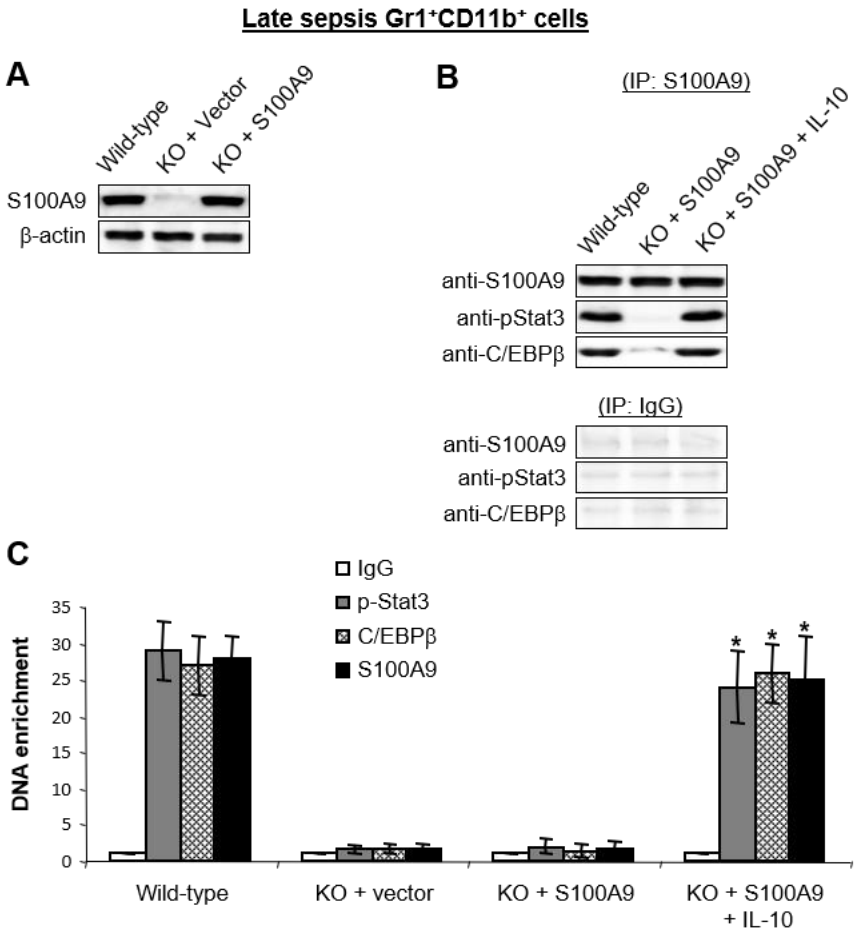

Figure 3.3: Ectopic expression of S100A9 in late sepsis Gr1$^+$CD11b$^+$ cells from the S100A9 knockout mice restores the pStat3-C/EBPβ protein complex formation and binding at the miRNA promoter. Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of S100A9 knockout mice during late sepsis response. Cells were transfected with S100A9 expression plasmid or control vector for 36 hr and then stimulated with 10 ng/ml of recombinant murine IL-10 for 6 hr. Cells from WT mice were used as a control. (**A**) Cell lysates were prepared, and S100A9 protein expression was determined by western blot. (**B**) Cell lysates were immunoprecipitated with an anti-S110A9 antibody, and proteins were detected by western blot. To obtain enough cell lysates for immunoprecipitation, cells were pooled from 2-3 mice per group. (**C**) Analysis of pStat3 (tyrosine 705), C/EBPβ and S100A9 binding at the miRNA promoter by chromatin immunoprecipitation (ChIP). ChIP DNA was extracted and amplified by quantitative real-time PCR to measure the level of miR-21 promoter DNA enrichment in chromatin. Samples values were normalized to the "input" DNA (DNA isolated before immunoprecipitation) and are presented as fold enrichment relative to the IgG-immunoprecipitated samples (set at 1-fold). Data are mean ± s.d. (n = 3-4 mice per group). *$p < 0.05$ vs. KO + S100A9. The results are representative of three independent experiments. KO, knockout.

Reconstituting late sepsis $Gr1^+CD11b^+$ cells from the S100A9 knockout mice with S100A9 activates expression of miR-21 and miR-181b, and induces NFI-A

Expression of miR-21 (**Figure 3.4A**) and miR-181b (**Supplementary Figure 4A- Appendix B**) decreases in the S100A9 knockout mice in late sepsis. We next examined whether reconstituting late sepsis $Gr1^+CD11b^+$ cells from the S100A9 knockout mice with S100A9 can restore the miRNA expressions, concurrently with the pStat3-C/EBPβ complex formation and binding at their promoters (**Figure 3.3**). Transfection with S100A9 plasmid restored expression of miR-21 (**Figure 3.4B**) and miR-181b (**Supplementary Figure 4B- Appendix B**) in the presence of IL-10.

Because S100A9 transfection induced miR-21 and miR-181b expressions in MDSCs from the S100A9 knockout mice, and because S100A9 also is required for the production of immunosuppressive MDSCs (Dai *et al.*, 2017), we investigated whether overexpression of miR-21 and miR-181b in these cells can restore their immunosuppressive function. MDSCs were transfected with control or miR-21 and miR-181b precursors, and stimulated with bacterial LPS, which induces IL-10 expression in MDSCs (McPeak *et al.*, 2017b). Overexpression of miR-21 and miR-181b could not induce IL-10, whereas LPS stimulation in S100A9-trasfected cells significantly induced IL-10 (**Figure 3.4C**). These results show that S100A9, in addition to inducing the miRNA expressions, also play a role in promoting MDSC immunosuppressive function.

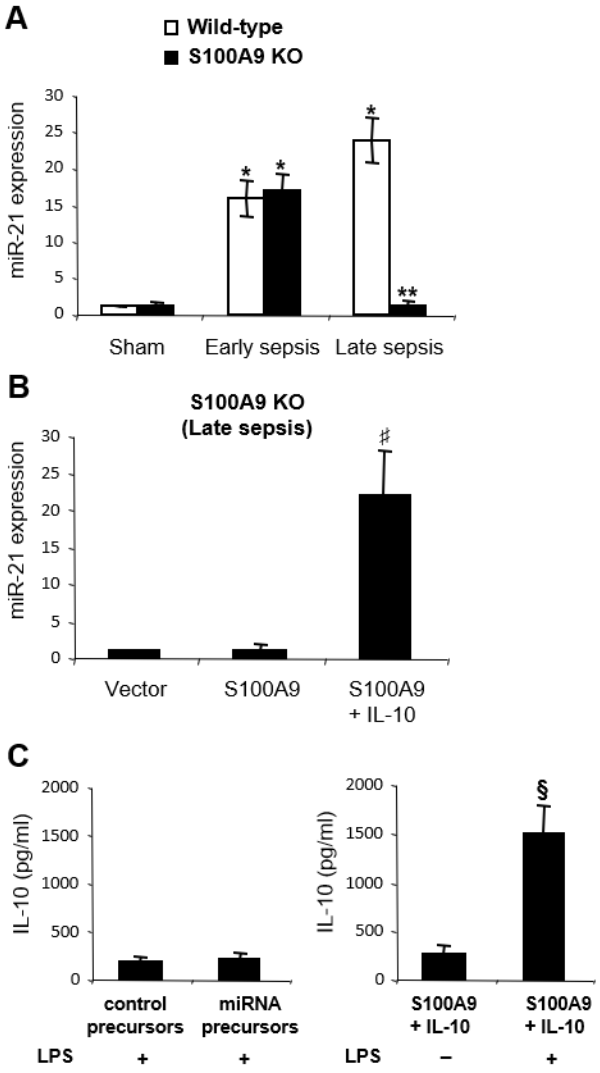

Figure 3.4: Ectopic expression of S100A9 in late sepsis Gr1$^+$CD11b$^+$ cells from the S100A9 knockout mice restores miR-21 expression. Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of sham and septic mice. **(A)** miR-21 expression. miRNA-enriched RNA was isolated, and levels of miR-21 were determined by quantitative real-time PCR using miR-21 specific primers. (**B**) Gr1$^+$CD11b$^+$ cells, isolated from the S100A9 knockout mice during late sepsis response (days 6-28 after sepsis induction), were transfected with S100A9 expression plasmid or control vector for 36 hr and then stimulated with 10 ng/ml of recombinant murine IL-10 for 6 hr. Expression of miR-21 was determined as in *A*. Sample values were normalized to U6 RNA as an internal control and are presented relative to values (set at 1-fold) from sham wild-type mice (*A*) or vector alone (*B*). (**C**) Overexpression of miR-21 and miR-181b does not restore Gr1$^+$CD11b$^+$ cell suppressive function. Late sepsis Gr1$^+$CD11b$^+$ cells from the S100A9 knockout mice were transfected with control precursors or a mixture of miR-21 and miR-181b precursors for 36 hr. The cells, along with S100A9-transfected cells, were washed and stimulated with 1 μg/ml of gram-negative bacterial LPS for 12 hr. Levels of IL-10 in the culture supernatants were determined by ELISA. Data are means ± s.d. (n = 4-6 mice per group). *$p < 0.05$ vs. sham; **$p < 0.05$ vs. wild-type; $^{\sharp}p < 0.05$ vs. S100A9; $^{\S}p < 0.05$. The results are representative of three independent experiments. KO, knockout.

S100A9 protein resides in the cytosol in early sepsis $Gr1^+CD11b^+$ cells, which are not immunosuppressive (Brudecki *et al.*, 2012), but moves to the nucleus during late sepsis (**Figure 3.5A**). We have shown that IL-10 signaling shuttles S100A9 protein from the cytosol to the nucleus in naive $Gr1^+CD11b^+$ cells from wild-type mice (Bah *et al.*, 2018). Here, we observed in late sepsis $Gr1^+CD11b^+$ cells from S100A9 knockout mice that, concurrent with the activation of miR-21 and miR-181b expressions (**Figure 3.4**), IL-10 induced translocation of the ectopically expressed S100A9 protein to the nucleus (**Figure 3.5B**).

Activation of miR-21 and miR-181b expressions during sepsis leads to induction of NFI-A, and subsequently promotes $Gr1^+CD11b^+$ cell expansion (McClure *et al.*, 2016). Similar to the miRNA expression pattern (**Figure 3.4**), NFI-A increased in early, but not late, sepsis in $Gr1^+CD11b^+$ cells in S100A9 knockout mice (**Figures 3.5C and D**). Introducing S100A9 plasmid into these cells restored NFI-A expression in the presence of IL-10 (**Figure 3.5D**). Collectively, the results presented above show that S100A9 induces expression of miR-21 and miR-181b in late sepsis $Gr1^+CD11b^+$ cells, leading to NFI-A expression and that IL-10 signaling mediated these effects by shuttling S100A9 from the cytosol to the nucleus.

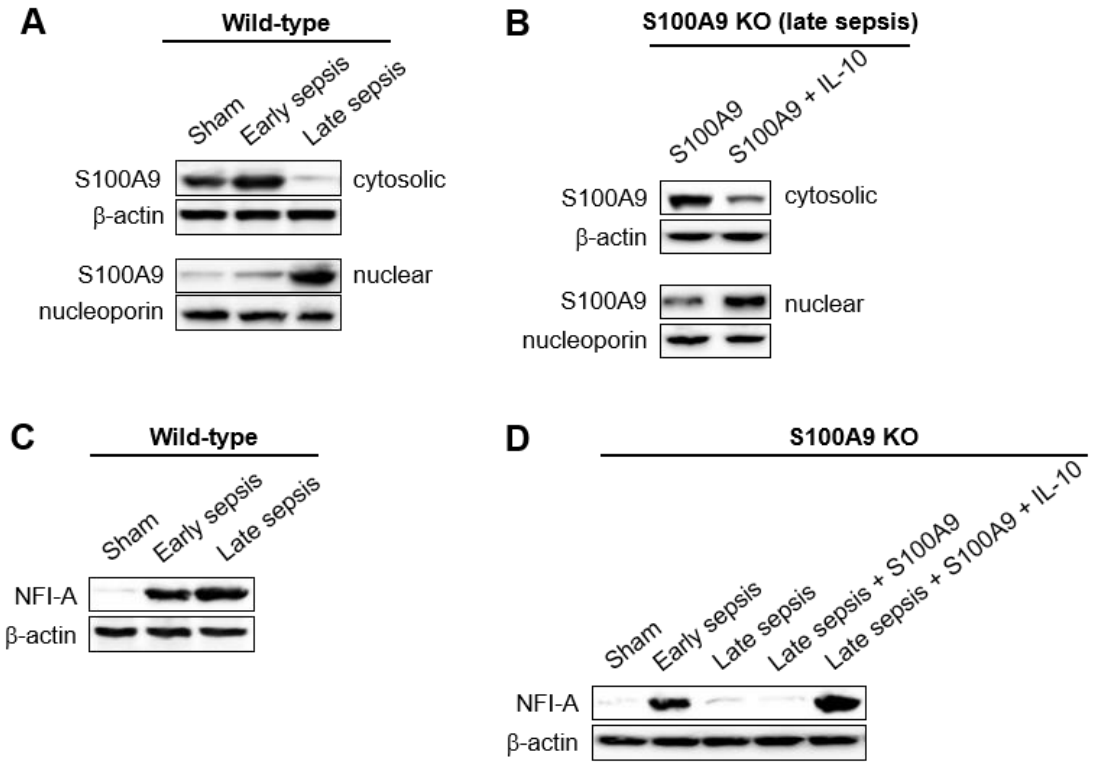

Figure 3.5: Nuclear S100A9 protein promotes NFI-A expression in $Gr1^+CD11b^+$ cells during late sepsis. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of sham and septic mice. S100A9 or NFI-A protein levels were determined by western blot. (**A**) Levels of S100A9 protein in the nucleus and cytosol of $Gr1^+CD11b^+$ cells from wild-type mice. (**B**) Levels of S100A9 proteins in the nucleus and cytosol of $Gr1^+CD11b^+$ cells from S100A9 knockout mice. The cells were isolated during the late sepsis phase (days 6-28 after sepsis induction) and transfected with S100A9 expression plasmid for 36 hr and then stimulated with 10 ng/ml of recombinant murine IL-10 for 6 hr. Cells were pooled from 2 mice per group. (**C**) Levels of NFI-A protein in $Gr1^+CD11b^+$ cells from wild-type mice. (**D**) Levels of NFI-A proteins in $Gr1^+CD11b^+$ cells from S100A9 knockout mice. The cells were isolated during the late sepsis phase and transfected and treated as in *A*. Cells from sham and sepsis mice, and without transfection were used as controls. Cells were pooled from 2 mice per group. The results are representative of three independent experiments. KO, knockout.

S100A9 promotes an immunosuppressive phenotype in $Gr1^+CD11b^+$ cells

$Gr1^+CD11b^+$ cells generated during late sepsis are immunosuppressive (i.e., MDSCs), as they can suppress T cell proliferation and activation (McPeak *et al.*, 2017a). We have shown that S100A9 knockout mice still generate $Gr1^+CD11b^+$ cells at baseline levels during late sepsis, and that these cells are not immunosuppressive (Dai *et al.*, 2017), i.e., like normal myeloid precursors that are produced under normal conditions. We examined whether introducing S100A9 into $Gr1^+CD11b^+$ cells from S100A9 knockout mice with late sepsis can promote an immunosuppressive phenotype, concurrently with the induction of miRNA expressions (**Figure 3.4B** and **Supplementary Figure 3- Appendix B**). $Gr1^+CD11b^+$ cells from sham and late septic wild-type mice were used as positive and negative controls, respectively. As shown in **Figure 3.6**, $Gr1^+CD11b^+$ cells from wild-type, control mice significantly suppressed T cell proliferation and activation (IFNγ production). Late sepsis $Gr1^+CD11b^+$ cells derived from S100A9 knockout mice and transfected with S100A9 plasmid could not suppress T cells, whereas S100A9 plasmid transfection with IL-10 stimulation led to significant suppression of T cell proliferation and IFNγ production (**Figure 3.6**). These results suggest that S100A9 promotes an immunosuppressive phenotype in $Gr1^+CD11b^+$ cells during late sepsis and that IL-10 signaling mediates these effects.

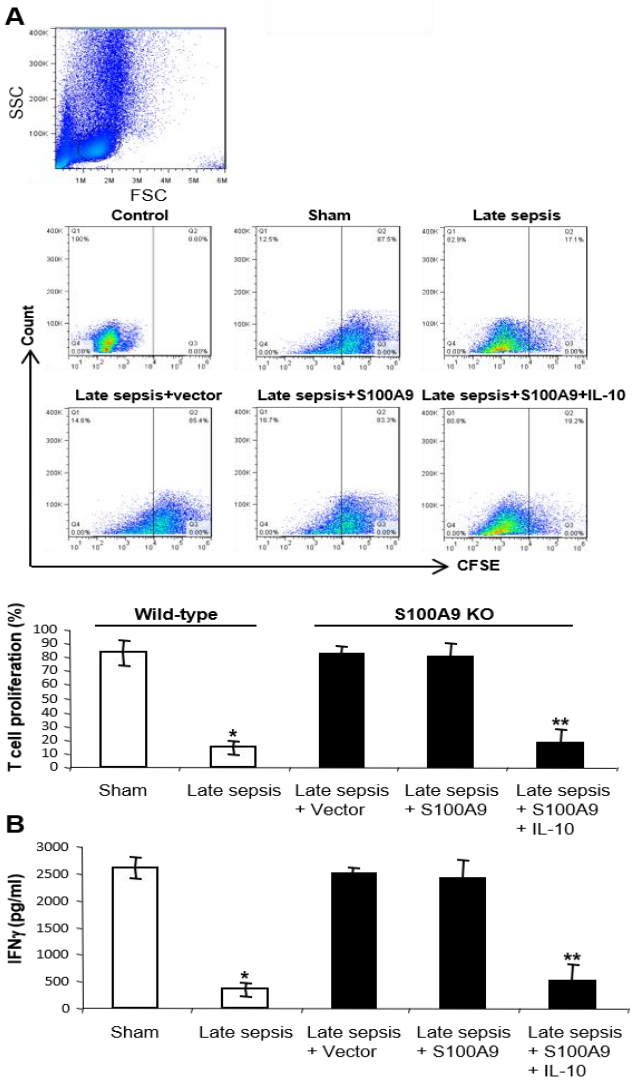

Figure 3.6: Reconstituting late sepsis Gr1$^+$CD11b$^+$ cells from the S100A9 knockout mice with S100A9 restores their immunosuppressive functions. Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of S100A9 knockout mice during the late sepsis phase. The cells were transfected with S100A9 expression plasmid for 36 hr and then stimulated with 10 ng/ml of recombinant murine IL-10 for 6. (**A**) Effect of Gr1$^+$CD11b$^+$ cells on CD4$^+$ T cell proliferation. Spleen CD4$^+$ T cells were isolated from normal (naive) mice by positive selection and labeled with the fluorescent dye CFSE for 10 min at room temperature. Gr1$^+$ CD11b$^+$ cells were then cocultured (1:1 ratio) with T CD4$^+$ cells. The culture was stimulated with anti-CD3 plus anti-CD28 antibodies (1 μg/ml/each). After three days, CD4$^+$ T cell proliferation was determined by the step-wise dilution of CFSE dye in dividing CD4$^+$ T cells by flow cytometry. Representative dot plots (upper panels) of CFSE positive T cells gated on CD4 are shown. Percentages of cell proliferation (lower) were calculated as follow: % cell proliferation = 100 x (count from T cell + Gr1$^+$CD11b$^+$ cell culture/count from T cell culture). *p < 0.001 vs. sham or early sepsis; **p < 0.001 vs. late sepsis WT. **(B)** ELISA determined levels of IFNγ in the culture supernatants. Cells from sham and sepsis wild-type mice, and without transfection were used as controls. Cells were pooled from 2-3 mice per group. Data are means ± s.d. (n = 3-4 mice per group). *p < 0.05 vs. sham wild-type; **p < 0.05 vs. late sepsis + vector or late sepsis + S100A9. The results are representative of three independent experiments. KO, knockout.

Discussion

The initial/acute phase of sepsis, if not resolved early to restore immune homeostasis, can progress to a late/chronic stage with protracted immunosuppression (Hotchkiss *et al.*, 2013b; Patil *et al.*, 2016) and continued mortality and morbidity (Delano and Ward, 2016; Hotchkiss *et al.*, 2013a). Increases in functional $Gr1^{+}CD11b^{+}$ cells accumulate within a few days of sepsis onset, but then switch their phenotypes to become MDSCs later (Brudecki *et al.*, 2012). We reported that nuclear S100A9 accumulation in $Gr1^{+}CD11b^{+}$ cells parallels increases in miR-21 and miR-181b levels during late sepsis in wild-type mice, but not in S100A9 deficient mice (Dai *et al.*, 2017). The present study's new discovery is that S100A9 mediates the expression of miR-21 and miR-181b during late sepsis concomitant with increasing MDSCs repressor function. These findings suggest that S100A9 is a significant regulator of MDSC expansion during late/chronic sepsis in mice.

Activation of miR-21 and miR-181b expressions is a dynamic process. In the steady-state, transcription factor C/EBPα, which supports normal myeloid cell development and differentiation (Hirai *et al.*, 2006), binds the miR-21 and miR-181b promoters (McClure *et al.*, 2014; McClure *et al.*, 2017). After sepsis initiation, C/EBPα is replaced by C/EBPβ, which forms a complex with pStat3 (McClure *et al.*, 2017). **Figure 3.7** depicts the dynamic binding process of pStat3 and C/EBPβ. The protein complex then activates the expression of miR-21 and miR-181b. The current study shows that pStat3 and C/EBPβ do not bind miR-21 and miR-181b promoters in $Gr1^{+}CD11b^{+}$ cells during late sepsis in the S100A9 knockout mice (Dai *et al.*, 2017). Thus, these findings indicate that S100A9 protein plays an essential role in miR-21 and miR-181b expression induction during late sepsis. In support of this, we showed that ectopic

expression of S100A9 in these cells restored pStat3 and C/EBPβ bindings at the miRNA promoters and induced their expression.

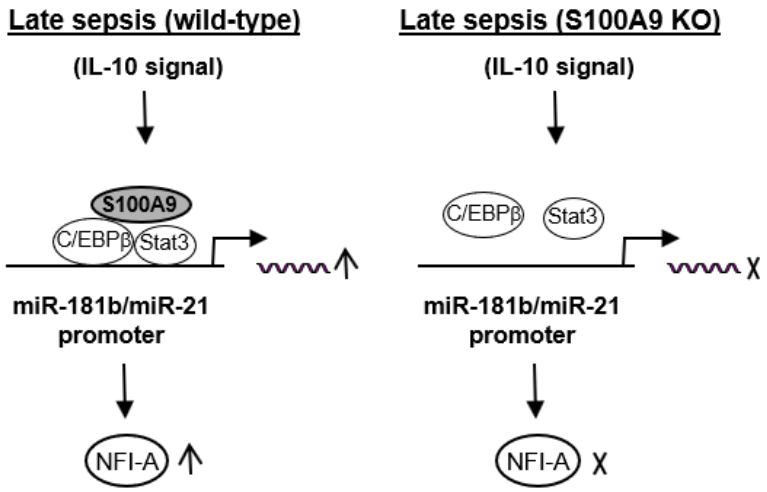

Figure 3.7: A proposed model for regulation of miR-21 and miR-181b promoters by S100A9 in $Gr1^+CD11b^+$ cells during late sepsis response. During late sepsis, S100A9 protein promotes assembly of pStat3 and C/EBPβ into one protein complex and induces its binding at the miR-181b and miR-21 promoters in $Gr1^+$ $CD11b^+$ cells, leading to activation of the miRNA expressions and subsequent upregulation of NFI-A. This process is mediated by IL-10 signaling, which promotes S100A9 protein translocation from the cytosol to the nucleus (Bah *et al.*, 2018). In the absence of S100A9 (S100A9 KO), pStat3 and C/EBPβ cannot bind/activate the miRNA promoters. Not shown is the regulatory node downstream of NFI-A that leads to $Gr1^+CD11b^+$ cell expansion and suppressive functions.

The mechanism by which S100A9 contributes to the pStat3-C/EBPβ protein complex binding at the miR-21 and miR-181b promoters only during late sepsis remains unclear. In early sepsis $Gr1^+CD11b^+$ cells, S100A9 resides in the cytosol and does not affect miR-21 and miR-181b promoters, because they are expressed in the S100A9 knockout mice (Dai *et al.*, 2017). The current study shows that nuclear S100A9 may stabilize pStat3-C/EBPβ complex binding at the miRNA promoters. Moreover, we found that IL-10, which induces S100A9 nuclear translocation during late sepsis, supports Stat3 phosphorylation (Bah *et al.*, 2018). Nuclear S100A9 acting as a coregulator might modify the chromatin structure around the miRNA promoters and facilitate pStat3-C/EBPβ complex binding. In this regard, we previously reported that HMGB1 protein, which like S100A9 functions as a proinflammatory mediator when secreted by immune cells

(Andersson *et al.*, 2000; Park *et al.*, 2006), modifies chromatin at promoters of proinflammatory genes in endotoxin-tolerant monocytes (El Gazzar *et al.*, 2009).

MiR-21 and miR-181b couple to promote NFI-A expression, which propagates its effects downstream in the path leading to $Gr1^+CD11b^+$ cell expansion in sepsis (McClure *et al.*, 2016; McClure *et al.*, 2014). This regulatory mechanism operates during the early and late sepsis phases in wild-type mice. However, $Gr1^+CD11b^+$ cells that expand during early sepsis are not immunosuppressive and can differentiate and mature *ex vivo* with growth factors (Brudecki *et al.*, 2012; McPeak *et al.*, 2017a). In contrast, late sepsis $Gr1^+CD11b^+$ cells, in which S100A9 resides in the nucleus (Dai *et al.*, 2017), are immunosuppressive (Brudecki *et al.*, 2012). On the other hand, the lack of $Gr1^+CD11b^+$ cell expansion and immunosuppressive functions in the S100A9 knockout mice during late sepsis suggests that S100A9 not only is vital for $Gr1^+CD11b^+$ cell expansion, but also for switching them into the immunosuppressive, MDSC phenotype.

S100 proteins released from leukocytes can act as proinflammatory mediators and acute phase proteins (Ehrchen *et al.*, 2009; Foell *et al.*, 2004a; Foell *et al.*, 2004b; Vogl *et al.*, 2012). S100A9 protein released at the site of inflammation can serve as an "alarmin" by binding to and activating toll-like receptor 4 (TLR4) on innate immunity cells, which increases phagocyte activation and recruitment (Foell *et al.*, 2004a; Hiratsuka *et al.*, 2006; Vogl *et al.*, 2007). A previous study reported that S100A9 promotes MDSC expansion in tumor-bearing mice by inhibiting myeloid progenitor differentiation via increasing production of reactive oxygen species (Cheng *et al.*, 2008). In our polymicrobial sepsis model, S100A9 protein is expressed throughout sepsis but its secretion markedly decreases concurrently with its nuclear accumulation in $Gr1^+CD11b^+$ cells during the late/immunosuppressive phase (Dai *et al.*, 2017). Together, the new data from this study support the notion that S100A9 is a multifunctional

protein and promote or repress inflammation, depending on the context (Foell *et al.*, 2004b; Ikemoto *et al.*, 2007; Vogl *et al.*, 2012; Vogl *et al.*, 2007). Our novel findings that S100A9 contribute to sepsis phenotype shift from proinflammatory to immunosuppressive at the molecular level are significant. Post-sepsis immunosuppression attenuates both innate and adaptive immune responses (Delano *et al.*, 2016; Hotchkiss *et al.*, 2013b), which leads to the acquisition of secondary, opportunistic infections, and thus elevates mortality (Otto *et al.*, 2011; Torgersen *et al.*, 2009).

Our findings that genetically targeting S100A9 in the mouse can inhibit MDSC expansion during late sepsis could be biologically significant. There are no molecular-based treatments for sepsis. Limiting MDSC-mediated support of immune suppression or chronic sepsis by targeting S100A9 might restore immune competency and improve survival during the post-sepsis chronic illness syndrome.

Conflicts of interest

The authors declare no conflicts of interest.

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CHAPTER 4

LONG NON-CODING RNA HOTAIRM1 PROMOTES S100A9 SUPPORT OF MDSC EXPANSION DURING SEPSIS

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Running title: Hotairm1 induces sepsis immune suppression

Summary Sentence: Hotairm1 modifies S100A9 function to induce MDSCs

Keywords: Sepsis, MDSC, Immune suppression, S100A9, Hotairm1

Abstract

Myeloid-derived suppressor cells (MDSCs) expand during mouse and human sepsis, but the mechanism responsible for this is unclear. We previously reported that nuclear transport of S100A9 protein programs $Gr1^+CD11b^+$ myeloid precursors into MDSCs in septic mice. Here, we show that long non-coding RNA Hotairm1 converts MDSCs from an activator to repressor state. Mechanistically, increased Hotairm1 expression in MDSCs in mice converted S100A9 from a secreted proinflammatory mediator to an immune repressor by binding to and shuttling it from cytosol to nucleus during late sepsis. High Hotairm1 levels were detected in exosomes shed from MDSCs from late septic mice. These exosomes inhibited lipopolysaccharide-stimulated secretion of S100A9 from early sepsis $Gr1^+CD11b^+$ cells. Importantly, Hotairm1 knockdown in late sepsis $Gr1^+CD11b^+$ MDSCs prevented S100A9 cytosol to nuclear transfer and decreased repression of proimmune T cells. Notably, ectopic expression of Hotairm1 in early sepsis $Gr1^+CD11b^+$ cells shuttled S100A9 to the nucleus and promoted the MDSC repressor phenotype. In support of translating the mechanistic concept to human sepsis, we found that Hotairm1 binds S100A9 protein in $CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs during established sepsis. Together, these data support that Hotairm1 is a plausible molecular target for treating late sepsis immune suppression in humans and its immune repressor mechanism may be cell autonomous.

Abbreviations:

CFSE, carboxyfluorescein diacetate, succinimidyl ester; CLP, cecal ligation and puncture; Hotairm1, Hox antisense intergenic RNA myeloid 1; IL-10, interleukin 10; lncRNA, long non-coding RNA; LPS, lipopolysaccharide; MDSCs, myeloid-derived suppressor cells

Introduction

Life threatening sepsis dysregulates the host response to uncontrolled infection [1]. The immune response to sepsis enhances myelopoiesis to compensate for innate immune cell loss during excessive inflammation [2, 3]. However, progenitors and precursors of monocyte, neutrophil and dendritic cells fail to differentiate into competent immune cells and may be reprogrammed into immunosuppressive phenotypes [2, 4, 5]. These myeloid-derived suppressor cells (MDSCs) suppress the innate and adaptive immune responses and thus hamper inflammation resolution and immune homeostasis [4, 6-8]. MDSC numbers increase in blood in mice with sepsis [2, 6, 9], and are found in septic patients that may become chronic (defined as having sepsis for 14 days or longer) and develop critical illness [4, 10]. The molecular mechanisms underlying MDSCs in sepsis remain unclear.

Sepsis is initiated by acute proinflammatory reaction, which may be rapidly lethal, or progress to a protracted immunosuppressive stage characterized by decreases in competent innate and adaptive immune cells and continued increases in mortality [11-13]. We reported that S100A9 contributes to post-sepsis protracted immunosuppressive state [14, 15]. S100A9 protein is a common constituent of myeloid-derived circulating leukocytes and amplifies inflammation [16, 17]. It is induced in myeloid cells by inflammation-derived signals [16, 18, 19]. We found that MDSCs expansion decreases in S100A9-deficient mice during polymicrobial sepsis [15]. S100A9 plasma protein levels increase in acutely septic mice, but decrease in mice with protracted immunosuppression [15]. $Gr1^{+}CD11b^{+}$ myeloid cells generated during acute sepsis are proinflammatory [6]. Notably, $Gr1^{+}CD11b^{+}$ myeloid cells generated during chronic sepsis (i.e., MDSCs) fail to secrete S100A9 upon ex vivo stimulation with bacterial lipopolysaccharide [15]. S100A9 localizes mainly in cytosol during acute sepsis, but moves to the nucleus as

immunosuppressive $Gr1^+CD11b^+$ MDSC emerge [15]. Thus, S100A9 can contribute to the proinflammatory or anti-inflammatory states typical of sepsis.

The role of long non-coding RNAs (lncRNAs) in sepsis is unclear. LncRNAs contain >200 nucleotides, which like protein-coding mRNAs, are polyadenylated, capped and spliced [20, 21]. Recent evidence indicates that lncRNAs control production of inflammatory mediators and cell fate [22, 23]. They are expressed in a cell- and tissue-specific manner [22], with differential expression during cell activation [23]. While most LncRNAs interact with protein, RNA, or DNA [24, 25], immune-related lncRNAs prefer protein binding [22, 23], protein modifications and transport [23, 24, 26]. In this study, we profiled expression of lncRNAs in $Gr1^+CD11b^+$ MDSCs during sepsis. Here, we found differential expression of myeloid-related lncRNA Hotairm1 in late sepsis. The results show that it controls MDSC repressor cell function by binding to and transferring S100A9 to the nucleus. Hotairm1 may provide a molecular targeting site for post-acute sepsis correction of immune suppression.

Materials and Methods

Mice

Male C57BL/6 mice (8 to 10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in a pathogen-free facility, and were acclimated to the new environment for a week before surgery. 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.

Polymicrobial sepsis model

Sepsis was induced by cecal ligation and puncture (CLP) in mice as described previously [27]. Briefly, a midline abdominal incision was made and the cecum was ligated distal to the ileocecal valve, and punctured twice with a 23-gauge needle. A small amount of feces was extruded into the abdominal cavity. The abdominal wall and skin were sutured in layers with 3-0 silk. Sham-operated mice were treated identically except that the cecum was neither ligated nor punctured. Mice received (i.p.) 1 ml of lactated Ringers plus 5% dextrose for fluid resuscitation. To establish intra-abdominal infection and approximate the clinical conditions of human sepsis where there is a delay between the onset of sepsis and the delivery of therapy [28], mice were treated (s.c) with antibiotic (imipenem; 25 mg/kg body weight) diluted in saline (0.9% sodium chloride) at 8 and 16 hr after CLP. The level of injury and manipulation create prolonged peritoneal infections with high mortality (~60-70%) during the late/chronic phase [27], as monitored for 28 days. Mice moribund during acute/early and chronic sepsis were euthanized and analyzed. For comparison purposes, we define early sepsis as the first 5 days after CLP and late sepsis post day 6 [27]. A corresponding number of mice from the control/sham group were also analyzed at the same time points.

Patients

Twenty-one patients 18 years of age or older who were admitted to Johnson City Medical Center and Franklin Woods Hospital in Johnson City, Tennessee, and who were diagnosed with sepsis or septic shock were included in the study. Criteria for patient selections were as described previously [15]. Patients were divided into two categories: early septic and late septic, relative to the day of sepsis diagnosis. The early/acute septic group included patients within 1-5 days of

sepsis diagnosis. Those who have been septic for more than 6 days were considered late/chronic septic. For this latter group, blood was drawn at days 6-68 after sepsis diagnosis. Blood from healthy control subjects was supplied by Physicians Plasma Alliance (Gray, TN). The study was approved by the Institutional Review Board (IRB) of the East Tennessee State University (IRB#: 0714.6s). Signed informed consent was obtained from all subjects.

Cell isolation

$Gr1^+CD11b^+$ cells were isolated from bone marrow using magnetic beads according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). Briefly, the bone marrow was flushed out of the femurs with RPMI-1640 medium (without serum) under aseptic conditions. A single cell suspension was made by pipetting up and down and filtering through a 70-μm nylon strainer, followed by incubation with erythrocyte lysis buffer and washing. The cell suspension was subjected to a positive selection of the $Gr1^+$ cells by incubating with biotin-coupled mouse anti-Gr1 antibody (Clone RB6-8C5; eBioscience, San Diego, CA) for 15 min at 4ºC. Cells were then incubated with anti-biotin magnetic beads for 20 min at 4ºC and subsequently passed over an MS column. The cell purity was more than 90% as determined by flow cytometry.

To isolate $CD33^+CD11b^+HLA\text{-}DR^-$ cells from peripheral blood, PBMCs were first isolated by gradients of Histopage-1077 and Histopaque-1119 per manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO), and then depleted of the $HLA\text{-}DR^+$ cell subset using magnetic beads. The $CD33^+CD11b^+$ cell subset was then positively selected by incubating with biotin-coupled human anti-CD33 and anti-CD11b antibodies (Miltenyi Biotech) and isolated with anti-biotin magnetic beads.

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% CO_2.

Purification of exosomes

We assessed whether exosomes contain mediators that modify S100A9 protein localization in MDSCs. Exosomes were purified from blood plasma or MDSC culture supernatants. For use in cell culture, exosomes were purified using exoEasy Maxi Kit per manufacturer's protocol (Qiagen, Valencia, CA). For use in RNA expression analysis, exosomes were purified, and RNA was isolated using exoRNeasy Starter Kit (Qiagen).

Protein extracts

For whole cell lysates, the $Gr1^+CD11b^+$ cells were lysed 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. After 30 min on ice, cell lysates were cleared by centrifugation for 5 min at 4ºC and 14,000 rpm. Protein concentrations were determined by Bradford assay (Bio-Rad), and aliquots were kept at -20ºC.

Cytoplasmic and nuclear protein extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) per the manufacturer's instructions. Immediately after harvesting, the 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 removed by

centrifugation for 5 min at 4ºC 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 4ºC and 14,000 rpm. Protein aliquots were kept at -20ºC.

RNA immunoprecipitation

RNA immunoprecipitation was performed to detect Hotairm1 RNA binding to S100A9 protein. Briefly, $Gr1^{+}CD11b^{+}$ cells (~$10x10^{6}$) were washed with warm PBS and incubated with 0.2% formaldehyde (in PBS) for 10 min at room temperature, to preserve RNA-protein complexes. Cross-linked cells were incubated for 30 min on ice in lysis buffer containing: 250 mM sucrose, 10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5% NP-40, 0.5% deoxycholate, 30 U/ml RNase inhibitor, and 1x protease inhibitor cocktail. After DNase I treatment for 10 min at 37C, cell lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4˚C. Cell lysates were immunoprecipitated with anti-S100A9 antibody according to a published method [29]. Aliquots of S100A9-immunoprecipitated protein complexes were used for western blot or subjected to RNA isolation using TRIzol reagent (Invitrogen, Carlsbad, CA) and measurement of Hotairm1 levels by PCR.

Hotairm1 expression plasmid

Hotairm1 was cloned in pReceiver-M02 expression vector downstream of the CMV promoter. An empty control vector (pReceiver-M02CT) served as a negative control (GeneCopoeia, Rockville, MD).

Cell transfection

For Hotairm1 expression, Hotairm1 plasmid was suspended in HiPerFect reagent at a 0.5 μg/ml final concentration (Qiagen, Valencia, CA). For Hotairm1 knockdown, Hotairm1-specific or scrambled siRNAs were suspended in HiPerFect reagent at a 0.5 μM final concentration. $Gr1^+CD11b^+$ cells were transfected according to the manufacturer's instructions and incubated with RPMI-1640 medium for 24-36 hr.

Real-time PCR

Real-time qPCR (RT-qPCR) was used to determine the levels of Hotairm1 in exosomes, $Gr1^+CD11b^+$ cells, or S100A9 immunoprecipitates. Exosomes were purified from plasma or cell culture supernatants, and exosomal RNA was isolated using exoRNeasy Starter Kit (Qiagen). Total cellular RNA was isolated from $Gr1^+CD11b^+$ cells using TRIzol reagent (Invitrogen). Levels of Hotairm1 expression were measured using QuantiTect PCR Mastermix and RT^2 lncRNA qPCR Assay primers (Qiagen). The expression level was calculated using the $2^{-\Delta\Delta Ct}$ cycle threshold method. Values were normalized to GAPDH RNA for total RNA or 18S RNA for exosomal RNA, amplified with QuantiTect Primer Assays. The PCR was performed in duplicate.

T cells proliferation assay

We used a co-culture system to test the effects of MDSC-derived exosomes or MDSCs lacking Hotairm1 on spleen CD4 T cell proliferation and activation. Splenocytes were isolated from naive wild-type mice. $CD4^+$ T cells were purified from splenocytes by positive selection using biotinylated anti-CD4 magnetic beads (Myltenyi) and labeled with carboxyfluorescein diacetate,

succinimidyl ester (CFSE) dye using the Vybrant CFDA SE Cell Tracer Kit (Invitrogen Molecular Probes, Eugene, OR). The cells were incubated for 10 min at room temperature with 10 μM CFSE dye. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of sham and septic mice and cultured for 24 hr in serum-free medium, and exosomes were purified from the culture supernatants as described above. The labeled $CD4^+$ cells were cultured in 12-well plates with naive $Gr1^+CD11b^+$ cells in the presence of exosomes (50 μg/well). In other experiments, $CD4^+$ T cells were co-cultured in a 1:1 ratio with late sepsis $Gr1^+CD11b^+$ cells in which Hotairm1 was first knocked down for 36 hr with a pool of siRNAs. Anti-CD3 antibody plus anti-CD28 antibody (1 μg/ml each) were added to the cultures to stimulate T cell activation and proliferation. After 3 days, the culture supernatants were collected and used to measure IFN-γ levels by ELISA. The cells were harvested and $CD4^+$ T cell proliferation was determined by the stepwise dilution of CFSE dye in dividing $CD4^+$ T cells using flow cytometry.

Arginase assay

Arginase 1 activity in $Gr1^+CD11b^+$ cells was determined by measuring urea concentration (a by-product of arginase 1 activity) in the cell lysates using an arginase assay kit (Abnova, Walnut, CA) as described previously [30]. One unit of arginase 1 converts 1 μmol of L-arginine to ornithine and urea per minute at pH 9.5 and 37ºC.

ELISA

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the levels S100A9 (MyBioSource, San Diego, CA) and IFNγ (eBioscience) in cell culture supernatants. Samples were run in duplicate.

Statistical analysis

Data were analyzed with Microsoft Excel, V3.0. Data are expressed as mean ± SD. Differences among groups were analyzed by a two-tailed student's *t* test for two groups and by one-way ANOVA for multiple groups. Statistical significance with p-values < 0.05 are reported.

Results

MDSC-derived exosomes contain mediators that inhibit S100A9 secretion.

Exosomes are nano-sized (30-100 nm) vesicles that are released to the extracellular space when fused with the plasma membrane [31, 32]. Exosomes facilitate the direct transfer of their cargos of proteins, non-coding RNA and DNA between parent cell and recipient cell in vivo and in vitro [33, 34]. We have shown that inhibition of S100A9 secretion and shuttling to the nucleus in $Gr1^+CD11b^+$ cells during late sepsis is associated with the development of MDSCs [15]. Because exosomes contents share some of the transcriptomic and proteomic signature of the parent cell [33, 35], we wondered whether exosomes shed from late sepsis $Gr1^+CD11b^+$ MDSCs could be used to identify mediators that promote S100A9 nuclear localization.

To test this, we cultured $Gr1^+CD11b^+$ cells from bone marrow of early septic mice - where S100A9 resides in cytosol and is readily secreted [15] - with exosomes purified from cultured $Gr1^+CD11b^+$ cells isolated from sham or septic mice. Then, the cells were stimulated with gram-negative bacterial lipopolysaccharide (LPS) to induce S100A9 secretion. S100A9 secretion significantly increased following stimulation with LPS, and was increased further slightly in the presence of exosomes from sham or early sepsis $Gr1^+CD11b^+$ cells (**Figure 4.1**). Notably, exosomes derived from late sepsis $Gr1^+CD11b^+$ MDSCs decreased S100A9 secretion significantly (**Figure 4.1**). These results suggest that late sepsis MDSC-derived exosomes contain inhibitors of S100A9 secretion.

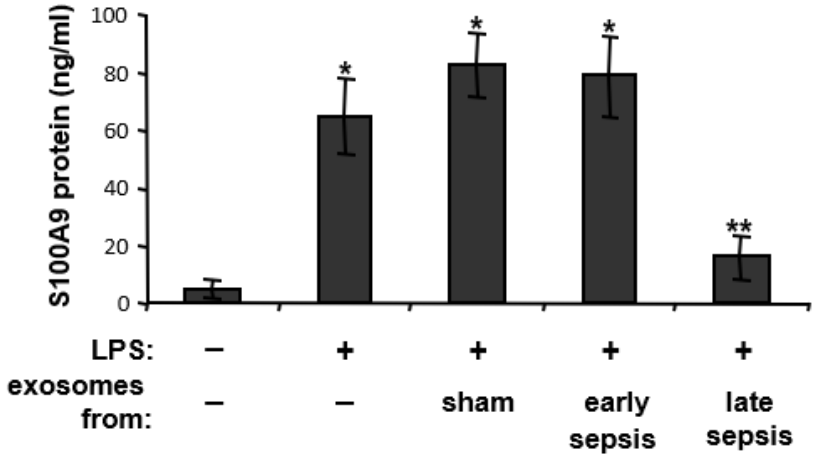

Figure 4.1: Exosomes shed from late sepsis MDSCs inhibit LPS-induced secretion of S100A9 protein from early sepsis MDSCs. $Gr1^+CD11b^+$ cells were isolated from bone marrow of sham and septic mice by positive selection using anti-Gr1 antibody and magnetic beads. The cells were cultured for 24 hr in serum-free media. Culture supernatants were harvested, and exosomes were purified using exoEasy Maxi kit. Early sepsis $Gr1^+CD11b^+$ cells were cultured in 12-well plates with exosomes (50 μg/well) for 24 hr with or without 0.1 μg/ml of E. coli LPS (serotype 0111:B4). Levels of S100A9 protein in the culture supernatants were measured by ELISA. Data are expressed as means ± SD of 6-8 mice (6-8 cultures/group) from three experiments. *p/**p < 0.05, versus exosomes from sham or early sepsis.

MDSC-derived exosomes switch naive $Gr1^+CD11b^+$ cells into suppressive $Gr1^+CD11b^+$ MDSCs.

MDSCs from late septic mice suppress T cell activation and proliferation [15]. We investigated whether MDSC-derived exosomes can induce an immunosuppressive phenotype in naive $Gr1^+CD11b^+$ cells, which cannot suppress T cell. Spleen $CD4^+$ T cells were isolated from naive mice and cultured with naive $Gr1^+CD11b^+$ cells in the presence of exosomes derived from cultures of sham or sepsis $Gr1^+CD11b^+$ cells. The T cells were stimulated with anti-CD28 and anti-CD3 antibodies. Flow cytometry analysis showed that exosomes derived from early sepsis $Gr1^+CD11b^+$ cells did not significantly affect T cell proliferation, as compared to sham $Gr1^+CD11b^+$ cells (**Figures 4.2A and B**). In addition, these exosomes had no significant effect on T cell activation as determined by IFNγ production (**Figure 4.2C**). Notably, exosomes from late sepsis $Gr1^+CD11b^+$ cells significantly inhibited both T cell proliferation and IFNγ production, suggesting that they contain mediators that render naive $Gr1^+CD11b^+$ cells immunosuppressive.

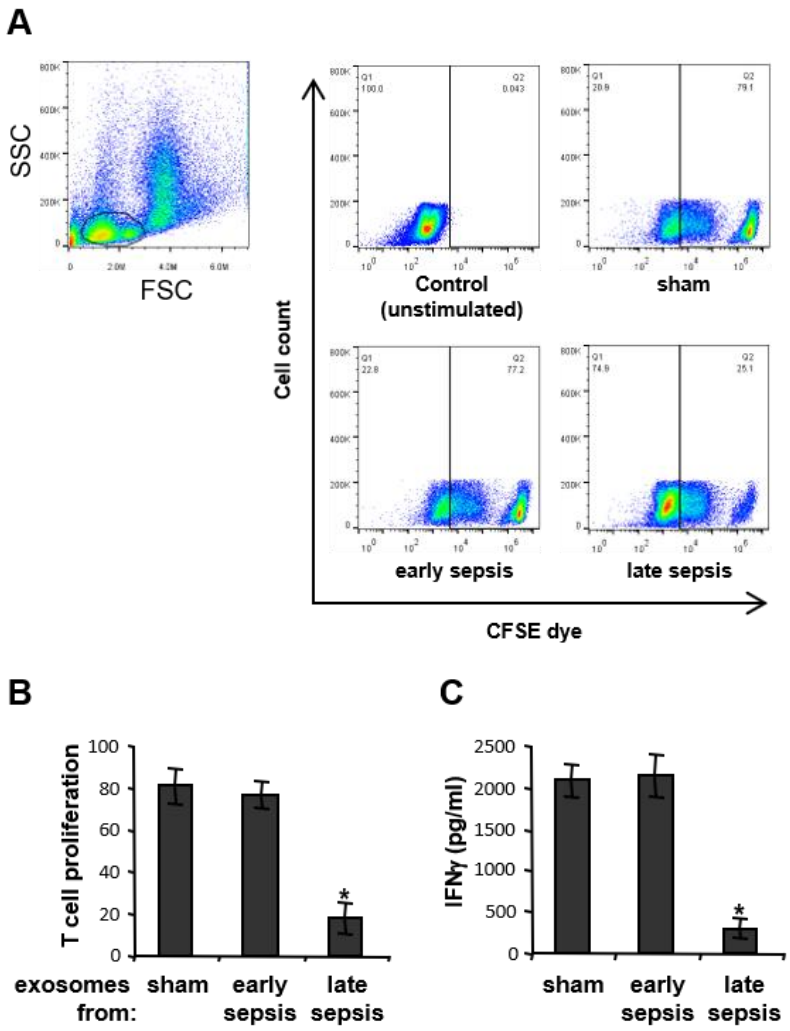

Figure 4.2: Late sepsis MDSC-derived exosomes carrying Hotairm1 switch naïve Gr1$^+$CD11b$^+$ cells into the immnosuppressive phenotype. Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of sham and septic mice by positive selection using anti-Gr1 antibody and magnetic beads. The cells were cultured for 24 hr in serum-free media, and exosomes were purified from the culture supernatants using exoEasy Maxi kit. CD4$^+$ T cells were isolated from splenocytes of naive mice with anti-CD4 antibody and labeled with the fluorescent dye CFSE. The CD4$^+$ T cells were cultured (1:1 ratio) with naive Gr1$^+$CD11b$^+$ cells in the presence of exosomes (50 μg/well) for 3 days in the presence of anti-CD3 plus anti-CD28 antibodies (1 μg/ml each), to stimulate T cells. The cells were harvested, and T cell proliferation was determined by the step-wise dilution of CFSE dye in dividing CD4$^+$ T cells using flow cytometry. (**A**) Representative dot plots of CFSE positive T cells gated on CD4 are shown. (**B**) Summary data of flow cytometry. (**C**) The culture supernatants were used to determine IFNγ levels by ELISA. Data are expressed as means ± SD of 6-9 mice (6-9 cultures/group) from three experiments. *$p < 0.05$.

Expression of lncRNA Hotairm1 is upregulated in late sepsis $Gr1^+CD11b^+$ MDSCs.

Re-localization of S100A9 protein from cytosol to nucleus of $Gr1^+CD11b^+$ cells occurs during late sepsis [15]. Because exosomes derived from late sepsis $Gr1^+CD11b^+$ MDSCs blocked S100A9 secretion and switched naive $Gr1^+CD11b^+$ cells to MDSCs (**Figures 4.1 and 4.2**); and because most immune-related lncRNAs can bind to and affect protein modifications and transport [23, 24, 26], we hypothesized that lncRNAs in exosomes contribute to S100A9 nuclear localization in $Gr1^+CD11b^+$ cells after early sepsis response. To test this, we profiled lncRNA expression in exosomes derived from sham and sepsis $Gr1^+CD11b^+$ cells, using mouse lncRNA expression Array V3.0 (Arraystar; Cat #AS-S-LNC-M). Sixteen lncRNAs simultaneously increased or decreased by ~2- to 4-fold in early and late sepsis $Gr1^+CD11b^+$ cells. Notably, lncRNA Hotairm1 (Hox antisense intergenic RNA myeloid 1) markedly increased in $Gr1^+CD11b^+$ cells after early sepsis. Using qPCR, we observed an 18-fold increase in Hotairm1 in exosomes from late sepsis $Gr1^+CD11b^+$ cells versus 2-fold in early sepsis cells (**Figure 4.3A**). A 29-fold increase in Hotairm1 transcripts occurred in total RNA extracted from late sepsis $Gr1^+CD11b^+$ cells (**Figure 4.3B**) and a 13-fold increase in plasma exosomes from late septic mice (data not shown). In addition, Hotairm1 was detected at high levels in naive $Gr1^+CD11b^+$ cells after culturing with exosomes derived from late sepsis $Gr1^+CD11b^+$ cells (**Figure 4.3C**).

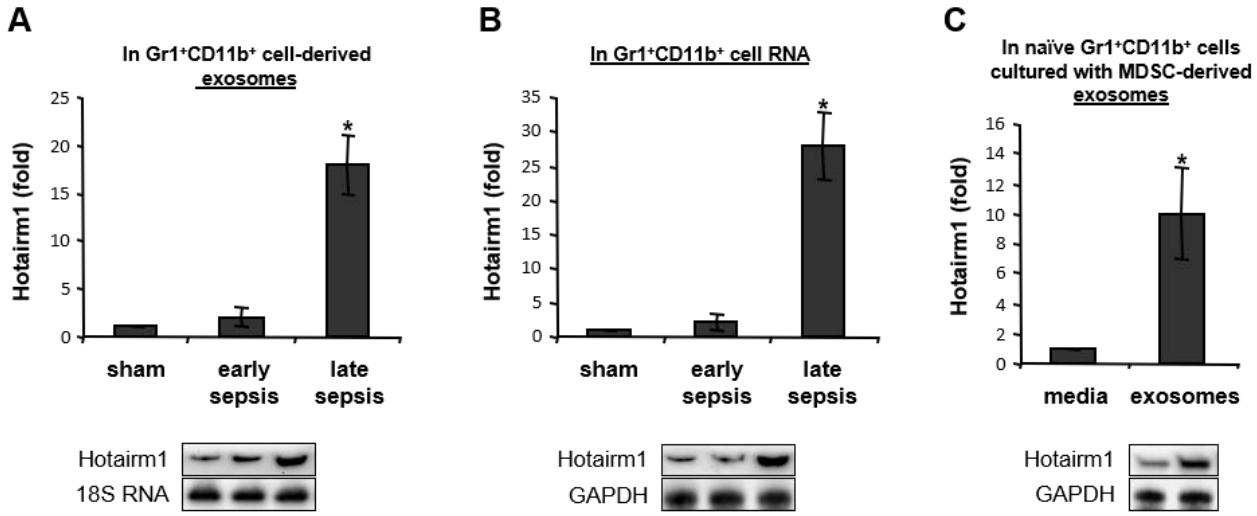

Figure 4.3: Hotairm1 expression is increased in late sepsis MDSCs. $Gr1^{+}CD11b^{+}$ cells were isolated from the bone marrow of sham and septic mice by positive selection using anti-Gr1 antibody and magnetic beads. (**A**) The cells were cultured for 24 hr in serum-free media. Exosomes were purified from the culture supernatants, and exosomal RNA extracted with exoRNeasy Starter kit. Levels of Hotairm1 were determined by RT-qPCR using RT lncRNA qPCR Assay Primers (Qiagen). Values were normalized to 18S RNA. (**B**) Total RNA was isolated from $Gr1^{+}CD11b^{+}$ cells using TRIzol reagent, and levels of Hotaim1 were determined as in *A*. Values were normalized to GAPDH RNA. (**C**) $Gr1^{+}CD11b^{+}$ cells isolated from naïve mice were cultured for 24 hr without or with exosomes (50 μg/well), purified from late sepsis MDSC culture. The cells were harvested, total RNA was extracted and levels of Hotairm1 were determined as in *B*. PCR was performed in duplicate. Data are presented relative to sham or media control (1-fold). Data in *A* and *B* are expressed as means ± SD of 6-9 mice/group from three experiments. Data in C are expressed as means ± SD of 7 cultures rom two experiments. $*p < 0.05$.

Knockdown of Hotairm1 attenuates immunosuppressive functions of late sepsis $Gr1^+CD11b^+$ MDSCs.

We then asked the question whether Hotairm1 knockdown affects $Gr1^+CD11b^+$ MDSC suppressive functions. Late sepsis $Gr1^+CD11b^+$ cells with Hotairm1 knockdown were co-cultured with $CD4^+$ T cells isolated from spleens of naive mice. T cell suppression assay showed that Hotairm1 knockdown significantly reduced the inhibitory effects of $Gr1^+CD11b^+$ MDSCs on $CD4^+$ T cell proliferation (**Figure 4.4A**). In addition, T cell activation, as measured by IFNγ production, increased significantly. Because $Gr1^+CD11b^+$ cells can take up purified exosomes (**Figure 4.3C**), we next examined immunosuppressive effects of exosomes lacking Hotairm1 on T cells. The $CD4^+$ T cells were co-cultured with naive $Gr1^+CD11b^+$ cells, which cannot suppress T cell, in the presence of exosomes purified from cultures of late sepsis $Gr1^+CD11b^+$ MDSCs in which Hotairm1 was knocked down first. Real-time PCR showed that the knockdown significantly reduced Hotairm1 levels in late sepsis MDSC exosomes (**Figure 4.4B**). Exosomes from late sepsis $Gr1^+CD11b^+$ MDSCs with control knockdown switched the naive $Gr1^+CD11b^+$ cells into immunosuppressive cells, as demonstrated by inhibition of T cell proliferation and IFNγ production (**Figure 4.4C**). Importantly, the inhibitory effects of these exosomes on T cell proliferation and IFNγ production decreased significantly after Hotairm1 knockdown (**Figure 4.4C**). These results support that Hotairm1 promotes the suppressive functions of late sepsis $Gr1^+CD11b^+$ MDSCs.

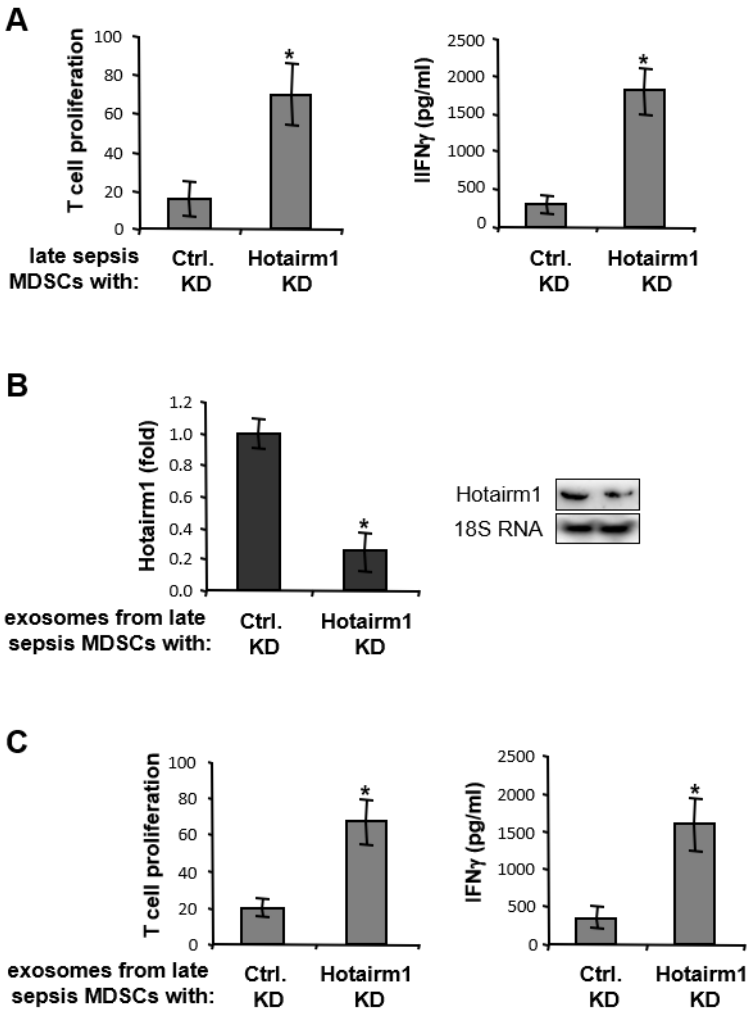

Figure 4.4: Knockdown of Hotairm1 in late sepsis MDSCs attenuates their immunosuppressive functions. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of late septic mice by positive selection using anti-Gr1 antibody and magnetic beads. The cells were transfected with Hotairm1-specific or scramble siRNAs for 36 hr. (**A**) Effects of MDSCs on T cell proliferation and activation. $CD4^+$ T cells were isolated from splenocytes of naive mice with anti-CD4 antibody and labeled with the fluorescent dye CFSE. The late sepsis $Gr1^+CD11b^+$ cells with Hotairm1 knockdown were then co-cultured (1:1 ratio). T cells were stimulated with anti-CD3 plus anti-CD28 antibodies (1 μg/ml each). After 3 days, the cells were harvested, and T cell proliferation and IFNγ production were determined as in Fig. 2. (**B and C**) Effect of exosomes lacking Hotairm1 on T cells cultured with naive $Gr1^+CD11b^+$ cells cultured. Late sepsis $Gr1^+CD11b^+$ cells with Hotairm1 knockdown were cultured for 24 hr in serum-free media. Culture supernatants were harvested, and exosomes were purified using exoEasy Maxi kit. (**B**) Levels of Hotairm1 in exosomal RNA was determined by RT-qPCR. Values were normalized to 18S RNA. (**C**) Spleen $CD4^+$ T cells were labeled and cultured (1:1 ratio) with naive $Gr1^+CD11b^+$ cells in the presence of the Hotairm1-lacking exosomes as described in Fig. 2. T cell proliferation and IFNγ production were measured as in *A*. Data are expressed as means ± SD of 5-6 mice/group from three experiments. *$p <$ 0.05. KD, knockdown.

Hotairm1 shuttles S100A9 protein to the nucleus in late sepsis $Gr1^+CD11b^+$ MDSCs.

Most immune-related lncRNAs function through interactions with proteins [24, 36]. To test whether Hotairm1 can bind S100A9 protein, we performed RNA-immunoprecipitation with cell lysates from late sepsis $Gr1^+CD11b^+$ cells. As shown in **Figure 4.5A**, PCR of cross-linked RNA extracted from S100A9 immunoprecipitates showed that Hotairm1 binds to S100A9 protein and transfers it from cytosol to nucleus in late sepsis $Gr1^+CD11b^+$ cells [15]. We tested the possibility that Hotairm1 shuttles S100A9 to the nucleus using western blot analysis, and found S100A9 moved from the nucleus to the cytosol after Hotairm1 knockdown (**Figure 4.5B**).

Next, we investigated Hotairm1-S100A9 interactions using early sepsis $Gr1^+CD11b^+$ cells, where S100A9 protein resides mainly in the cytosol [15]. The cells were transfected with Hotairm1 expression plasmid. Immunoblotting of S100A9 IP showed that Hotairm1 plasmid transfection did not change S100A9 protein levels (**Figure 4.5C**). RNA-immunoprecipitation showed that Hotairm1 binding to S100A9 increased significantly after Hotairm1 plasmid transfection (**Figure 4.5C**). Notably, Hotairm1 expression resulted in S100A9 protein shuttling from the cytosol to the nucleus (**Figure 4.5D**). Together, these results indicate that Hotairm1 mediates S100A9 protein localization in nucleus in $Gr1^+CD11b^+$ MDSCs in late sepsis.

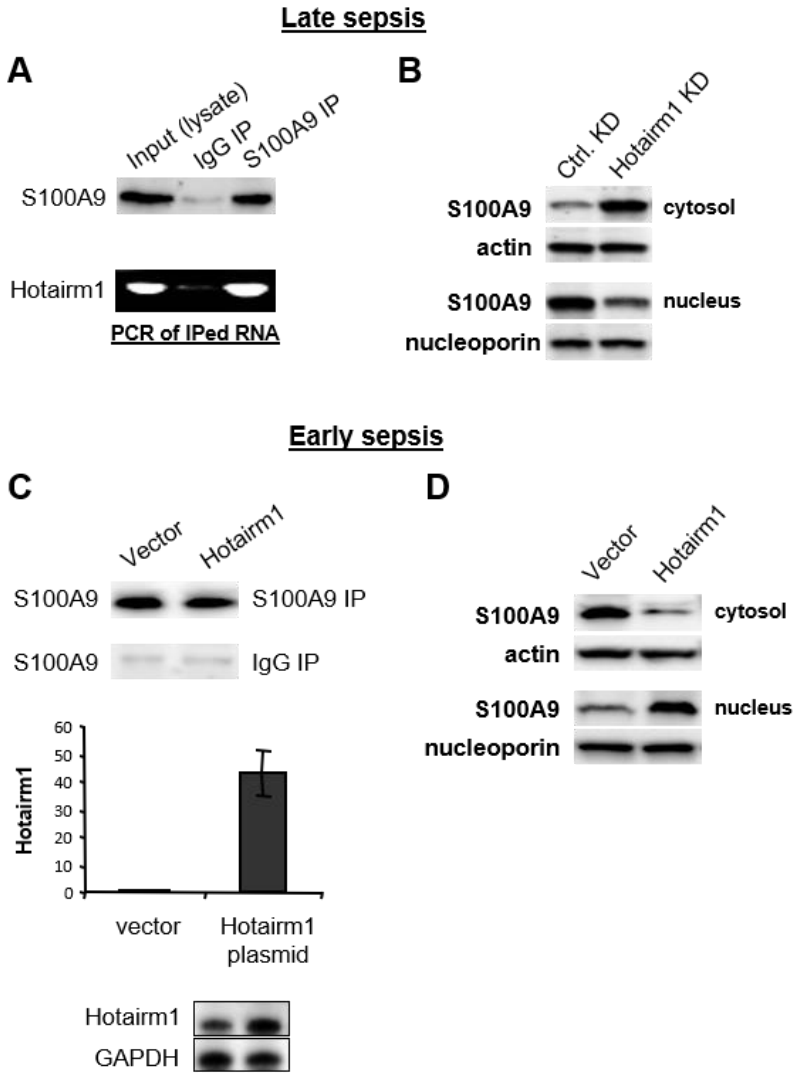

Figure 4.5: Hotairm1 binds to S100A9 in late sepsis MDSCs. $Gr1^+CD11b^+$ cells were isolated from the bone marrow of late septic mice using anti-Gr1 antibody and magnetic beads. (**A**) The cells (pooled from 2 mice) were treated with formaldehyde for reversible cross-linking of RNA-protein complexes. Cell lysates were prepared and immunoprecipitated with S100A9 or IgG antibody, and S100A9 levels were determined by western blot. The cross-linked RNA was extracted from the immunoprecipitated complexes using TRIzol reagent, and Hotairm1 levels were determined by standard PCR. (**B**) Hotairm1 knockdown relocalizes S100A9 in cytosol. Late sepsis $Gr1^+CD11b^+$ cells were transfected with Hotairm1-specific or scramble siRNAs for 36 hr. Cytoplasmic and nuclear proteins were extracted, and levels of S100A9 were determined by western blot. (**C**) Ectopic expression of Hotairm1 in early sepsis $Gr1^+CD11b^+$ cells moves S100A9 to the nucleus. The cells were transfected with Hotairm1 plasmid or control vector for 24 hr. Cell lysates were prepared and immunoprecipitated with S100A9 or IgG antibody, and S100A9 levels were determined by western blot. RNA was extracted from S100A9 IP and Hotairm1 levels were determined by qPCR. Values were normalized to IgG IP samples and presented relative to vector. (**D**) Protein extracts were prepared, and levels of S100A9 were determined by western blot. The results are representative of three experiments.

Increased Hotairm1 expression in early sepsis $Gr1^+CD11b^+$ MDSCs promotes an immunosuppressive phenotype.

Knockdown of Hotairm1 in late sepsis $Gr1^+CD11b^+$ MDSCs attenuated their suppressive effects on $CD4^+$ T cells (**Figure 4.4**). $Gr1^+CD11b^+$ MDSCs suppress T cells through the production of immunosuppressive mediators such as Arginase 1 and IL-10 [6]. We tested whether increasing Hotairm1 levels in early sepsis $Gr1^+CD11b^+$ MDSCs could affect Arginase 1 and IL-10 expressions. $Gr1^+CD11b^+$ cells from early septic mice were transfected with Hotairm1 expression plasmid, incubated with recombinant IL-10, which promotes S100A9 protein translocation into the nucleus [14] , and stimulated with bacterial LPS. Hotairm1 expression was assessed by qPCR (**Figure 4.6A**). Importantly, Hotairm1 upregulation significantly increased Arginase 1 expression and IL-10 production significantly compared with cells transfected with an empty vector (**Figures 4.6B and 4.6C**). In contrast, levels of the proinflammatory cytokine TNFα were decreased (**Figure 4.6D**). In addition, transfection of Hotairm1 expression plasmid into $Gr1^+CD11b^+$ cells from late septic mice had no significant effects on the production of these inflammatory mediators, indicating that further increasing Hotairm1 in late sepsis MDSCs does not impact their immunosuppressive phenotype (**Supplementary Figure 5 – Appendix C**). These results suggest that Hotairm1 upregulation during late sepsis induces the immunosuppressive phenotype of $Gr1^+CD11b^+$ MDSCs.

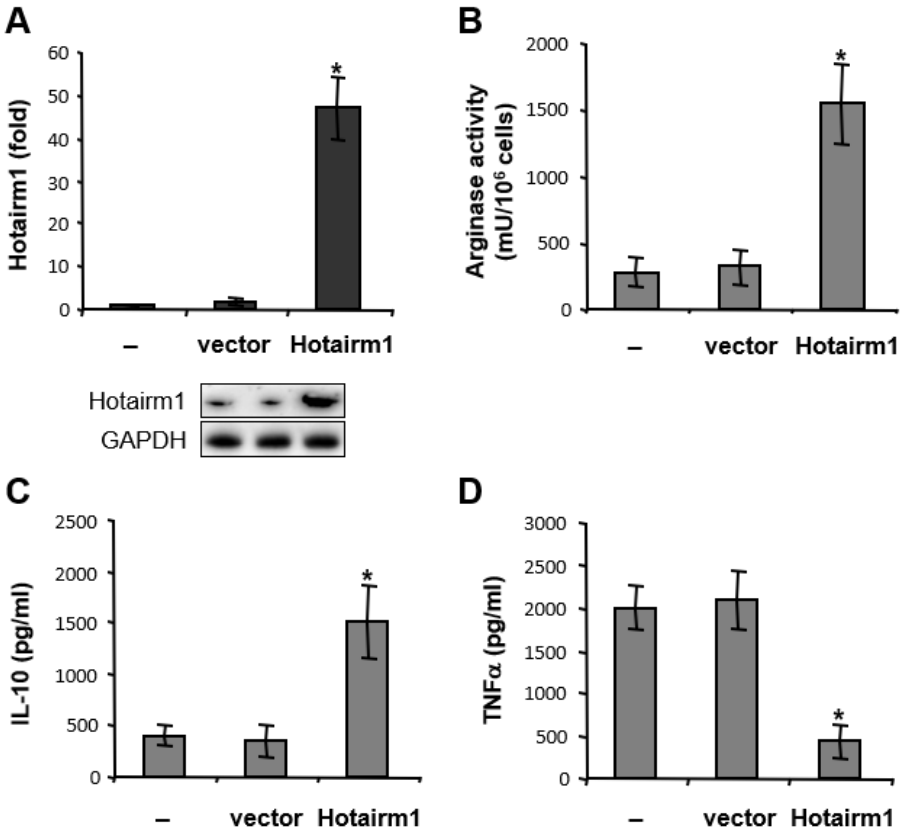

Figure 4.6: Overexpression of Hotairm1 in early sepsis Gr1$^+$CD11b$^+$ cells switches them to the immunosuppressive phenotype. Gr1$^+$CD11b$^+$ cells were isolated from the bone marrow of early septic mice using anti-Gr1 antibody and magnetic beads. The cells were transfected with a Hotairm1 expression plasmid or an empty vector and cultured with 10 ng/ml of recombinant mouse IL-10. (**A**) After 24 hr, a portion of the cells was used for Hotairm1 expression measurement by RT-qPCT. Then, the cells were washed and stimulated with bacterial lipopolysaccharide (*E. coli*; 0111:B4, Sigma, St. Louis, MO) were added (1 μg/ml) for 24 hr. (**B**) The cells were harvested, lysed and analyzed for arginase activity. (**C** and **D**) Levels of IL-10 and TNFα in the culture supernatants were measured by ELISA. Data are expressed as means ± SD of 5-6 mice (5-6 cultures/group) from two experiments. *$p < 0.05$.

Hotairm1 expression correlates with S100A9 localization in human MDSCs during sepsis. We have previously demonstrated that, similar to mice, S100A9 protein secretion is decreased significantly in late/chronic septic patients [15], pointing out to changes in S100A9 protein localization. We tested the possibility that Hotairm1 could modify S100A9 protein. First, we determined Hotairm1 levels in exosomes purified from patients plasma and in RNA from $CD33^{+}CD11b^{+}HLA\text{-}DR^{-}$ MDSCs, the equivalent of mouse $Gr1^{+}CD11b^{+}$ MDSCs [4, 37]. PCR analysis showed that Hotairm1 levels were significantly higher in plasma exosomes from late septic compared with early septic patients (**Figure 4.7A**). Hotairm1 levels were also significantly elevated in $CD33^{+}CD11b^{+}HLA\text{-}DR^{-}$ MDSCs in late septic patients (**Figure 4.7B**). Notably, higher Hotairm1 expression in late septic patients correlated with S100A9 protein accumulation in the nucleus (**Figure 4.7C**). In addition, RNA-immunoprecipitation showed Hotairm1 binding to S100A9 protein in $CD33^{+}CD11b^{+}HLA\text{-}DR^{-}$ MDSCs in late septic patients (**Figure 4.7D**). These results show that Hotairm1 promotes S100A9 protein accumulation in the nucleus in human MDSCs during chronic sepsis.

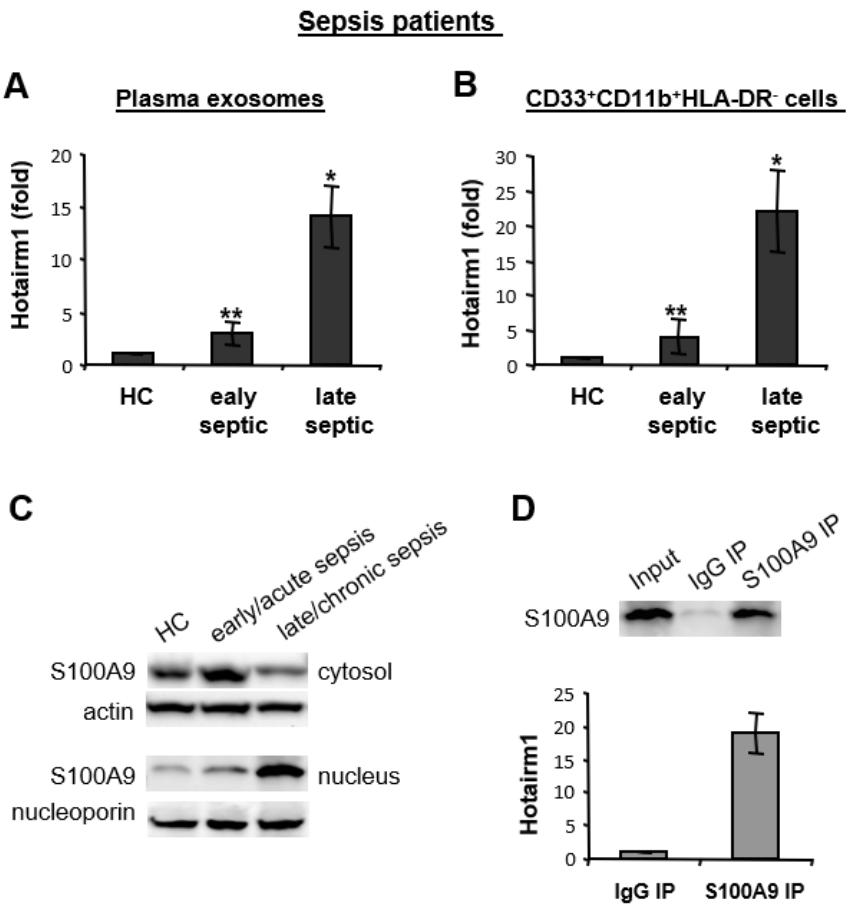

Figure 4.7: High levels of Hotairm1 in plasma exosomes and MDSCs from late septic patients. (**A**) Exosomes were purified from plasma and RNA was extracted using exoRNeasy Starter kit. (**B**) Peripheral blood $CD33^+CD11b^+HLA\text{-}DR^-$ cells were isolated by magnetic cell separation. PBMCs were first purified and depleted of the $HLA\text{-}DR^+$ cells. The $HLA\text{-}DR^-$ cell population was then subjected to positive selection with biotin-coupled anti-CD33 antibody, followed by anti-CD11b antibody. Total RNA was extracted using TRIzol reagent. Levels of Hotairm1 were determined by RT-qPCR as in Fig. 3. Values in *A* were normalized to 18S RNA, and values in *B* were normalized to GAPDH RNA. Data are expressed as means ± SD of 7-9 subjects/group and are presented relative to HC (1-fold). *$p < 0.05$, versus HC or early septic; **$p < 0.05$, versus. (**C**) S100A9 accumulates in cytosol in $CD33^+CD11b^+HLA\text{-}DR^-$ cells during late sepsis. Cytoplasmic and nuclear proteins were extracted from $CD33^+CD11b^+HLA\text{-}DR^-$ cells, and levels of S100A9 were determined by western blot. The results are representative of two western blots. (**D**) Hotairm1 binds S100A9 protein during late sepsis. Cell lysates were prepared from $CD33^+CD11b^+HLA\text{-}DR^-$ cells isolated from late septic patients (n=4) and immunoprecipitated with S100A9 or IgG antibody. S100A9 levels were determined by western blot. RNA was extracted from S100A9 IP, and Hotairm1 levels were determined by qPCR. Values were normalized to the input samples and presented relative to IgG IP. The results are representative of two immunoprecipitations.

Discussion

The major finding of this study is that Hotairm1 dependent translocation of S100A9 protein from the cytosol to the nucleus in $Gr1^+CD11b^+$ myeloid precursors predominates during the late/chronic stage of sepsis and supports MDSC repressor function. Our previous study showed that genetic deletion of S100A9 in mice improved late sepsis survival [15]. Mechanistically, increased expression of Hotairm1 associates with its binding to and shuttling S100A9 protein from cytosol to nucleus in MDSCs after early sepsis response. Ectopic expression of Hotairm1 in $Gr1^+CD11b^+$ cells from early/acutely septic mice with cytosolic S100A9 are proinflammatory [15]. Notably and of translational value, we found Hotairm1 binding to S100A9 in $CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs from chronically septic patients. Together, this study is compatible with the new concept that Hotairm1 acts as a molecular chaperone to modify S100A9 subcellular localization and switch $Gr1^+CD11b^+$ myeloid precursors into $Gr1^+CD11b^+$ MDSCs.

S100A9 expression chronically increases during early/acute sepsis, but significantly decreases in late septic mice concurrently with its translocation to the nucleus in $Gr1^+CD11b^+$ MDSCs [15]. In this study we made the novel observation that exosomes shed from late sepsis $Gr1^+CD11b^+$ MDSCs inhibited the LPS-induced secretion of S100A9 from early sepsis $Gr1^+CD11b^+$ cells. Notably, these exosomes also promoted an immunosuppressive phenotype in $Gr1^+CD11b^+$ cells, as evidenced by suppressing T cell activation and proliferation; a main feature of MDSCs [38]. Although mouse MDSCs have similar phenotype ($Gr1^+CD11b^+$) throughout sepsis, $Gr1^+CD11b^+$ cells that expand during early/acute sepsis for up to 5 days are not immunosuppressive [6, 27]. In contrast, and after day 6 in our model, sepsis $Gr1^+CD11b^+$ cells are immunosuppressive, and have higher levels of S100A9 protein in the nucleus [15]. Importantly, we observed significant increases in Hotairm1 levels in late sepsis $Gr1^+CD11b^+$

cells as well as in exosomes derived from their culture compared with early sepsis $Gr1^+CD11b^+$ cells. In addition, late sepsis $Gr1^+CD11b^+$ MDSCs with Hotairm1 knockdown could not inhibit T cells activation and proliferation. These findings suggest that Hotairm1 plays a pivotal role in generating immunosuppressive $Gr1^+CD11b^+$ after acute sepsis in mice and in established chronic sepsis in humans.

Hotairm1 is located at the 3' end of the HOXA cluster between HOXA1 and HOXA2 genes [39]. Limited studies on Hotairm1 show its expression is specific to the myeloid lineage [39]. Hotairm1 is low in human normal myeloid progenitors, but significantly increases in more mature myeloid leukocytes, with highest levels in neutrophils [39]. MDSCs are progenitors and precursors of monocytes, granulocytes and dendritic cells, and are considered intermediates in the myeloid differentiation program and unable to give rise to mature cells [2]. Thus, the elevated levels of Hotairm1 suggest that its expression is part of the immunopathological response to sepsis. In contrast, Hotairm1 expression is decreased in $CD33^+$ MDSCs from patients with lung cancer [40]. In that study, the common myeloid marker CD33 identified MDSCs, which is not sufficient to phenotype human MDSCs [37, 41]. In addition, Hotairm1 is significantly increased during retinoic acid-induced granulocytic differentiation of the NB4 human acute promyelocytic leukemia cell line [39]. Together, these data support that Hotairm1 expression pattern in immature myeloid cells depends on the underlying pathological conditions. In support of this, MDSCs expansion and suppressive functions depends largely on the inflammatory microenvironment in which they arise [37, 42].

In summary, we identified a novel molecular pathway leading to MDSC expansion during late/chronic sepsis. We discovered that following increased expression, lncRNA Hotairm1 binds to and transfers S100A9 protein from the cytosol to the nucleus, where its

promotes development of $Gr1^{+}CD11b^{+}$ MDSC repressor phenotype. Since genetic targeting of S100A9 in mice limits MDSC expansion and sepsis-associated immunosuppression [15], molecular targeting of Hotairm1 is a plausible drug target for reversing sepsis-induced chronic immunosuppression.

Authorship

T.A., I.B., and A.K. performed the experiments; D.Y. and Z.Q.Y. discussed the results and commented on the manuscript; C.E.M. discussed the results and reviewed the manuscript; and M.E. supervised the experimental design and research progress, and wrote the manuscript.

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Conflicts of Interest Disclosure

The authors declare no conflicts of interest.

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CHAPTER 5

SUMMARY

MDSCs contribute to high lethality rates in sepsis, but how sepsis induces and programs MDSCs is unclear. Previously, our lab showed that miR-21 and miR-181b are required to increase NFIA expression that elevate MDSCs (McClure et al. 2014). Also, the knockout of NFI-A in myeloid cells decreased MDSC development. Meanwhile, S100A9 was found to not only be critical for Gr1+CD11b+ cell expansion, but also for programming them into the immunosuppressive MDSC phenotype (Dai et al. 2017). The research presented here reveals and discusses some of the underlying mechanisms behind MDSC expansion and reprogramming in late sepsis. The first manuscript discusses the pathway that miR-21 and miR-181b stabilize NFI-A mRNA to induce MDSCs expansion in late sepsis (Bah et al. 2020). In the second manuscript, we demonstrate the regulatory mechanism in which S100A9 promotes miR-21 and miR-181b expression to expand immunosuppressive MDSCs in late sepsis (Alkhateeb et al. 2019). In the third manuscript, we present findings indicating the role of a lncRNA, Hotairm1, to translocate S100A9 from the cytosol to the nucleus. These results suggest that targeting NFI-A, S100A9, and Hotairm1 in MDSCs during sepsis may enhance resistance to uncontrolled infection.

MDSC Expansion

Post-Transcriptional Regulation of NFI-A

Post-transcriptional regulation of genes plays a critical role in the immune response during inflammatory conditions. It can either improve or worsen the disease progression as well as prevent autoimmunity or immunodeficiencies (Anderson 2008, 2010). By controlling mRNA stability and translation, it may influence myeloid gene expression and function during the

immune response (Chang et al. 2012; Gerstberger et al. 2014). Also, the interactions between various RNA binding proteins (RBPs) and miRNAs can determine the levels of target mRNA expression (Anderson 2008; Kafasla et al. 2014).

Human antigen R (HuR) is a universally expressed RBP that affects post-transcriptional gene regulation in a wide range of cell types, including myeloid cells (Chang et al. 2012; Poria et al. 2015; Siang et al. 2020). HuR regulates genes important for inflammation, tumor survival, embryonic development, apoptosis, and immune responses (Abdelmohsen and Gorospe 2010; Abdelmohsen et al. 2008; Gupta et al. 2011; Katsanou et al. 2009; Poria et al. 2015; Zhu et al. 2015). For instance, HuR regulates immune homeostasis by stabilizing mRNAs of proinflammatory cytokines (Anderson 2008; Silanes et al. 2004). In mice with myeloid cells lacking HuR, inflammatory conditions were exacerbated due to an elevated release of proinflammatory cytokines (Yiakouvaki et al. 2012). This was suggested to be due to an absence of inhibitory mechanisms that influence the cytokines' mRNA stability or translation (Yiakouvaki et al. 2012). In contrast, in mice with HuR upregulation in myeloid cells, suppression of the inflammatory response occurred with lower inflammatory mediators secreted from macrophages (Katsanou et al. 2005; Rajasingh et al. 2006; Yiakouvaki et al. 2012). These studies suggest that although HuR stabilizes mRNAs of proinflammatory cytokines, HuR can also inhibit mRNA translation of these AU rich transcripts by working with mRNA translational silencers (Katsanou et al. 2005; Rajasingh et al. 2006; Yiakouvaki et al. 2012). Thus, HuR may act by inducing post-transcriptional silencing (Yiakouvaki et al. 2012). In most chronic inflammatory diseases, HuR upregulation is associated with a persistent increase in pro-inflammatory mediators (Esnault and Malter 2003; Katsanou et al. 2005; Zhou et al. 2007). On the other hand, immunosuppressive mediators, such as IL-10 and IL-19, have been shown to

influence HuR function (Cuneo et al. 2010; Gracias and Katsikis 2011; Krishnamurthy et al. 2009; Oconnell et al. 2012; Rajasingh et al. 2006). In myeloid cells, IL-10 inhibits HuR function and stabilizes mRNAs of inflammatory cytokines (Krishnamurthy et al. 2009; Rajasingh et al. 2006). Thus, this response may be beneficial in suppressing inflammation when needed (Srikantan and Gorospe 2012). HuR may also interact with miRNAs to control mRNA translation and thus, the function of immune cell mediators (Srikantan et al. 2012). This interaction can be either in an agonistic or antagonistic manner. For instance, if HuR binding affinity to a mRNA transcript prevails, this leads to increased gene expression, but if a miRNA interacts with HuR, it will decrease gene expression (Srikantan and Gorospe 2012). In this context, interaction of HuR and miRNA will destabilize the target mRNA (Srikantan and Gorospe 2012).

HuR regulates mRNA stability and translation via binding to regulatory elements rich in AU within the 3' UTR sequence of the target mRNA transcript (Brennan and Steitz 2001; Casolaro et al. 2008; Kafasla et al. 2014; Mukherjee et al. 2011; Techasintana et al. 2015). In our polymicrobial sepsis model, the interaction between HuR and miR-21 and miR-181b increased NFI-A mRNA stability and gene expression in Gr1+CD11b+ cells. In the absence of miR-21 and miR-181b, the RBP - CUG-binding protein 1 (CUGBP1), which promotes mRNA decay, binds to a GU-rich sequence of NFI-A 3' UTR and destabilizes its mRNA (Moraes et al. 2006; Rattenbacher et al. 2010). Mechanistically, we found that argonaute 2 (Ago2), which forms the RISC protein complex that induces target mRNA decay, bind to and interacts with CUGBP1 at the NFI-A 3'UTR (Lal et al. 2004; Pare et al. 2013; Valencia-Sanchez et al. 2006). Thus, these results reveal a mechanistic pathway that leads to the upregulation of NFI-A in sepsis. Importantly, previous studies in our lab have shown that septic mice deficient in NFI-A

displayed significant decreases in MDSC accumulation (McClure et al. 2014, 2016). In parallel, the ectopic expression of NFI-A in myeloid precursors promoted MDSC expansion. Thus, increases in NFI-A mRNA stability expression underlies MDSC development in sepsis.

MDSC Programming

S100A9 Axis

We found that translocation of the S100A9 protein from the cytosol to the nucleus in Gr1+CD11b+ cells during the late sepsis phase maintains miR-21 and miR-181b expressions through a feedback loop involving STAT3 and C/EBPb, leading to NFI-A upregulation and MDSC expansion (McClure et al. 2017). Importantly, the *S100a9* gene ablation decreased MDSC accumulation, promoted normal myeloid differentiation, and improved survival in late, but not early septic mice (Dai et al. 2017). In these knockout mice, expression of miR-21 and miR-181b decreased in the late sepsis Gr1+CD11b+ cells, which were generated at baseline levels, as found in wild-type mice, and had no immunosuppressive functions (Dai et al. 2017). It was found that NFI-A was absent in the S100A9 knockout mice during late sepsis only (Dai et al. 2017). However, p-STAT3 and C/EBPβ levels were similar in both types of mice, with and without S100A9. These results reveal that S100A9 may be responsible for inducing/maintaining MDSC expansion during the late sepsis by sustaining miR-21 and miR-181b expressions and subsequent NFI-A upregulation.

In this manuscript, the pathway of S100A9-mediated expansion of MDSCs during late sepsis was described (Alkhateeb et al. 2019). S100A9 induces the pStat3-C/EBPβ protein complex binding at the miR-21 and miR-181b promoters to induce their expression, which further elevates MDSC repressor expansion and immunosuppression in late sepsis (Alkhateeb et

al. 2019). Mechanistically, S100A9 may change chromatin architecture around miRNA promoters to allow pStat3- C/EBPβ complex binding. In this context, the anti-inflammatory cytokine IL-10 was necessary for promoting S100A9 translocation to the nuclear compartment in Gr1+CD11b+ cells, and thus enhancing MDSC expansion (Alkhateeb et al. 2019).

S100A9 and its dimerization partner S100A8 are constitutively expressed in myeloid cells, but their expression generally decreases in differentiated cells (Cheng et al. 2008). This may partially explain the high levels of S100A9 in late sepsis MDSCs, which are characteristically immature myeloid cells that do not follow the normal differentiation and maturation pathway, and instead acquire immunosuppressive functions (Dai et al. 2017). During late (i.e., post-acute) sepsis, nuclear S100A9 exerts its anti-inflammatory action by programming Gr1+CD11b+ cells into the MDSC phenotype (Dai et al. 2017). Unlike the proinflammatory role of cytosolic/secreted S100A9 in early sepsis, nuclear build-up of S100A9 in Gr1+CD11b+ MDSCs, during late sepsis, prevents its secretion and pro-inflammatory effects, which further highlight its role as an anti-inflammatory mediator in late sepsis (Dai et al. 2017). Consequently, we suggest that nuclear S100A9 build up during late sepsis may act as a transcription co-factor by modifying the chromatin structure of immunosuppressive mediators such as Agr1, IL-10, NO, and TGF-b, thus increasing their expression in MDSCs (Alkhateeb et al. 2019). In conclusion, the results brought forth in this manuscript bring attention to the transcriptional role that nuclear S100A9 may play in sepsis-induced immunosuppression and increasing the suppressive MDSC phenotype.

Previous studies in this laboratory have shown that the immunosuppressive cytokine IL-10 is elevated and sustained during the late phase of sepsis (Brudecki et al. 2012). Interestingly, major metabolic and epigenetic modifications were found to coordinate the programming of

myeloid cells during sepsis shift from the early/acute hyperinflammatory phase to the late anti-inflammatory/immunosuppressive phase (Vachharajani and McCall 2019). This may increase and maintain IL-10 during the late phase of sepsis permitting heterochromatin modifications to proceed. Alterations in DNA methylation status were found to be correlated with elevated IL-10 levels, which are also increased in sepsis patients (Hedrich and Bream 2010; Hedrich et al. 2010; Lorente-Sorolla et al. 2019). Under chronic inflammatory conditions, IL-10 levels were increased and correlated with site-specific DNA demethylation while DNA methylation suppressed IL-10 secretion (Hwang et al. 2018). On the other hand, histone acetylation promoted STAT3 binding on IL-10 promoters, increasing its transcription (Hossain et al. 2013). This may further support the hypothesis that elevated IL-10 in myeloid cells may contribute to epigenetic programming of Gr1+CD11b+ myeloid precursors into immunosuppressive MDSCs.

Hotairm1 Axis

Like S100A9, we found Hotairm1 accumulates in the nucleus in MDSCs. LncRNAs nuclear localization may be involved in the metabolic and epigenetic reprogramming of specific gene transcription by altering DNA methylation, histone acetylation, and chromatin structure (Budhwar et al. 2018; El Gazzar 2015; Tian et al. 2018; Zhang et al. 2016). Importantly, lncRNAs may also influence myeloid cell differentiation (Chen et al. 2016). In some cancer studies, Hotairm1 levels inversely correlated with MDSC development (Montoya et al. 2019; Tian et al. 2018).

In sepsis, we found that Hotairm1 plays a pivotal role in MDSC expansion. In our third manuscript, we reported that Hotairm1 levels in MDSCs were significantly increased compared to other lncRNAs during late sepsis. Hotairm1 acted as a molecular chaperone to recruit S100A9

to the nucleus during late sepsis. Previous studies in this laboratory have shown that inhibition of S1009 secretion and its transfer to the nucleus in late sepsis correlates with MDSC development (Dai et al. 2017). Our results suggested that Hotairm1 programs Gr1+CD11b+ cells from an activator to MDSC repressor state, thus inhibiting T-cell proliferation and activation (i.e., IFN-g production). Notably, we found that exosomes shed from late septic MDSCs contained high levels of Hotairm1, and transfection of these exosomes into early sepsis Gr1+CD11b+ cells, which unlike MDSCs are not immunosuppressive, disrupted their normal ability to secrete S100A9 after LPS stimulation. Also, knockdown of Hotairm1 in MDSCs inhibited S100A9 transfer from the cytosol to the nucleus and decreased their immunosuppressive activities. Translationally, in MDSCs isolated from late sepsis patients, Hotairm1 levels were elevated and increased S100A9 build up in the nucleus by binding to S100A9 and controlling its relocation. Collectively, these results suggest that Hotairm1 programs Gr1+CD11b+ myeloid precursors into MDSCs, which may inform a new molecular switch in the pathway leading to post-sepsis immunosuppression.

Future Studies

Future studies should build on these findings and investigate whether S100A9 acts as a transcription co-factor to modify gene expression of immunosuppressive mediators in sepsis environment and whether epigenetic mechanisms drive this process in late sepsis.

The mechanism that induces Hotairm1 expression in late sepsis is unknown and may be of interest to elaborate on. On the other side, it is likely possible that a relationship exists between HuR and Hotairm1 in promoting MDSC expansion in sepsis through a somewhat divergent mechanism. HuR is important for nuclear localization of various proteins that may

function as transcription factors or co-factors (Zhang et al. 2016). Also, HuR has been shown to play a critical role in the nuclear import of retinoic acid, which stabilizes HuR transcripts (Liepelt et al. 2016; Zhang et al. 2016). In two separate studies, Hotairm1 was induced by retinoic acid (Lemons and McGinnis 2006; Rinn et al. 2007). Thus, HuR may be vital for nuclear localization of Hotairm1 in late sepsis to permit MDSC programming.

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APPENDICES

APPENDIX A

Supplementary Figures

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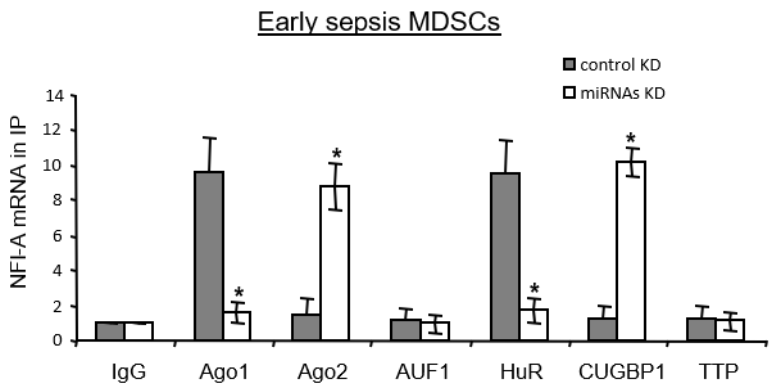
7561 TACAATTACT GGAAGCAAGT ACTGCATTTC CTATGCAACA AAAAAAGGAA AAATAAAAAA
7621 AAAATTGCTA ATGCTACGGA AAAAAAAAAA AAAAAA
```

Supplementary Figure 1: Mouse Nfia 3'UTR Sequence: 7641 nt, which span the region from +1634 to +9274 bp of the mRNA sequence.

The miR-21a-5p and miR-181b-5p binding site squences are highlighted in yellow and blue respectively. The PCR primers used to amplify the UTR sequence are shown in italic underlined. The GRE (GU-rich element) is highlighted in pink.

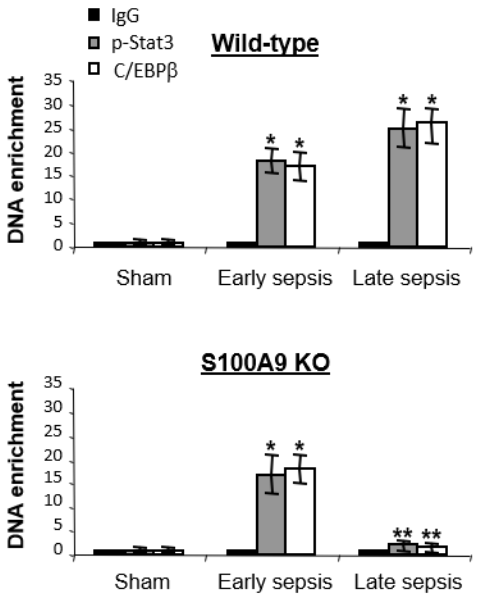
The sequence was downloaded from UCSC genome browser (RefSeq: NM_001122952.1).



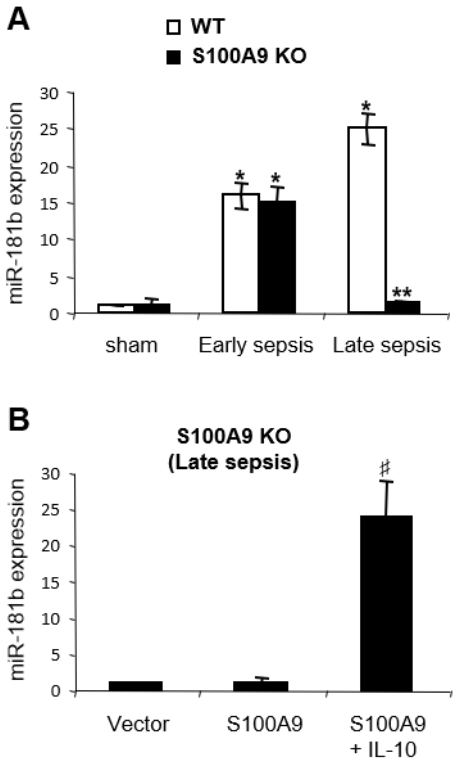
Supplementary Figure 2: Binding of RNA regulatory proteins to NFI-A mRNA in early sepsis Gr1+CD11b+ cells after miR-21 and miR-181b knockdowns. Gr1+CD11b+ cells were isolated from the bone marrow of early septic mice. The cells were transfected with miR-21 and miR-181b inhibitors or negative control inhibitor, and cultured for 36 hr. Whole cell lysates were immunoprecipitated with antibody against Ago1, Ago2, HuR, AUF1, TTP, CUGBP1 or IgG isotype control antibody. RNA was extracted from the immunoprecipitates and analyzed by RT-qPCR for the presence of NFI-A mRNA using primers that amplify the 3'UTR sequence. Values were normalized to GAPDH mRNA. Data are means ± SD from 7 mice per group and are presented relative to IgG samples (1-fold). *$p < 0.05$ vs. control KD. IP, immunoprecipitate; KD, knockdown.

APPENDIX B

Supplementary Figures



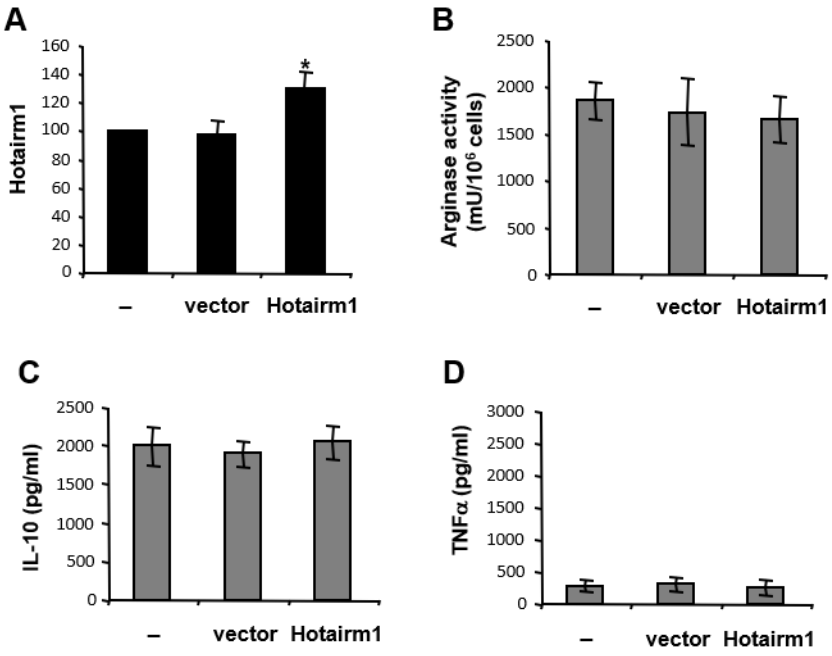
Supplementary Figure 3: Lack of S100A9 expression prevents assembly of the pStat3-C/EBPb protein complex at the miR-181b promoter in Gr1+CD11b+ cells in late sepsis. Gr1+CD11b+ cells were isolated from the bone marrow of sham and septic mice (n = 3-4 mice per group), and ChIP and PCR were performed as described in Fig. 1. *$p < 0.05$ vs. sham; **$p < 0.05$ vs. early sepsis. The results are representative of three independent experiments. KO, knockout.



Supplementary Figure 4: Ectopic expression of S100A9 in late sepsis Gr1+CD11b+ cells from the S100A9 knockout mice restores miR-181b expression. Gr1+CD11b+ cells were isolated from the bone marrow, treated, and miR-181b expression was determined as described in Fig. 4. Data are means ± s.d. (n = 4-5 mice per group). *$p < 0.05$ vs. sham; **$p < 0.05$ vs. wild-type; ♯$p < 0.05$ vs. S100A9 or S100A9 + IL-6. The results are representative of three independent experiments. KO, knockout.

APPENDIX C

Supplementary Figure



Supplementary Figure 5: Production of inflammatory mediators by late sepsis Gr1+CD11b+ cells after transfection with Hotairm1 expression plasmid. Gr1+CD11b+ cells were isolated from the bone marrow of late septic mice. The cells were transfected with Hotairm1 expression plasmid. **(A)** After 24 hr, a portion of the cells was used to measure Hotairm1 level by RT-qPCR. Values were normalized to GAPDH RNA and are presented relative to cells without transfection (100 %). The cells were washed and stimulated with 1 mg/ml of bacterial lipopolysaccharide (LPS) for 24 hr. (**B**) The cells were harvested, and cell lysates were used for determining levels of arginase activity. (**C and D**) Levels of IL-10 and TNFa in the culture supernatants were measured by ELISA. Data are expressed as means ± SD of 5 mice (5 cultures/group). *$p < 0.05$.

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Abstracts and Publications:

Alkhateeb T, Bah I, Kumbhare A, McCall CE, El Gazzar M. S100A9-Induced Reprogramming of Myeloid Cells During Sepsis. Abstract. Critical Care Medicine. (2020) 48(1):12 https://doi.org/10.1097/01.ccm.0000618592.67319.a3

Bah I, **Alkhateeb T**, Kumbhare A, Youssef D, Yao ZQ, Hawkins G, McCall CE, El Gazzar M. HuR promotes miRNA-mediated upregulation of NFI-A protein expression during murine sepsis. Molecular Immunology. (2020) 123:97-105 https://doi.org/10.1016/j.molimm.2020.04.014

Alkhateeb T, Bah I, Kumbhare A, Youssef D, McCall CE, El Gazzar M. HuR promotes miRNA-mediated upregulation of NFI-A protein expression during murine sepsis. Abstract. Midwest Clinical and Translational Research Meeting of CSCTR and MWAFMR. The Journal of Investigative Medicine. (2020) 68:1107. http://dx.doi.org/10.1136/jim-2020-MW

Alkhateeb T, Kumbhare A, Bah I, Youssef D, Yao ZQ, McCall CE, El Gazzar M. Long non-coding RNA Hotairm1 promotes S100A9 support of MDSC expansion during sepsis. *Manuscript under review*

Alkhateeb T, Kumbhare A, Bah I, Youssef D, Yao ZQ, McCall CE, El Gazzar M. S100A9 maintains myeloid-derived suppressor cells in chronic sepsis by inducing miR-21 and miR-181b. Molecular Immunology. (2019) 112:72–81. https://doi.org/10.1016/j.molimm.2019.04.019

Presentations:

Invited Speaker- Star Research Oral Presentation Alkhateeb T, Bah I, Kumbhare A, McCall CE, El Gazzar M. S100A9-Induced Reprogramming of Myeloid Cells during Sepsis. *Society of Critical Care Medicine, Critical Care Congress*. Feb 2020

Oral & Poster Presentations Alkhateeb T, Kumbhare A, Bah I et al. S100A9 sustains myeloid-derived suppressor expansion and immunosuppression during chronic murine sepsis. *Appalachian Student Research Forum, ETSU.* Apr 2019

Honors and Awards:

Trainee Travel Award Midwest Clinical and Translational Research Meeting. Apr 2020

Star Research Achievement Award Society of Critical Care Medicine. Feb 2020

Exceptional First Place Winner in Doctoral -Medicine & Diseases- Oral + Poster Presentations at the ETSU Appalachian Student Research Forum. Apr 2019