

FUNCTIONAL ROLE OF THE TLR4 SIGNALING PATHWAY IN
THE BONE MARROW RESPONSE TO SEPSIS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Medical and Molecular Genetics,
Indiana University

August 2015

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENTS

First and foremost, I want to express my sincere gratitude to my mentor Dr. Nadia Carlesso for granting me the opportunity to study in her lab. Dr. Carlesso is a passionate scientist whose enthusiasm greatly inspires me. Throughout the years, she has guided and supported me with tremendous patience and caring. I enjoyed very much when we work together on my fellowship proposal and manuscript, from which my ability of data interpretation and troubleshooting have been greatly improved. Dr. Carlesso's meticulousness and diligence towards science have influenced me and will guide me toward becoming an independent scientist in the future. Again, I am very grateful for all the merits I learned from Dr. Carlesso and I would not have made it without her guidance.

I am truly grateful for all the help and support from my committee members: Dr. Angelo Cardoso, Dr. Mircea Ivan, Dr. Janice S. Blum, Dr. Yunlong Liu and Dr. Brittney Herbert. They always provide me with enlightening ideas and suggestions from a different perspective on my projects. I never fail to have a great time discussing scientific ideas and problems with them. Especially, I want to thank Dr. Angelo Cardoso for proof reading my thesis with significant details; Dr. Janice S. Blum for giving me good advices on the current project and future academic pursuits; Dr. Yunlong Liu for helping me to improve my biostatistic analysis; Dr. Mircea Ivan for kindly sharing the miR-21flox/flox mice with us to pursuit my project and Dr. Brittney Herbert for her patient and kind support.

I would like to thank American Heart Association for awarding me the pre-doctoral fellowship. I still remember the joy when I find out my proposal was

approved. I felt much rewarded for my hard work and encouraged to work even harder.

I would love to thank my brilliant and kind collaborators: Dr. Henrique Serezani and Dr. Suojuan Wang for helping me with the polymicrobial sepsis model.

I also would love to thank my current and previous labmates: Sonia Rodruiguez, Lin Wang, Christen Mumaw, Liyun Cao, Amy Zollman, Jonathan Buchler, Yukiko Kitagawa and Youngwoong Kim. I want to thank them for supporting me whenever I need help. Thank you for making my everyday life in the lab so enjoyable.

I am forever grateful for the unconditional love from my parents. Their unwavering support gave me the courage to undertake this long journey of academic pursuit. I also want to thank my grandparents who are retired biology teachers. I would not have fell in love with biology since when I was a child without their enlightenment.

I am also very thankful for my friends outside the department: Suizhen Lin, Chong Yan, Yi-chun Chen, Claire Li, Liren Tu, Xin Tong, Sam Gilmore, Ameya Sharma, Lingnan Li and Huimin Lou for their friendship and great accompany during my stay in Indianapolis.

My optimism originates from all the wonderful people around me and I believe it will help me to move forward to the next chapter of my scientific pursuit.

Huajia Zhang

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Marrow RESPONSE TO SEPSIS

Sepsis is a clinical syndrome due to a systemic inflammatory response to severe microbial infection. Little is known about the changes in the bone marrow (BM) and how they affect the hematopoietic response to bacterial infection. Using an animal model of severe sepsis induced by *Pseudomonas aeruginosa*, we have previously reported that hematopoietic stem cells (HSC) undergo a significant expansion in the BM accompanied with myeloid suppression. This bone marrow response was Toll-like Receptor 4 (TLR4)-dependent. TLR4 is activated by bacterial lipopolysaccharide (LPS) and signals through two major independent downstream molecules: TRIF and MyD88. In the present study, I found that the TLR4/TRIF and the TLR4/MyD88 pathways contribute in a distinct manner to the BM response to *P. aeruginosa*'s LPS. TRIF plays a major role in the expansion of the HSC pool, whereas MyD88 is required for myeloid suppression. Following LPS stimulation, HSCs enter in the cell cycle, expand and exhaust when transplanted in healthy mice. Loss of TRIF rescued completely the long-term engraftment and multilineage reconstitution potential of septic HSCs, but did not affect myeloid differentiation. Conversely, MyD88 deficiency prevented completely the myeloid suppression in the myeloid progenitors, but conferred limited protective effects on the HSC function.

It is of great therapeutic value to identify the downstream molecules involved in TLR4/MyD88 dependent myeloid suppression. I found miR-21, a microRNA that is involved in inflammation, was up-regulated upon LPS challenge in a MyD88-dependent manner. However, deletion of miR-21 in the BM did not rescue LPS-induced bone marrow dysfunction, demonstrating that miR-21 is not a critical regulator in these processes. Further studies are warranted to determine the precise molecular mechanisms involved in the complex pathogenesis of BM response to sepsis. Taken together, my results show for the first time that the TLR4/TRIF signaling as a key mediator of HSC damage during acute LPS exposure and that activation of the TLR4/MyD88 signaling pathway play a dominant role in myeloid suppression. These results provide novel insights into our understanding of the molecular mechanisms underlying bone marrow injury during severe sepsis and may lead to the development of new therapeutic approaches in this disease.

Nadia Carlesso, M.D., Ph.D.

TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
CHAPTER ONE.....	1
INTRODUCTION	1
SEPSIS	1
ANIMAL MODEL OF SEPSIS	3
PATHOGENESIS OF SEPSIS	8
INFLAMMATORY MODULATION OF HEMATOPOIESIS	22
INFLAMMATORY REGULATION OF MICRORNA	30
SUMMARY AND SIGNIFICANCE	33
HYPOTHESIS	35
CHAPTER TWO.....	38
MATERIALS AND METHODS.....	38
MATERIALS.....	38
METHODS	44
CHAPTER THREE	49
ROLE OF MYD88 AND TRIF IN MEDIATING BM RESPONSE DURING <i>PSEUDOMONAS AERUGINOSA'S</i> LPS CHALLENGE.....	49

INTRODUCTION.....	49
RESULTS.....	50
3.1 <i>P. aeruginosa</i> 's LPS induced LSK expansion in the BM is TLR4/TRIF dependent.....	50
3.2 The TLR4/TRIF-dependent pathway is required for the LSK expansion in polymicrobial sepsis.....	53
3.3 Expansion of LSK is marginally due to upregulation of Sca-1 in the myeloid progenitors.....	55
3.4 <i>P. aeruginosa</i> 's LPS induced cell cycle activation in LSK cells in a TRIF and MyD88 dependent manner.....	58
3.5 Absence of MyD88 but not TRIF prevents LPS-induced myelosuppression in vivo.....	61
3.6 Absence of MyD88 but not TRIF prevents myelosuppression during polymicrobial sepsis.....	63
3.7 Activation of MyD88 impairs CMP's ability to differentiate	65
3.8 <i>P. aeruginosa</i> 's LPS induced distinct cytokine production profile in WT, MyD88 ^{-/-} and TRIF ^{-/-} mice.....	67
3.9 Both cell-autonomous and non-cell autonomous activation of MyD88 contributes to myelosuppression and neutropenia induced by LPS.....	69
3.10 <i>P. aeruginosa</i> 's LPS increased BM vascular permeability in a MyD88 dependent manner.....	72

CONCLUSION AND DISCUSSION.....	74
CHAPTER FOUR	82
ABLATION OF THE TRIF and MYD88 PATHWAY PROTECTS HSC FUNCTION DURING <i>P. AERUGINOSA</i> 'S LPS CHALLENGE	82
INTRODUCTION.....	82
RESULTS.....	84
4.1 Loss of TRIF and MyD88 rescue the long-term engraftment efficiency of HSC after LPS challenge.	84
4.2 Increased contribution of LPS-MyD88 ^{-/-} and LPS-TRIF ^{-/-} HSC to the recipient BM following transplantation.....	87
4.3 Different impact of TRIF and MyD88 deletion on HSC multilineage reconstitution following endotoxic injury.	92
4.4 MyD88 ^{-/-} and TRIF ^{-/-} LSK cells were able to maintain the expression of essential regulators for myelopoiesis.	94
4.5 Downregulation of essential regulators for HSC function and myelopoiesis persisted at 24 weeks post-transplantation.	96
CONCLUSION AND DISCUSSION.....	98
CHAPTER FIVE	104
FUNCTIONAL ROLE OF MIR-21 IN LPS-INDUCED DYSFUNCTIONAL HEMATOPOIESIS.....	104
INTRODUCTION.....	104

RESULTS.....	106
5.1 miR-21 is differentially expressed in the BM hematopoietic subsets....	106
5.2 <i>P. aeruginosa</i> 's LPS upregulates miR-21 in the LSK cells in a MyD88-dependent manner.	108
5.3 Development of tissue-specific miR-21 deletion mouse model.....	110
5.4 miR-21 is dispensable for the BM response to endotoxemia.	112
CONCLUSIONS AND DISCUSSION	114
CHAPTER SIX.....	115
FUTURE DIRECTIONS.....	115
REFERENCES.....	120
CURRICULUM VITAE	

LIST OF TABLES

Table 1.1	25
Table 2.1	40
Table 2.2	40
Table 2.3	40
Table 2.4	41
Table 2.5	42
Table 2.6	43
Table 2.7	46
Table 4.1	89

LIST OF FIGURES

Figure 1.1 Simplified view of the TLR4 signaling pathway.....	18
Figure 1.2 Hierarchy of hematopoiesis in the BM of adult mice.....	26
Figure 1.3 Working model	37
Figure 3.1 <i>P.aeruginosa</i> 's LPS-induced LSK expansion in the BM in a TLR4/TRIF dependent manner.....	52
Figure 3.2 BM responses in the HSPC compartment during polymicrobial sepsis.	54
Figure 3.3 GFP expressions in different cellular subsets in the BM during LPS challenge.	57
Figure 3.4 Acute LPS exposure increase LSK proliferation in a TRIF and MyD88 dependent manner.	60
Figure 3.5 LPS induced myeloid suppression in a MyD88 dependent manner. .	62
Figure 3.6 Polymicrobial sepsis induced myeloid suppression in the BM in a MyD88 dependent manner.	64
Figure 3.7 CMP and GMP differentiation assay.....	66
Figure 3.8 Cytokine productions in the serum of LPS-challenged WT, MyD88 ^{-/-} and TRIF ^{-/-} mice.....	68
Figure 3.9 Contribution of cell-autonomous and non-cell autonomous effect of MyD88 activation to myeloid suppression in the BM during LPS exposure.	71

Figure 3.10 Loss of MyD88 prevents calvarium BM vascular injury during LPS challenge.	73
Figure 3.11 Signaling pathways engage with the MyD88 adaptor protein.	81
Figure 4.1 Loss of TRIF and MyD88 protects LSK engraftment potential.....	86
Figure 4.2 Loss of TRIF and MyD88 provide functional protection to LPS challenged LSK cells.	90
Figure 4.3 Loss of TRIF protected the multilineage reconstitution ability of septic LSK cells.	93
Figure 4.4 LPS downregulates transcription factors essential for myeloid differentiation in the LSK cells in a MyD88 dependent manner.	95
Figure 4.5 LPS-induced irreversible long-term genetic alterations in the LSK cells.....	97
Figure 5.1 miR-21 is differentially expressed in different hematopoietic populations and during myeloid differentiation.	107
Figure 5.2 LPS-induced miR-21 upregulation in the LSK cells in a TLR4/MyD88 dependent manner.	109
Figure 5.3 Generation of transgenic mice with inducible miR-21 deletion specific in the hematopoietic and stroma cells.....	111
Figure 5.4 Loss of miR-21 did not affect the BM response induced by LPS.....	113
Figure 6.1 Summary of final conclusion.....	119

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AP-1	Activator protein 1
APC	Activated protein C
BM	BM
BrdU	Bromodeoxyuridine
CARS	Compensatory anti-inflammatory response syndrome
C/EBP α	CCAAT/enhancer-binding protein alpha
CLP	Cecal ligation and puncture
CLP	Common lymphoid progenitors
CMF	Common myeloid progenitors
CXCL10	C-X-C motif chemokine 10
DGCR8	DiGeorge syndrome chromosomal region 8
dpc	Days post conception
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GARG16	Glucocorticoid attenuated response gene 16
G-CSF	Granulocyte-colony stimulating factor
GMP	Granulocytic monocytic progenitors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GFP	Green fluorescent protein
HMGB1	High mobility group box 1 protein
HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem and progenitor cells
ICU	Intensive care unit
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
IL	Interleukin
IL-RN	Interleukin-1 receptor antagonist
i.p.	Intraperitoneal

IRAK	Interleukin-1 receptor-associated kinase
IRF-3	Interferon regulatory factor 3
IRG1	Immune responsive gene 1
i.v.	Intravenous
IVFM	Intravital fluorescence microscopy
KO	Knock-out
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LSK	Lin-Sca1+cKit+
LT-HSC	Long-term hematopoietic stem cells
MARS	Mixed antagonist response syndrome
MD-2	Myeloid differentiation factor-2
ME	Microenvironment
MEP	Megakaryocyte erythroid progenitor
miRNA	microRNAs
MPP	Multipotent progenitors
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa B
NK cells	Nature killer cells
NO	Nitric oxide
nt	Nucleotides
SIRS	Systemic inflammatory response syndrome
ST-HSC	Short-term hematopoietic stem cells
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCDC4	Programmed cell death protein 4

PI	Propidium iodide
pIpC	Poly (I:C)
qRT-PCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SEM	Standard error
SNP	Single-nucleotide polymorphism
TGF	Transforming growth factor
TIR	Toll/interleukin-1 receptor
TNF- α	Tumor necrosis factors alpha
TRAF6	TNF receptor associated factors 6
TRIF	TIR-domain-containing adapter-inducing interferon- β
TLR	Toll-like receptor
U6 snRNA	U6 small nuclear RNA
UTR	Untranslated regions
VCAM-1	Vascular cell adhesion protein 1
WT	Wild type

CHAPTER ONE

INTRODUCTION

SEPSIS

Sepsis is defined as Systemic Inflammatory Response Syndrome (SIRS) induced by pathogen infections [1,2]. The SIRS criteria were first put forth by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in 1992, and include two or more of the following symptoms: 1) Body temperature $> 38^{\circ}\text{C}$ or $< 36^{\circ}\text{C}$; 2) Heart rate > 90 beats per minute; 3) Respiratory rate > 20 breaths per minute or arterial CO_2 tension less than 32mmHg ; and 4) white blood cell count greater than $12,000/\text{mm}^3$ or less than $4000/\text{mm}^3$ [3]. Severe sepsis develops when one or more organ system become dysfunctional upon the onset of infection-related SIRS. Septic shock is severe sepsis accompanied by hypotension [4]. A recent study revealed that gram-negative bacteria accounts for the majority (62%) of infections in intensive care units worldwide [5].

Despite the long history of the battle between sepsis and human [2], the prevalence and incidence of the disease remains high, especially in emergency rooms and intensive care units [6]. Listed as the 11th leading cause of death in the United States in 2009 by the Centers for Disease Control and Prevention [7], severe sepsis was recently estimated to cost over twenty billion dollars annually to the U.S. health care system [8]. In the developed world, the rate of sepsis climbs annually due to increased aging population and the development of

antibiotic-resistant bacterial infections. In the developing countries, malnutrition, poverty, poor hygiene conditions and lack of timely treatment contributes to high incidence of sepsis. The difficulty in managing sepsis lies mainly on the high level of heterogeneity of the disease. For instance, pathogens, affected organs and the time of intervention for each individual patient can be different and require distinct therapeutic strategies. Cardiovascular, respiratory and renal dysfunctions were found to be the most common affected organ systems in sepsis [9-12]. While concerted efforts are ongoing to elucidate the molecular and cellular mechanism underlying the dysfunctional alterations in these organ systems, limited progress has been made on the study of sepsis induced alterations in the bone marrow (BM). The BM is poised to respond quickly to local changes elicited by trauma or inflammation. In fact, it was not until recently when the BM, where most immune cells derive from, was studied as one of the organs that is also dramatically affected by sepsis and inflammation [13]. The study of BM response during sepsis will provide further therapeutic opportunities to improve the prognosis and survival of patients with sepsis.

ANIMAL MODEL OF SEPSIS

Rodent models provide a significant tool for studying sepsis pathology while satisfying reproducibility and consistency. The sepsis model can be divided into three categories: 1) endotoxin administration; 2) bacterial inoculum model and 3) fecal pellet mode [14-17].

Endotoxin administration

Systemic administration of endotoxin can be also referred to as the LPS (Lipopolysaccharide) model [17]. LPS or endotoxins were first identified in 1941 by Andre Boivin [18], and are glycolipids found in the cell wall of gram-negative bacteria [19]. LPS can initiate innate immune response in affected individuals mainly through the Toll-like Receptor (TLR) pathways [20]. Previous studies have shown that low dose injection into healthy volunteers triggered pathophysiological changes similar to septic patients [21,22]. The LPS model is usually performed with intraperitoneal (i.p.) or intravenous (i.v.) infusion into the animal. It can induce a series of septic events observed in human, such as hematological alterations and increase of pro-inflammatory cytokines in the serum [23]. The LPS model provides a simple and reproducible way to determine many pathophysiology changes during sepsis. Compared to other sepsis models, it is easier to tightly control the experimental system with the use of specific strain and dose of the endotoxins [16]. Experimental data generated from endotoxin models are usually more reproducible and consistent between different independent groups due to the simplicity of the experimental process [23]. The

caveat of the LPS model is that it does not always reflect key events that occur in septic patients. For instance, cytokine levels peaked in a prolonged manner and at lower levels in the septic patients while LPS-infusion led to a transient and high levels of cytokine production in animal models [24]. The endotoxin model was first developed based on the hypothesis that endotoxin is the key component that triggers septic shock in patients, which was later discovered not true in multiple studies [25]. In fact, the level of endotoxin discovered in septic patients is frequently lower than the dose administered to the experimental animals [26]. This also may explain the failure of several therapeutic trials aiming to block or eliminate the endotoxins in septic patients [27]. The endotoxin model does not represent the complexity of the actual disease where more than one pathogen can be present and are replicating within the host. In short, one needs to be mindful when incorporating findings from the endotoxin models into testing the efficiency of therapeutic interventions and into treatment recommendations for patients. Despite the limitations, the LPS model is no doubt a valuable tool for identifying the cellular and molecular mechanisms of many septic pathological events during the early stage of research.

Bacterial inoculum model

The bacterial inoculum model is generated via intravenous administration of concentrated live pathogen organisms into the recipient animal. Besides rodents, bacteremia model animals also include non-human primates, canine and swine [28-37]. It has been criticized that the bacterial inoculum model does not mimic human sepsis. Host animals are usually infused with a high dose of

live bacteria at one time whereas in human sepsis, patients are not exposed to such massive loads of pathogens. As Wichterman et al. noted, septic patients usually harbor a septic focus which is intermittently, but persistently, showering the body with bacteria [18]. Another caveat of this model is that the complement response could lead to rapid lysis of the high doses of bacteria administered and prevent them from colonizing and replicating within the host, leading the model to somewhat become an endotoxin model rather than a true model of infection [38]. Finally, there are several factors that could significantly interfere with the host response such as bacterial load, bacteria strain specificity, and route of infection among others [16]. It is of great importance to limit the variables when performing experiments and to consider these variables when interpreting and comparing results generated from other groups to one's study. Despite the inherent limitations of the bacterial inoculum models, they remain a useful tool to unravel the mechanisms of the host response during sepsis.

Fecal pellet model

Well-known as a gold-standard model for polymicrobial sepsis, the cecal ligation and puncture model (CLP) has been extensively used for more than three decades since its development by the Chaudry group [18,27,39]. The CLP technique involves creating fecal leakage from the normal protective barriers of the caecum distal. It is a model induced by peritoneal contamination by the microflora from the gut of the animals. This model achieves its popularity because of the following advantages. First, it is simple to perform. Instead of growing high concentration of bacteria prior to bacterial inoculum, the CLP model

only requires a straightforward surgical procedure [27]. Second, the ability of the CLP model to recapitulate the human disease progression is very attractive for therapeutic development. For instance, the CLP model can reproduce a hemodynamic profile and cytokine elevation pattern similar to the actual disease [27,31,39]. While the other sepsis models allow for the study of septic response to one particular pathogen or endotoxin, the CLP model creates a condition that is induced by polymicrobial infection, which is more often the real situation in septic patients. Finally, this model has good versatility. By adjusting the size of needle, the number of punctures (one-hit or two-hit), and the location of the puncture, one can induce a wide spectrum of sepsis severity, ranging from chronic and moderate to acute and lethal [31]. However, there is no perfect model in this research field, as the CLP model has its own caveats. Disparities in results derived from CLP model in different laboratories have been the most significant limitation noted in the model [40]. Such discoveries could be due to several factors: 1) surgical operator variability; 2) different anesthetic agents used; 3) animal housing condition; 4) gut flora differences from distinct mice strains [16,18,21,39,40]. Unlike the LPS model, it is very difficult to control the magnitude of the septic challenge induced by the CLP model; therefore, results generated from this model can have a significant variability. One way to overcome the variability is to increase the sample size, especially in small animals. The Chaudry group has proposed the criteria for a model of sepsis: 1) The mice should be toxic and have positive blood cultures; 2) it should manifest true characteristic metabolic and physiologic perturbations; 3) the septic insult

should occur over time and; 4) it should be reproducible and inexpensive [32].

The CLP model fulfills a lot of the criteria for a biologically relevant sepsis model.

Since there is not one sepsis model that can completely recapitulate the human disease with good standardization, reproducibility and versatility, it is important to select the right model suitable for solving the research problem. The current project is a further pursuit of the work published from the Carlesso group. In the previous research, burn and inoculation of *Pseudomonas Aeruginosa* was performed in a mouse model [13]. Our group has previously found that the i.p. injection of *P. aeruginosa*'s LPS was able to recreate the hematological and BM response observed in the bacteremia model. Therefore, it is plausible that the LPS model can be used as a simplified tool to study the cellular and molecular mechanism underlying the BM response during *P. aeruginosa* sepsis. Furthermore, I also introduced the CLP model in my current study to demonstrate that similar BM pathological alterations were also found in polymicrobial sepsis.

PATHOGENESIS OF SEPSIS

SIRS, CARS or MARS

In 1972, Lewis Thomas proposed an innovative theory about infectious disease. He stated that instead of the pathogens being viral, it was our hyperactive immune response that do harm to ourselves and trigger lethal events such as organ injury [41]. A considerable amount of findings support this theory. Tumor necrosis factor alpha (TNF- α) was the first inflammatory cytokine identified to indicate poor prognosis outcome for septic patients [42,43]. Interleukin 1 (IL-1) was found to induce similar inflammatory response such as TNF- α [44]. Both IL-1 and TNF- α are able to activate target cells such as endothelial cells, macrophages, neutrophils and monocytes to produce more inflammatory mediators, leading to amplification of the inflammatory signal [22,45,46]. More cytokines were later found to be involved and increased during SIRS, such as IL-6, IL-8, IL-12 and interferon gamma (IFN- γ) [22,47,48]. Simultaneously, the complement system is activated, leading to C5a production, which is able to further enhance cytokine and chemokine production [49]. The normal function of the coagulation system is also hampered during SIRS, with formation of blood clots and reduction of blood flow. Frequently in septic patients intravascular disseminated coagulation occurs, leading to ischemic injury to organs and tissues, which is associated often with organ failure [50,51]. Coagulation disorder in sepsis is due to the failure to generate adequate Activated Protein C (APC), a proteolytic inhibitor of clotting factors. Besides regulating normal coagulation, APC can also inhibit TNF- α , IL-1 and IL-6

production from monocytes. Reduced APC activity therefore promotes the expansion of the pro-inflammatory status [52,53]. At this point, the body of the septic patient is showered with high level of pro-inflammatory cytokines, chemokines, and acute phase protein. These mediators activate phagocytic cells (neutrophils and macrophages) to release granular enzymes, nitric oxide (NO) and reactive oxygen species (ROS). These molecules are essential for pathogen clearance. However, excessive release of proteases and oxidants can cause tissue damage and lead to organ failure [1,54]. The theory that the body was unable to turn off the indefinitely amplifying inflammatory reaction during sepsis led to numerous trials aiming at attenuating the effects of pro-inflammatory mediators. Most of these studies, unfortunately, were not successful in improving patient survival. Although two independent groups have found that TNF- α blockade protected mice and baboons from endotoxic shock or bacteremia, no survival benefits in human patients were found in subsequent clinical trials [55-57]. Similarly, inhibition of IL-1 through recombinant IL-1 receptor antagonist did not confer protective effects in human patients even though it reduced the mortality rate in animal models [58,59]. One explanation for the different results found in human and animal model is that the cytokine production pattern is different, being prolonged and accumulative in human patients while transient and dramatic in animal. Therefore, after multiple trials examining the efficacy of counteracting pro-inflammatory mediators turned futile, researchers start to reconsider Lewis Thomas' theory.

Roger Bone was a pioneer researcher in sepsis who first put forth the term compensatory anti-inflammatory response syndrome (CARS). He pointed out that Lewis Thomas' theory was one-sided; it overlooked the anti-inflammatory response that was also incited in response to the inciting event [60]. It is now known that the host response to sepsis involves not only immune stimulation but also immunosuppression. Several reviews stated that as sepsis progress, CARS become predominant. Anti-inflammatory mediators such as IL-4, IL-10, transforming growth factor- β (TGF- β) and granulocyte colony-stimulating factor (G-CSF) are produced. T-cell anergy occurs at the same time and Th2 cell (anti-inflammatory) differentiation is favored [61]. Apoptosis in B cells becomes prevalent [62]. Neutropenia, one of the poor prognosis factors for patient survival, is commonly found in CARS. Immune paralysis renders sepsis patients even more vulnerable to secondary infection and is associated with lethality [63]. One study found reduced TNF- α and IL-6 production in intensive care units (ICU) patients [64]. Another group found that patients with severe burns and sepsis exhibit T cell proliferation and fail to produce IL-2 and IL-12 [65,66]. Based on these results, therapeutic interventions trying to boost the suppressed immune system were examined. Administration of stimulation agents such as G-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF) to sepsis patients, however, showed no improvement in survival [67]. The difficulty in finding a magic bullet to cure this complex disease lies greatly in the heterogeneity of a patient's immune status. Roger Bone proposed a delicate summary of the interaction between SIRS and CARS: if pro-inflammatory and anti-inflammatory

responses balance each other locally, then homeostasis is restored; otherwise either pro-inflammatory or anti-inflammatory mediators will spill over to the circulation system and render the patients either SIRS or CARS. Many times, a heterogeneous pattern of SIRS and CARS can occur simultaneously and the patients will suffer from the mixed antagonistic response syndrome (MARS) [60,68]. In summary, it is now generally accepted that the pathology of sepsis is the failure to maintain a homeostatic balance between hyperactive and inadequate inflammation. Therefore, accurate diagnosis of the stages of SIRS and CARS is essential for providing the proper medical intervention for each individual septic patient.

Cellular responses during sepsis

Cellular dysfunctional changes in sepsis can be divided into two groups: excessive activation and depressed function [4]. The cellular component of the sepsis response includes phagocytes (neutrophils and monocytes) and lymphocytes (T, B, NK cell) [46].

Neutrophils are a critical component of the innate immune response. They are the primary effector cells for pathogen clearance. Originated from the hematopoietic stem and progenitor cells in the BM, neutrophils are released into the circulation system with a short lifespan of 5.4 days in human under normal conditions [69]. The mobilization of neutrophils is mainly directed by G-CSF and GM-CSF [70]. Circulating neutrophils move along the blood vessel luminal surface through low-affinity interaction between L-selectin/CD62L on the

neutrophils and E-/P- selectin (CD62E/CD62P) on the endothelium [71]. When inflammation occurs, inflammatory regulators stimulate the expression of β -integrins on the surface of neutrophils, which then bind intensively with ICAM-1/CD54 and VCAM-1/CD106 on the endothelial cells, facilitating firm adhesion of neutrophils to the endothelium near the infected site [72]. Adherent neutrophils then transmigrate through the tight junction of the endothelium along a gradient of chemokines, complement factors and chemoattractant, for example; these factors finally guide the neutrophils to the site of infection to perform their phagocytosis function for pathogen clearance [73]. Besides phagocytosis, neutrophils also release antimicrobial products such as protease enzymes, oxygen radicals and NO. Robust and appropriate function of neutrophils is crucial for an effective functional innate immune response. During sepsis, however, neutrophils often become either hyperactive or defective, both leading to destructive outcomes. Pro-inflammatory signals can extend the half-life of neutrophils in the circulation by delaying apoptosis, leading to overly exuberant neutrophil activation [70,74]. Excessive production of antimicrobial products such as proteolytic enzymes initiates local tissue injury, which ultimately can develop into organ failure [14,71]. In septic patients that are immunosuppressed, blunted neutrophil function renders them more vulnerable to secondary infections [75]. It has been found that neutrophils from septic patients have increased expression of integrins on the surface, augmenting the intensity of endothelium adherence but lowering the ability to transmigrate into the infection sites [71]. Neutropenia is a common poor prognosis factor and is associated with high mortality rate [13].

Neutrophils are a double-edged sword in the pathogenesis of sepsis. The fact that neutrophil functions are over active in some patients while suppressed in others demonstrates the heterogeneous nature of sepsis. Just as a homeostatic balance between pro-inflammatory response and anti-inflammatory response is of crucial importance for a proper host response, a tight regulation of neutrophil activity is also of great importance to prevent tissue injury and infectious complications.

Lymphocytes include T cells, B cells and NK cells. T and B cells are required for adaptive immune response while NK cells are effectors in the innate immunity. Systemic apoptosis of lymphocytes has been reported in septic patients infected with *P. aeruginosa* [62,76]. Massive depletion of CD4, CD8, B cells as well as of dendritic cells were found in septic patients who died in the ICU [63,77]. CD4⁺ T cells can differentiate into either pro-inflammatory type 1 helper Th1 cells or anti-inflammatory Th2 cells. Th2 cells have anti-inflammatory properties and are predominant in the blood of septic patients with burns or trauma [66]. T cell anergy is another cellular mechanism that contributes to immune paralysis, as T cells become nonresponsive to specific antigens. Anergic T cells neither proliferate nor produce cytokines in response to cognate antigens. In summary, T and B cells depletion, T cell anergy and Th2 dominance are key cellular mechanisms underlying adaptive immune response suppression during sepsis.

Endotoxin and Toll-Like receptor 4

More than 60% of infections in the ICU worldwide are due to gram-negative bacteria [5]. As early as 1892, Pfeiffer et al. have found that a heat-stable material associated with gram-negative bacteria was able to induce shock and death in animals, and coined the term endotoxin [78]. Nowadays we know that the endotoxin is LPS, which comprise the outer leaflet of the membrane of gram negative bacteria. The molecular structure of LPS can be divided into Lipid A, the core and the O polysaccharide. Lipid A is composed of a β -D-glucosaminyl-(1-6)- α -D-glucosamine disaccharide and is highly conserved among different gram-negative bacteria. It is responsible for the major toxicity of LPS because it can be recognized by the host's immune cells [79]. The core oligosaccharide is a hetero-oligosaccharide with limited variability within different bacterial species. Finally, the O polysaccharide consists of repeating oligosaccharide units that determine the specificity of the bacteria cell wall [80].

The hyposensitivity of C3H/HeJ and C57BL/10ScCr mice to endotoxin led to the identification of the TLR4 as the receptor for LPS [81,82]. The TLR4 is a member of the TLR family. Toll receptors were first discovered in *Drosophila* as a developmental receptor required for dorsal-ventral embryonic polarity [83,84]. The discovery by the Hoffmann lab that flies with Toll mutants were more susceptible to fungi infection first brought the awareness that this receptor is also important in immune responses [85]. The mammalian homologues of the Toll protein discovered later were then designated as the TLRs. Up to date, 10 TLRs in human and 13 in mice have been identified [86]. In humans, TLR3 and TLR5

are expressed ubiquitously. A large repertoire and variety of TLRs have been found in the spleen, peripheral blood leukocytes, colon, small intestine and lung, all tissues that are either involved in immune responses or have a direct counteract with microbes [87]. The location of TLR expression in mammals significantly reflects their biological functions. TLRs function as pattern recognition receptors. They are the molecular basis for immune cells to distinguish foreign microbial molecules (non-self) from molecules that arise from the host itself. During infection, TLRs interact with pathogen-associated molecular patterns (PAMPs) to initiate host response. The cytoplasmic domain of TLRs is called the Toll-Interleukin 1 Receptor (TIR) domain because it has high similarity with the IL-1 receptor family. The extracellular region of TLRs is composed of leucine-rich repeats (LRRs) [88]. The extracellular domain is responsible for PAMP recognition. Different TLRs can form heterodimers or homodimers in response to different PAMPs to activate corresponding inflammatory signaling pathways. For instance, TLR3 and TLR5 forms homodimers and recognize virus double-stranded RNA and bacterial flagellin respectively [89,90]. Lipoteichoic acid from Gram-positive bacteria induces potent immune response through interaction with TLR2 and TLR6/1 heterodimers [91]. TLR4 forms homodimers upon LPS ligation and is the only known TLR that is responsive to endotoxin [88].

The LPS-TLR4 interaction involves multiple acceptor proteins including LPS-binding protein (LBP) and CD14. A recent study showed that R-form LPS can activate TLR4 in a CD14-independent manner [92]. Myeloid differentiation

factor-2 (MD-2) forms a complex with TLR4 and is required for LPS signaling [93]. A study of crystal structure revealed that LPS interacts with a hydrophobic pocket in MD-2 and induce the homodimer formation of two copies of the TLR4/MD-2/LPS complex [94]. LPS-induced TLR4 signaling can be mediated in a Myeloid Differentiation factor 88 (MyD88)-dependent and MyD88-independent manner. MyD88 is an adaptor protein composed of the TIR domain in the C-terminus and a death domain in the N-terminus. MyD88 associates with TLR4 via the TIR domain and upon LPS stimulation recruits IL-1 receptor-associated kinase (IRAK) to TLR4 through the death domain. IRAK is then phosphorylated and becomes activated for TNF receptor-associated factor 6 (TRAF6) recruitment, leading ultimately to the activation of the JNK-pathway and nuclear factor kappa B (NF- κ B) pathway. Studies with MyD88-deficient mice study revealed that besides TLR4, MyD88 is also an adaptor protein for TLR2, TLR5, TLR7, and TLR9. [95] The MyD88-independent pathway was identified when a group of genes were expressed in MyD88-deficient macrophages upon LPS stimulation, including several interferon (IFN)-inducible genes like glucocorticoid-attenuated response gene 16 (GARG16), immunoresponsive gene 1 (IRG1) and CXC-chemokine ligand 10 (CXCL10) [96]. Expression of these IFN-inducible genes was eliminated in TLR4-deficient macrophages, demonstrating the existence of a TLR4/MyD88-independent pathway. Subsequent work showed that LPS-induced TLR4/MyD88-independent pathway activates the transcription factor interferon responsive factor 3 (IRF-3) and thereby induces IFN- β , leading to the production of IFN-induced co-stimulatory molecules [97,98]. Laboratory mice with defective

TIR domain-containing adaptor (TRIF or TICAM-1) led to the discovery of the molecular basis for the MyD88-independent pathway. TRIF-knockout mice do not express IFN-inducible genes in response to LPS. Therefore, TRIF is essential for TLR4/MyD88-independent pathway [99-101]. Similar to MyD88, TRIF is also an adapter protein containing the TIR domain. Besides TLR4, TRIF also associates with TLR3 to induce IFN-inducible gene expression [102,103]. TRIF knockout led to defective TLR4-mediated cytokine production, and overexpression of TRIF in 293T cells induced NF- κ B activation [103]. Therefore, the TLR4/MyD88-independent pathway can also induce NF- κ B signaling. It is now known that the TLR4/MyD88 dependent pathway leads to early activation of NF- κ B whereas the TLR4/MyD88-independent pathway induces late activation of NF- κ B. Both MyD88 and TRIF are required for the complete activation of the TLR4 pathway [6,88,95]. Tight regulation of the TLR4, as well as other TLRs, is therefore of great importance (Figure 1.1). Inadequate TLR4 activity will hamper the host's ability to clear pathogen during infection while uncontrolled activation will lead to immune disorder including sepsis.

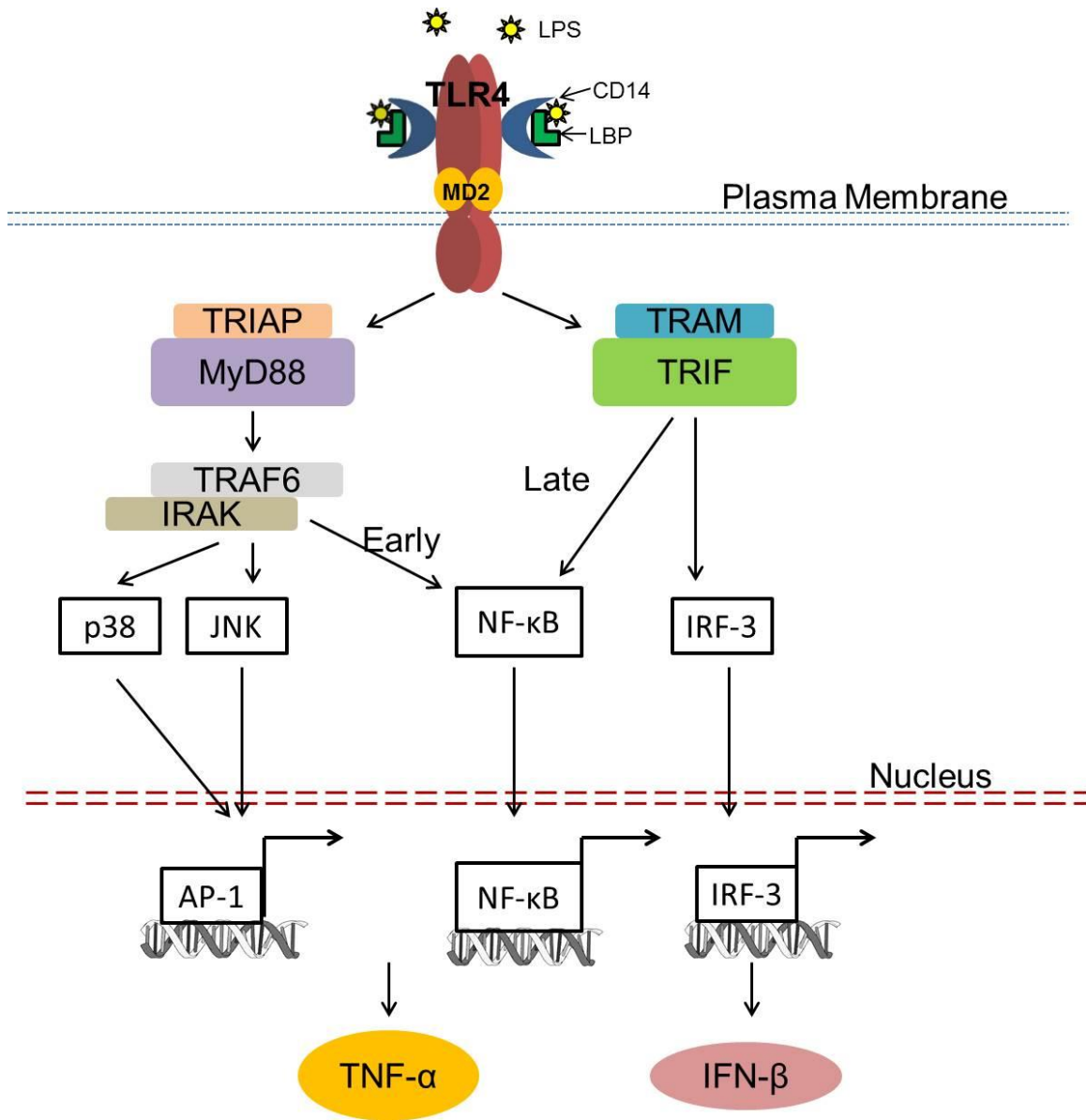


Figure 1.1 Simplified view of the TLR4 signaling pathway.

LPS binding to TLR4 leads to activation of the MyD88-dependent and /or MyD88-independent (TRIF) pathway. TLR4/MyD88-dependent pathway activates p38, JNK and NF-κB signaling. TLR4/TRIF pathway activates delayed activation of NF-κB signaling and IRF-3 signaling.

Genomic storm

A recent study conducted by the Tompkins group revealed that a large fraction (greater than 80% of the human genome) of the leukocyte transcriptome was significantly changed in patients with severe blunt trauma, severe burn and healthy adults exposed to low-dose endotoxin [104]. This study brought about a new term “genomic storm”, which describes a situation where the leukocyte transcriptome is dramatically affected by severe injury/inflammatory stress in human. Results from this study demonstrated that both pro-inflammatory and anti-inflammatory genes were elevated in leucocytes upon the onset of injury, which is different from the traditional concept of SIRS/CARS. The Tompkins group also found a uniformed gene expression profile among patients with trauma, burn or healthy adults exposed to endotoxin. Interestingly, instead of observing that a second genomic hit was promoting poor outcomes in these patients, they discovered that it was the duration and magnitude of this gene profile alteration that was associated with detrimental complications, including organ failure. This novel and elegant study provides a new paradigm for sepsis pathogenesis at a genetic level.

Contribution of genetic polymorphisms to sepsis patients' outcome

The genetic background of the host affects the systemic response to infection [105,106]. With the development of genome sequencing, single-nucleotide polymorphisms (SNPs) in human genes are largely revealed. SNPs that are identified in the exon regions or intron regulatory regions of genes that encode proteins regulating immune responses are likely to affect the susceptibility and outcome of sepsis [107]. For example, SNPs in the promoter and coding region of the TLR4 gene are associated with higher incidence of gram-negative sepsis in humans and increased mortality [108,109]. Figueroa et al. reported that a TLR4 polymorphism interfered with the recruitment of MyD88 and TRIF to TLR4 upon LPS binding, which would likely affect the outcome of sepsis in human patients [110]. Similarly, polymorphisms in the TLR2 gene were reported to be associated with severe staphylococcal infections [111]. Pro-inflammatory cytokine TNF- α played a pivotal role in the initial activation of inflammatory response during the early onset of sepsis. Various reports showed that polymorphisms of the TNF- α gene were linked to increased severity of inflammatory diseases such as chronic obstructive pulmonary disease and sepsis [112,113]. IL-6 is another highly elevated cytokine that is found in the serum level of sepsis patients [114]. A SNP in the promoter region for IL-6 has been reported to be associated with low serum level of IL-6 in premature neonates [115]. Genetic variations were also identified in sepsis-related cytokines/mediators such as IL-1 receptor antagonist (IL-1RN), IL-1 β and high mobility group box 1 protein (HMGB1) [116-118]. Intriguingly, although the IL-1 β

polymorphism correlates with high secretion of IL-1 β , it does not associate with the outcome of sepsis [116]. The variation identified in the IL-1RN is associated with decreased production of IL-1Ra and hence, it is hypothesized to correlate with increased mortality risk of severe sepsis; however, this remains to be established [119]. Kornblit et al. demonstrated that a genetic variant in the promoter region of the HMGB1 gene or a SNP in the exon region was highly associated with reduced long-term survival rate or increased early death frequency [118].

In conclusion, the development of the Next-generation sequencing will help to further identify additional genetic polymorphisms in the coding regions of regulatory domains of essential mediators of sepsis, allowing for the development of specific personalized therapeutic strategies and accurate disease prognostic and diagnostic methods in sepsis.

INFLAMMATORY MODULATION OF HEMATOPOIESIS

Hematopoietic Stem Cells

The hematopoietic stem cells (HSC) are a rare (~0.01-0.001% of total BM cells) population of cells with the capacity to self-renewal and to replenish the entire blood system [120,121]. During embryonic development in mouse, hematopoiesis occurs by 8 days post conception (dpc 8) in the yolk sac blood island [122]. Lifelong hematopoiesis depends on the yolk sac HSC, which not only provide local hematopoiesis during development but also is the root of hematopoiesis in the BM and spleen [123].

In the past 20 years, advances in flow cytometry, *in vitro* colony assays and *in vivo* reconstitution assays have led to the identification and purification of murine HSC, as well as of their progenies [121,124]. In 1988, Spangrude et al. enriched a fraction of the mouse BM cells that were able to rescue 50% of lethally irradiated mice, when injected via tail vein. This fraction was identified as Lineage marker (Lin; including TER119, Mac1, Gr1, CD45R/B220, CD3, CD4, CD8)-Thy-1.1^{Low}Sca-1⁺ [125]. In 1991, the Ogawa group found that the multilineage reconstitution ability of the mouse BM cells was exclusive to the cells expressing a receptor for stem cell factor (SCF) named c-Kit [126]. The LSK (Lin⁻Sca-1⁺c-Kit⁺) fraction have since then become the canonical marker for hematopoietic stem cell enrichment [127]. It was later recognized that the LSK is a heterogeneous population that encompasses long-term HSC (LT-HSC), short-term HSC (ST-HSC) and multipotent progenitors (MPP) [127-129]. The LT-HSC

are the most primitive HSC with the capacity to self-renewal and life-long reconstitution. The LT-HSC make up only about 10% of the LSK fraction [121]. The ST-HSC, on the other hand, can provide transient reconstitution in lethally irradiated recipients and sustain self-renewal capacity for about 8 weeks [127]. The identification of the signaling lymphocytic activation molecule (SLAM) family proteins allowed further discrimination of the various populations within the LSK fraction. Morrison et al. demonstrated that LT-HSC highly express the SLAM marker CD150, but not CD48. The expression levels of CD48 increases as HSC differentiate into less primitive populations (ST-HSC and MPP). A simple gating of CD150⁺CD48⁻ within the LSK fraction could enrich LT-HSC to about 1 in 2 cells [130]. High degree of LT-HSC purity could also be achieved using the gating criteria of Lin⁻Sca-1⁺c-Kit⁺CD34⁻Flt3⁻ [128,131,132]. An alternative strategy utilized the HSC's characteristic of effluxing the Hoechst 33342 dye, which identified the side population (SP) in combination with the LSK markers as the LT-HSC [133-135].

A clear-cut phenotypic definition of the ST-HSC and MPP remain to be proposed. Multiple models have been proposed regarding the differentiation hierarchy. Akashi et al. suggested that the ST-HSC and MPP are heterogeneous transitional intermediates between LT-HSC and oligopotent progenitors [136]. Despite the lack of consensus agreement in differentiating ST-HSC and MPP, various groups have used different yet proper gating strategies for functional studies for these subsets [132,137]. There are two gating criteria for LT-HSC and ST-HSC identification: the first one, is LT-HSC that are designated as Lin⁻Sca-

1⁺c-Kit⁺CD34⁻Flt3⁻ and ST-HSC as Lin⁻Sca-1⁺c-Kit⁺CD34⁺Flt3⁺ [13,132]; the second one, using the SLAM markers, is LT-HSC that are Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁻, MPP1 are Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁺ and MPP2 are Lin⁻Sca-1⁺c-Kit⁺CD150⁻CD48⁺ [138,139].

The differentiation pathways in adult murine hematopoiesis

Since the LSK cells are enriched with HSC and MPP, the identification of lineage-specific progenitors were sought outside of the LSK fraction. The common lymphocyte progenitors (CLP) were the first lineage-committed progenitors isolated [140]. They were found to be the earliest population to express IL-7R α , the receptor for IL-7, an essential cytokine for T cell and B cell development [141]. Therefore, the BM fraction isolated as IL-7R α ⁺Lin⁻Sca-1^{Low}c-Kit^{Low} CLP possess T, B, and NK cell potential but failed to undergo myeloid differentiation. The IL-7R α ⁻Lin⁻Sca-1⁺c-Kit⁺ fraction (commonly known as the LK cells) are mainly myeloid progenitor cells [136]. Common myeloid progenitors (CMP), megakaryocyte-erythrocyte progenitors (MEP), and granulocyte-macrophage progenitors (GMP) all reside within the LK population and can be fractionated based on the expression level of FcR γ II and FcR γ III (Fc γ RII/III) and CD34. CMP are Fc γ RII/III^{Low}CD34⁺, GMP are Fc γ RII/III^{High}CD34⁺ and MEP are Fc γ RII/III^{Low}CD34⁻ [142] (Table 1.1). The CMP differentiates into GMP and MEP. GMP further produce granulocytes and macrophages while MEP differentiate into megakaryocytes and erythrocytes. The CMP are able to generate all myeloid colonies but GMP and MEP have more restricted lineage potentials. Unlike the HSC, these progenitors do not have self-renewal capacity upon *in vivo*

transplantation. Instead, they possess the potential to transiently reconstitute the specific lineage as shown by their *in vitro* studies [143] (Figure 1.2).

Table 1.1 Hematopoietic subsets and immunophenotypic markers.

Abbreviation	Hematopoietic subsets	Immunophenotypic markers
LSK	Hematopoietic stem and progenitor cells	Lin ⁻ Sca-1 ⁺ c-Kit ⁺
LK	Myeloid progenitor cells enriched	Lin ⁻ Sca-1 ⁻ c-Kit ⁺
LT-HSC	Long-term hematopoietic stem cells	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD150 ⁺ CD48 ⁻
MPP1	Multipotent progenitors 1	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD150 ⁺ CD48 ⁺
MPP2	Multipotent progenitors 2	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD150 ⁻ CD48 ⁺
CMP	Common myeloid progenitors	Lin ⁻ IL-7R α ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ RII/III ^{Low} CD34 ⁺
GMP	Granulocytic monocytic progenitors	Lin ⁻ IL-7R α ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ RII/III ⁺ CD34 ⁺
MEP	Megakaryocyte erythroid progenitors	Lin ⁻ IL-7R α ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ RII/III ^{Low} CD34 ⁻
CLP	Common lymphoid progenitors	IL-7R α ⁺ Lin ⁻ Sca-1 ^{Low} c-Kit ^{Low}
NE	Neutrophils	Gr1 ⁺ Mac1 ⁺
M ϕ	Macrophages	F480 ⁺ Mac1 ⁺
Ery	Erythrocytes	Ter119 ⁺
T	T cells	CD3 ⁺ or CD4 ⁺ or CD8 ⁺
B	B cells	B220 ⁺

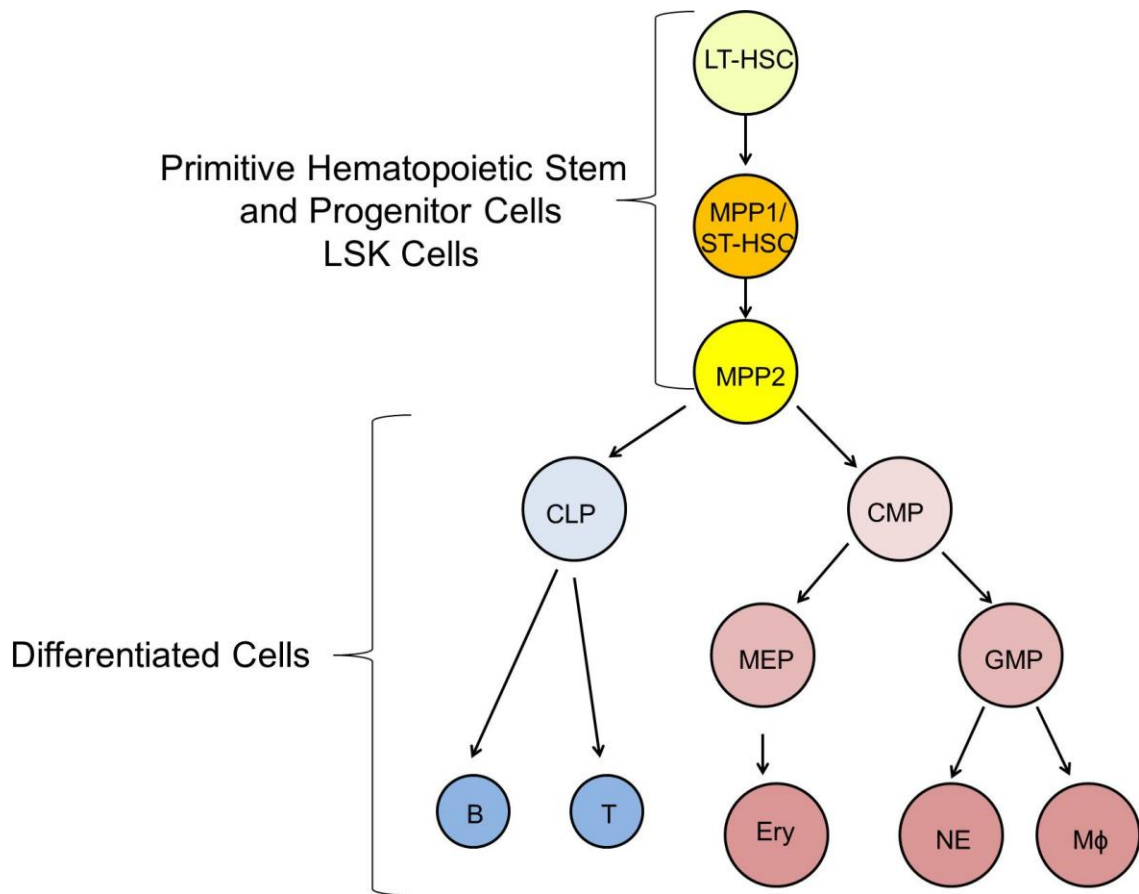


Figure 1.2 Hierarchy of hematopoiesis in the BM of adult mice.

Hematopoietic differentiation pathway in adult mice and corresponding immunophenotypic markers commonly used to distinguish each hematopoietic subsets (LT-HSC, Long-term hematopoietic stem cells; MPP, Multipotent progenitors; CLP, Common lymphoid progenitors; CMP, Common myeloid progenitors; MEP, Megakaryocyte erythroid progenitors; GMP, Granulocytic monocytic progenitors; B, B cells; T, T cells; Ery, Erythrocytes; NE, Neutrophils; M ϕ , Macrophages).

The hematopoietic stem cell niche

A niche is a local tissue microenvironment that directly regulates a defined type of stem and progenitor cells [144]. In adult human and mice, the BM is the primary site of HSC maintenance [145]. The discovery of the SLAM marker has greatly improved the specificity of murine HSC localization in the BM via fluorescence microscopy. Most Lin⁻CD150⁺CD48⁻CD41⁻ localize adjacent to sinusoid vessels in the endosteum of the trabecular bone [130,146,147]. Essential cell types identified in the BM HSC niche include the sinusoidal osteoblasts, vascular and perivascular endothelial cells, reticular cells, stromal cells and BM adipocytes [148-150]. Increasing knowledge of the roles of these cell types on HSC function have been generated using mouse models with tissue specific gene deletion. For instance, Jagged-1 ligands expressed in the BM endothelial cells trigger the activation of Notch signaling and promote HSC maintenance [151]. Depletion of osteoblastic cells impaired HSC niches formation and indirectly affected normal hematopoiesis in the BM [152]. Whether these cellular components in the HSC niche affect HSC functions directly or via indirect mechanism still require more investigation. It is critical take into consideration the microenvironment niche when studying HSC and hematopoiesis during homeostasis or inflammation.

Hematopoietic stem cells response to inflammatory signals

Cell cycle analysis on the HSC has demonstrated that higher than 70% of these cells remain in a quiescent, dormant state. Moreover, it has been found that only the dormant HSC were able to reconstitute the entire hematopoietic system upon transplantation. As the quiescent HSC enter cell cycle, they also lose their long-term engraftment potential [153]. While HSC remain in dormancy during homeostasis, they can proliferate and differentiate upon hematopoietic stress or infection. In a hemorrhage model, HSC expanded to restore the erythrocytes [154], and severe infection was shown to induce CLP proliferation as a compensatory response to T cell apoptosis [155]. Similarly, emergency granulopoiesis occurs when the granulocytes are rapidly consumed during bacterial infection [156]. It was previously believed that the alteration in HSC proliferation and differentiation pattern in the HSC was an indirect response to infections, aiming to replenish the mature blood cells. Recent studies, however, have proved that this hypothesis overlooked a large portion of the whole picture. Though residing in the seemingly isolated BM niche, HSC can in fact respond to various inflammatory signals directly. Inflammatory cytokines including IFN α , IFN γ , TNF- α and G-CSF affect HSC proliferation, differentiation, mobilization and engraftment activity [157-162]. Expansion of the LSK population has been reported in polymicrobial, bacterial and endotoxin models of systemic inflammation [13,163]. Continuous treatment of IFN α and IFN γ elevated HSC cell cycle activity *in vivo* [157,164]. It has been found that alongside promoting the LT-HSC to enter the cell cycle, Type 1 IFNs also have proapoptotic effects on

the activated HSC, which hampered their regenerative potentials [165]. Similarly, TNF- α , a major pro-inflammatory cytokine produced during sepsis, also mediate important effects on the HSC. On one hand, TNF- α inhibits murine HSC growth *in vitro* and excessive TNF- α signaling is associated with BM failure [161,166-168]. On the other hand, TNF- α produced by CD8⁺ cells prevented HSC apoptosis *in vivo* and *in vitro* [169], and loss of baseline TNF- α signaling impaired HSC's self-renewal capacity [170]. Expression of TLRs has been found on the surface of HSC, providing molecular evidence that HSC can sense the pathogens and pathogenic materials directly [171]. The TLR4 ligand LPS stimulates HSC to enter cell cycle and skew for myeloid differentiation [171]. Similar to the impact of IFNs, chronic infection or low dose LPS exposure impaired the reconstitution capacity of the HSC [172]. In a mouse model of severe endotoxemia, acute and high dose LPS exposure triggered LSK cell expansion and myeloid differentiation deficiency, ultimately resulting in neutropenia [13]. In summary, the HSC are primary sensor of infection-induced inflammation. Cytokines and endotoxin significantly alter the hematopoiesis dynamics by affecting the HSC. The physiological response of the hematopoietic system to environmental factors is of crucial importance for immune cell replenishment or pathogen clearance. It is of great importance to return to a homeostasis when the danger signal is no longer present. However, what is often the case during sepsis, the cytokine storm and endotoxemia is so severe that the hematopoietic system was traumatized and unable to regain homeostasis.

INFLAMMATORY REGULATION OF MICRORNA

microRNAs

First identified in *Caenorhabditis elegans*, microRNAs (miRNA) are endogenous non-coding RNA with 19-22 nucleotides (nt) [173]. miRNA genes are commonly found to co-localize with other protein-encoding genes and share the same promoter regulatory machine with the protein-encoding gene. They can also be found as an independent gene in the genome. miRNA genes are transcribed by the Polymerase II in the nucleus. The primary transcripts of miRNA genes are termed pri-miRNA. Pri-miRNA are processed by a microprocessor complex composed by the ribonucleas (RNase) III Drosha enzyme and the DiGeorge Syndrome Critical Region 8 (DGCR8) protein. Processed pri-miRNA become 70-100 nt pre-miRNA, which are then exported into the cytoplasm where they are integrated into the RNA-induced silencing complex (RISC). Pre-miRNA manifests a hairpin topology structure and is cleaved by the RISC with one strand being the passenger strand that is released and degraded, while the other strand, termed the guide strand, remains in the RISC and guide the complex to the 3' untranslated regions (3'-UTR) of target messenger RNAs (mRNA) for target repression [174-176]. miRNA are key components of the gene regulatory network in all multicellular organisms. Phylogenetic studies showed high conservation of primary sequences of miRNA during evolution [177]. The transcriptional regulation of miRNA provide an additional regulation layer for complex biological processes.

Sepsis and microRNAs

Altered miRNA have been implicated in various disease conditions such as cancer, cardiovascular diseases and inflammatory diseases [178]. Identification of differential expression of miRNA in the blood of septic patients suggested their involvement in sepsis pathogenesis [179]. For instance, decreased levels of plasma miR-150 in critically ill patients correlated with chances of organ dysfunction and mortality [180]. miR-146a and miR-233 were found to be decreased in sepsis patients when compared to healthy individuals [181]. Upregulation of miR-133 was found to be correlated with the severity of sepsis [182]. Extracellular miRNA are potential diagnostic and prognostic biomarkers for sepsis and intracellular miRNA targeting the inflammatory signaling pathway are also putative regulators of the disease. Consistent with its correlation with disease severity in the serum, miR-146 negatively modulates the TLR4 pathway by targeting TLR4 itself and the downstream adapter proteins MyD88, IRAK1 and TRAF6 [183,184]. miR-155 is another well-known inflammatory miRNA because it is upregulated in a NF- κ B dependent manner in macrophages and stromal cells during inflammation. Endotoxin treatment greatly increased miR-155 expression in liver tissues [185]. A recent study by our group revealed that miR-155 is significantly elevated in a chronic inflammatory microenvironment induced by loss of Notch signaling, which ultimately contributes to myelodysplasia syndrome [139]. Interestingly, the pro-inflammatory cytokine TNF- α was identified to be a target of miR-125b *in silico* [186], and decreased transcriptional activity of TNF- α induced by miR-125b has been

demonstrated *in vitro* [185]. The role of miR-21 during inflammation has been shown to be cell-type specific. Increased levels of miR-21 have been reported in the muscle tissue of sepsis patients [187]. Similarly, miR-21 promotes the production of pro-inflammatory mediators in non-hematopoietic cells, and LPS, IL-6 and TNF- α can induce miR-21 expression [188-190]. Also, miR-21 has been found to target PCDC4, a pro-inflammatory protein, and therefore it has been regarded also as a negative regulator to tune down the activated NF- κ B signaling pathway [191]. Currently, miR-21 is regarded as a switch that could either promote or inhibit the inflammatory response depending on whether is an early or late phase [192].

The discovery that many miRNA are altered during sepsis provides potential prognostic and diagnostic approaches to better classify septic patients, and potentially lead to more personalized treatment. Moreover, intracellular miRNA regulating the inflammatory pathways could have therapeutic potentials for the treatment of sepsis.

SUMMARY AND SIGNIFICANCE

Sepsis arises when an individual's immune system respond to infection in an uncontrolled manner. It is a global healthcare problem with high risk of death. In industrialized countries like the US, sepsis accounts for more deaths than breast cancer, prostate cancer and AIDS combined [193]. With an increased number of elderly patients, immune-compromised patients and the emergence of antibiotics-resistance superbug, the incidence of sepsis in the future is likely to remain the same, or even increased. In developing countries, the situation is worrisome due to poor hygiene conditions and the lack of advanced medical armamentarium. The rate of hospitalized neonatal infection in developing countries have been reported to be 3 to 20-fold higher than in developed countries, and did not include the large portion of newborns that are delivered at home, which is common in the developing world [194]. Sepsis has imposed and continues to impose an enormous economic burden on the global health care system. Tremendous amounts of money and efforts have been spent to improve the life of patients with sepsis, yet the incidence and rate of mortality in patients with sepsis remain high. Most current therapeutic interventions are focused on reversing the complications that come along with sepsis instead of targeting the fundamental pathological changes in the affected individuals. Despite multiple trials put forward to identify the panacea for sepsis in the past, none was able to remain on the market due to low efficiency or severe side effects. The highly complicated pathology of sepsis and the highly diverse clinical contexts in each patient make the journey to conquer the disease very rough. For a long period of

time, sepsis studies have been focusing on reversing cytokine storm, coagulation dysfunction, and lung/cardiovascular injury. The BM, as an organ which is also greatly affected by inflammation, was largely neglected. In fact, BM failure and hematopoietic defects have been found in animal and human induced by shock and injury [195]. Altered hematopoiesis is a common event occurs upon infection. Adequate supply of immune cells requires normal function of the BM. Therefore, identifying the BM alteration upon sepsis or septic-like stimulation will broaden the scope of therapeutic development.

HYPOTHESIS

Numerous studies have revealed that hematopoietic cells including HSC can directly respond to infection by expressing TLR4, the receptor for Gram-negative bacteria's LPS. Previous work in our lab showed TLR4 is indispensable for the BM response induced by *P. aeruginosa*'s LPS, where immunophenotypic hematopoietic stem and progenitor cells (HSPC) were found to expand but with jeopardized stem cell function and myeloid differentiation was blocked. TLR4 signaling can be mediated by either the MyD88-dependent pathway or the TRIF-dependent pathway. In this project, my hypothesis is that both MyD88 and TRIF are crucial during the BM response to bacterial infections and I sought to determine their distinct contributions to HSC regulation during sepsis (Figure 1.3).

The specific aims of my project are listed as the following:

1. To determine whether BM myeloid suppression induced by *P. aeruginosa*'s LPS is mediated by the TLR4/MyD88 pathway or the TLR4/TRIF pathway.
2. To determine whether the *P. aeruginosa*'s LPS-induced HSPC injury requires the TLR4/MyD88 pathway or the TLR4/TRIF pathway.
3. To examine putative mediators downstream of the TLR4/MyD88 or TLR4/TRIF pathway in contributing to the BM dysfunction during *P. Aeruginosa*'s LPS challenge.

Identification of the molecular pathway underlying BM myeloid suppression and HSC injury will provide novel therapeutic strategies for preventing neutropenia and protecting HSC injury during sepsis.

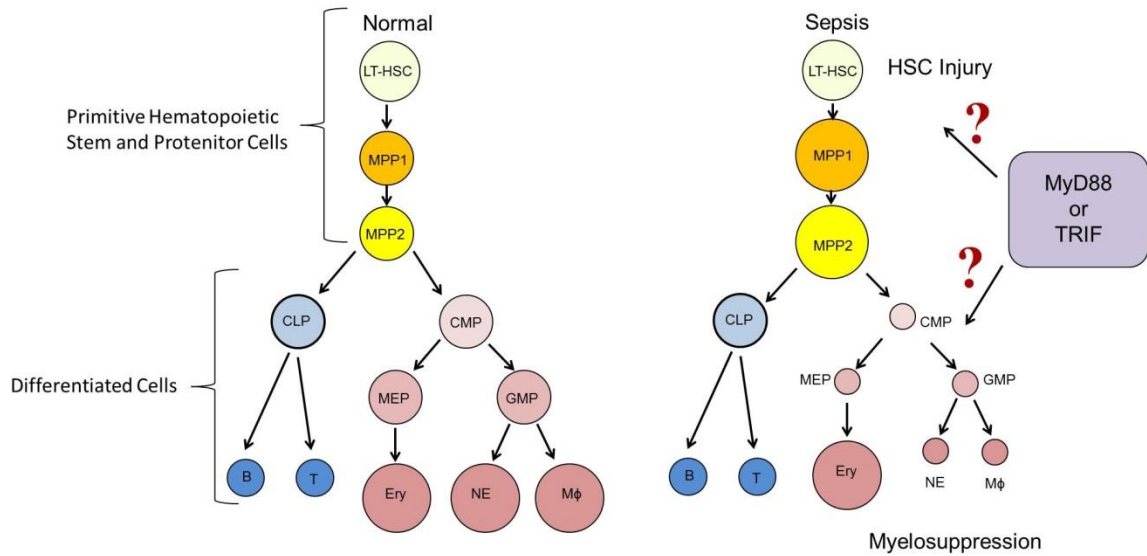


Figure 1.3 Working model

This diagram shows that the general hypothesis is that both TLR4/MyD88 and TLR4/TRIF is important for the BM responses during sepsis. The aim is to elucidate the distinct contribution of TLR4/MyD88-dependent and TLR4/MyD88-independent (TRIF) pathway to hematopoietic responses in the BM during sepsis (LT-HSC, Long-term hematopoietic stem cells; MPP, Multipotent progenitors; CLP, Common lymphoid progenitors; CMP, Common myeloid progenitors; MEP, Megakaryocyte erythroid progenitors; GMP, Granulocytic monocytic progenitors; B, B cells; T, T cells; Ery, Erythrocytes; NE, Neutrophils; Mφ, Macrophages).

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS

1. Mice

All mice studied were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine study protocol # 10252 and 10275 (Principle Investigator: Nadia Carlesso). Gender-matched mice of both sexes between the ages of 8 and 12 weeks were used.

a. C57BL/6 mice

C57BL/6 mice were purchased from The Jackson Laboratory and the In Vivo Therapeutics Core at Indiana University School of Medicine.

b. MyD88^{-/-} (MyD88KO and B6.129P2(SJL)-Myd88^{tm1.1Defr}/J) mice

The MyD88 KO mice originally generated by the Akira Lab (Hyogo, Japan) were a generous gift from Dr. Mark Kaplan (Indiana University School of Medicine).

The B6.129P2(SJL)-Myd88^{tm1.1Defr}/J mice were purchased from The Jackson Laboratory. Both strains have eliminated MyD88 function.

c. TRIF^{-/-} (C57BL/6J-Ticam1^{Lps2}/J) mice

The TRIF^{-/-} were purchased from The Jackson Laboratory.

d. FVB/N-TgN Lys-EGFP reporter mice

The Lys-EGFP reporter mice were obtained from Dr. Graf from the Center for Genomic Regulation, Barcelona, Spain.

e. BoyJ (B6.SJL-PtrcaPep3b/BoyJ) mice

The BoyJ mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

f. miR-21^{flox/flox} mice

The miR-21^{flox/flox} mice were a generous gift from Dr. Mircea Ivan for collaborative projects. They were crossed with the Mx1-Cre mice.

g. Mx1-Cre mice

The Mx1-Cre mice were a generous gift from Dr. Reuben Kapur.

2. Primers

Standard polymerase chain reaction (PCR) reactions for genotyping (Table 2.1) and quantitative reverse transcription-PCR (qRT-PCR) for gene expressions were performed using oligonucleotides synthesized by Invitrogen (Table 2.2). qRT-PCR for miRNA expression (Table 2.3) were performed using commercialized primers from TaqMan® (Applied Biosystems).

Table 2.1 Genotyping Primers

Gene	Forward (5'-3')	Reverse (5'-3')	Mutant (5'-3')
MyD88	TGGCATGCCTC C ATCATAGTTAAC C	GTCAGAAACAA C CACCACCATGC	ATCGCCTTCTA TCGCCTT CTTGACG
miR21 flox	ACAGCTTTCTTT CCTAGAATTGG C	GTCTCACAAGA CATAAGGACCA CAA	
Cre	CATTTGGGCCA GCTAAACAT	ATTCTCCCACC GTCAGTACG	

Table 2.2 Quantitative RT-PCR Primers

Gene	Forward (5'-3')	Reverse (5'-3')
PU.1	AGAAGCTGATGGCTTGGAGC	GCGAATCTTTTTCTTGCTGCC
CEBP/α	AAAGCCAAGAAGTCGGTGGAC	CTTTATCTCGGCTCTTGCGC
TLR4	TCTGAGCTTCAACCCCTTGA	TGCCATGCCTTGTCTTCAATT
MYD88	TAGTCGCAGACAGTGATGAAC	CTGCAGAGCAAGGAATGTGA
TRIF	CAGCTCAAGACCCCTACAGC	CTCCCACACAGCCTCGTC
GAPDH	AAGCCCATCACCATCTTCCA	TAGACTCCACGACATACTCA

Table 2.3 miRNA RT-PCR Primers

Gene	Company	Catalog Number
hsa-miR-21	Applied Biosystems	4331182
U6 snRNA	Applied Biosystems	4427975

3. Antibodies

Table 2.4 Antibodies for Flow Cytometry

Primary Antibody	Company	Clone
CD3-FITC	BD Biosciences	145-2C11
CD4-FITC	BD Biosciences	RM4-5
CD8-FITC	BD Biosciences	53-6.7
B220-FITC	BD Biosciences	RA3-6B2
Gr-1-FITC	BD Biosciences	RB6-8C5
Mac1-FITC	BD Biosciences	M1/70
Ter119-FITC	BD Biosciences	TER-119
CD45.2-FITC	BD Biosciences	104
CD3-PE	BD Biosciences	145-2C11
CD4-PE	BD Biosciences	H129.19
CD8-PE	BD Biosciences	53-6.7
B220-PE	BD Biosciences	RA3-6B2
Gr-1-PE	BD Biosciences	RB6-8C5
Mac1-PE	BD Biosciences	M1/70
Ter119-PE	BD Biosciences	TER-119
CD45.2-PE	BD Biosciences	104
Flt3-PE	BD Biosciences	A2F10.1
Sca1-PE	BD Biosciences	D7
CD48-PE	eBioscience	HM48-1
CD150-PE	Biolegend	TC15-12F12.2
CD48-APC	eBioscience	HM48-1
CD150-APC	eBioscience	9D1
cKit-APC	BD Biosciences	2B8
CD11b-APC	BD Biosciences	M1/70
CD16/CD32-APC	eBioscience	93
Sca1-PE-Cy5.5	Invitrogen	SCA1
CD45.2-PE-Cy5.5	eBioscience	104
B220-PE-Cy5.5	eBioscience	RA3-6B2
IL7-R-PE-Cy7	eBioscience	A7R34
Sca-1-PE-Cy7	eBioscience	D7
cKit-APC-Cy7	eBioscience	2B8
CD11b-APC-Cy7	Biolegend	M1/70
CD34-Pacific Blue	eBioscience	RAM34
CD48-Pacific Blue	eBioscience	HM48-1
Annexin V- PE	BD Biosciences	
Annexin V-APC	BD Biosciences	

4. Commercial Kits

Table 2.5 Commercial Kits

Kit	Company	Catalog Number
APC BrdU Flow Kit	BD Pharmingen	557892
Lin- Cell Depletion Kit, mouse	Miltenyi Biotec	130-090-858
Fast Universal PCR Master Mix (2X)	TaqMan®	4352042
MicroRNA Reverse Transcription Kit	TaqMan®	4366596
RNAqueous®-Micro Total RNA Isolation Kit	Life Technologies	AM1931
Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix	Agilent Technologies, Inc.	600882
iScript™ cDNA Synthesis Kit	Bio-Rad	1708891
GoTaq® DNA Polymerase	Promega	M3005
Mouse G-CSF Quantikine ELISA Kit	R & D Systems	MCS00
Mouse TNF-alpha Quantikine ELISA Kit	R & D Systems	MTA00B
Mouse IFN-alpha ELISA Kit	R & D Systems	42120-1

5. Reagents

Table 2.6 Reagents

Reagent	Company	Catalog number
Lipopolysaccharides from <i>Pseudomonas aeruginosa</i> 10	Sigma Aldrich	L8643
IL-3, mouse	R & D systems	403-ML-010
SCF, mouse	R & D systems	455-MC-010
TRITC dextran 150 100mg	TdB Consultancy	TD150-100mg

METHODS

1. Endotoxemia model

Mice were subjected with i.p. inoculation of 2mg/kg (approximately 20-50µg/mouse) of *P. aeruginosa* LPS (Sigma) and euthanized 24 hours after LPS challenge for BM analyses. Inoculation with this dose of LPS was able to recapitulate the BM responses induced by inoculation with the *P. aeruginosa* [13]. Endotoxemia model was used as the major mouse sepsis model in the current project to simplify the experimental procedure.

2. Polymicrobial sepsis induction

Sepsis was induced by cecal ligation and puncture (CLP) [196]. Briefly, mice were anesthetized with ketamine:xylazine (100 mg/kg:10 mg/kg, i.p.), and severe sepsis was induced by perforating their cecum with one superficial puncture using a 21G1 needle. Sham mice received cecal ligation but no perforation of the cecum. Mice were euthanized 24 hours after the CLP surgery for BM analyses.

3. Generation of chimeras

To develop chimeras with MyD88^{-/-} microenvironment and WT hematopoietic cells (MyD88^{-/-} ME), total BM cells (1 to 2 x 10⁶) from donor C57BL/6-CD45.1 (Boy/J) mice were transplanted via tail-vein injection into lethally irradiated (a split dose of 12Gy) MyD88^{-/-} (CD45.2) recipients. To develop chimeras with MyD88^{-/-} hematopoietic cells and WT microenvironment (MyD88^{-/-} donor), total

BM cells (1 to 2×10^6) from donor MyD88^{-/-} (CD45.2) mice were transplanted via tail-vein injection into lethally irradiated (a split dose of 12Gy) Boy/J recipients.

4. Reconstitution competitive assay

Boy/J recipients were lethally irradiated with a split dose of 12Gy 8 hours before transplantation. LSK donor cells were derived from PBS or LPS challenged WT, MyD88^{-/-} or TRIF^{-/-} mice. Competitive BM cells were prepared as a single-cell suspension from Boy/J CD45.1⁺ mice and admix with the CD45.2⁺ LSK donor cells. A total of 2500-5000 LSK control or septic LSK cells were transplanted into recipients together with 10^5 competitive CD45.1⁺ cells. Engraftment was evaluated at 4-week intervals until week 24 by collecting the peripheral blood for analysis of CD45.2 and lineage markers. At week 24, all recipients were sacrificed for BM analysis.

5. Homing assay

LSK cells were sorted from PBS- or LPS- challenged C57BL/6-CD45.2 mice. LSK cells (2.5 to 5×10^4) were transplanted into lethally irradiated Boy/J-CD45.1 mice. Recipient mice were sacrificed 16 hours post-transplantation, and BM cells were recovered and analyzed for CD45.2 distribution in the BM by FACS.

6. Flow cytometry analysis

BM and peripheral blood cell suspensions were labeled with monoclonal antibodies to define distinct hematopoietic subsets (Table 2.7). Cells were

acquired on an LSR II (BD Biosciences). Events (0.5 to 5 x 10⁶ per staining) were analyzed with FlowJo 7.6.5 and FlowJo 7.2.5.

Table 2.7 Identification of distinct hematopoietic subsets

Hematopoietic Subsets	Surface Marker
Long-term hematopoietic stem cells (LT-HSC)	Lin ⁻ Sca1 ⁺ c-Kit ⁺ CD150 ⁺ CD48 ⁻
Multipotent progenitors 1 (MPP1)	Lin ⁻ Sca1 ⁺ c-Kit ⁺ CD150 ⁺ CD48 ⁺
Multipotent progenitors 2 (MPP2)	Lin ⁻ Sca1 ⁺ c-Kit ⁺ CD150 ⁻ CD48 ⁺
LSK	Lin ⁻ Sca1 ⁺ c-Kit ⁺
Common myeloid progenitors (CMP)	Lin ⁻ IL-7R α ⁻ Sca1 ⁻ c-Kit ⁺ CD34 ⁺ Fc γ RII/III ^{lo}
Granulocytic monocytic progenitors (GMP)	Lin ⁻ IL-7R α ⁻ Sca1 ⁻ c-Kit ⁺ CD34 ⁺ Fc γ RII/III ^{hi}
Megakaryocyte erythroid progenitors (MEP)	Lin ⁻ IL-7R α ⁻ Sca1 ⁻ c-Kit ⁺ CD34 ⁻ Fc γ RII/III ^{lo}
Common lymphoid progenitors (CLP)	Lin ⁻ IL-7R α ⁺ Sca1 ^{lo} c-Kit ^{lo}
Granulocytes and monocytes	Gr1 ⁺ Mac1 ⁺
B cells	B220 ⁺
T cells	CD4 ⁺ /CD8 ⁺ /CD3 ⁺

7. Cell sorting

For hematopoietic stem and progenitor cell sorting, BM cells were incubated with Biotin-antibody cocktail (Miltenyi Biotec) followed by incubation with Anti-biotin microbeads (Miltenyi Biotec) and negative selection by magnetic-activated cell sorter (MACS) separation system. Lineage-depleted BM cells were labeled with anti-c-Kit, anti-Sca1, anti-IL-7R, anti-Fc γ , anti-CD34 and sorted. For mature hematopoietic cell sorting, BM cells were stained with anti-Gr1, anti-Mac1. All the cells were sorted with the FacsAria (BD Biosciences).

8. Cell cycle and apoptosis analysis

PBS or LPS challenged mice were injected with bromodeoxyuridine (BrdU) (1-2mg/mouse) 18 hours prior to sacrifice (6 hours after LPS or PBS injection). Total BM cells were harvested 24hours from LPS (or PBS) challenge. BM cell cycle characterization was performed using the BrdU-APC Flow Kit (BD Pharmingen) in combination with markers for LSK and Gr1⁺Mac1⁺ cells. Cell death was measured by labeling BM cells with Annexin V and Propidium iodide (PI) in combination with markers for LSK, Gr1 and Mac1. Samples were analyzed by FACS.

9. *In vitro* differentiation assay

BM cells from PBS or LPS-challenged WT, MyD88^{-/-} and TRIF^{-/-} mice were harvested from femurs by crushing with phosphate-buffered saline (PBS)/0.2mM EDTA. Cells were lineage depleted using the lineage cell depletion kit (Miltenyi Biotec) and sorted for CMP and GMP. Cells were cultured in IMDM + 10%FBS supplemented with SCF (50 ng/mL) and IL-3 (20 ng/mL), seeded at 1 x 10⁴ cells/mL in 96-well plates and cultured at 37 °C, 5% CO₂ for up to 6 days. Cells were collected at day 2 and day 4 and stained with Gr1 and Mac1 to determine the myeloid differentiation potential of sorted cells.

10. qRT-PCR and miRNA quantification

RNA from sorted BM cells was purified with the RNAqueous micro kit (Ambion). qRT-PCR was performed with SYBR green II Brilliant (Stratagene) and the primers shown in Table 2.2 (Invitrogen). miRNA expression was assessed by

qRT-PCR (Applied Biosystems) with miRNA-specific TaqMan® miRNA Assays (has-miR-21, U6snRNA) on an ABI 7900HT FAST platform (Applied Biosystem).

11. Imaging acquisition and image analysis

Two-photon images of calvarium BM were collected using Olympus XLUMPLFL 20xW, NA 0.95 objective. Z-stacks were collected through the depth of tissue (60 µm Z-stacks) from 6 regions of calvarium BM, at step size settings of 1µm and 512x512 pixels frame size. Projection images were created using MetaMorph imaging software (Molecular 135 Devices). Volume rendering software Voxx was used to create 3D reconstructions of BM.

12. Statistical analysis

Results were evaluated by performing unpaired t-tests or ANOVA using GraphPad Prism Version 6.0 software (GraphPad Software) and Excel 2010. Data were expressed as mean ± SEM unless indicated otherwise. *p < 0.05 was determined significant, and **p < 0.01 were considered highly significant.

CHAPTER THREE

ROLE OF MYD88 AND TRIF IN MEDIATING BM RESPONSE DURING *PSEUDOMONAS AERUGINOSA*'S LPS CHALLENGE

INTRODUCTION

In the BM, the myelosuppression and neutropenia induced by *P. aeruginosa* inoculation are recapitulated by the injection of *P. aeruginosa*'s LPS (20-50µg/mouse). These responses are largely due to a differentiation block in the HSPC. Mice with functionally depleted TLR4 are resistant to LPS-induced myeloid suppression [13].

TLR4 is one of the extracellular receptors for LPS sensing. TLR4 activation can be mediated by MyD88 and/or by TRIF. The MyD88-dependent pathway induces early activation of NF-κB and Activation Protein 1 (AP-1), resulting in production of inflammatory cytokines. The TRIF pathway, also known as the delayed-MyD88-independent pathway, leads to the activation of IRF3 and late NF-κB activation, which induces the production of IFN-β [197].

Here, I evaluated the distinct contribution of TRIF and MyD88 to the BM response during LPS-induced endotoxemia (Figure 1.3). The ultimate objective is to identify the cellular and molecular mechanisms underlying such alterations to develop new therapeutic approaches for the treatment of sepsis.

RESULTS

3.1 P. aeruginosa's LPS induced LSK expansion in the BM is TLR4/TRIF dependent

It has been previously reported that *P. aeruginosa's* LPS induced an expansion of LSK cells in a TLR4-dependent manner. LSK cell is a cell subset enriched with HSPC [13]. To identify whether TLR4-mediated HSPC expansion upon LPS stimulation is dependent on MyD88 or TRIF, we challenged WT, MyD88^{-/-} and TRIF^{-/-} mice with LPS for 24 hours and performed immunophenotypic analysis of the BM cells. As expected, WT mice responded to LPS challenge with a significant increase in the percentage of LSK cells. LPS also induced a dramatic expansion of LSK cells in the MyD88^{-/-} mice. Interestingly, we observed a significantly smaller increase (2-fold increase) in LSK cells in the TRIF^{-/-} mice compared to WT and MyD88^{-/-} mice (Figure 3.1 A, C). To further identify the subsets within the HSPC that were affected by LPS stimulation, we used SLAM markers to distinguish LT-HSC (Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD45⁻), MPP1 (Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD45⁺) and MPP2 (Lin⁻Sca-1⁺c-Kit⁺CD150⁻CD48⁺) (Figure 1.2 and Figure 3.1 B). LPS leads to a significant increase in the percentage of LT-HSC in WT and MyD88^{-/-} mice but no change was seen in the TRIF^{-/-} mice (Figure 3.1 D). Similar to the results from a Type-I IFN study [165], I did not find alteration in the absolute number of LT-HSC upon LPS challenge in WT mice (Figure 3.1 D), despite an increased frequency observed in both WT and MyD88^{-/-}. Significant expansion of MPP1 and MPP2 in LPS-challenged WT and MyD88^{-/-} mice was observed (Figure 3.1 E-F). In

contrast, in TRIF^{-/-} mice, absolute number of MPP1 and MPP2 following LPS challenge were similar to PBS control (Figure 3.1E-F).

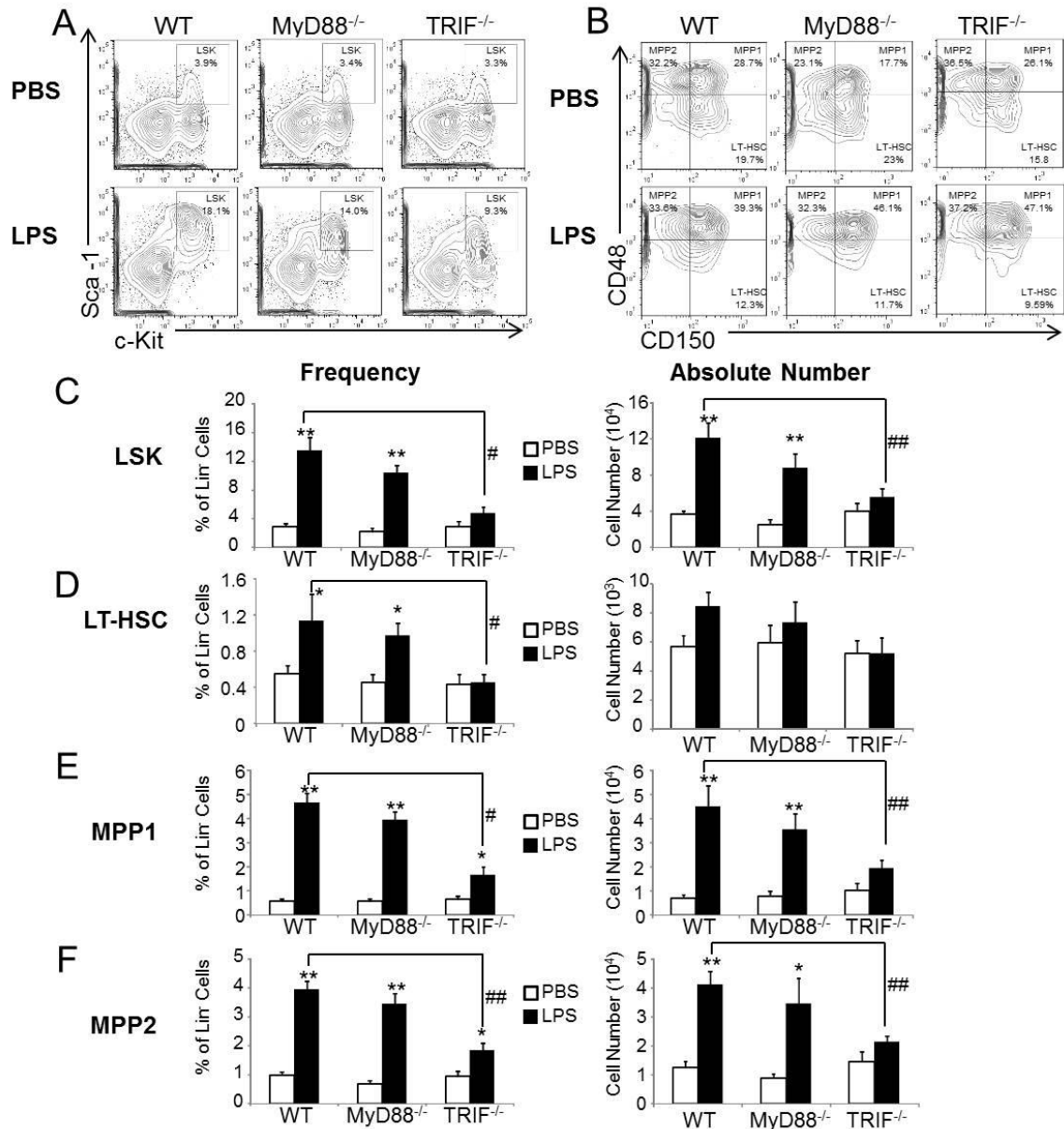


Figure 3.1 *P. Aeruginosa*'s LPS-induced LSK expansion in the BM in a TLR4/TRIF dependent manner.

(A-B) Representative FACS plots for LSK populations (A) or HSPC (B) labeled with SLAM family marker (LT-HSC: Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁺; MPP1: Lin⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁺; MPP2: Lin⁻Sca-1⁺c-Kit⁺CD150⁻CD48⁺). (C-F) Percentage in the gated Lin⁻ population (left) or absolute cell numbers per femur (right) in PBS versus LPS challenged mice of LSK (C), LT-HSC (D), MPP1 (E) and MPP2 (F) (n = 5-14). Data represent mean ± SEM. LPS vs. PBS: * p < 0.05, ** p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, unpaired t test.

3.2 The TLR4/TRIF-dependent pathway is required for the LSK expansion in polymicrobial sepsis.

Next, I examined whether the LSK expansion observed during endotoxemia was a representative response during polymicrobial sepsis. To this end, we used a model of severe peritonitis induced by CLP in WT, MyD88^{-/-} and TRIF^{-/-} mice. 4 out of 16 WT mice died within 24 hours after the CLP surgery and were unsuitable for BM analysis due to massive cell death in the marrow. Analysis of the HSPC fraction in CLP and Sham control WT mice revealed a BM response similar to the mice that were challenged with LPS. Absolute number of both LSK and MPP1 fraction increased significantly upon CLP in WT mice, and this response required both MyD88 and TRIF (Figure 3.2). No significant alteration in the LT-HSC absolute numbers was observed except for a mild incline trend in the MyD88^{-/-} mice. Surprisingly, I also found that there was a four-fold increase in the MPP1 absolute numbers in the TRIF^{-/-} mice (Figure 3.2). The discrepancy observed in the CLP model could be contributed to the activation of other inflammatory pathways such as the TLR2 and TLR9 [198,199]. Despite this divergence, I confirmed that expansion of HSPC were also present in the CLP polymicrobial sepsis model, which better recapitulate the actual clinical scenario.

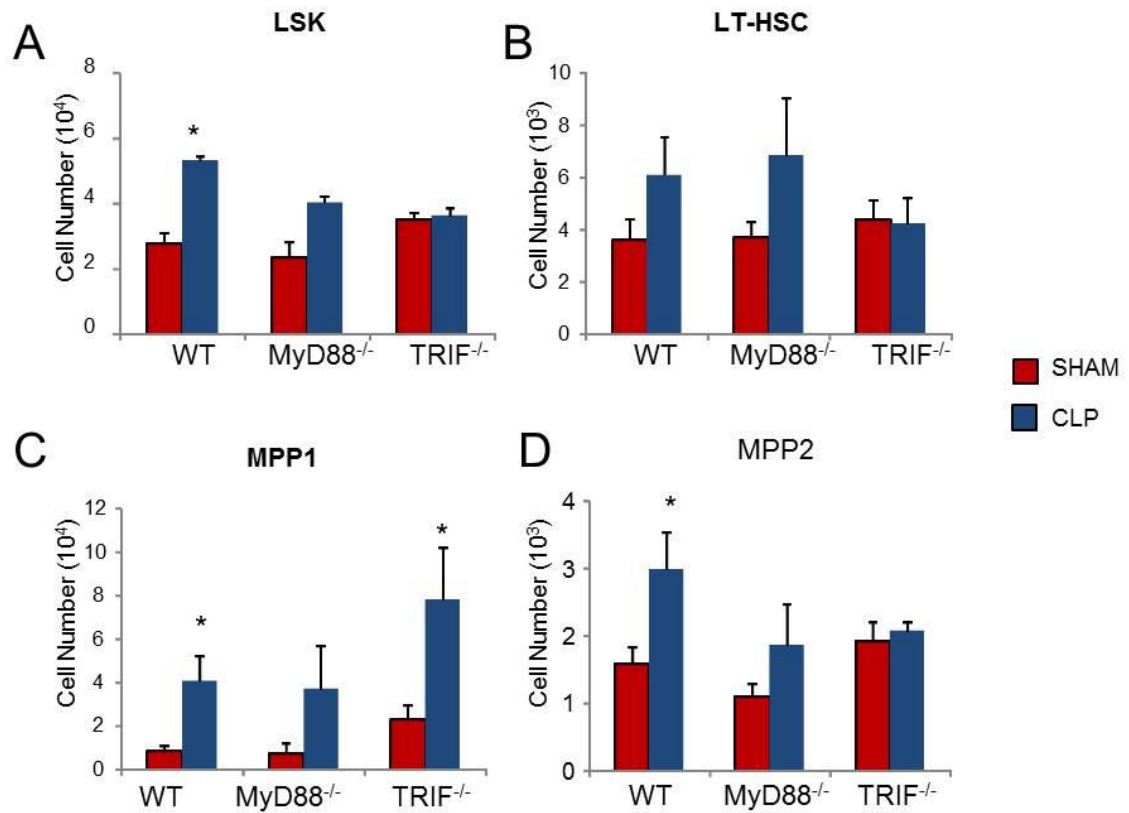


Figure 3.2 BM responses in the HSPC compartment during polymicrobial sepsis.

(A-D) Absolute numbers of cells per femur in CLP-challenged versus Sham-treated mice in BM LSK (A), LT-HSC (B), MPP1 (C) and MPP2 (D) (WT: n = 6-11; MyD88^{-/-} and TRIF^{-/-}: n = 3-5). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, unpaired t test.

3.3 Expansion of LSK is marginally due to upregulation of Sca-1 in the myeloid progenitors.

It is widely known that LPS induces IFN and TNF- α production in mouse and human [200-204]. Following LPS and sepsis challenge, the levels of Sca-1 expression on HSPC were overall increased (Figure 3.3A). Previous research found that the expression of the canonical HSPC marker Sca-1 in progenitors can be induced by IFNs and TNF- α [205,206]. Therefore, one could argue that the LSK expansion observed in the current endotoxin model is due to re-expression of Sca-1 by Sca-1-negative myeloid progenitor cells. To examine this hypothesis, I used the Lys-EGFP reporter mice to track the myeloid compartment in control and LPS-mice. In the Lys-EGFP reporter mice, green fluorescent protein (GFP) expression is driven by the myeloid-specific lysozyme promoter [207]. The GFP⁺ fraction should reside only in the myeloid lineage cells. If the hypothesis stated above were true, I would expect to see increased level of GFP in the expanded LSK fraction. The GFP fluorescence intensity were examined in the LSK, LT-HSC, MPP, CMP, GMP and Gr1⁺Mac1⁺ compartments. The percentage of GFP⁺ cells in the myeloid subsets were comparable with results derived by Miyamoto et al. [208]. Consistent with the published results [13], the GFP⁺ fraction in the Gr1⁺Mac⁺, GMP and CMP compartments were significantly reduced, confirming the myeloid differentiation block (Figure 3.3 B). Interestingly, higher expression of GFP was observed in the LSK, LT-HSC and MPP from LPS treated mice (Figure 3.3 B), indicating that the expanded LSK cells were indeed “contaminated” with some myeloid progenitors that re-express Sca-1.

Comparison of the absolute number of the total HSPC with those that are GFP⁺, however, demonstrated that the contaminating myeloid progenitor cells only make up a very small fraction of the expanded HSPC during LPS challenge (Figure 3.3 C).

In summary, this result showed that although Sca-1 re-expression in myeloid progenitors occurs during LPS challenge *in vivo*, it is not the key cellular mechanism underlying the HSPC expansion. In other words, a large fraction of the expanded LSK cells are not derived from myeloid progenitors that re-express Sca-1, but rather primitive LSK cells.

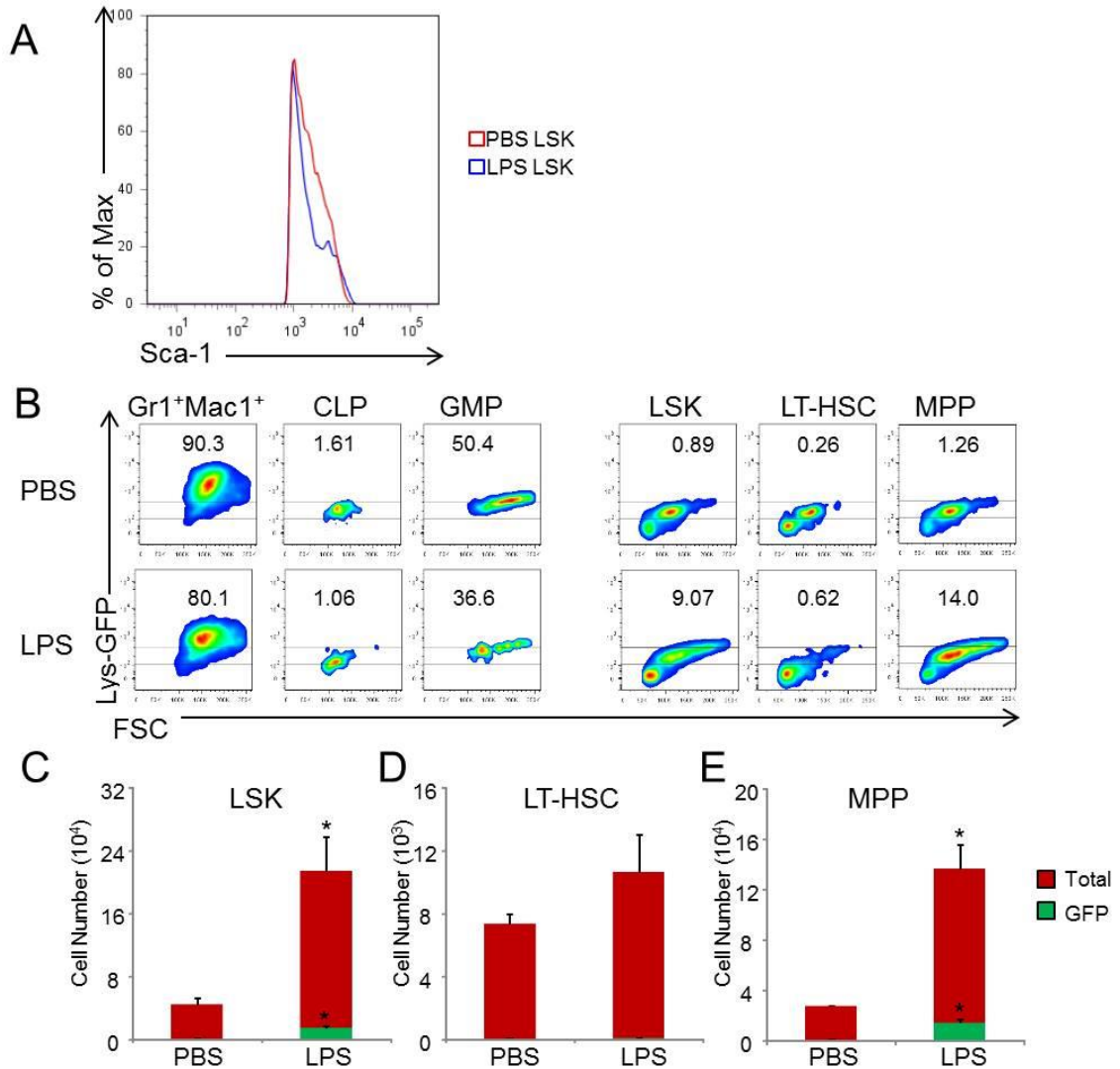


Figure 3.3 GFP expressions in different cellular subsets in the BM during LPS challenge.

(A) Histogram shows the intensity of Sca-1 in LSK cells from cells from the BM of LPS or PBS challenged WT, MyD88^{-/-} and TRIF^{-/-} mice (n = 4-5). (B) Representative FACS plots show GFP expression on granulocytes, CLP, GMP, LSK, LT-HSC and MPP (n = 3-4). (C-E) Absolute number per femur of total LSK, LT-HSC and MPP (Red) and GFP⁺ LSK, GFP⁺ LT-HSC and GFP⁺ MPP (Green) (n = 3-4). Data represent mean \pm SEM. *p < 0.05, **p < 0.01, unpaired t test.

3.4 *P. aeruginosa*'s LPS induced cell cycle activation in LSK cells in a TRIF and MyD88 dependent manner.

In order to study the cellular mechanism underlying the expansion of LSK cells in response to LPS exposure, I used *in vivo* BrdU incorporation assay to analyze the cell cycle activity of LSK cells from WT, TRIF^{-/-} and MyD88^{-/-} mice challenged with either LPS or PBS (control). BrdU was injected into the mice 6 hours after LPS challenge (18 hours prior to sacrifice). *In vivo* cell cycle analysis showed that LSK cells in the WT mice challenged with LPS had a significantly increased cycling activity and reduced quiescence compared to PBS controls (Figure 3.4 A). In accordance with the LSK pattern in TRIF^{-/-} mice, there were no alterations in cell cycle activity in the LSK cells from LPS TRIF^{-/-}. Surprisingly, however, unlike LPS WT mice, we did not observe any increase of cell cycle in MyD88^{-/-} LSK cells and the percentage of quiescent cells remained unchanged in the expanded LSK cells. The cell cycle analyses suggested that the expanded septic LSK cells from WT and MyD88^{-/-} mice maintain different cellular characteristics (Figure 3.4 A).

In order to determine whether LPS exposure induce apoptosis in the primitive HSPC population, LSK cells from normal and septic mice from all three genotypes were studied for cell death using both Annexin/PI and Sub-G1. Both criteria showed that a very low percentage of LSK cells undergo apoptosis or necrosis, and there were no significant differences between normal and septic LSK cells in all three genotypes (Figure 3.4 B).

Collectively, these data showed that LPS-induced expansion of HSPC, especially LT-HSC, was dependent on the TLR4/TRIF pathway but not the MyD88 pathway. The expansion of the LSK cell pool from WT septic mice was largely due to cell cycle activation. However, quiescence is preserved in expanded MyD88^{-/-} septic LSK cells, suggesting that mechanisms other than the cell cycle activation, such as delayed HSC differentiation resulting in slower egression from the primitive to the differentiated pool, may participate to this process following LPS.

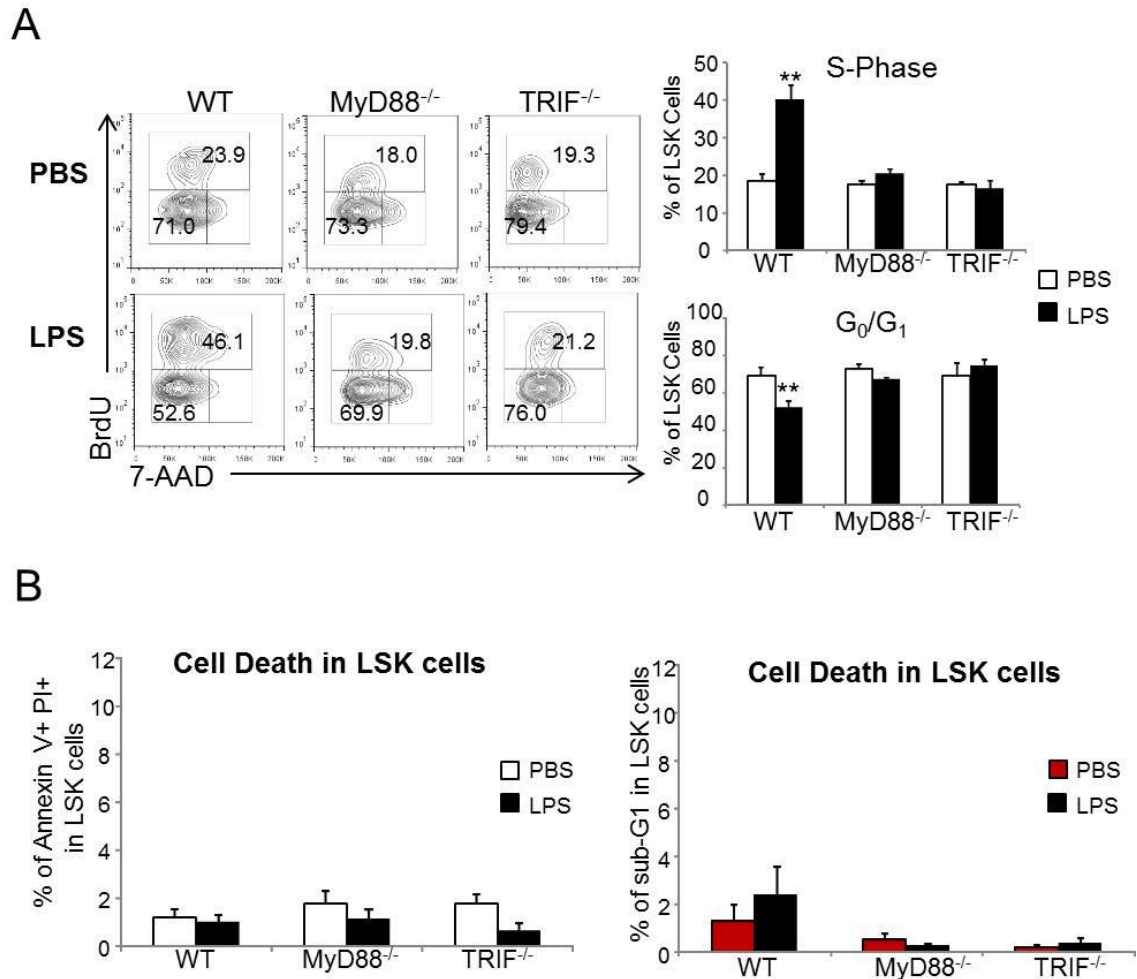


Figure 3.4 Acute LPS exposure increase LSK proliferation in a TRIF and MyD88 dependent manner.

(A) Left: Representative FACS plots of BrdU/7-AAD incorporation in LSK cells from PBS or LPS challenged WT, TRIF^{-/-} and MyD88^{-/-}; Right: Bar graph shows mean percentage of BrdU-incorporated S-phase and G₀/G₁ phase in LSK cells (n = 4-9). (B) Left: Bar graph shows the mean percentage of apoptotic cells in LSK cells from PBS or LPS challenged WT, TRIF^{-/-} and MyD88^{-/-}; Right: Bar graph shows the mean percentage of Sub-G1 subsets in LSK cells (n = 4-5). Data represent mean ± SEM. *p < 0.05, **p < 0.01, unpaired t test.

3.5 Absence of MyD88 but not TRIF prevents LPS-induced myelosuppression in vivo.

To further address the molecular mechanisms underlying myeloid suppression, we characterized the myeloid progenitors and mature myeloid cells in WT, TRIF^{-/-} and MyD88^{-/-} mice stimulated with LPS or PBS. Multiparameter immunophenotypic analyses showed that LPS caused a reduction of CMP, GMP and MEP in the WT and TRIF^{-/-} mice. While the CMP were decreased in LPS-challenged MyD88^{-/-} mice, the GMP subsets were greatly protected by the loss of MyD88 signaling (Figure 3.5 A, C, D). Flow cytometry analyses of the BM Gr1⁺/Mac1⁺ population, which include monocytes and neutrophils, revealed that LPS-induced neutropenia required activation of the MyD88 pathway but not TRIF (Figure 3.5 B, E). This effect was not due to increased survival as following LPS cell death within the LK was negligible whereas a notable increase was observed in the Gr1⁺/Mac1⁺ population (15-20%); however, cell death rates were similar in all genotypes (Figure 3.5 F). In conclusion, activation of the TLR4/MyD88 pathway played a dominant role in mediating myeloid suppression in the BM upon septic endotoxemia.

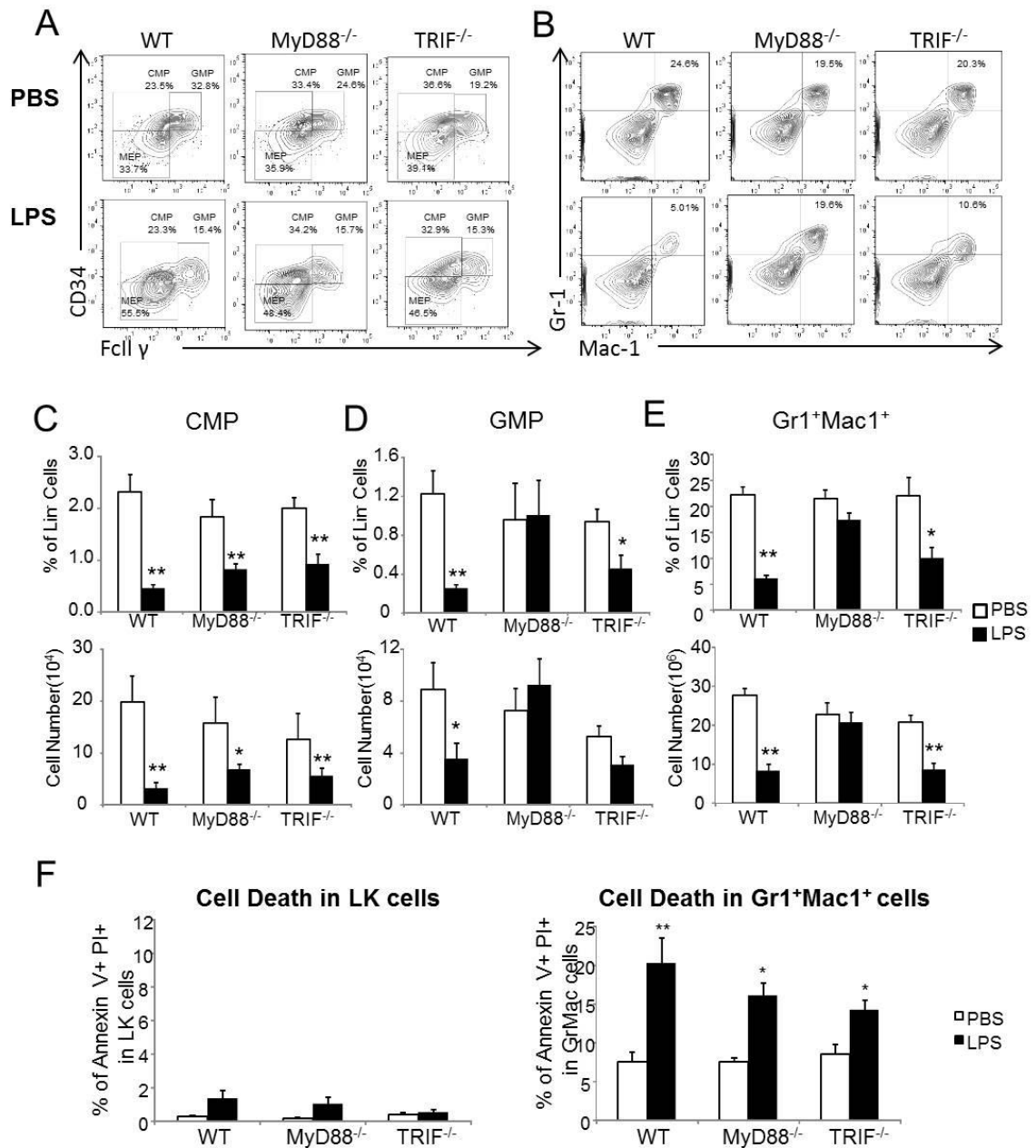


Figure 3.5 LPS induced myeloid suppression in a MyD88 dependent manner.

(A-B) Representative dot-plots for myeloid progenitors CMP, GMP (A) and Gr1⁺Mac1⁺ (B) in the BM of PBS and LPS-treated WT, TRIF^{-/-} and MyD88^{-/-} mice. (C-D) Percentage and absolute number/mouse of CMP (C) and GMP (D) (n = 7-16). (E) Average of the percentage and absolute number/mouse of granulocytes (n=7-28). (F) Percentage of apoptotic cells in LK and Gr1⁺Mac1⁺ cells (n = 4-5). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, unpaired t test.

3.6 Absence of MyD88 but not TRIF prevents myelosuppression during polymicrobial sepsis.

Next, we examined the myeloid differentiation in the BM of CLP challenged mice. WT and TRIF^{-/-} mice exhibited loss of CMP, GMP and granulocytes 24 hours after CLP challenge. In contrast, MyD88^{-/-} mice stimulated with CLP showed normal myeloid differentiation and with normal level of CMP, GMP and neutrophils (Figure 3.6). Thus, loss of MyD88 prevents myelosuppression also in the settings of polymicrobial sepsis.

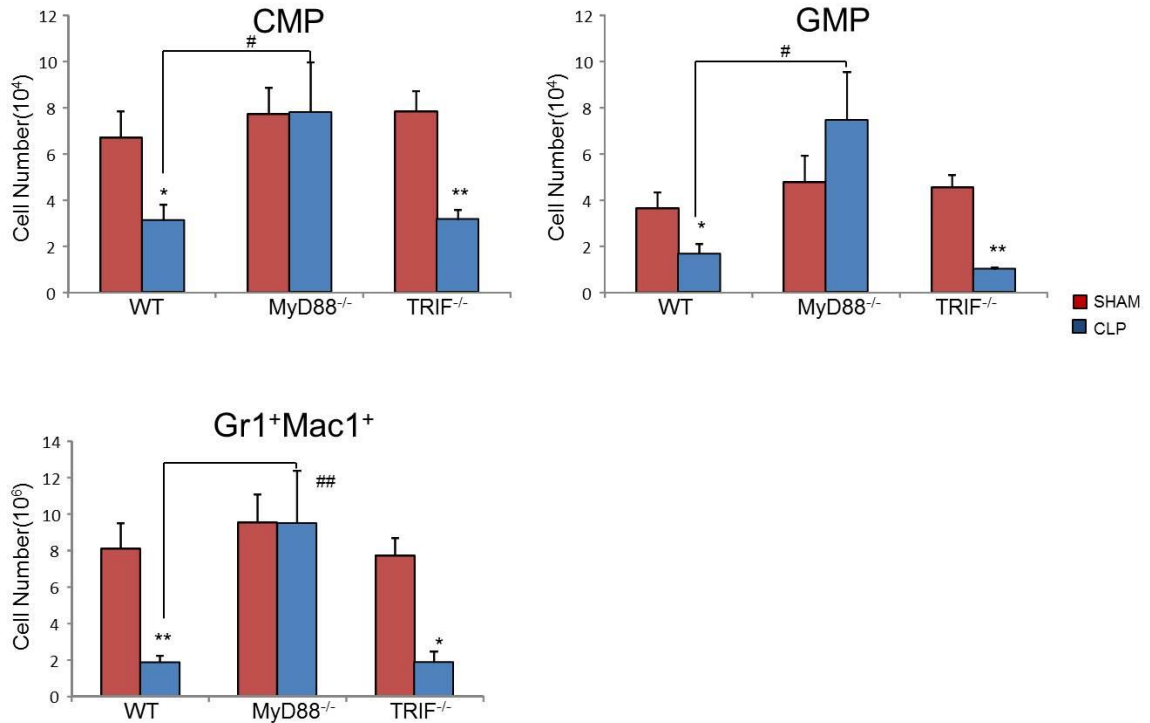


Figure 3.6 Polymicrobial sepsis induced myeloid suppression in the BM in a MyD88 dependent manner.

Bar graphs indicate the mean of absolute number of BM CMP, GMP and Gr1⁺Mac1⁺ per femur in CLP challenged versus Sham treated mice (n = 3-10). Data represent mean \pm SEM. LPS vs. PBS: *p < 0.05, **p < 0.01 unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} vs. LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, one-way ANOVA.

3.7 Activation of MyD88 impairs CMP's ability to differentiate

Next, I characterized at what stage of the hematopoietic hierarchy did the activation of the MyD88 pathway block myeloid differentiation. Since the HSPC phenotype was not affected by the loss of MyD88 as shown in previous results (3.1-3.2), I focused on the myeloid progenitors, CMP and GMP. CMP and GMP were sorted from WT, MyD88^{-/-} and TRIF^{-/-} mice challenged with LPS or PBS. Sorted cells were cultured with IL-3 and SCF for differentiation assay *in vitro* (Figure 3.7 A). At day 4, cells isolated from normal or endotoxemia mice were subject for flow cytometry analyses to compare their myeloid differentiation capacity. CMP isolated from WT and TRIF^{-/-} mice challenged with LPS demonstrated a delayed myeloid differentiation. In contrast, loss of MyD88 prevented this delay (Figure 3.7 B). Interestingly, GMP (collected at day 2) from LPS-challenged mice from WT, MyD88^{-/-} and TRIF^{-/-} manifested no differences in their myeloid differentiation dynamics compared to respective PBS control (Figure 3.7 C). Collectively, these *in vitro* data show that activation of the TLR4/MyD88 signal in the BM impairs CMP's ability to differentiate.

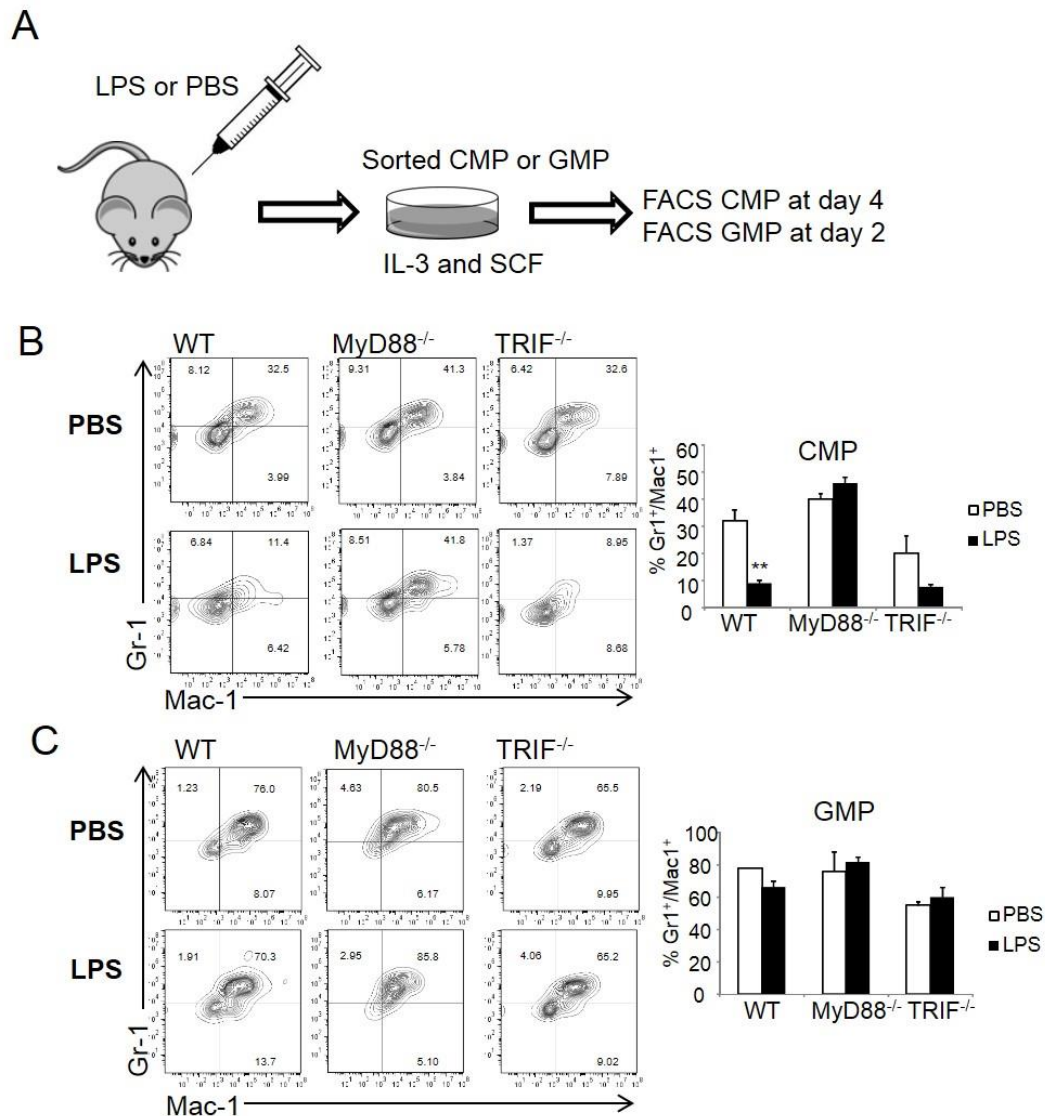


Figure 3.7 CMP and GMP differentiation assay.

(A) Experiment design for the differentiation assay for CMP and GMP sorted subsets from LPS or PBS challenged WT, MyD88^{-/-} and TRIF^{-/-} mice. (B) Representative contour plots for myeloid differentiation from CMP at day 4; bar graphs show average of mature myeloid cells differentiated from plated CMP (n = 3-4). (C) Contour plots for myeloid differentiation from GMP at day 4; bar graphs show average of mature myeloid cells differentiated from plated GMP (n = 2-4). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, unpaired t test.

3.8 P. aeruginosa's LPS induced distinct cytokine production profile in WT, MyD88^{-/-} and TRIF^{-/-} mice.

In addition to affecting the intrinsic properties of hematopoietic cells, loss of MyD88 in the microenvironment can affect hematopoiesis, in particular through production of cytokines [209]. G-CSF, TNF- α and IFN α , are critical hematopoietic cytokines strongly induced during sepsis and endotoxemia. Measurement of these cytokines in the serum showed that MyD88^{-/-} mice were defective in inducing all these three cytokines following LPS, in contrast to WT mice, which induced all of them, and TRIF^{-/-} mice, which were defective in induction of TNF- α and IFN α (Figure 3.8 B, C) but maintained induction of G-CSF (Figure 3.8 A).

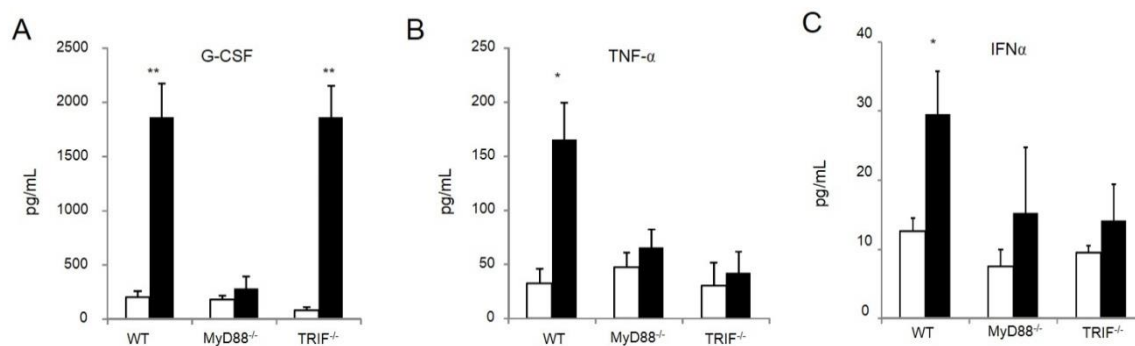


Figure 3.8 Cytokine productions in the serum of LPS-challenged WT, MyD88^{-/-} and TRIF^{-/-} mice.

(A) Average concentration of G-CSF in the serum of LPS or PBS challenged WT, TRIF^{-/-} and MyD88^{-/-} mice (n = 3-5). (B) Average concentration of TNF-α in the serum of LPS or PBS challenged WT, TRIF^{-/-} and MyD88^{-/-} mice (n = 3-5). (C) Average concentration of IFNα in the serum of LPS or PBS challenged WT, TRIF^{-/-} and MyD88^{-/-} mice (n = 3-5). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test.

3.9 Both cell-autonomous and non-cell autonomous activation of MyD88 contributes to myelosuppression and neutropenia induced by LPS.

Our observations indicate that MyD88 and TRIF contribute in different ways to two different ways to the BM response to sepsis. While TRIF leads to LSK expansion, MyD88 is required for myeloid differentiation block. Since loss of MyD88 could prevent LPS-induced BM neutropenia in our mouse model, we hypothesize that manipulation of MyD88 could provide a therapeutic target for the prevention of neutropenia in sepsis. To further understand the contribution of direct (cell-autonomous) and indirect (non cell-autonomous) MyD88 activation to myeloid suppression, we generated chimeric mice. Reciprocal transplantations were performed to generate BM chimeric mice with MyD88 KO (CD45.2⁺) hematopoiesis in a WT (CD45.1⁺) microenvironment background (MyD88^{-/-} Donor) and WT (CD45.1⁺) hematopoiesis in a MyD88 KO (CD45.2⁺) microenvironment background (MyD88^{-/-} ME) (Figure 3.8 A). As expected, neither MyD88^{-/-} Donor nor MyD88^{-/-} ME were able to revert the LSK expansion after LPS challenge (Figure 3.9 B). However, a reduced CMP, GMP and MEP populations were seen in both chimeras (Figure 3.9 C-E). Comparative analysis of the mature myeloid cells revealed that although LPS challenge lead to a significant decrease in mature myeloid cells (Gr1⁺/Mac1⁺) in both chimeras, the Gr1⁺/Mac1⁺ population in the LPS-MyD88^{-/-} Donor and MyD88^{-/-} ME was significantly ($p < 0.01$) higher than in the LPS-WT. Moreover, we noticed that the combination of myeloid protective effects by both chimeras was similar to the parental MyD88^{-/-} mice (Figure 3.9 F). The Gr1⁺/Mac1⁺ population among WT,

MyD88^{-/-} and both chimeras under normal (PBS) conditions were compared and no significant differences were found, thus excluding the artifact that the partial rescue arose from myeloid expansion in the chimeras due to irradiation (Figure 3.9 F). Collectively, I found that both cell-autonomous and non-cell autonomous activation of MyD88 contributes to myelosuppression and neutropenia.

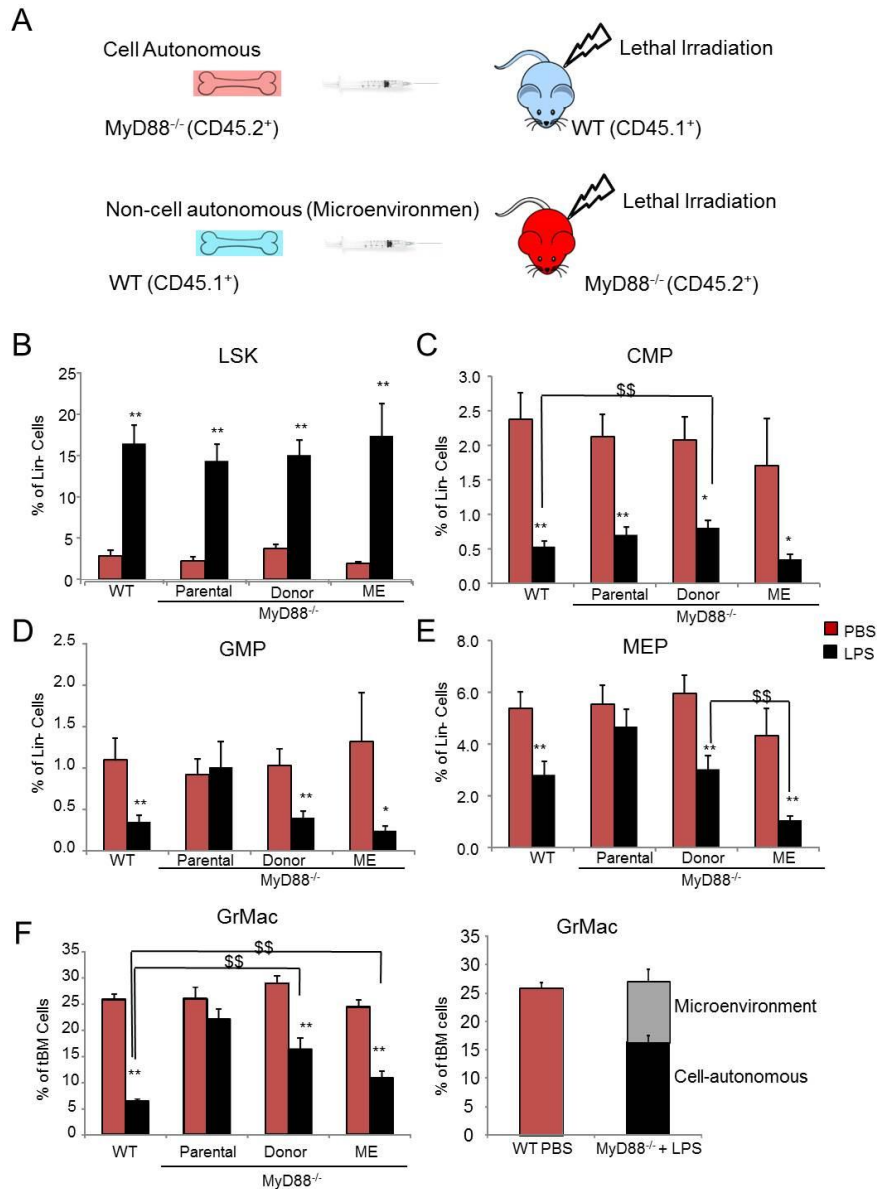


Figure 3.9 Contribution of cell-autonomous and non-cell autonomous effect of MyD88 activation to myeloid suppression in the BM during LPS exposure.

(A) Generation of MyD88^{-/-} Donor and MyD88^{-/-} ME chimeras through reciprocal transplantation. (B) Mean percentage of LSK cells in donor-derived Lin⁻ population from WT, MyD88^{-/-}, MyD88^{-/-} Donor and MyD88^{-/-} ME chimeras. (C-E) Mean percentage of CMP, GMP and MEP cells in donor-derived Lin⁻ population from WT, MyD88^{-/-}, MyD88^{-/-} Donor and MyD88^{-/-} ME chimeras. (F) Mean percentage of donor-derived mature myeloid cells from WT, MyD88^{-/-}, MyD88^{-/-} Donor and MyD88^{-/-} ME chimeras (n = 7-14). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01; LPS-WT vs. LPS-chimeras: \$\$p < 0.01, two-way ANOVA.

3.10 P. aeruginosa's LPS increased BM vascular permeability in a MyD88 dependent manner.

Results from the reciprocal transplantation showed that MyD88 ablation in the BM microenvironment could partially preserve mature myeloid cells, suggesting that activation of the TLR4/MyD88 pathway in the BM niche cells might also contribute to myeloid differentiation block. Indeed, a recent study reports the importance of endothelial TLR4 in replenishing emergency granulopoiesis during mild infection [210]. To better understand the potential role of MyD88 in the BM microenvironment, we used intravital microscopy. Our group has optimized intravital multiphoton fluorescence microscopy (IVFM) of the BM from mouse calvarium, and this technique has allowed us to study the change in the microenvironment, in particular the function of the BM vascular niche. Following LPS, the vasculature changed its properties: vessels are more dilated and there is increased permeability. We could observe these effects on WT and TRIF^{-/-} mice. Interestingly, I found that loss of MyD88 preserved the integrity of the BM vasculature and the vessels were less dilated after 24 hours of LPS challenge (Figure 3.10 A). I further performed permeability analysis on WT and MyD88^{-/-} vasculature by measuring the intensity ratio of the dextran in the intersected tissue and the dextran inside the vessels. Indeed, I found a significant increase of vasculature permeability in LPS-WT mice while there was an unaltered vasculature intensity ratio in LPS-MyD88^{-/-} mice (Figure 3.10 B).

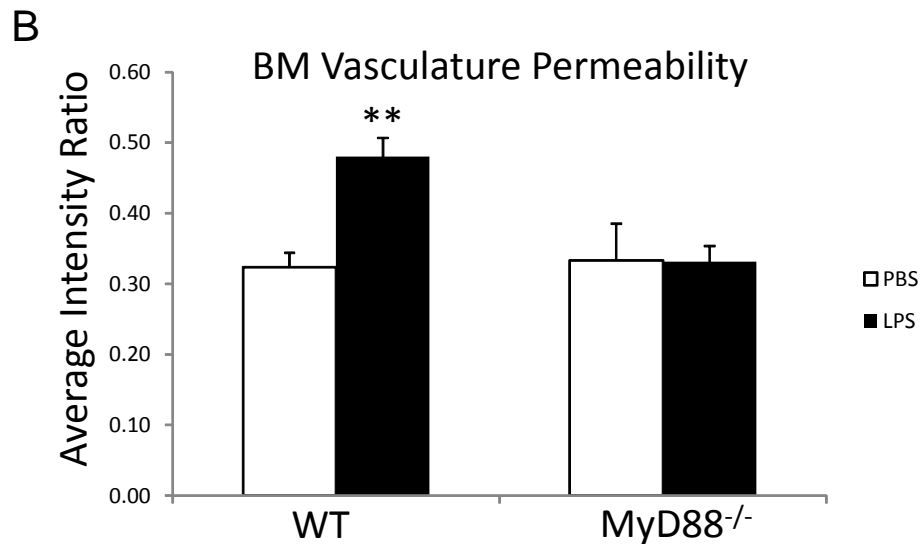
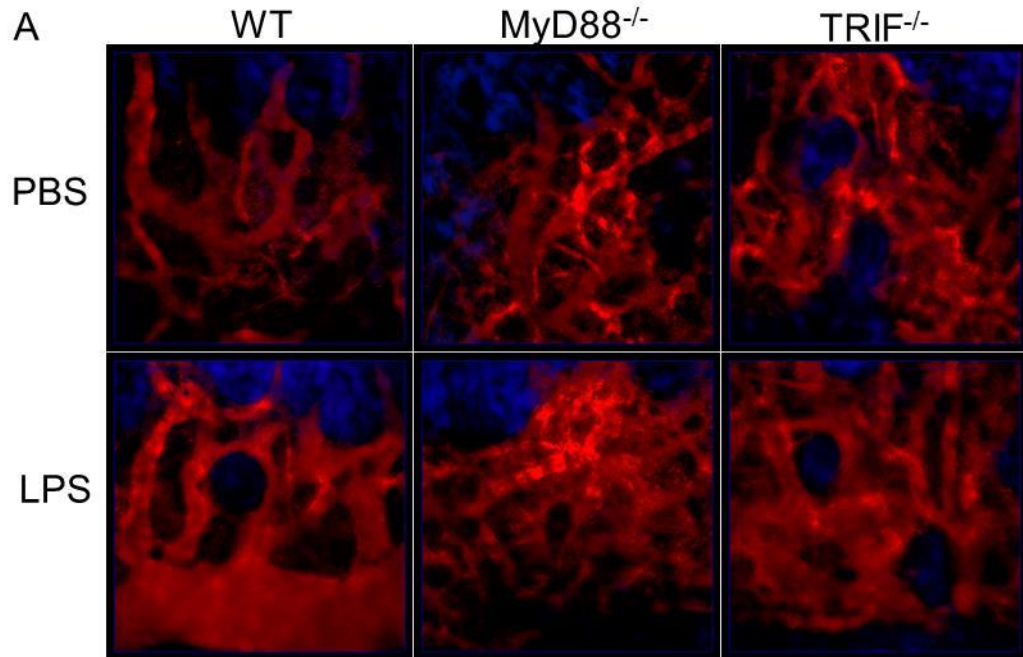


Figure 3.10 Loss of MyD88 prevents calvarium BM vascular injury during LPS challenge.

(A) IVFM imaging of calvarium BM vasculature in Wild-type, MyD88^{-/-} and TRIF^{-/-} animal challenged with LPS or PBS as control. Vasculature was defined by injection of Dextran-Texas Red. (B) Calvarium bone BM vasculature permeability measured by the average intensity ration in WT and MyD88^{-/-} mice (n = 4-5). Data represent mean \pm SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test.

CONCLUSION AND DISCUSSION

Myelosuppression has been reported in burn *P. aeruginosa* models as well as in LPS endotoxin models [13,211]. Such alteration was also present in the severe CLP polymicrobial sepsis model, demonstrating that the BM response I characterized here can be extended to different bacterial models of sepsis. It is important to note that various studies have reported the expansion of myeloid progenitor and mature cells, namely “emergency granulopoiesis” in the BM of mice challenged with *E. coli*'s LPS or during mild CLP polymicrobial sepsis [210,212]. A previous study noted distinct patterns of cardiovascular response induced by *E. coli*'s LPS and *P. aeruginosa*'s LPS in rats. In particular, *P. aeruginosa*'s LPS induced higher level of serum TNF- α than *E. coli*'s LPS [213]. Therefore, the different BM response induced by *E. coli*'s LPS and *P. aeruginosa*'s LPS is likely due to the different microorganisms the endotoxin is derived from, and also to the different experiment models used. Indeed, myeloid expansion is observed in certain models of CLP, where the CLP procedure performed with a 25-gauge needle induces a milder polymicrobial sepsis [212]. In the current study, the colon puncture was performed using a 21G1 gauge needle and induced an acute and severe peritonitis. Therefore, the model I used was more lethal and recapitulated the stage of acute and severe sepsis characterized by immunoparalysis and neutropenia.

It was previously reported that the *P. aeruginosa*'s LPS triggered BM response required intact TLR4 function. In order to further identify whether this response in the BM is mediated by MyD88-dependent pathway or MyD88-

independent pathway (TRIF-dependent pathway), I compared the BM response of WT, MyD88^{-/-} and TRIF^{-/-} after LPS challenge. I observed that the dysfunctional expansion of HSPC and the myeloid differentiation block were mediated by two different pathways. LSK expansion is dependent on the TRIF-dependent pathway whereas myelosuppression and neutropenia are dependent on the MyD88 pathways.

Further characterization of the LSK fraction by the SLAM markers revealed that the percentage of the LT-HSC, MPP1 and MPP2 compartment were all increased by LPS in a TLR4/TRIF dependent manner. However, similar to the response to IFN-1 exposure [165], the absolute number of LT-HSC was not affected by LPS. Sca-1 is one of the canonical markers used to define HSC [125]. It is an 18-kDa mouse glycosyl phosphatidylinositol-anchored cell surface protein (GPI-AP) of the *Ly6* gene family [214]. All HSC express Sca-1 [215,216]. During normal hematopoiesis, Sca-1 expression is downregulated as HSC differentiate into progenitors and mature blood cells [142]. However, during inflammation, cytokines can reactivate Sca-1 in myeloid progenitor cells [205,206]. A recent study by the Passegue group showed that IFN-1 exposure reactivates Sca-1 expression in myeloid progenitors *in vitro* [165]. Because the absolute number of LT-HSC was not increased by LPS like the LSK cells, it was reasonable to think that the LSK expansion in our LPS model could be due to Sca-1 reactivation by Sca-1-negative myeloid progenitors. To better define this effect, I used Lys-EGFP mice, in which only myeloid cells express the GFP protein, and can be tracked by flow cytometry analyses. Analysis of the GFP

distribution within the primitive LSK subsets revealed that while there were indeed a small number of the expanded LSK cells were GFP positive contaminating myeloid progenitors with reactivated Sca-1 expression, the majority of the expanded LSK cells were GFP negative. The results from the Lys-EGFP mice showed that Sca-1 re-expression in the myeloid progenitors is not the main mechanism for expansion of the LSK fraction during LPS challenge. Next, I investigated the cell cycle activity in the LSK cells. *In vivo* BrdU analysis showed that LPS induced quiescent LSK cells to enter S-phase. This response requires both MyD88 and TRIF pathway to be intact. This is not surprising for the TRIF^{-/-} mice but it is intriguing in the case of MyD88^{-/-} mice, as LSK expansion was also observed in LPS-challenged MyD88^{-/-} mice. Cell cycle analyses in LPS-MyD88^{-/-} LSK cells suggest that mechanism(s) other than cell cycle activation is/are involved in the LSK expansion in MyD88^{-/-} mice. Cell pool expansion without cell cycle activation has been reported in other context such as Skp2^{-/-} mice [217]. One possible mechanism is that symmetrical division is favored over asymmetrical division in the MyD88^{-/-} LSK cells after LPS challenge. Paired-daughter cell assay on these LSK cells is required to further test this hypothesis. In summary, LSK expansion during LPS is likely due to increased cell cycle activity in the WT mice. However, the reason why MyD88^{-/-} LSK was expanded without change in the cell cycle activity remains elusive. Increased proliferation rate and reduced quiescence in LPS-WT LSK cells suggest they could have lower regenerative function and engraftment rate compared to LPS-MyD88^{-/-} LSK and LPS-TRIF^{-/-} LSK cells [153]. This aspect is further pursued in Chapter Four.

Our group has previously shown that bacterial sepsis induces TLR4-mediated depletion of myeloid progenitors and neutropenia [13]. Here, I further identified that the myeloid differentiation block during LPS challenge or CLP polymicrobial sepsis requires the TLR4/MyD88-dependent signaling pathway but not the TLR4/TRIF-dependent pathway. Interestingly, following LPS, MyD88^{-/-} mice exhibited normal number of GMP and neutrophils, but decreased CMP levels. Therefore, I hypothesize that LPS-induced activation of the TLR4/MyD88 pathway blocked the egress from CMP to GMP and ultimately Gr1⁺/Mac1⁺ cells. This hypothesis was confirmed in *in vitro* differentiation assay of CMP and GMP from PBS- or LPS- challenged WT, MyD88^{-/-} and TRIF^{-/-} mice: septic WT and TRIF^{-/-} CMP showed a delayed differentiation that was rescued by loss of MyD88, whereas LPS-GMP showed no difference compared to the PBS control and among the different genotypes. In summary, our *in vitro* data strongly suggests that although a reduced CMP population was observed in the MyD88^{-/-} mice challenged with LPS, these CMP are functionally different from the septic WT and TRIF^{-/-} CMP in the sense that they are able to egress through the myeloid differentiation pathway to generate GMP and neutrophils. Further transplantation experiments with CMP from different genotypes are necessary to consolidate this finding.

Multiple *in vivo* and *in vitro* studies showed TLR ligands could impact HSC functions and hematopoiesis directly [218] [172]. In the CMP *in vitro* differentiation assay, I showed activation of TLR4/MyD88 pathway blocked myeloid differentiation in a cell-autonomous manner. Hematopoietic cells can

also be affected by the microenvironment mediators that are activated by TLR ligands. It is reported that BM mesenchymal stem cells in the BM niche express TLR4 and sense circulating TLR ligands to regulate monocyte migration [219]. Another study showed that TLR4-expressing nonhematopoietic cells in the BM were required for emergency myelopoiesis upon LPS stress [210]. Besides microenvironment cells, cytokines induced by LPS also contribute to the non-cell autonomous effect. Indeed, I found that LPS challenge significantly increased the serum level of G-CSF, TNF- α and IFN α in a Myd88-dependent manner. These pro-inflammatory cytokines are known for their roles in regulating hematopoiesis. Whether the protective effect of MyD88 deficiency on myeloid differentiation block is mediated by a cell-autonomous or non-cell autonomous mechanism remained elusive. Using a reciprocal transplantation method, I found that deletion of MyD88 in hematopoietic cells or in the microenvironment alone could only partially rescue the myeloid differentiation following LPS challenge. The *in vitro* differentiation data and *in vivo* chimera data shows that activation of MyD88 both in the myeloid cells and in the microenvironment contributes to myelosuppression during sepsis.

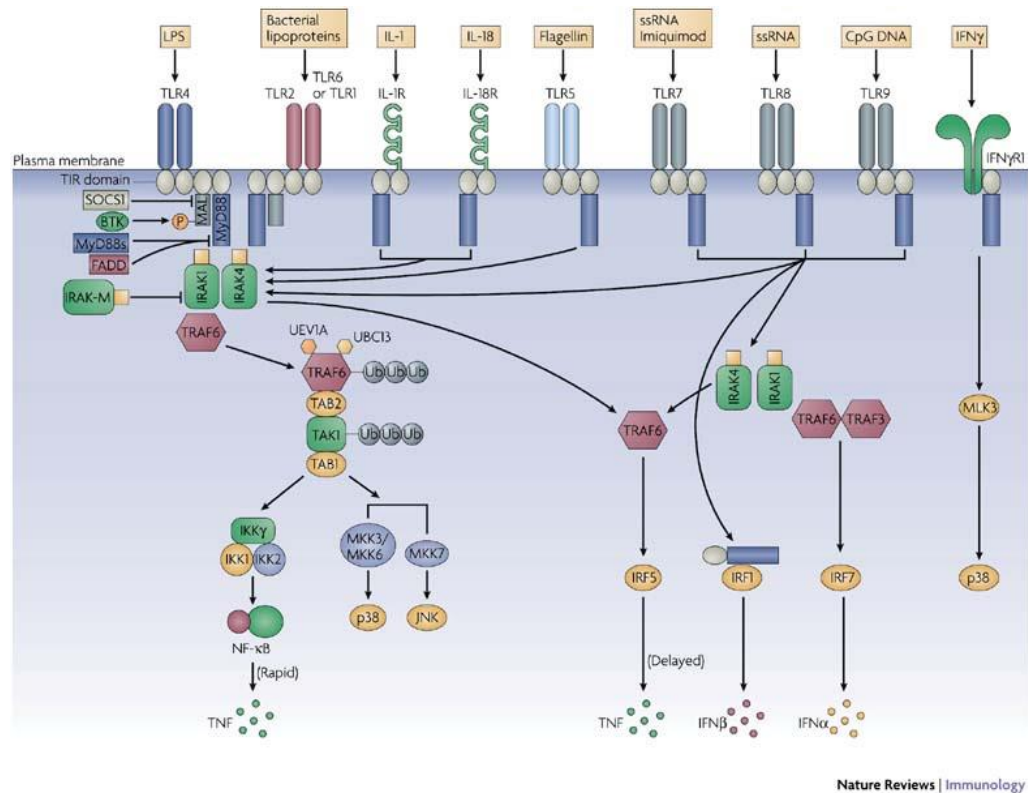
Because activation of the TLR4/MyD88 pathway in the microenvironment also contributed to myeloid suppression, I proceeded to identify potential alterations in the BM niche following LPS challenge. Using the IVFM method, I showed that the BM vascular niche is significantly altered by acute LPS exposure. Enlarged vessels were seen in WT and TRIF^{-/-} mice following LPS. Quantitative analysis of vascular permeability revealed that loss of MyD88 can

prevent capillary leakage induced by endotoxin. Vasodilatation and capillary leakage are two important pathological events during septic shock that induce hypotension in patients [220]. Here, I found that MyD88 deletion can prevent these events in the BM vasculature. Thus, the TLR4/MyD88 pathway mediates both myelosuppression and endothelial dysfunction following LPS. This observation suggests the possibility of a link between these two processes and thus then highlights the importance to study the interplay between the microenvironment and hematopoietic function in sepsis.

While studying the functional role of TLR4/MyD88-dependent pathway in the BM response following LPS challenge, it is important to consider the possible indirect activation of MyD88-pathway by cytokines induced by inflammation, such as IL-1 and IL-18, as MyD88 is a downstream adaptor of their receptors [221,222] (Figure 3.11). Global cytokine profiling with cytokine array, however, showed that IL-1 levels in the serum of LPS-challenged mice in the current study were not significantly elevated (data not shown). Therefore, it is unlikely that IL-1R/MyD88-signaling pathway play a role in the BM response during LPS challenge. Assuming that other yet-to-be-identified cytokine(s) X induced by LPS stimulation were playing a major role in inducing MyD88-dependent myeloid suppression, a complete rescue effect of Gr1⁺/Mac1⁺ loss should have been observed in the MyD88^{-/-} Donor chimera, because the production of cytokine X by the WT microenvironment cells after LPS challenge remained intact. However, my chimera data showed loss of MyD88^{-/-} in the hematopoietic cells alone could only partially prevent BM neutropenia, suggesting that cytokine-activation of

MyD88-dependent pathway did not play a dominant role in BM neutropenia during LPS stimulation. However to definitively validate this point further experiments need to be performed to determine the impact of recombinant IL1b on BM and the effects of LPS challenge in mice null for the IL1B receptor.

In conclusion, this part of the study dissected the functional role of TLR4/MyD88 and TLR4/TRIF pathway in mediating the BM response during *P.aeruginosa*'s LPS challenge and CLP polymicrobial sepsis. The TLR4/TRIF pathway played a dominant role in mediating the LSK expansion during sepsis. While Sca-1 re-expression in the myeloid progenitors leads to a small fraction of contaminating LSK cells, the majority of expanded LSK cells are real HSPC. Cell cycle activity is significantly increased in septic LSK cells both in MyD88 and TRIF dependent manner. Loss of MyD88 significantly prevents myeloid differentiation block essentially at the stage of CMP and ultimately prevents neutropenia upon endotoxemia or sepsis. Interestingly, such protective effect required a complete ablation of the TLR4/MyD88 pathway both in the microenvironment and in the hematopoietic cells. Finally, I made a novel discovery that the activation of TLR4/MyD88 lead to BM vasculature niche damage *in vivo*. Collectively, my results suggest the TLR4/MyD88 signaling is a very good therapeutic candidate for protecting the BM from inflammatory damage during sepsis. Studies involving the use of MyD88 decoy peptide are underway.



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Figure 3.11 Signaling pathways engage with the MyD88 adaptor protein.

MyD88 is the direct adaptor protein of TLR4, TLR2, TLR7, TLR8 and TLR9. It is the recruiter of IRAK4 to activate downstream signaling pathways including NF- κ B and/or p38 pathway. MyD88 is also a key adaptor molecule for IL-1R, IL-18R for IRAK4 recruitment and associate with IFN γ R1 for p38 activation.

CHAPTER FOUR

ABLATION OF THE TRIF and MYD88 PATHWAY PROTECTS HSC FUNCTION DURING *P. AERUGINOSA*'S LPS CHALLENGE

INTRODUCTION

Profound BM dysfunction and hematopoietic failure have been observed during sepsis both in human patients and in experimental animals [223-226]. Administrations of CD34⁺ HSPC greatly improved the survival rate of mice with polymicrobial sepsis likely by attenuating the hypoinflammatory response [227]. It has been reported that the regenerative potential of the LT-HSC resides mainly in the quiescent fraction [153]. Cell cycle activity increases as HSC differentiate along the hematopoietic hierarchy restricting their self-renewal activity [153]. Several studies have shown that chronic inflammation, LPS, IFNs or G-CSF exposure diminish the self-renewal and reconstitution potential of HSC while activate their cell cycle entry [157,162,172]. One common BM response to these stimuli is the expansion of the LSK fraction. Previous work by our group showed that the expanded LSK cells isolated from the WT mice challenged with *P. aeruginosa*'s LPS lost their engraftment and reconstitution potential in transplantation experiments [13]. Results from the previous chapter showed that LPS induced cell cycle activation in WT LSK fraction in a TLR4/MyD88 and TRIF dependent manner. The different cell cycle activity in septic WT LSK and MyD88^{-/-} LSK, TRIF^{-/-} LSK cells suggested they may have different stem cell functions. Here, I found that loss of TRIF fully preserved HSC function following LPS, while

loss of MyD88 only partially protected HSC from endotoxic damage. Consistently, ablation of the TLR4/TRIF or MyD88 pathway prevented the irreversible downregulation of crucial transcription factors required for myelopoiesis and HSC functions in septic LSK cells.

RESULTS

4.1 Loss of TRIF and MyD88 rescue the long-term engraftment efficiency of HSC after LPS challenge.

To determine the impact of the immunophenotypic expansion of the LSK pool following LPS, and the potential role of MyD88 and TRIF, we tested their function in HSC by performing the competitive reconstitution assay. Briefly, 2500 CD45.2⁺ LSK cells containing equivalent amount of LT-HSC were sorted from the BM of PBS- or LPS challenged WT, MyD88^{-/-} or TRIF^{-/-} mice, and were transplanted into lethally irradiated CD45.1⁺ recipient mice together with 10⁵ competitor cells. Changes in PB chimerism were followed up to 24 weeks (Figure 4.1 A). We found that the ability of normal (PBS) LSK to engraft at short-term and long-term was similar in WT, My88^{-/-} and TRIF^{-/-} genotypes (70-77% chimerism at 24 weeks). MyD88^{-/-} LSK cells showed a slight lower short-term engraftment efficiency which was not statistically significant. In contrast, WT, MyD88^{-/-} and TRIF^{-/-} LPS LSKs displayed great differences in their ability to compete and engraft at short- and long-term. The engraftment ability of LPS-WT LSK at short- and long-term was very poor, and chimerism was 9% at weeks 8 and 16, respectively, and 7% and 15% at weeks 20 and 24, respectively (Figure 4.1 B). The low engraftment of LPS-WT cells was not due to decreased homing (Figure 4.1 C). Loss of TRIF greatly improved short-term engraftment (51% and 63% chimerism at week 8 and 16) and fully restored long-term reconstitution at 24 weeks (68%). Interestingly, loss of MyD88 enhanced significantly short- and

long-term engraftment, but less robustly than TRIF deletion (35%, 48% and 58% chimerism, respectively at week 8, 16 and 24).

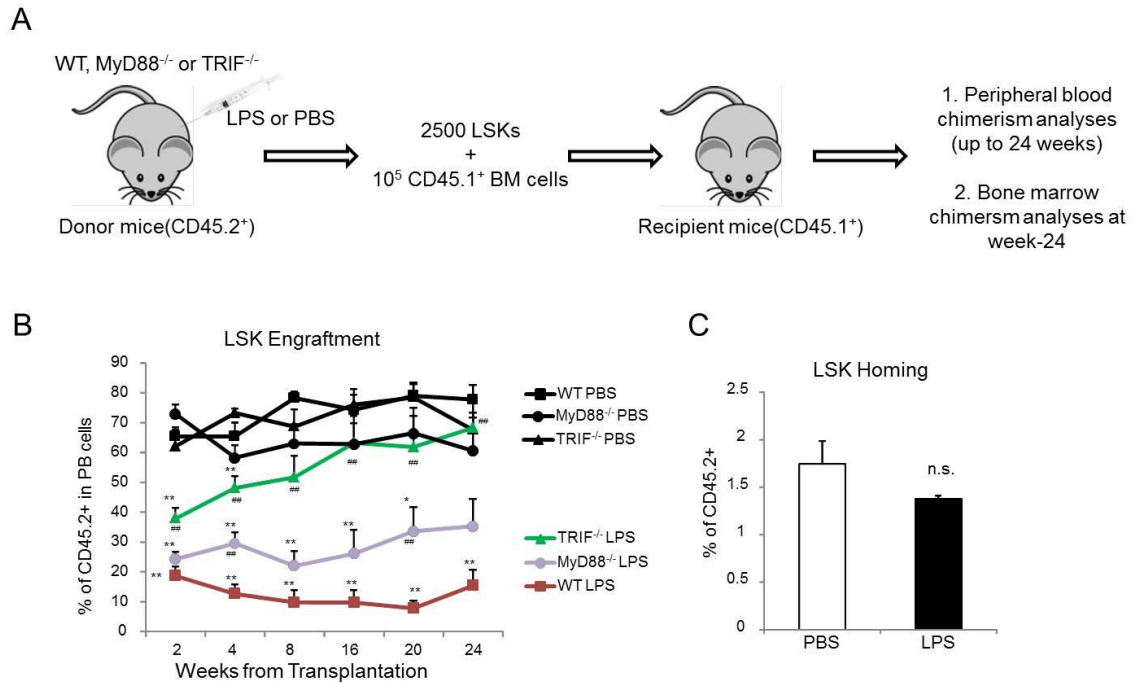


Figure 4.1 Loss of TRIF and MyD88 protects LSK engraftment potential.

(A) Experimental design for LSK competitive reconstitution assay. (B) Donor engraftment (CD45.2⁺) in the PB at indicated time points in BoyJ mice transplanted with WT PBS-LSK (Black square solid bar; n = 6-15), MyD88^{-/-} PBS-LSK (Black circle solid bar; 2 weeks: n = 3; 4-24 weeks: n = 8-12), TRIF^{-/-} PBS-LSK (Black triangle solid bar; n = 7-8), WT LPS-LSK (Red; n = 5-14), MyD88^{-/-} LPS-LSK (Purple; n = 6-12) and TRIF^{-/-} LPS-LSK (Green; n = 7-8). (C) Mean percentage of CD45.2⁺ cells in the BM of CD45.1⁺ Boy/J mice 16 hours after receiving 25,000 LSK cells derived from PBS or LPS challenged C57BL6/J mice (n = 3). Data represent mean ± SEM. For (B), LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ## p < 0.01, unpaired t test. For (C), LPS vs. PBS: n.s., not significant, unpaired t test.

4.2 Increased contribution of LPS-MyD88^{-/-} and LPS-TRIF^{-/-} HSC to the recipient BM following transplantation.

In order to further study the reconstitution capability of septic LSK in the BM at long-term (24 weeks), I analyzed the hematopoietic compartments in the recipients of WT, TRIF^{-/-} and MyD88^{-/-} LSK cells (septic vs. normal) at 24 weeks after transplantation. Consistent with the PB result, I found that only 10% of all BM cells were CD45.2 donor-derived in mice transplanted with LPS-WT LSK, whereas CD45.2 donor-derived cells represented 50%-60% of total BM when LPS-MyD88^{-/-} were transplanted (Figure 4.2 A). In particular, in mice transplanted with TRIF^{-/-} LSK cells, the contribution of septic LSK was similar to normal LSKs, indicating a complete protection. To examine the donor LSK's capacity to regenerate new LSK cells in the recipients at long-term, I compared the mean intensity of the CD45.2-PE within the LSK population from all experimental groups. Sorted LPS-WT LSK cells were unable to give rise to new LSK cells in the recipients as shown by the dramatic left shift of the histogram peak compared to the control recipients that received normal LSKs. In contrast, LPS-TRIF^{-/-} LSKs were able to derive new LSK cells upon transplantation. At the same time, the capacity of LPS-Myd88^{-/-} LSK cells' capacity to regenerate new LSK cells was in between WT and TRIF^{-/-}, indicating a partial rescue (Figure 4.2 B). Consistent with the histogram data, flow cytometry analyses in the primitive subpopulations showed a low contribution of LPS-WT LSK to the LT-HSC, MPP1 and MPP2 subsets ($\leq 30\%$). Transplanted LPS-TRIF^{-/-} LSK cells contributed more than 80% to all the primitive subsets. Again, loss of MyD88 in the LSK subsets after acute

LPS exposure could only partially maintain the contribution to LT-HSC, MPP1 and MPP2 primitive subsets (Figure 4.2 C).

After 24 weeks from transplant the absolute number of donor LT-HSC in the recipient BM was compared with the parental mice as input control. Healthy donor LT-HSC from WT, MyD88^{-/-} and TRIF^{-/-} maintained a similar regeneration potential. Consistent with the PB engraftment data, a dramatic decrease in LPS-LT-HSC number was observed (5-fold). On the contrary, LPS-TRIF^{-/-} LSK cells showed the capacity to generate comparable number of LT-HSC cells as the normal LSK cells 24 weeks after transplantation. Moreover, the absolute number of LPS-TRIF^{-/-} LT-HSC in the recipients was significantly higher than the LPS-WT LT-HSC, while LPS-MyD88^{-/-} LT-HSC exhibited an intermediate regeneration potential between LPS-WT and LPS-TRIF^{-/-} LT-HSC (Figure 4.2 D).

The data from the analysis of absolute number indicated that the self-renewal potential of LPS-TRIF^{-/-} LT-HSC was not hampered. To test this hypothesis, we calculated the self-renewal quotient. LT-HSC self-renewal capacity (self-renewal quotient) was measured as described by Challen et al. [228]. Total absolute number of LT-HSC at 24 weeks post-transplantation was established and divided by the absolute number of injected LT-HSC (Table 4.1). As shown in Figure 4.2, LPS-WT LT-HSC's self-renewal was significantly lowered than the normal WT LT-HSC whereas the self-renewal capacity of LPS-TRIF^{-/-} LT-HSC was fully maintained. Surprisingly, although the LPS-MyD88^{-/-} LSK cells demonstrated a partial rescued stem cell function, the self-renewal quotient were comparable to the normal control and significantly higher than the

WT septic cells. The cellular mechanism underlying these differences requires further investigation. In summary, these data showed that loss of TRIF^{-/-} can completely preserve the engraftment and regeneration potential of endotoxin-challenged HSC. In other words, activation of the TLR4/TRIF pathway during acute inflammation significantly hampers the LT-HSC self-renewal potential.

Table 4.1 Percentage of LT-HSC in LSK cells.

	% of LT-HSC in LSK cells				Cells/2500 LSK cells	
	PBS	SEM	LPS	SEM	PBS	LPS
WT	20.5	±0.66	8.87	±0.91	512	211
MyD88 ^{-/-}	19.1	±1.79	9.19	±0.97	479	229
TRIF ^{-/-}	15.9	±2.46	9.09	±0.57	397	227

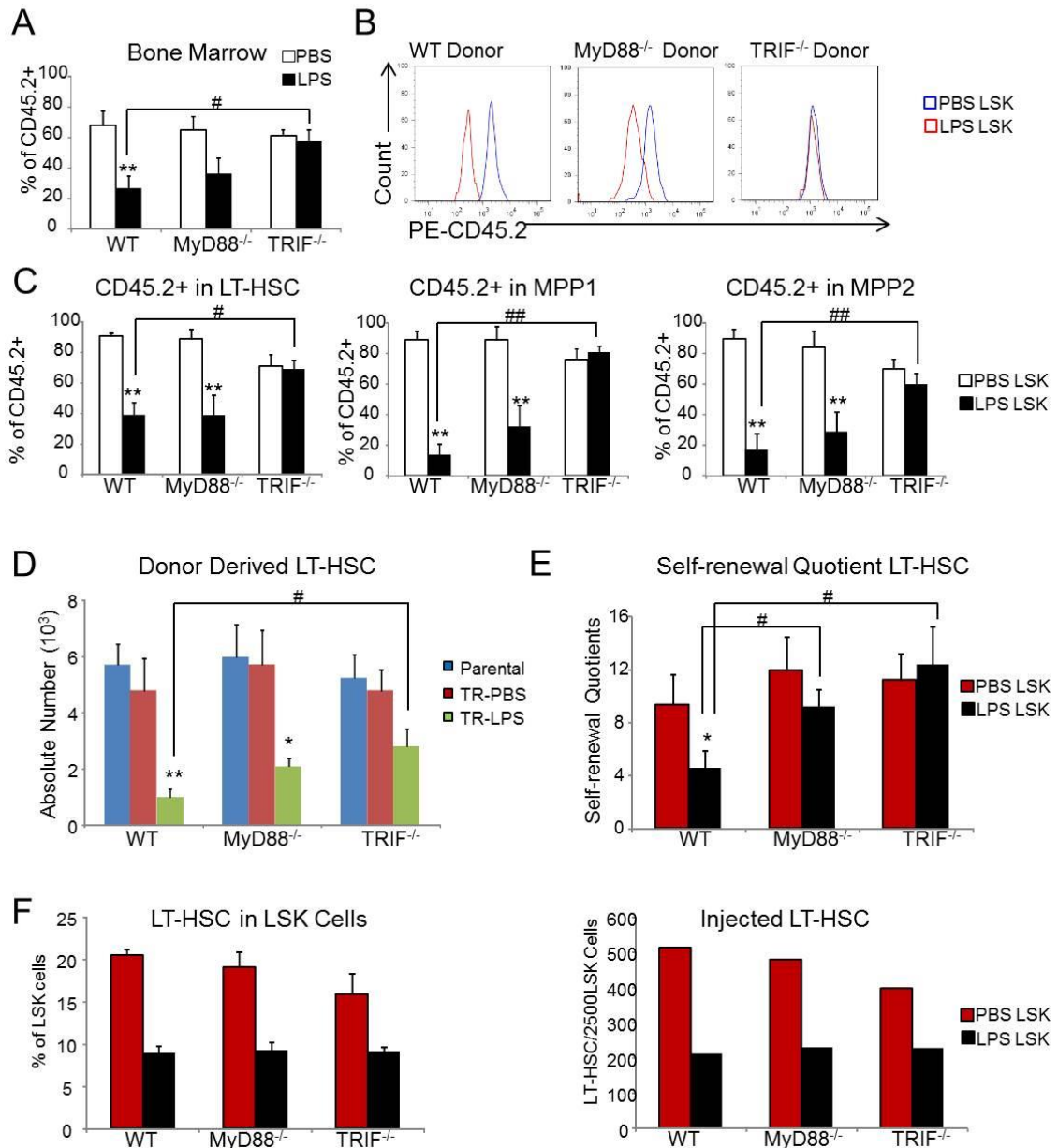


Figure 4.2 Loss of TRIF and MyD88 provide functional protection to LPS challenged LSK cells.

(A) Percentage of donor engraftment of WT, MyD88^{-/-} and TRIF^{-/-} PBS or LPS LSK cells (CD45.2⁺) in the BM of recipient mice at 24 weeks post-transplantation (n = 7-9). (B) Representative histogram of intensity of PE-CD45.2 within the LSK population of recipient mice (n = 7-9). (C) Percentage of donor-derived CD45.2⁺ BM LT-HSC, MPP1 and MPP2 in recipient mice (n = 7-9). (D) Absolute number of donor LT-HSC per femur in: Blue, normal WT, MyD88^{-/-} and TRIF^{-/-} mice; Red in mice receiving LSK cells from PBS-challenged WT, MyD88^{-/-} and TRIF^{-/-} mice

24 weeks post-transplantation; and Green, in mice receiving LSK cells from LPS-challenged WT, MyD88^{-/-} and TRIF^{-/-} mice 24 weeks post-transplantation (n = 7-9).still need to decide color. (E) Self-renewal quotients of LT-HSC cells from PBS and LPS challenged WT, MyD88^{-/-} and TRIF^{-/-} mice at 24 weeks post-transplantation. Quotient is calculated by dividing the absolute number of LT-HSC by the average number of LT-HSC input at the time of transplantation (n = 7-9). (F) Number of LT-HSC was estimated based on the general percentage of LT-HSC within the LSK population from four independent experiments and the number of LSK cells used for transplantation (Table 4.1). Data represent mean ± SEM. For (A), (C), and (E), LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, ##p < 0.01, unpaired t test. For (D), LPS vs. PBS: *p < 0.05, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} vs. LPS-TRIF^{-/-}: #p < 0.05, one-way ANOVA.

4.3 Different impact of TRIF and MyD88 deletion on HSC multilineage reconstitution following endotoxic injury.

To determine whether acute LPS exposure altered the ability of HSC to contribute to multilineage progenitors, and to evaluate whether MyD88 and/or TRIF contribute to such function, I compared the progenitor and mature myeloid subsets in the BM from all the recipient mice. Flow cytometry analyses indicated that LPS-WT LSK contributed little to these subsets compared to the control: CMP (20% vs. 90%); GMP (20% vs. 90%); MEP (15% vs. 97%); CLP (25% vs. 60%) and Gr1⁺ myeloid cells (5% vs. 98%). Loss of TRIF rescued the ability of the septic LSK cells to contribute to these subsets: 80% in CMP, GMP and MEP; 65% to CLP and 75% to Gr1⁺ cells. Loss of MyD88 had some protective effect and increased the contribution to CMP, GMP and MEP to up to 40%; CLP up to 25% and Gr1⁺ cells up to 55% (Figure 4.3 A). Analyses of multilineage contribution in the PB were consistent with these observations. LPS-WT HSC showed low contribution to myeloid, B and T cells at early and late time points (Figure 4.3 B, C); LPS-TRIF^{-/-} HSC contributed robustly to all 3 lineages since the early time points of engraftment and MyD88 showed an intermediate behavior, with progressive improvement overtime (Figure 4.3 B, C).

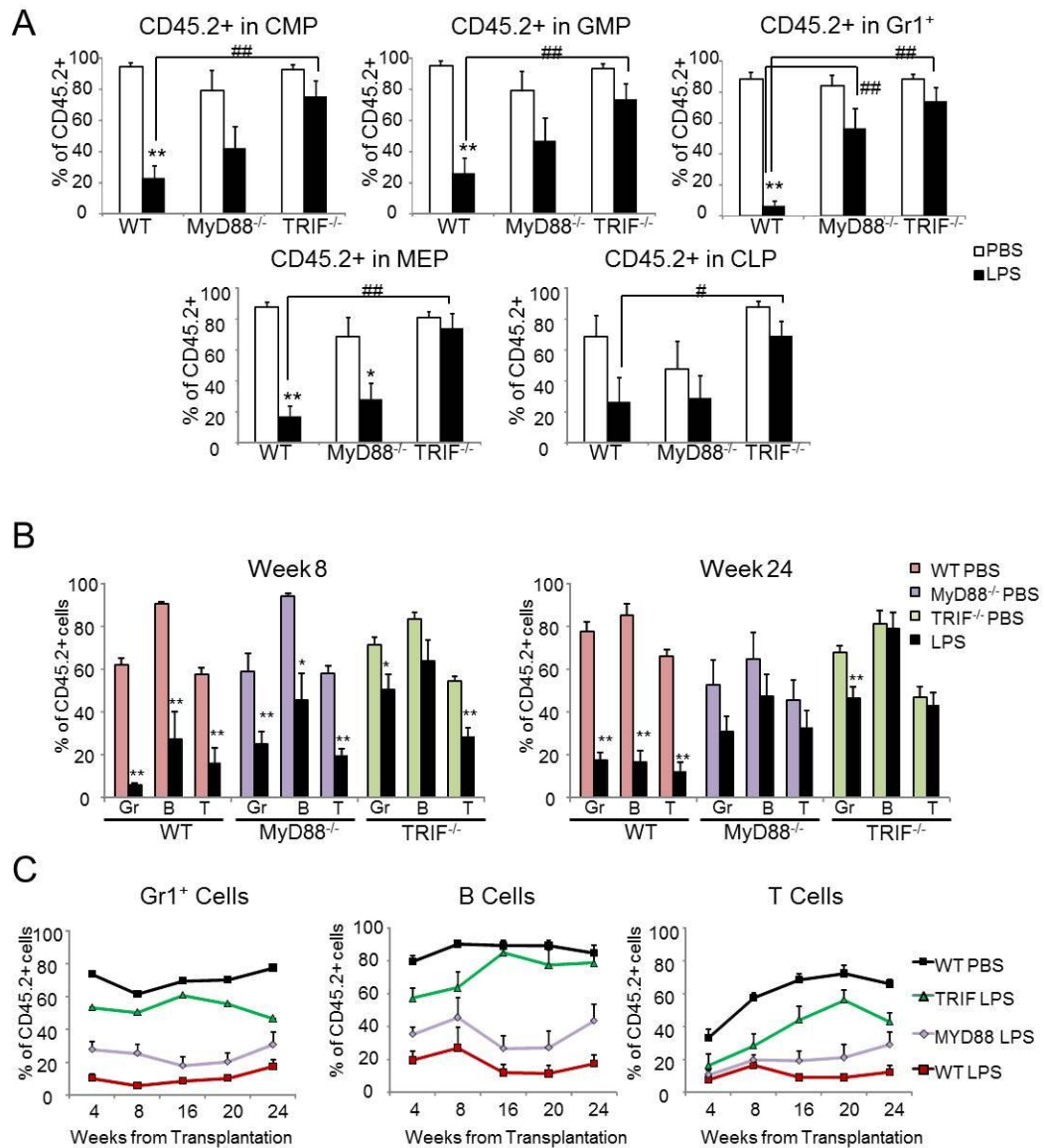


Figure 4.3 Loss of TRIF protected the multilineage reconstitution ability of septic LSK cells.

(A) Mean percentage of donor derived CD45.2⁺ BM CMP, GMP, Gr1⁺, MEP and CLP populations in recipient mice (n = 8-10). (B) Mean percentage of CD45.2⁺ PB cells in myeloid cells (Gr1⁺), B cells (B220⁺) and T cells (CD4⁺ or CD8⁺) in recipient mice at 8 weeks and 24 weeks post-transplantation (n = 7-16). (C) Line graph shows mean percentage of CD45.2⁺ cells in in myeloid cells (Gr1⁺), B cells (B220⁺) and T cells (CD4⁺ or CD8⁺) in recipient mice at indicated timepoint post-transplantation (n = 7-16). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} vs. LPS-TRIF^{-/-}: #p < 0.05, ## p < 0.01, one-way ANOVA.

4.4 MyD88^{-/-} and TRIF^{-/-} LSK cells were able to maintain the expression of essential regulators for myelopoiesis.

Evaluation of the BM response to endotoxemia in MyD88^{-/-} and TRIF^{-/-} mice revealed a distinct role of MyD88 and TRIF on myeloid progenitors and stem cells. To determine whether this effect was also associated with disruption of the transcriptional regulators, we analyzed the expression of PU.1 and C/EBP α , two transcription factors important for myeloid differentiation. Levels of PU.1 and C/EBP α expression were determined in LSK cells sorted from LPS or PBS-treated WT, MyD88^{-/-} and TRIF^{-/-} mice (Figure 4.4A).

PU.1 encoded by *Spi1* is a transcription factor that acts upstream of regulators controlling myelopoiesis [229,230]. qRT-PCR analyses showed a 3-fold decrease of PU.1 transcripts in LPS-WT LSK cells and a 2-fold decrease in the LPS-TRIF^{-/-}. PU.1 gene expression level was preserved in LPS-MyD88^{-/-} LSK cells (Figure 4.4 B). C/EBP α is another important transcription factor required for myelopoiesis, which is encoded by *CEBPA* [136]. qRT-PCR data showed that both MyD88^{-/-} and TRIF^{-/-} can prevent the downregulation of *CEBPA* induced by LPS, as observed in WT mice (Figure 4.4 C). Collectively, my data is in agreement with the knowledge that loss of MyD88 can prevent myeloid differentiation block during LPS stress. It suggests that loss of MyD88 can protect the myeloid differentiation regulatory machinery from being disrupted by LPS at the level of primitive HSPC.

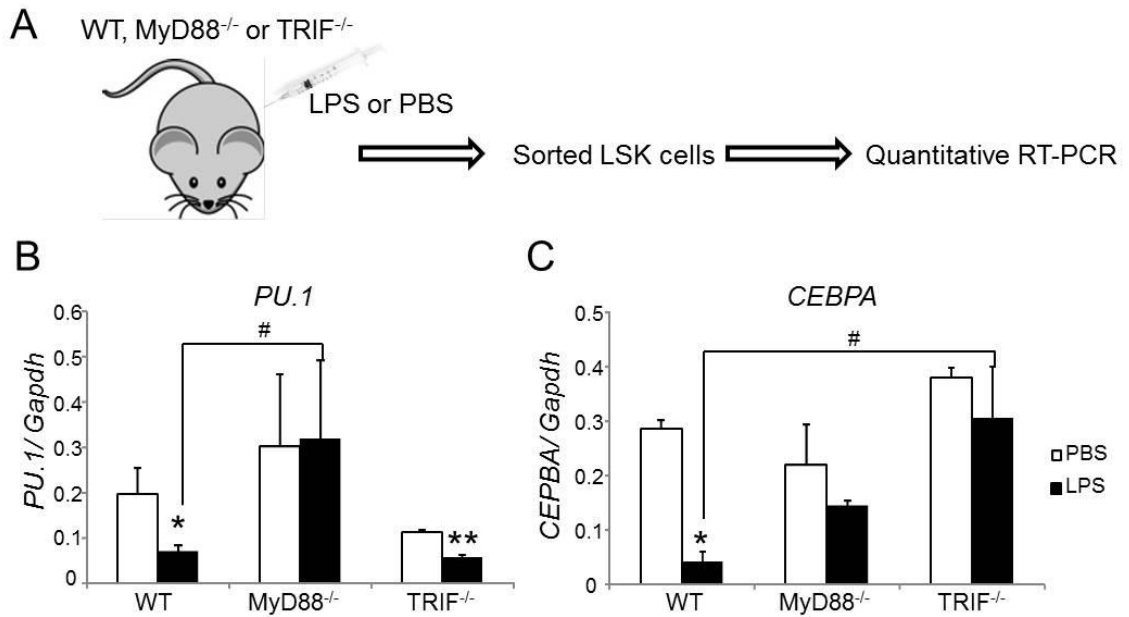


Figure 4.4 LPS downregulates transcription factors essential for myeloid differentiation in the LSK cells in a MyD88 dependent manner.

(A) Experiment design for gene expression analyses in normal and septic LSK cells from WT, MyD88^{-/-} and TRIF^{-/-} mice. (B) Relative mRNA level of PU.1 (*Spi1*) compared to *Gapdh* in sorted LSK cells (n = 3-9). (C) Relative mRNA expression level of *CEBPA* compared to *Gapdh* in sorted LSK cells (n = 3-9). Data represent mean ± SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, unpaired t test.

4.5 Downregulation of essential regulators for HSC function and myelopoiesis persisted at 24 weeks post-transplantation.

Acute LPS exposure induced long-term HSC cell function damage as shown by the competitive repopulation assay. TRIF deficiency completely rescued HSC from the LPS-induced HSC injury while MyD88 deficiency had an intermediate protective effect. My hypothesis is that short-term endotoxin stress leads to a permanent alteration of HSC at the genetic level. To test this hypothesis, I further examine the gene expression levels of the PU.1 and C/EBP α transcription factors in sorted donor-derived LSK cells from recipient mice, at 24 weeks after transplantation (Figure 4.5 A).

Downregulation of PU.1 and C/EBP α was found in sorted donor-derived LSK from LPS-WT LSK recipients 24 weeks after transplantation, suggesting that these alterations are irreversible (Figure 4.5 B). On the contrary, mRNA levels of these molecules were either upregulated or unchanged in donor-derived LSK cells from LPS-MyD88^{-/-} or LPS-TRIF^{-/-} LSK recipients (Figure 4.5 C-D). PU.1 is required for maintaining the balance between HSC self-renewing division and quiescence [229]. Depletion of C/EBP α is associated with HSC exhaustion [231]. Collectively, these published results and our data suggested that acute LPS exposure leads to long-term irreversible downregulation of key transcription factors required for HSC maintenance and myelopoiesis. Loss of TRIF and/or MyD88 can partially, if not completely, prevent such disruption. The preservation of the gene expression of key transcription factors is likely to be critical for the protective effect of TRIF and MyD88 deficiency on HSC function.

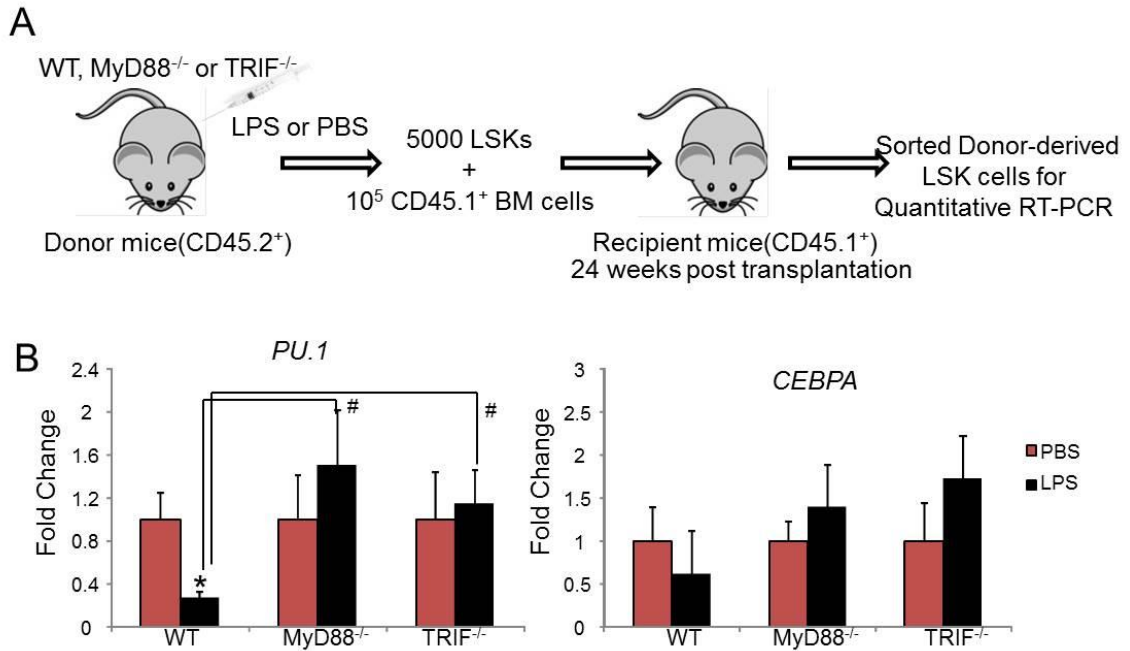


Figure 4.5 LPS-induced irreversible long-term genetic alterations in the LSK cells.

(A) Experiment design for gene expression comparison between normal and septic LSK cells after 24 weeks of transplantation. (B) Fold change of expression levels of *PU.1* and *CEBPA* in parental mice and recipient mice reconstituted with normal or septic LSK cells (n = 3-4). Data represent mean \pm SEM. LPS vs. PBS: *p < 0.05, **p < 0.01, unpaired t test; LPS-WT vs. LPS-MyD88^{-/-} or LPS-TRIF^{-/-}: #p < 0.05, unpaired t test.

CONCLUSION AND DISCUSSION

How inflammation affects HSC function has been an intriguing question that still lacks a comprehensive answer. A few studies revealed that acute or chronic infection greatly impairs the short- and long-term engraftment potential of HSC following transplantation [157,172]. The discovery that TLR4 is expressed by HSPC provides the molecular evidence that HSPC participate directly in the primary BM response to infections [171]. LSK cells from mice challenged with *P. aeruginosa*'s LPS were unable to engraft after transplantation into lethally irradiated mice [13]. Functional damage of the HSPC by *P. aeruginosa*'s LPS is associated with myeloid differentiation block and neutropenia, which are dependent on the TLR4 pathway. In the previous chapter, I have shown that activation of TLR4/MyD88 signaling leads to myeloid suppression in the BM. In the current chapter, I further dissected the contribution of TLR4/MyD88 and TLR4/TRIF signaling in mediating HSC injury after acute exposure to LPS.

In vivo BrdU analysis showed that *P. aeruginosa*'s LPS activated quiescent LSK cells and forced them into cell cycle. Loss of MyD88 or TRIF prevented cell cycle activation of LSK cells (Figure 3.3). Previous studies showed that actively proliferating HSC lose their post-transplantation engraftment capacity and that HSC's self-renewal and multilineage potential resides mainly in the quiescent fraction [153]. The different cell cycle dynamics of the LSK cells from septic WT, MyD88^{-/-} and TRIF^{-/-} mice suggest that they exhibit different stem cell functions when transplanted into lethally irradiated recipients. Indeed, in the competitive reconstitution assay, WT septic LSK cells' engraftment potential was

very low at short- and long-term. Since loss of MyD88 was able to preserve mature myeloid cell development in the BM, I expected the LPS-MyD88^{-/-} LSK cells were also functionally protected. However, LPS-MyD88^{-/-} LSK cells were only partially rescued, but their low engraftment activity at short-term was gradually improved at long-term. LPS-MyD88^{-/-} LSK also displayed a partial protective effect in the multilineage regenerative potential. The regenerative function of LSK cells from septic TRIF^{-/-} mice was significantly preserved compared to WT and MyD88^{-/-}. Similarly, when the BM compartments of different recipients were examined 24 weeks after transplantation, I observed that the LPS-WT LSK cells have a rather poor reconstitution capacity in regenerating any subset in the BM. This is consistent with the finding that LT-HSC exhaustion is observed in LPS-WT LSK cell recipients. Similar to the peripheral blood data, LPS-TRIF^{-/-} LSK cells displayed the same capacity in generating primitive HSPC subsets and mature myeloid cells as the normal LSK cells. In fact, loss of TRIF prevented LT-HSC exhaustion in septic LSK cells after transplantation. In contrast, LPS-MyD88^{-/-} LSK cells did not show advantageous contribution to the primitive LT-HSC and could only partially preserve the ability to regenerate myeloid progenitors and mature myeloid cells. The fact that MyD88^{-/-} partially prevented LPS-induced LT-HSC exhaustion at 24 weeks after transplantation suggested that LPS-MyD88^{-/-} LSK cells are slowly “recovering” from the damage induced by acute LPS exposure. This hypothesis requires a secondary transplantation experiment to be confirmed. The finding that TRIF deletion fails to prevent myeloid suppression after acute LPS exposure, but protects HSC from

injury, indicates that TLR4/TRIF signaling has a different immediate impact on these two cell types (myeloid progenitors and HSC) and that these two effects are independent. This point is further re-enforced by the observation that following LPS challenge, MyD88 has a dominant protective effect on myeloid progenitors' differentiation function, but only a mild protective effect on HSPC functions following LPS challenge. In summary, LPS-induced TLR4/TRIF activation mainly affects HSC's stem cell function while TLR4/MyD88 activation is the major mediator of myelosuppression.

Unlike MyD88, which is a common adaptor for all TLR signaling except for TLR3, TRIF participates in the signal transduction of TLR3 and TLR4 [78]. The TLR4/TRIF signaling leads to type I interferon production via IRF-3 activation [103]. TRIF function has been intensively studied in immune response in different infection scenarios. Hematopoietic TRIF activity was found to be crucial for bacterial clearance and survival in a model of murine gram-negative pneumonia. Interestingly, in the same study, hematopoietic TRIF expression significantly contributed to cellular injury during late-stage of infection [209]. Upon TLR4 ligation by LPS, TRIF is involved in macrophage inflammatory cytokine production and dendritic cell activation [103,232]. While TRIF function is extensively studied in mature immune cells, how it affects the primitive hematopoietic subsets has remained largely unknown. In a recent study, the TLR4/TRIF pathway significantly contributed to allogeneic BM cell rejection by mediating the production of pro-inflammatory cytokines by macrophages and NK cells [233]. The result from this study was in agreement with my finding that

TRIF^{-/-} could protect BM cell engraftment activity upon inflammation. However, since the transplantation was performed using whole BM cells, the scope of this study was limited, failing to provide knowledge specific of HSC function. The observation that TRIF^{-/-} septic LSK cells displayed protected short-term and long-term engraftment potential and multilineage regenerative activity both in the peripheral blood and BM could be explained by two different mechanisms. The first was that in TRIF^{-/-} mice, the lack of TRIF-mediated production of pro-inflammatory cytokines including Type I IFN by resident and/or mature immune cells induced by LPS prevented the damage to HSC. Another scenario was that the lack of TRIF^{-/-} directly prevented the activation of TLR4/TRIF signaling pathway in the HSPC induced by LPS ligation, hence preventing the LPS damage. To address this question, it will be of great importance to use the model of TRIF^{-/-} chimera with TRIF loss of function only in the BM microenvironment or in the hematopoietic cells, and then examine how LPS affects the HSC function of these chimeras post-transplantation. Although this open question remains to be studied, my findings provide the evidence that TLR4/TRIF signaling plays a dominant role in mediating HSC damage induced by endotoxemia.

Because acute LPS exposure leads to a prolong impairment in WT HSC function even when they are transplanted into a healthy microenvironment, I hypothesize that permanent alterations in these HSC must have occurred. First, I determined whether the gene expressions of transcriptional factors were affected in the LSK fractions following LPS exposure. PU.1 and C/EBP α were selected for preliminary study because they were required for both myeloid cell differentiation

and HSC self-renewal maintenance. Dramatic downregulation of both transcription factors was found in WT septic LSK cells. This alteration persisted when septic LSK cells were isolated from recipients 24 weeks after transplantation, confirming the hypothesis that irreversible alteration in the genetic level indeed occurs in response to LPS challenge. Both loss of MyD88 and TRIF were able to maintain the expression levels of C/EBP α in parental animals or after transplantation. Interestingly, PU.1 was downregulated in LSK cells sorted from LPS challenged TRIF^{-/-} mice but unaltered PU.1 expression was observed in septic TRIF^{-/-} LSK cells 24 weeks after transplantation. I hypothesize that in the absence of TRIF^{-/-}, the transient downregulation of PU.1 by acute LPS challenge returns to normal level after transplantation. In conclusion, acute LPS exposure significantly and likely permanently disrupts the gene profile required for normal myelopoiesis and HSC function maintenance. Future studies from our group are ongoing to address the role of epigenetic regulation of HSC following sepsis.

Collectively, all our data show that loss of MyD88 and TRIF have beneficial impact on the BM during severe sepsis. However, it is of great importance to bear in mind that MyD88 and TRIF are necessary for the innate immune cells to respond to infection. Human and mice with MyD88 or TRIF deficiency are highly susceptible to infections [209,234]. Therefore, normal function of the MyD88 and TRIF is required for defending pathogens. It is only when these pathways go awry in conditions like hyperinflammation or sepsis that they confer damage effects to tissues including the BM. Although blocking the

TRIF and MyD88 signaling pathway seems a tempting therapeutic strategy to prevent BM damage during sepsis, one would reach higher level of specificity and safety by targeting their downstream molecules.

CHAPTER FIVE

FUNCTIONAL ROLE OF MIR-21 IN LPS-INDUCED DYSFUNCTIONAL HEMATOPOIESIS

INTRODUCTION

miRNA are double-stranded non-coding RNA molecules of 19-24 nt. They control gene expression post-transcriptionally by binding to the 3'-UTR of target mRNA sequences [235]. Each miRNA potentially has a broad influence over several diverse genetic pathways [235]. Emerging studies showed that miRNA played a delicate regulatory function in HSC differentiation in the bone as well as in mature hematopoietic cells that constituted the immune system. miRNA expression and function can be regulated at three levels, from transcription, processing and subcellular localization, and each of these step can be influenced by inflammation and stress [236]. Importantly, it has been reported that activated TLR signaling regulates various miRNA including miR-21, miR-155, miR-146a and miR-9 [236]. Moreover, several miRNA have been found to be upregulated in the serum of patients with severe sepsis [237,238].

In this study, I focused on miR-21, as I found that its expression is MyD88-dependent. Since its discovery, miR-21 has been known as an onco-mir due to its overexpression in multiple types of solid tumor and leukemia [239]. In a clinical study, miR-21 was found to have increased expression level in the skeletal biopsy tissue of septic patients compared to normal individuals [187]. miR-21 is also involved in normal immune cell differentiation during

hematopoiesis. Overexpression of miR-21 together with miR-196b in BM lineage-depleted cells blocked granulopoiesis *in vitro* [240]. Previous work by F.J. Sheedy et al. revealed that miR-21 was induced via TLR4/MyD88-NF- κ B pathway in human peripheral blood mononuclear cells upon LPS challenge; they further showed one of the major miR-21 targets, the tumor suppressor gene programmed cell death 4 (PDCD4), finely tuned the production of pro-inflammatory factors [191]. Study of the promoter region of pri-miR-21 revealed a conserved binding site for AP-1, which can be activated by TLR4/MyD88 pathway [241]. Taken together, all these results support the hypothesis that miR-21 could be regulated by the TLR4 signaling and that it plays a role in the immune response to sepsis.

The aim of this chapter was to investigate the functional role of miR-21 in mediating the dysfunctional hematopoietic response in the BM after LPS exposure.

RESULTS

5.1 miR-21 is differentially expressed in the BM hematopoietic subsets.

To determine the basal expression level of miR-21 in different hematopoietic BM subsets, I investigated the level of mature miR-21 in sorted LSK, CMP, GMP, MEP, Gr1⁺/Mac1⁺ cells, T cells and B cells from the BM by qRT-PCR. I observed that miR-21 was differentially expressed in these subsets, with higher expression of miR-21 was observed in the more mature subsets (Gr1⁺/Mac1⁺, T cells, B cells) whereas immature progenitors (CMP, GMP) and stem cells (LSK) showed lower levels (Figure 5.1 A). Consistent with this result, miR-21 expression was upregulated as immature progenitors (Lin⁻ cells) progressed toward myeloid differentiation into Gr1⁺/Mac1⁺ cells *in vitro* (Figure 5.1 B, C).

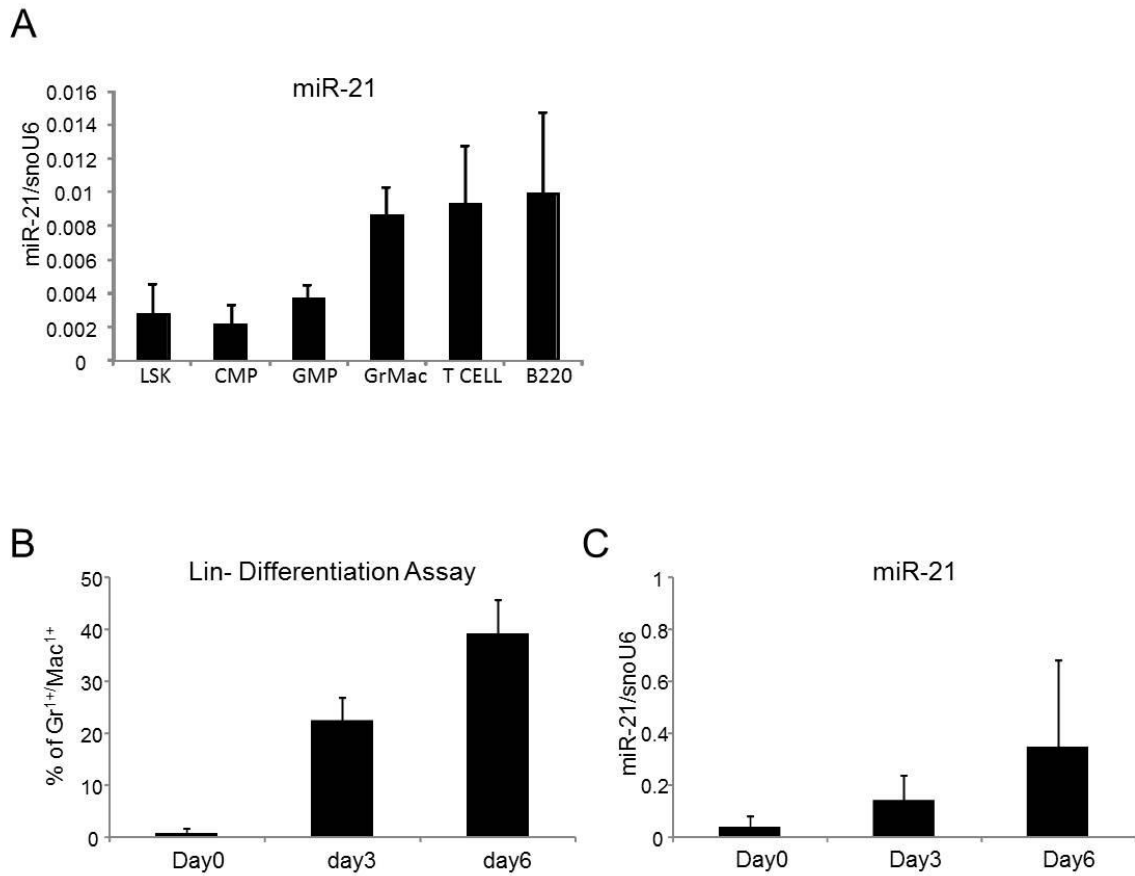


Figure 5.1 miR-21 is differentially expressed in different hematopoietic populations and during myeloid differentiation.

(A) miR-21 expression levels in sorted LSK, CMP, GMP, Gr1⁺/Mac1⁺, T cell and B cells. (B) Average percentage of Gr1⁺/Mac1⁺ cells in culture during Lin⁻ differentiation assay. (C) miR-21 expression level upon Lin⁻ cells differentiating into myeloid cells (n = 3). Data represent mean ± SEM.

5.2 P. aeruginosa's LPS upregulates miR-21 in the LSK cells in a MyD88-dependent manner.

Under homeostatic conditions, miR-21 expression in the LSK cells is low. Previous research has shown miR-21 induction by LPS through activation of the TLR4 pathway [191]. To determine whether miR-21 level in HSPC was affected by LPS challenge, I sorted LSK cells from LPS- or PBS-challenged WT mice. qRT-PCR analysis revealed a 4-fold increase in the level of mature miR-21 in septic LSK cells (Figure 5.2 A).

Interestingly, such elevation was absent in TLR4^{-/-} mice and MyD88^{-/-} mice (Figure 5.2 B-C), indicating that the upregulation of miR-21 in LSK cells was mediated by the TLR4/MyD88-dependent pathway.

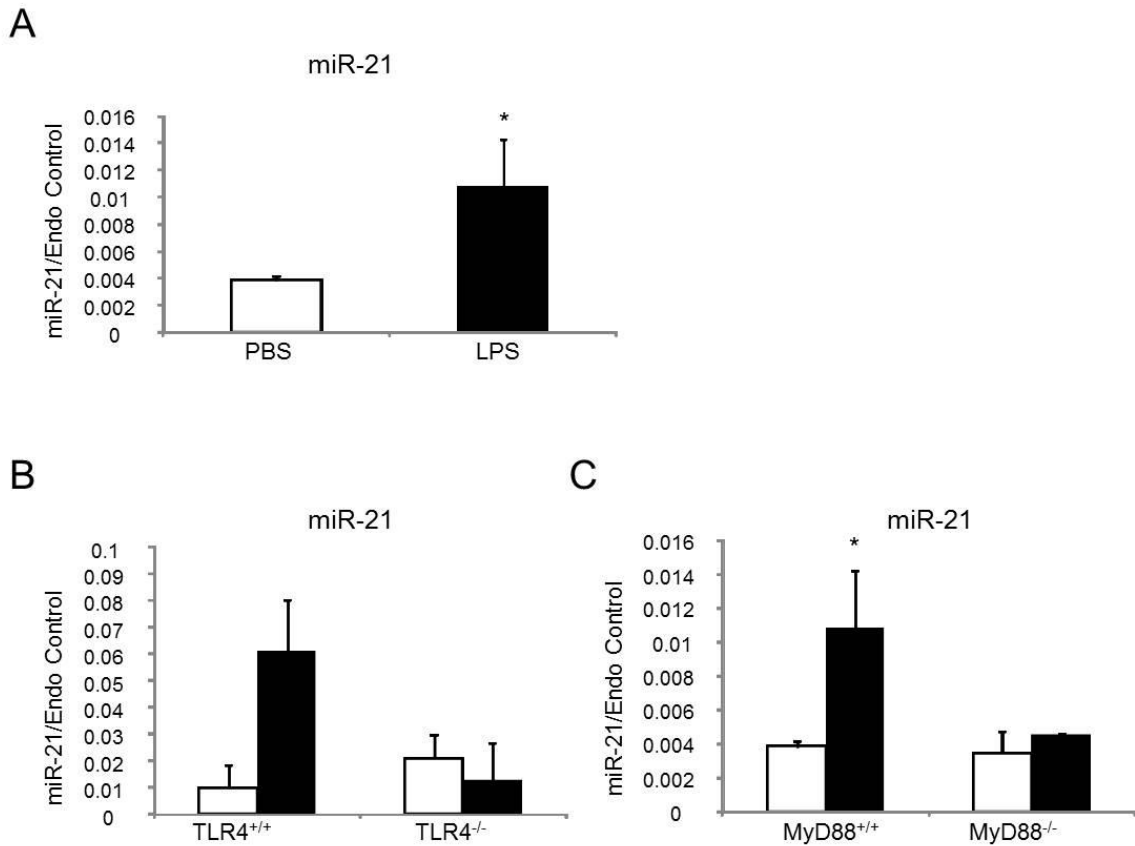


Figure 5.2 LPS-induced miR-21 upregulation in the LSK cells in a TLR4/MyD88 dependent manner.

(A) miR-21 was upregulated in LSK cells after LPS challenge for 24 hours (n = 4). (B) miR-21 was not upregulated in LSK cells from TLR4^{-/-} animals challenged with LPS (n = 2, error bar indicates standard deviation). (C) miR-21 was not upregulated in LSK cells from MyD88 KO animals challenged with LPS (n = 4). Data represent mean \pm SEM, *p < 0.05, unpaired t test.

5.3 Development of tissue-specific miR-21 deletion mouse model.

Because the upregulation of miR-21 was mediated in a TLR4/MyD88 dependent manner and miR-21 played a crucial role in regulating the inflammatory circuit [192], I sought to determine whether miR-21 played a dominant role in the BM myeloid suppression induced by *P. aeruginosa*'s LPS. To this end, I generated mice with specific miR-21 deletion in the hematopoietic and stromal cells by crossing the Mx1-Cre mice with miR-21^{flox/flox} mice (Figure 5.3 A). Mx1-Cre⁺miR-21^{flox/flox} and Mx1-Cre⁻miR-21^{flox/flox} mice were identified by genomic DNA PCR (Figure 5.3 B) and subject to Poly (I:C) (pIpC) injection to induce specific miR-21 deletion. Mx1 promoter is active specifically in BM hematopoietic and non-hematopoietic cells and is activated by IFN α , induced by pIpC injection (Figure 5.3C). qRT-PCR analyses on the BM cells isolated from induced-miR-21^{-/-} mice showed greater than 90% deletion of the miRNA (Figure 5.3 D).

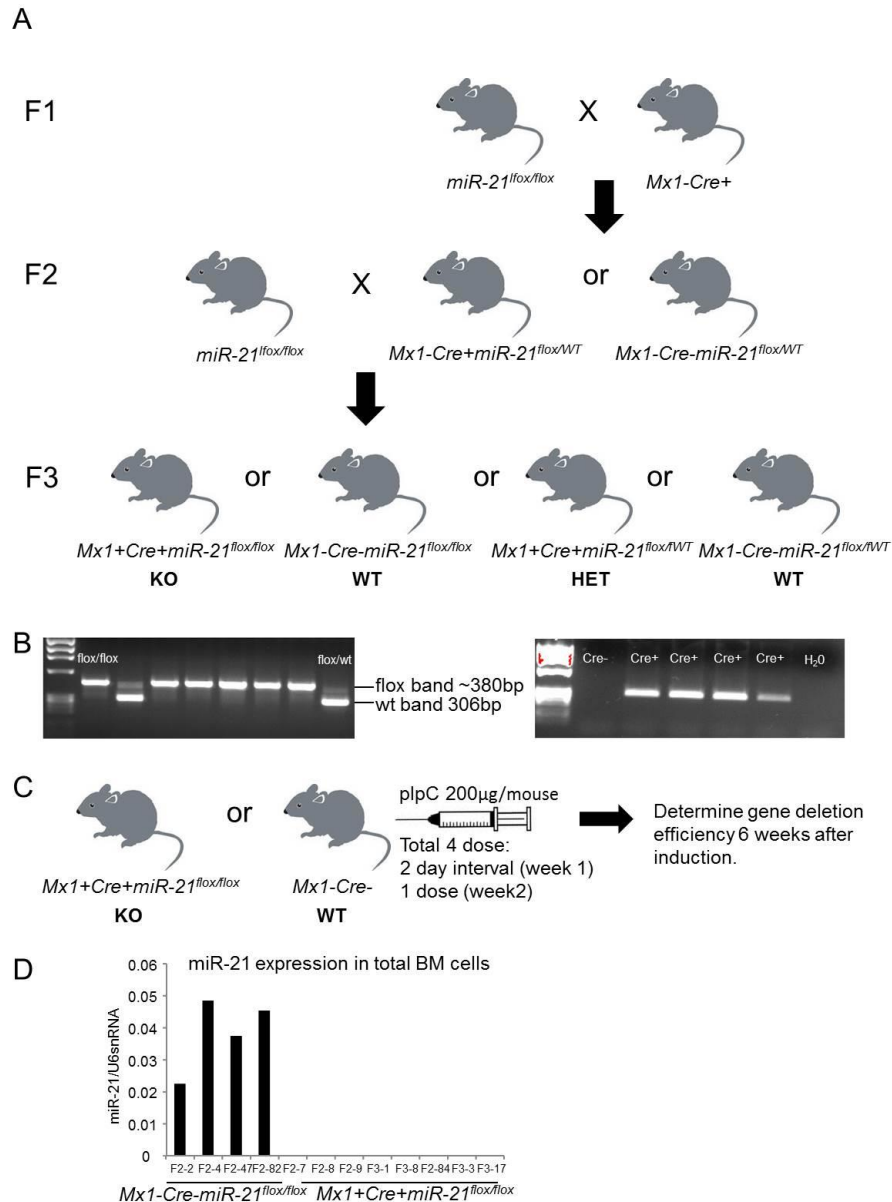


Figure 5.3 Generation of transgenic mice with inducible miR-21 deletion specific in the hematopoietic and stroma cells.

(A) Diagram shows the crossing strategy to develop *Mx1-Cre⁺miR-21^{flox/flox}* and *Mx1-Cre⁻miR-21^{flox/flox}* mice. (B) Genotype PCR band for flox allele or wt allele and *Mx1-Cre⁺* or *Mx1-Cre⁻* band. (C) Diagram shows the induction strategy. (D) miR-21 Taqman qRT-PCR validation of loss of miR-21 expression in *Mx1-Cre⁺miR-21^{flox/flox}* mice induced with plpC.

5.4 miR-21 is dispensable for the BM response to endotoxemia.

To determine whether miR-21 plays a role in MyD88 mediated myelosuppression, I challenged miR-21^{-/-} mice and litter mate WT control with LPS. If miR-21 was critical in the process we would expect to observe a rescue of myeloid cells in its absence. However, loss of miR-21 had no impact on the hematopoietic response induced by *P. aeruginosa*'s LPS (Figure 5.4 A). Surprisingly, I noticed a slight but significant increase in the percentage of LT-HSC in miR-21^{-/-} mice. However, sorted expanded LT-HSC showed no significant advantage in hematopoietic reconstitution upon transplantation short-term and long-term (Figure 5.4 B-C).

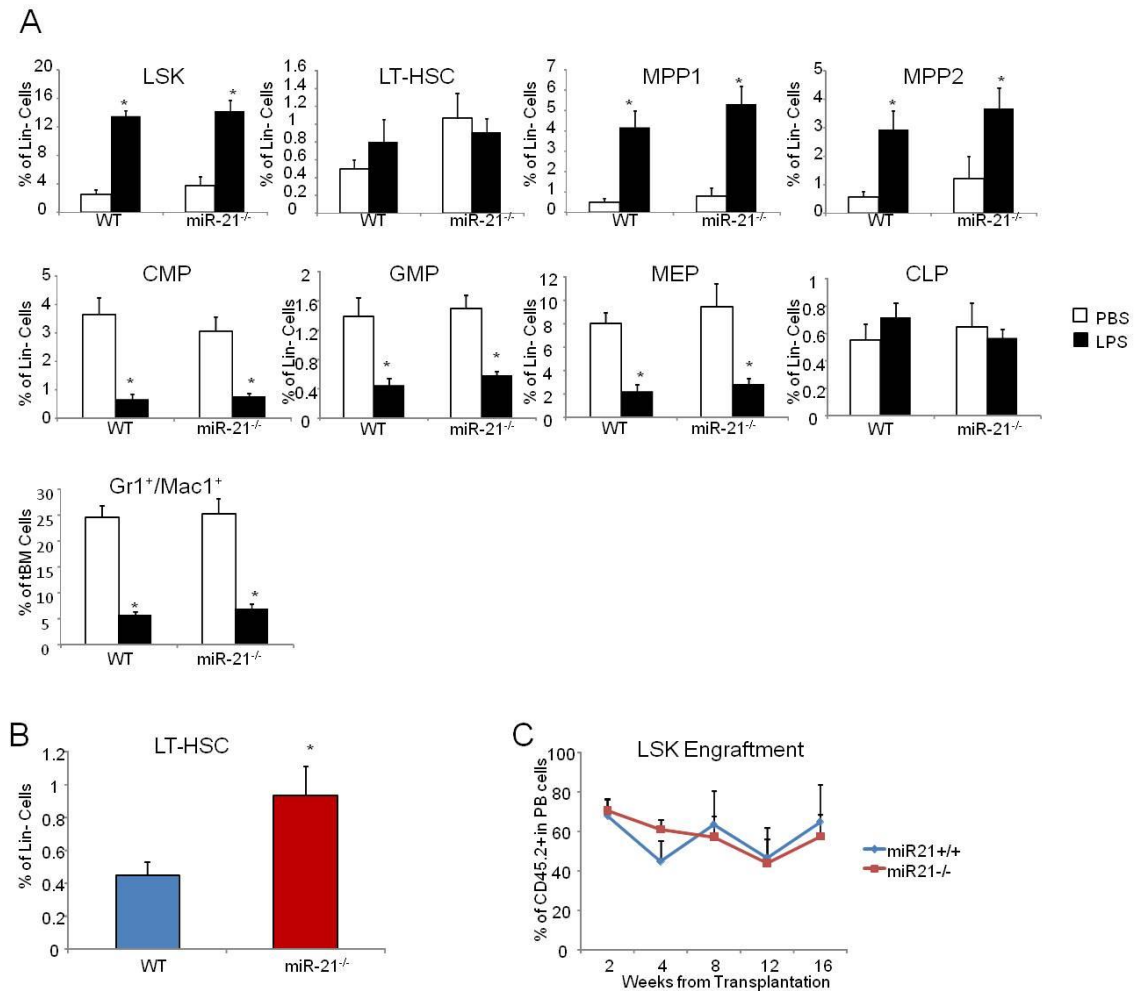


Figure 5.4 Loss of miR-21 did not affect the BM response induced by LPS.

(A) Bar graphs show average percentage of LSK, LT-HSC, MPP1, MPP2, CMP, GMP, MEP, CLP in Lin^- cells and $\text{Gr1}^+/\text{Mac1}^+$ cells in total BM cells from WT and $\text{miR-21}^{-/-}$ mice challenged with PBS or LPS ($n = 6-7$). (B) Bar graph shows average percentage of LT-HSC cells in Lin^- cells from WT and $\text{miR-21}^{-/-}$ mice under homeostatic conditions ($n = 7$). (C) Average percentage of donor-derived PB cells is shown in recipients of WT and $\text{miR-21}^{-/-}$ LSK cells ($n=5$). Data represent mean \pm SEM, * $p < 0.05$, unpaired t test.

CONCLUSIONS AND DISCUSSION

Results from this chapter demonstrated that miR-21 was upregulated during myeloid differentiation in homeostatic conditions. However, miR-21 depletion in the BM did not cause obvious hematopoietic defects. Loss of miR-21 led to a mild increase in the LT-HSC, yet how this functionally affected the LT-HSC function remained to be determined. Although miR-21 was upregulated in the LSK cells by LPS in a TLR4/MyD88-dependent manner, it did not play a dominant role in contributing to hematopoietic dysfunction or to myelosuppression during acute LPS exposure. This negative result reflects the molecular mechanism underlying the myeloid suppression as far more complicated than I expected and targeting one single molecule will likely be insufficient to prevent neutropenia. In a recent review, it has been suggested that miR-21 upregulation by pro-inflammatory stimulus was involved in a negative regulatory loop where miR-21 targeted pro-inflammatory mediators to tune down the inflammatory response during late stage of infection so that the amplified response could be resolute [192]. This could explain why deletion of miR-21 did not confer protective effects to the BM upon LPS challenge. However, the reason why loss of miR-21 did not worsen the condition remains to be determined.

In summary, miR-21 does not seem to exert a dominant regulatory role in BM response during acute inflammation induced by *P. aeruginosa*'s LPS. Further studies are required to identify the functional role of miR-21 during sepsis.

CHAPTER SIX

FUTURE DIRECTIONS

In the current project, I found that loss of MyD88 prevented myeloid differentiation block and neutropenia during sepsis. This is of great clinical importance because modulation of the MyD88 pathway could confer protective effects to sepsis patients that develop neutropenia. However, because the TLR4/MyD88 pathway is required for innate immune response, targeting the MyD88 adapter protein itself may not be an ideal therapeutic approach. Therefore, the future directions of this study include identifying the downstream molecules involved in myeloid suppression. In Chapter Four, the mRNA levels of two myeloid differentiation regulators (PU.1 and C/EBP α) were found to be significantly downregulated in HSPC by LPS and were greatly preserved when MyD88 was absent. It will be interesting to determine whether overexpression of PU.1 or C/EBP α in mouse will bypass the myeloid differentiation defects during LPS challenge. The molecular link between MyD88 pathway and PU.1 and C/EBP α downregulation remains elusive and require further investigations.

Reciprocal transplantation assays between WT and MyD88^{-/-} mice showed that loss of MyD88 in the BM microenvironment could partially rescue the loss of mature myeloid cells. Consistently, loss of MyD88^{-/-} can protect the BM vasculature from undergoing pathological alterations characterized by dilated vessels and increased leakage during LPS induction. The influence of the BM microenvironment on hematopoiesis is well recognized [242-245]. Preserved BM

vasculature in the MyD88^{-/-} might be functionally linked to the hematopoietic protection. To examine this hypothesis, we would generate an endothelial cell-specific MyD88 knock-out mouse model and compare their BM response to WT and MyD88^{-/-} mice during LPS challenge.

Another focus of this project was to identify how the TLR4 signaling contributed to HSC injury during endotoxin challenge. Here, using the competitive reconstitution assay, I showed for the first time that loss of TRIF^{-/-} in the HSPC completely preserved their long-term and short-term multilineage engraftment potentials. Activation of the TRIF pathway on HSPC is the major contributor to HSPC injury upon TLR4 activation. This finding is not only important for sepsis pathology but it also provides insights to our understanding of BM engraftment following irradiation. To move this finding forward, we will continue to investigate which molecule(s) downstream of TLR4/TRIF is/are responsible for HSC injury upon inflammation. BM failure is commonly found in sepsis survivors and BM failure is largely due to defective HSC function. A complete elucidation of how TLR4/TRIF signaling pathway contributes to HSC injury could provide future therapeutic opportunities to prevent BM failure in sepsis survivors. The discovery that loss of TRIF prevents HSC injury during inflammation has potential clinical implication in other medical scenarios such as BM transplantation and graft-versus host disease. Inflammation induced by irradiation often accompanies patients undergoing BM transplantation. Since the TRIF-signaling is involved in inflammation, modulation of the TRIF pathway could provide a putative therapeutic strategy to improve BM engraftment and/or

reducing the possible development of graft-versus-host disease following BM transplantation.

The findings outlined in this thesis are also potentially relevant for cancer research. Chronic inflammation is found to predispose tissues or cells to become pre-cancerous [246]. Increased TLR4 activation is observed in tissue samples from patients with liver, head and neck, ovarian and breast cancer [247] and constitutive activation of TLR4 promotes tumorigenesis [248]. In this project, I find that activation of the TLR4/MyD88 and TLR4/TRIF signaling pathway disrupts normal hematopoiesis in the BM and induces HSC exhaustion, which are often associated with leukemia pathogenesis [249]. Therefore, it will be important to further characterize the role of TLR4/TRIF and/or TLR4/MyD88 activation in favoring a pre-leukemia state in HSPC.

Finally, although loss of miR-21 confers no significant protective effects on LPS-induced BM injury, it is established that miR-21 is significantly elevated during inflammation. Therefore, this miRNA could be a good putative diagnostic biomarker for sepsis. Besides an inflammatory regulator, miR-21 is also a well-characterized onco-miR that contributes to colon carcinoma and B cell lymphoma [250,251]. Association between chronic inflammation and cancer development has long been appreciated [252], and it has been shown that chronic inflammation contributes to tumor development [139]. Therefore, further pursuit of the functional role of miR-21 would also include determining whether prolonged upregulation of miR-21 induced by endotoxin or chronic inflammation will contribute to hematological malignancies.

To summarize, my project successfully dissected the various contribution of TLR4/MyD88 and TLR4/TRIF pathway in two different yet related hematopoietic responses in the BM during acute inflammation induced by exposure to high dose of *P. aeruginosa*'s LPS or severe polymicrobial sepsis. TLR4/MyD88 activation plays a dominant role in myeloid suppression and neutropenia but has limited impact on HSPC stem cell function. On the other hand, TLR4/TRIF signaling pathway significantly damages the engraftment and multilineage potentials of HSC upon acute inflammation (Figure 6.1). Taken together, my overall goal is to help gain a better understanding on the BM pathological alterations during sepsis and help move forward the long and difficult journey to tackle this complicated disease while leading to novel therapeutic approaches.

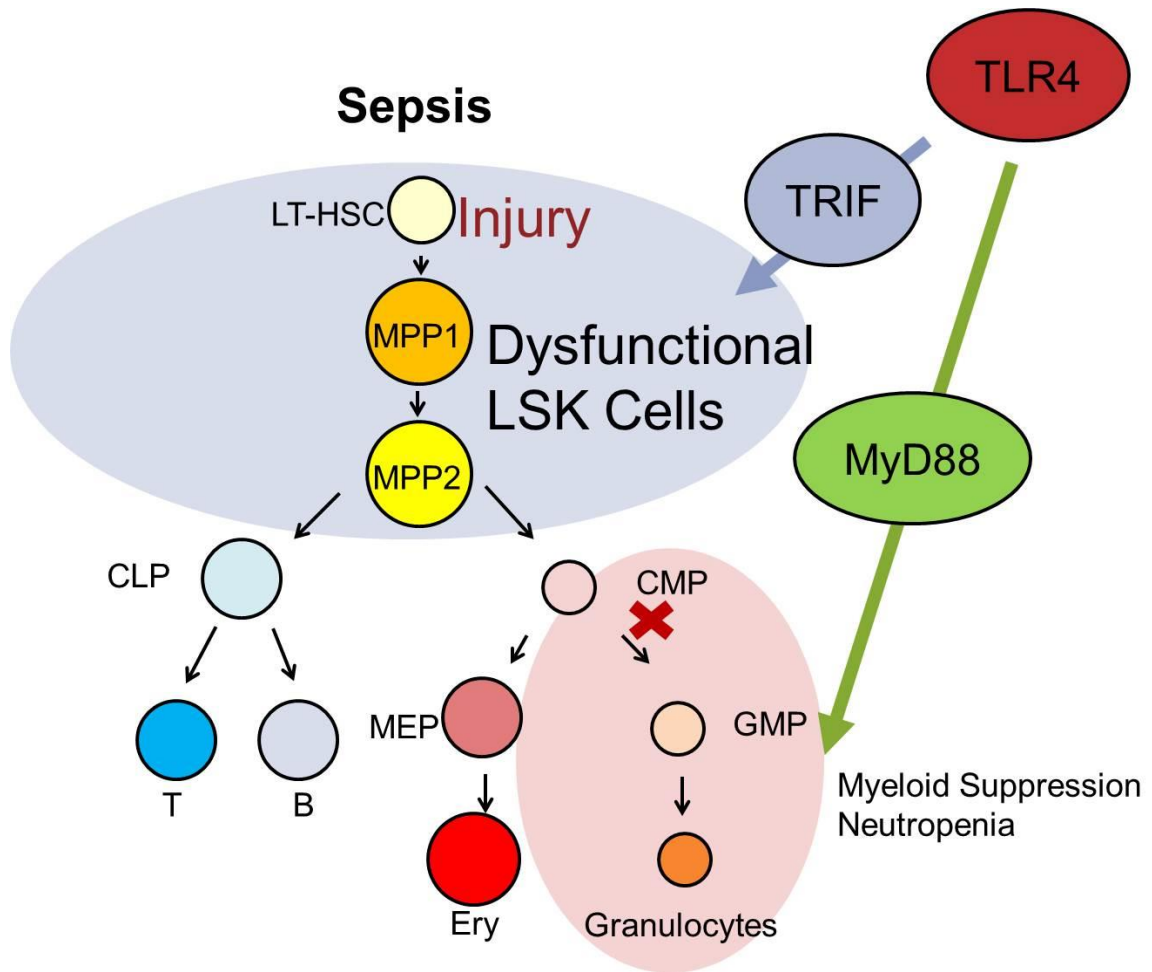


Figure 6.1 Summary of final conclusion.

Diagram show the distinct contribution of TLR4/MyD88 and TLR4/TRIF pathway to hematopoietic responses in the BM during LPS challenge. (LT-HSC, Long-term hematopoietic stem cells; MPP, Multipotent progenitors; CLP, Common lymphoid progenitors; CMP, Common myeloid progenitors; MEP, Megakaryocyte erythroid progenitors; GMP, Granulocytic monocytic progenitors; B, B cells; T, T cells; Ery, Erythrocytes; NE, Neutrophils; M ϕ , Macrophages).

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CURRICULUM VITAE

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EDUCATION

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Side Project: Functional role of miR-21 in the bone marrow response to inflammatory stress.

Collaborative Project: Molecular mechanism in myeloproliferative neoplasms induced by Notch loss-of-function.

PUBLICATIONS

Wang L., Zhang H., Rodriguez S., Cao L., Parish J., Mumaw C., Zollman A., Kamoka G., Mu J., Chen D., Srour E., Chitteti B., Esch H., Tu X., Bellido T., Boswell S., Manshouri T., Verstovsek T., Verstovsek S., Yoder M., Kapur R., Cardoso A., Carlesso N. Notch-dependent repression of miR-155 in the bone marrow niche regulates hematopoiesis in an NF- κ B-dependent manner. *Cell Stem Cell* 15, 51-65, July 3, 2014

Chitteti BR., Kobayashi M., Cheng Y., Zhang H., Poteat BA., Broxmeyer HE., Pelus LM., Hanenberg H., Zollman A., Kamocka MM., Carlesso N., Cardoso AA., Kacena MA., Srour EF. CD166 regulates human and murine hematopoietic stem cells and the hematopoietic niche. *Blood* 124(4): 519-29, July 24, 2014

Tu X., Calle J., Condon K., Cepeda M., Zhang H., Carlesso N., Taketo M., Plotkin L., Bellido T. Osteocytes mediate the anabolic actions of canonical Wnt/ β catenin signaling in bone. *PNAS* vol. 112 no.5, February 3, 2015

Zhang H., Rodriguez S., Wang L., Serezani H., Kapur R., Cardoso A., Carlesso N. Differential contribution of TRIF and MyD88 in regulating TLR4 induced exhaustion of hematopoietic stem and progenitor cells during sepsis. Submitted to *Stem Cell Reports*, 2015

ORAL PRESENTATIONS

Huajia Zhang, Sonia Rodriguez-Rodriguez, Lin Wang, Christen Mumaw, Amy Zollman, Yunlong Liu, Angelo A. Cardoso and Nadia Carlesso. Activation of TLR4-MyD88 Pathway Impairs HSC Function During Acute Inflammation. The 10th International Workshop on Molecular Aspects of Myeloid Stem Cell Development and Leukemia, May 4-7, 2014. Cincinnati, OH

ABSTRACTS

Zhang H., Rodriguez S., Wang L., Mumaw C., Zollman A., Liu Y., Cardoso A. and Carlesso N. Activation of TLR4-MyD88 Pathway Impairs HSC Function During Acute Inflammation. The 55th ASH Annual Meeting and Exposition. December 7-10, 2013. New Orleans, LA

Rodriguez S., Wang L., Zhang H., Zollman A., Cardoso AA., and Carlesso N. Absence of the E3 Ubiquitin Ligase SKP2 Attenuates Notch-induced T-cell Leukemogenesis. 55rd ASH Annual Meeting. December 07-10, 2013. New Orleans, LA

Rodriguez S., Wang L., Zhang H., Zollman A., Cardoso AA., and Carlesso N. The E3 Ubiquitin Ligase F-Box Protein SKP2 is a Critical Contributor for Notch-induced T-cell Leukemogenesis. Keystone Symposia: Stem Cell Regulation in Homeostasis and Disease. February 24-March 1, 2013. Banff, Canada

Zhang H., Rodriguez S., Wang L., Mumaw C., Zollman A., Cardoso AA., Liu Y. and Carlesso N. Functional Role of the TLR4/MyD88 Signaling Pathway in the Bone Marrow Response to Sepsis. Ninth International Workshop on Molecular Aspects of Myeloid Stem Cell Development and Leukemia. May 6-9, 2012. Cincinnati, OH

Wang L., Rodriguez S., Zhang H., Mumaw C., Cardoso AA., Carlesso N. Activation of the Canonical Notch Signaling Pathway in the Hematopoietic Microenvironment is Required to Maintain Normal Hematopoiesis. The 53rd ASH Annual Meeting and Exposition. December 10-13, 2011. San Diego, CA

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