I, Yi-Ting Tsai, hereby submit this original work as part of the requirements for the degree of Master of Science in Immunobiology.

It is entitled:
Myeloid Derived NFkappa;B Regulation of LPS-Induced Endotoxic Shock

Student's name: Yi-Ting Tsai

This work and its defense approved by:

Committee chair: Simon Hogan, Ph.D.
Committee member: Kasper Hoebe, Ph.D.
Committee member: Nives Zimmermann, Ph.D.
Myeloid Derived NFκB Regulation of LPS-Induced Endotoxic Shock

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Yi-Ting Tsai

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Committee Chair: Simon P. Hogan, Ph.D.
Kasper Hoebe, Ph.D.
Nives Zimmermann, M.D.
Abstract

Sepsis is a life-threatening event predominantly caused by gram-negative bacteria. Bacterial infection causes a pronounced macrophages (MΦs) and dendritic cells (DCs) activation which leads to excessive pro-inflammatory cytokine IL-1β, IL-6, and TNFα production (cytokine storm), resulting in endotoxic shock. Previous experimental studies have revealed that inhibition of NFκB signaling ameliorates disease symptoms, however, the contribution of myeloid p65 in endotoxic shock remains elusive. In this study, we demonstrate increased mortality in p65Δmye mice compared to WT mice upon ultra pure LPS (U-LPS) challenge. We show that increased susceptibility to endotoxic shock was associated with elevated serum level of IL-1β and IL-6 and increased recruitment of neutrophils to the peritoneal cavity following U-LPS challenge. Mechanistic analyses revealed that LPS-induced pro-inflammatory cytokine production was ameliorated in p65-deficient non-inflammatory bone marrow-derived macrophages (BMDMs), however, p65-deficient inflammatory peritoneal MΦs exhibited elevated IL-1β and IL-6. We show that the elevated IL-1β secretion in part was due to increased caspase-1 expression. Our data demonstrate a differential role of NFκB signaling in non-inflammatory and inflammatory MΦs in regulating host response toward LPS challenge and provide an insight to the MΦs/NFκB axis in sepsis.
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Table of Contents

Contents

ABSTRACT I

ACKNOWLEDGEMENT III

TABLE OF CONTENTS IV

LIST OF ABBREVIATIONS VII

CHAPTER 1. INTRODUCTION 1

1. Sepsis 1

2. Role of Toll-Like Receptors 1
   2.1 Overview of TLR activation 2

3. Role of IL-1β, IL-6, and TNFα 5
   3.1 TNFα 5
   3.2 IL-6 6
   3.3 IL-1β 7

4. Regulation of IL-1β production 7
   4.1 Transcriptional regulation of IL-1β 8
   4.2 Post-translational regulation of IL-1β 8
   4.3 Secretory regulation of IL-1β 9

5. Macrophages 10

6. LPS-induced MΦ cell death 12
   6.1 Programmed cell death 12
6.2 Lipopolysaccharide

7. NFκB signaling
   7.1 Non-canonical pathway
   7.2 Canonical pathway

Summary

CHAPTER 2. MATERIALS AND METHODS

Mice.
Bone marrow derived macrophages.
Peritoneal macrophages.
Macrophage stimulation
ELISA
FACS analysis
Western blot.
Real-time PCR analysis.
MTT assay.
IFNβ bioassay
Statistical analysis.

CHAPTER 3. RESULTS

1. Ultra pure LPS challenge decreases survival rate in p65Δmye mice
2. Ultra pure LPS challenge increases pro-inflammatory cytokines and alters cell recruitment in p65Δmye mice
3. Decreased inflammation and viability in p65Δmye BMDMs upon ultra pure LPS stimulation
4. Dirty LPS decreases pro-inflammatory cytokine secretion and viability in p65Δmye peritoneal cells
5. Ultra pure LPS stimulation results in increased pro-inflammatory cytokine production in p65Δmye peritoneal MΦs
6. p65 deletion does not induce surface TLR4 expression in MΦs upon thioglycollate challenge
7. MyD88-independent TLR4 signaling pathway is intact in p65Δmye peritoneal MΦs

FIGURES
Figure 1. *In vivo* ultra pure LPS (U-LPS) challenge results in increased mortality in p65Δmye mice.

Figure 2. *In vivo* ultra pure LPS (U-LPS) challenge results in increased pro-inflammatory cytokine production and altered cell recruitment in p65Δmye mice.

Figure 3. *In vitro* ultra pure LPS (U-LPS) stimulation decreases pro-inflammatory cytokine expression and viability in p65Δmye BMDMs.

Figure 4. *In vitro* dirty LPS (D-LPS) challenge decreases pro-inflammatory cytokine expression and viability in p65Δmye peritoneal cells.

Figure 5. *In vitro* ultra pure LPS (U-LPS) challenge increases pro-inflammatory cytokine expression in p65Δmye peritoneal MΦs.

Figure 6. TLR4 expression is not altered in p65Δmye MΦs at steady state or post thioglycollate activation.

Figure 7. MyD88-independent signaling pathway is intact in p65Δmye peritoneal MΦs following *in vitro* ultra pure LPS (U-LPS) challenge.

CHAPTER 4. DISCUSSION

REFERENCES
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>Activator protein</td>
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<tr>
<td>BMDM</td>
<td>Bone marrow–derived macrophages</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-associated recruitment domain</td>
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<tr>
<td>CPPD</td>
<td>Calcium pyrophosphate dehydrate</td>
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<td>CLRs</td>
<td>C-type lectin receptors</td>
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<td>DAMPs</td>
<td>Danger-associated molecular patterns</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DD</td>
<td>Death domain</td>
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<td>ERK</td>
<td>Extracellular signal–regulated kinase</td>
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<td>Fas-associated protein-containing death domain</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<td>Gfi1</td>
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<td>IκB kinase</td>
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<td>IκB</td>
<td>Inhibitor of kappa B</td>
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<td>Interleukin</td>
</tr>
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<td>IRAK-1</td>
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<td>IFN regulatory factor 3</td>
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<td>LRR</td>
<td>Leucine-rich repeat</td>
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<td>Lipopolysaccharides</td>
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<td>M-CSF</td>
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<td>Major histocompatibility complex</td>
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<td>Multiple organ dysfunction syndrome</td>
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<tr>
<td>MSU</td>
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<td>----------</td>
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<tr>
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<td>Myeloid differentiation factor 88</td>
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<td>Type III secretion system</td>
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Chapter 1. Introduction

1. Sepsis

Sepsis is a life-threatening event that can be complicated by shock, coagulopathy, and multiorgan dysfunction, causing millions of deaths globally each year [1]. Recent epidemiologic data show an increase in age-adjusted death rates due to bacteraemia from 4.2 per 100,000 population in 1980 to 13.2 per 100,000 in 1992 in the United States of America [2]. Multiple organ dysfunction syndrome (MODS) is the most frequent cause of death in patients admitted to the intensive care unit (ICU) with sepsis [3]. Sepsis is caused by bacterial infection, in particular Gram-negative bacteria [4]. Bacterial antigens are thought to stimulate the innate arm of the immune system resulting in a cytokine storm characterized by elevated level of interleukin-1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNFα), which cause endotoxic shock and death in few days [5].

2. Role of Toll-Like Receptors

Sepsis is primarily induced by danger-associated molecular patterns (DAMPs) [6]. Bacteria express various DAMPs called pathogen-associated molecular patterns (PAMPs), which are structural or secreted products including endotoxin, lipoteichoic acid, zymosan, and flagellin [7-9]. Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) that recognize common patterns in microbial molecules and PAMPs and provide
efficient and immediate immune responses to bacterial, fungal, and viral infections [10], [11]. There are ten TLRs, and each of them binds a distinct family of microbial molecules [12], [13]. For example, TLR2, together with TLR1 or TLR6, binds bacterial lipoproteins, whereas TLR4 and TLR5 recognize Lipopolysaccharides (LPS) and flagellin respectively. Bacterial or viral nucleic acids are recognized by TLR3 and TLR7–9 [7]. The engagement of TLRs with ligand molecules induces dimerization of the receptors, which leads to recruitment of intracellular signaling adaptors and kinases, such as TNF receptor associated factor 6 (TRAF6), inhibitor of kappa B (IκB) kinase (IKK), and mitogen-activated protein kinases (MAPKs), resulting in the synthesis of pro-inflammatory cytokines [14] (Figure 1).

2.1 Overview of TLR activation

Like other single transmembrane receptors, TLRs are activated by ligand-induced multimerization. The extracellular domain of each TLR has the characteristic horseshoe-like structure of the leucine-rich repeat (LRR) family of proteins [15], [16] and a cytoplasmic domain containing regions that are homologous to the intracellular signaling domain of Toll/interleukin-1 receptor (TIR). Most receptors in the TLR family signal through the adaptor protein myeloid differentiation factor 88 (MyD88). Binding of ligands to TLRs initiates dimerization of TLRs and interaction of the TLR TIR domain and MyD88. This interaction facilitates the binding of another death domain (DD)–containing molecule, IL-1 receptor-associated kinase-1 (IRAK-1), which results in autophosphorylation of IRAK-1. Autophosphorylated IRAK-1 dissociates from MyD88
and interacts with TRAF6, causing the activation of a downstream kinase cascade involving IKK, leading to IκB degradation and nuclear factor kappa B (NFκB) activation [17]. The NFκB family of proteins consist of five members, c-Rel, RelA/p65, RelB, NFκB1 (p50/p105), and NFκB2 (p52/p100) that all share conserved Rel-homology domains that modulate DNA-binding activity and protein dimerization [18]. Aside from MyD88-dependent signaling pathway, TLRs engagement can also activate a MyD88-independent pathway in which TIR domain-containing adaptor inducing IFNβ (TRIF) was identified to be important [19]. Signaling through TRIF results in activation of IFN regulatory factor 3 (IRF3) and expression of interferon (IFN)-inducible genes [20]. TRIF knockout mice displayed defective expression of IFN-inducible genes in response to the TLR4 ligand [21] whereas overexpression of TRIF induced activation of the IFNβ promoter in 293 cells [19].

2.2 TLRs and immune response

TLRs are widely expressed by different types of immune cells including dendritic cells (DCs), macrophages (MΦs), natural killer (NK) cells, mast cells, B cells, and T cells, permitting these cells to modulate both innate and adaptive immune responses [22]. MΦs are the major cell type involved in innate immune responses that utilize TLRs in response to infection and initiate antimicrobial activity [23]. TLR-mediated signaling is known to control antigen uptake, antigen presentation, and cytokine production in DCs and MΦs [24], [25]. Stimulation of TLRs can affect the function and phenotype of DCs [26], as TLR signaling upregulates expression of the costimulatory molecules CD40,
CD80, and CD86 on the DC surface, as well as type I IFNs, which together promote DC maturation [27], [28]. Furthermore, cytokines induced by TLR agonists, including IL-1, IL-2, IL-6, IL-12, and TNFα, have also been reported to act in an autocrine manner to activate differentiation and proliferation of DCs and also to promote T-cell differentiation and clonal expansion [29], [30]. Indeed, it is the type of TLRs activated and the response of DCs that can determine the differentiation of naive T helper (Th) cells toward Th1 or Th2 cells. For example, LPS enhances DC antigen presentation in vivo through upregulation of major histocompatibility complex class II (MHCII) and IL-12 production, which is essential for Th1 responses [31]. On the other hand, TLR2 activation in human DCs resulted in IL-10 production and blockade of IL-12, which favors Th2 response [32]. In fact, activation of TLRs on DCs shapes the function and phenotype of DCs and the T cells that these DCs are interacting with. Certain TLR ligands are known to induce IL-10 production that stimulates Th2 or T-regulatory responses as demonstrated by mice treated with Pam-3-cys, a TLR2 agonist, which promotes IL-10 production and Th2 skewed response [33]. Other ligands that induce strong production of transforming growth factor β (TGFβ), IL-6, and IL-23 promote Th17 cells [34], as exhibited by mice infected with Streptococcus pneumoniae and Candida albicans [35] evidencing a Th17 immune response.

Mouse CD4+ and CD8+ T cells have been reported to express TLR2, TLR3, TLR4, and TLR9 in association with their coreceptor (MD2) and adaptor MyD88 [36]. However, studies demonstrate that only TLR2 induced direct activation of Th1, but not Th2, cells in
the absence of T cell receptor (TCR) stimulation [36], [37]. The activation of CD8⁺ T cells is mainly through DC cross-presentation of exogenous antigens. For example, engagement of TLR3, 7, and 9 induces cross-presentation and stimulation of CD8⁺ T cells [38].

3. Role of IL-1β, IL-6, and TNFα

3.1 TNFα

TNFα is a member of TNF family and is involved in physiological processes, systemic inflammation, tumor lysis, apoptosis, and initiation of the phase reactions [39]. TNFα is mainly produced by MΦs, although other cells also express TNFα at low levels [39]. It appears that the expression of TNFα is NFκB dependent as there are several DNA-binding sites for NFκB within the promoter region of TNFA gene [40]. Interestingly, TNFα itself also triggers activation of IκB/NFκB and the MAPK/activator protein 1 (AP-1) pathway, and can promote expression of other pro-inflammatory cytokines [41]. TNFα signals through the TNF receptor type 1 (TNFR1) and TNFR2, which contain DDs used to recruit TNFR1-associated death domain protein (TRADD) and downstream adaptor proteins [42]. TNFα induces apoptosis via TNFR1/TRADD and Fas-associated death domain protein (FADD) interaction, which promotes pro-caspase 8 activation [43], leading to the cleavage of the pro-apoptotic protein BH3 interacting-domain death agonist (BID) and subsequent mitochondrial membrane potential loss and cytochrome c
release [44]. This ultimately leads to activation of caspase 3, 7, and 9, resulting in cell apoptosis [42].

3.2 IL-6

TLR signaling triggers NFκB activation and pro-inflammatory cytokines production [45]. One of the major NFκB target genes is IL-6, a member of the 4-helical cytokine family [46]. IL-6 signals via an 80-kDa cytokine receptor IL-6R and associates with the signaling receptor subunit glycoprotein 130 (gp130) [46]. IL-6 can also bind to the soluble IL-6R (sIL-6R) and signal through the gp130 molecule, indicating that sIL-6R/gp130 can serve as an alternative signaling pathway, trans-signaling pathway [47]. Serum IL-6 level is usually increased during acute and chronic inflammatory diseases; it has also been established as a clinically suitable biomarker for sepsis [48]. In seminal studies, Waage et al. observed high levels of IL-6 and showed an association between IL-6 levels and fatal sepsis in patients with meningococcal infection [49]. The pro-inflammatory activities of IL-6 are mainly driven by IL-6 trans-signaling via sIL-6R [50]. Trans-signaling induced in vascular endothelial cells plays a major role in sepsis [51]. IL-6 triggers redistribution of vascular endothelial (VE)-cadherin, leading to vascular leakage in human umbilical vascular endothelial cells [52]. Experimental investigations have also demonstrated a relationship between capillary leak and mortality in murine sepsis, as mortality levels decreased following sealing of the vascular leaks [53]. IL-6 has also been shown to be involved in intestinal epithelial barrier dysfunction during sepsis, where blocking IL-6 trans-signaling by soluble gp130 linked to the fragment crystallizable region (Fc)-portions
of a human IgG1 antibody (sgp130Fc) blocked intestinal epithelial cell apoptosis in the gut and epithelial barrier dysfunction and decreased mortality [54]. Collectively, evidence suggests that IL-6/siIL-6R trans-signaling is a valuable target for pharmaceutical intervention for sepsis.

3.3 IL-1β

IL-1β is the best characterized of the eleven IL-1 family members and is produced by a wide variety of cells including fibroblasts, keratinocytes, mesangial cells of the kidney, astrocytes, vascular endothelial and smooth muscle cells, NK cells, and lymphocytes; however, monocytes and MΦs are the major cellular source of IL-1β [55]. IL-1β is best known as an endogenous pyrogen due to its ability to cause fever in experimental settings [56]. Moreover, it has been shown to mediate inflammatory responses through directly activating lymphocytes and epithelial cells [57], [58]. IL-1β is also a potent inducer of intercellular and endothelial adhesion molecule expression and thus promotes the infiltration of inflammatory and immunocompetent cells from the circulation into the extravascular space [59]. IL-1β production has also been closely linked to a special type of cell death known as pyroptosis [60] and is produced in response to PAMPs and DAMPs as an inactive precursor, pro-IL-1β [61].

4. Regulation of IL-1β production

IL-1β activation and secretion is a tightly regulated process. IL-1β is regulated at the
transcriptional and translational level and utilizes a non-classical secretion pathway as it lacks a secretory signal peptide [59].

4.1 Transcriptional regulation of IL-1β

Activation of TLR-induced MyD88 recruitment and downstream NFκB activation is thought to be the primary pathway for Pro-il-1b gene transcription, however, other protein kinase activation cascades, such as MAPKs p38, p42 and p44 [p42/p44; also known as extracellular signal-regulated kinase (ERK) 1 and ERK2 (ERK1/2)], and JNK, can be involved. Notably ERK1/2 MAPK appears to regulate transcriptional activation, whereas p38 and JNK regulate ii-1b mRNA stabilization [62]. Activation of these protein kinase cascades is mostly mediated by TLRs and TIR domain-containing adaptor proteins activation of MyD88 and downstream effector molecules. Nevertheless, other receptors, such as CD11b or CD11c alpha chains of β2 integrins, have also been described as inducing IL-1β transcription resulting from phosphorylation and activation of ERK1/ERK2 and p38/MAPK in human monocytes [63].

4.2 Post-translational regulation of IL-1β

Proteolytic cleavage of pro-IL-1β requires caspase-1 activity, which is regulated by the inflamasome, which is composed of a PRR of the Nod-like receptor (NLR) family such as NACHT leucine-rich (LRR) pyrin domain (PYD) containing 1 (NLRP1), NLRP3, NLR family caspase-associated recruitment domain (CARD)-containing protein 4 (NLRC4), and retinoid acid-inducible gene 1 (RIG-I) receptors (RLRs) [64], [65]. The
best-characterized inflammasomes to date are NLRP3 and NLRC4 [64]. NLRP3 is activated in response to a variety of structurally diverse PAMPs and DAMPs and is also thought to be the main sensor for sterile inflammatory stimuli due to the evidence that it can be activated by ATP and crystalline materials, including monosodium urate (MSU) crystals and calcium pyrophosphate dehydrate (CPPD) crystals [66], [67]. In contrast, NLRC4 is thought to serve as a sensor of bacterial infection by sensing flagellin and the type III secretion system (T3SS) rod protein PrgJ, as they both contain a similar sequence motif that is essential for detection by NLRC4 [68]. Once the respective inflammasome components are engaged to its ligands, they oligomerize and recruit pro-caspase-1 via homotypic CARD-CARD interaction [69]. The formation of the inflammasome initiates autoactivation through autoproteolytic cleavage of pro-caspase-1 into its active form and thus further cleavage of pro-IL-1β [70] (Figure 2).

4.3 Secretory regulation of IL-1β

The secretion of IL-1β is reported to occur via a number of mechanisms [55]. IL-1β can be secreted through vesicles, which protect it from tryptic digestion [71]. It can also be secreted from the cell via the shedding of microvesicles from the plasma membrane [72] or via exosomes [73]. Another possible route for IL-1β secretion is through the inflammasome-induced activation of caspase-1-dependent pore formation, which provides a conduit through which IL-1β passes to reach the extracellular space [74]. It is suggested that these different mechanisms may be dependent on the strength of the inflammatory stimulus, cell type, species, concentration of DAMPs, and the nature and
duration of the stimulation [55].

5. Macrophages

MΦs are bone marrow (BM)-derived cells of the myeloid lineage that reside in lymphoid and non-lymphoid tissues and are believed to be involved in steady state tissue homeostasis via the clearance of apoptotic cells and the production of growth factor [75]. MΦs are differentiated from monocytes by stimulation with MΦ colony-stimulating factor (M-CSF) and granulocyte-MΦ colony-stimulating factor (GM-CSF). Monocytes are released into the peripheral blood and circulate for several days before entering tissues and replenishing resident tissue MΦ population [76].

MΦs are a very heterogeneous cell population and are divided into subpopulations on the basis of their anatomical location and functional phenotype. These specialized tissue-resident MΦs are osteoclasts (bone), alveolar MΦs (lung), Kupffer cells (liver), histiocytes (interstitial connective tissue), lamina propria MΦs (GI), and marginal zone MΦs (spleen) [75]. These cell populations constantly survey their immediate surroundings for signs of tissue damage or invading organisms through their PRRs, including TLRs, C-type lectin receptors (CLR), scavenger receptors, RLRs and NLRs [77]. Activation of the PRRs initiates transcriptional mechanisms that lead to phagocytosis, cellular activation, and the release of cytokines, chemokines, and growth factors [78-80]. MΦs also express numerous secreted molecules including complement
and Fc receptors that bind opsonin molecules, C3b, and antibodies, which activate the complement cascade and enhance the process of phagocytosis by tagging the pathogen surface [77].

In response to tissue injury or infection, peripheral monocytes enter the damaged organs and exhibit a pro-inflammatory phenotype, in that they secrete pro-inflammatory molecules such as IL-1, IL-6 and TNFα, which participate in activation of various antimicrobial mechanisms [81]. MΦs also secrete IL-12 and IL-23 that drive polarization of Th1 and Th17 cells [34]. The reactive oxygen and nitrogen intermediates that MΦs produce during infection are highly toxic to microorganisms but can also be very damaging to neighboring tissues and lead to aberrant inflammation [82].

MΦs are critical to the LPS-induced immune response [83]. In a lung inflammation model, mice that were depleted of MΦs by clodronate and exposed to *Escherichia coli* LPS exhibited significantly lower levels of TNFα production, NFκB activation and LPS-induced neutrophil recruitment in lung tissue [84]. Activation of MΦs is dependent largely on NFκB signaling. In BM-derived MΦs (BMDMs), activation of a MyD88-dependent pathway is required for sustained NFκB activation and the production of pro-inflammatory cytokines, such as IL-6 and TNFα. The importance of p65 in NFκB signaling in MΦs upon LPS challenge has also been emphasized by Sharif-Askari et al.. They demonstrated that inhibition of p65-mediated transcriptional transactivation by interfering with p65 binding to the DNA of target gene promoters decreases TNFα production by MΦs [85].
6. LPS-induced МΦ cell death

6.1 Programmed cell death

Apoptosis, one of the types of programmed cell death (PCD), is an ATP-dependent form of cell death characterized by specific morphological and biochemical changes of dying cells, including chromatin condensation, nuclear fragmentation, cell shrinkage, and blebbing of the plasma membrane [86], [87]. The end result of apoptosis is fragmentation of the cell into small, membrane-bound bodies that are quickly cleared by phagocytic cells [88]. Biochemical changes include chromosomal DNA cleavage, phosphatidylserine externalization, and a number of intracellular substrate cleavages by the specific proteolysis initiated by caspases [89]. Activation of caspases is a characteristic of apoptosis and leads to DNA fragmentation and the cleavage of key regulatory proteins, resulting in cell death [90]. Two major mechanisms of apoptosis have been characterized, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway involves transmembrane receptor–mediated interaction involving death receptors that are members of the TNFR superfamily and its ligands such as Fas ligand/Fas receptor and TNFα/TNFR1. The intrinsic pathway involves various intracellular signals that indicate that the cell is either lacking certain growth factors and apoptotic suppression or undergoing stress such as radiation, toxins, and hypoxia, and thus leads to the release of pro-apoptotic proteins from the intermembrane space of mitochondria to the cytosol [91].
Autophagy and necrosis are two other main types of PCD. Autophagy is characterized by the sequestration of cytoplasm and organelles in double-membrane or multimembrane vesicles and delivery of these vesicles to the cell’s own lysosomes for subsequent degradation; autophagy occurs when cells undergo excess stress or during starvation [92]. In contrast to apoptosis and autophagy, necrotic cell death is an energy-independent process characterized by cell swelling and lysis. Unlike apoptotic cells and autophagic cells, necrotic cells release cellular constituents that elicit an inflammatory reaction in surrounding viable tissue [91].

Cell death contributes to sepsis in regards of modulation of immune system and organ failure. There is evidence that patients suffering from sepsis show symptoms of immunosuppression and cell death of lymphocytes substantially impairs innate and adaptive immunity reducing the capacity to eliminate the invading pathogen [93], [94]. In addition, the uptake of apoptotic cells by MΦs induced production of TGFβ, prostaglandin E2 and PAF, which induces inhibition of inflammatory mediators via paracrine and autocrine effects [95]. However, adoptive transfer of apoptotic cells prior to the induction of polymicrobial sepsis markedly reduced septic survival associated with a decreased production of IFNγ by splenocytes when compared to necrotic cell material or buffer [96]. Thus, apoptotic cells might alter the immune system not only by modulating inflammation but also by direct influence on the adaptive immunity by providing a variable source of antigen for immune cells. Apoptosis of parenchymal cells (e.g. in the lung, liver and gut) may also promote organ failure and death [97].
6.2 Lipopolysaccharide

LPS is a major constituent of the outer cell wall of gram-negative bacteria and a highly potent activator of the innate immune system and thus constitutes a major marker for the recognition of intruding gram-negative pathogens by the host. The interaction of LPS with immune cells, such as MΦs, granulocytes, DCs or mast cells leads to the formation and release of a large spectrum of inflammatory mediators which are essential for the early innate and subsequent adaptive anti-bacterial defense [23][98]. Genetic studies have established that TLR4 is the LPS receptor as mice that carry mutations in the Tlr4 gene [C3H/HeJ [99], C57BL/10ScCr (Cr) [100] and C57BL/10ScN [101]] are resistant to LPS-induced inflammation. Conversely, insertion of the Tlr4 transgene reverses the LPS-resistant phenotype of C57BL/10ScCr mice [102]. In this study, investigation of the LPS-induced cytokine (IL-6) response also revealed a linear relationship between the IL-6 response and the logarithm of TLR4/MD-2 levels in MΦs, indicating a direct correlation between TLR4 expression and LPS sensitivity [102].

LPS also induces cell death in MΦs and contribute to MΦ dysfunction [103]. MΦ death after pathogen infection has been reported in several studies [104], and LPS-induced apoptosis is believed to contribute to the pathogenesis of sepsis [105]. MΦs derived from C3H/HeJ mice are resistant to LPS-induced apoptosis [106]. Studies have shown that LPS can activate caspase-1, 3, 6, and 8, among which caspase-8 is the most well-characterized molecule in apoptosis induction [107], [108]. LPS has also been reported to upregulate expression of the proapoptotic B-cell lymphoma 2 (BCL-2) family
members Bcl-2-associated X protein (BAX), Bcl-2-associated death promoter (BAD), and Bcl-2 homologous antagonist/killer (BAK) and to downregulate anti-apoptotic molecule BCL-2 and B-cell lymphoma-extra large (BCL-XL) [109], [110].

Several of the upstream signaling molecules involved in LPS activation of NFκB are similarly involved in promoting LPS-induced apoptosis. Expression of either MyD88 or IRAK-1 dominant negative (D/N) constructs inhibits LPS-induced NFκB activation and protects mononuclear phagocytes against LPS-induced apoptosis [111]. On the other hand, activation of TRAF6 in LPS-induced apoptosis involves JNK, which lies upstream of caspase activation [112]. Collectively, TLR4 can serve as a death receptor for LPS, and the signaling molecules involved in LPS-induced NFκB activation serve a dual role in promoting LPS-induced apoptosis.

7. NFκB signaling

The NFκB signaling system consists of NFκB heterodimers and homodimers of Rel homology domain (RHD) containing peptides, which are RelA, RelB, cRel, p50 and p52 and their inhibitor IκB proteins [113]. Some of the physiologically important dimers are p65:p50, cRel:p50, RelB:p52, RelB:50, and p65:p65 [114]. Activation of NFκB results in the induction of inflammatory, developmental and survival genes. The rapid and reversible inflammatory response typically occurs through the activation of the canonical pathway, while the slower and irreversible developmental response typically occurs
through the non-canonical pathway.

7.1 Non-canonical pathway

Non-canonical pathway is activated by TNF-family cytokines such as lymphotoxin b (TNFSF3), CD40 ligand, and B cell activating factor (BAFF and TNFSF13B), however, TNFα is not included [115]. Activation of non-canonical pathway leads to activation of RelB:p52 and RelB:p50, resulting in biological functions in cells including secondary lymphoid organogenesis and architecture organization, thymic epithelial cell differentiation, B-cell maturation and survival, DC maturation, and regulation of T cell differentiation [116].

7.2 Canonical pathway

The canonical NFκB pathway has been defined primarily in response to TNFα and IL-1 signaling [115]. This pathway is activated through pathogen and inflammatory signals resulting in the degradation of IκB, allowing for the nuclear translocation of p65:p50, p65:p65 and cRel:p50 dimers that bind to promoter region of genes responsible for inflammation. The importance of the p50 and p65 in the pathophysiology of sepsis has been demonstrated by increased nuclear binding activity of each subunit in both animal models and septic patient peripheral blood mononuclear cells (PBMCs) [117]. Early study has showed that p65:p50 heterodimers is the major transcription factor involved in LPS-induced pro-IL-1β transcription in MΦs [118], while other study has revealed the critical role of increased p65:p65 homodimer activity in neutrophilic lung
inflammation [119]. c-Rel:p50 has also been shown to be a key NFκB member required for host antimicrobial defenses as loss of c-Rel and p50 leads to defective MΦ phagocytosis, moderate cytokine response, decreased bacterial clearance and thus increased mortality in sepsis [120], [121].

While NFκB members are well accepted for the enhancive effect on inflammation, some studies have also demonstrated the repressive effects. p50 homodimer, which lacks transactivation domain, has been shown to repress expression of NFκB target genes and inhibit inflammation in MΦs[122]. In addition, Gadjeva et al. reported that p50-deficient mice that are heterozygous for p65 (p50\(^{-/-}\) p65\(^{+/-}\)) were extremely sensitive to LPS-induced shock, suggesting anti-inflammatory roles of p50 homodimer and p65:p50 heterodimers in septic shock [123]. Collectively, these studies suggest that p65 is rather intriguing to possess dual roles of both pro-inflammatory and anti-inflammatory responses. As p65-mediated transcriptional activity directly regulates pro-inflammatory cytokine production in response to LPS in MΦs [85], [118], p65 involvement in NFκB signaling in septic shock is worth of investigation.

**Summary**

In a murine model of endotoxic shock, investigators have demonstrated that the toxic effects of LPS are mostly related to TLR4 induced NFκB activation in activated MΦs and the release of pro-inflammatory mediators including IL-1β, IL-6, and TNFα.
However, the role of the NFκB subunit p65 in LPS-induced NFκB activation and pro-inflammatory cytokine production by MΦs is still unclear, and the importance of this pathway to the symptoms of the shock response is unknown.

The hypothesis of this dissertation is that myeloid-specific p65 regulates the inflammatory response and susceptibility to endotoxic shock. The specific aims of this dissertation are:

1. To examine the endotoxin-induced shock response in mice deficient in p65 in myeloid cells
2. To examine the cytokine response in mice deficient in p65 in myeloid cells
3. To examine the effect of myeloid p65 deletion on MΦ differentiation *in vitro*
4. To assess the consequence of p65 deletion in LPS/TLR4-induced MΦ activation *in vitro*
Figure 1. Toll-like receptor–mediated signaling pathway. Activation of TLRs can stimulate myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent signal activation. In the MyD88-dependent pathway, MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual Toll/interleukin-1 (IL-1) receptor (TIR) domains. Upon stimulation, IL-1 receptor-associated kinase (IRAK) is recruited to MyD88 through the death domain and autophosphorylated. Phosphorylated IRAK dissociates from MyD88 and recruits tumor necrosis factor (TNF) receptor–associated factor-6 (TRAF6). This induces phosphorylation and degradation of inhibitor of NFκB (IκB) and subsequent nuclear factor kappa B (NFκB) nuclear localization and pro-inflammatory cytokine gene transcription. In the MyD88-independent signaling, engagement of TLR triggers TIR domain-containing adaptor inducing IFNβ (TRIF) recruitment and activates interferon (IFN) regulatory factor 3 (IRF3), which translocates into nucleus and induces IFNβ gene transcription. p indicates phosphorylation in the figure.
Figure 2. Inflammasome activation leads to mature interleukin-1β (IL-1β) production. Two major signaling pathways are required for production of mature IL-1β. Signaling pathway 1: engagement of various pathogen-associated molecular patterns (PAMPs) or IL-1β to their receptors (e.g. Toll-like receptors (TLRs) and IL-1R) results in pro-IL-1β transcription. Signaling pathway 2: activation of inflammasome by various stimuli to release caspase-1. Binding of adenosine triphosphate (ATP) to its receptor P2X7 purinoceptor 7 (P2X7) triggers the activation of the inflammasome through association of NACHT leucine-rich (LRR) pyrin domain (PYD) containing protein (NLRP), apoptosis-associated speck-like protein containing a carboxy-terminal caspase-associated recruitment domain (CARD) (ASC), and pro-caspase-1. The other pathway of inflammasome activation is through phagocytosis of monosodium urate (MSU) or other crystals. The activation of inflammasomes results in the generation of caspase-1 from pro-caspase-1. This caspase-1 then cleaves pro-IL-1β to mature IL-1β, which acts through an autocrine manner, serving as a key mediator for inflammation. TIR, Toll/interleukin-1 receptor domains. IRAK, IL-1 receptor-associated kinase. TRAF6, tumor necrosis factor receptor–associated factor-6. NFκB, nuclear factor kappa B. IκB, inhibitor of NFκB. p indicates phosphorylation in the figure.
Chapter 2. Materials and methods

**Mice.** Male and female Lysozyme M (LysM)\(^{cre/cre}\) RelA/p65\(^{fl/fl}\) (RelA/p65\(^{Δmye}\), C57BL/6/129/SvEv) and LysM\(^{cre/cre}\) RelA/p65\(^{+/+}\) (WT line for RelA/ p65\(^{Δmye}\) mice). All mice were housed under specific pathogen-free condition and animal care were performed by experienced veterinary technicians, and all experimental techniques have been approved by the IACUC of Cincinnati Children’s Hospital.

**Bone marrow derived macrophages.** Bone marrow was obtained from mice femurs using 5 mL harvesting buffer (25 mM Hepes/ 50 µg/mL Gentamycin in 1x HBSS). Cells were passed through 70µM filter and spin down at 1200 rpm before resuspended in 20 mL BMDM media (20% M-CSF in 10% FBS containing complete DMEM) and cultured for 5 days (37°C, 5% CO\(_2\)).

**Peritoneal macrophages.** Mice were intraperitoneally injected with 1 mL 3.85% thioglycollate. 3 days following the i.p. injection, a peritoneal lavage was performed and cells were centrifuged and resuspended in 25 mL 10% FBS complete DMEM and plated for 4 hours adherence. Cells were then washed with PBS and detached with trypsin/EDTA (Life Technologies; Carlsbad, CA) at 37°C for 5 minutes and resuspended in 10% FBS complete DMEM. Alternative operational approach used only EDTA (2.5mM EDTA in 1x PBS) at 37°C for 20 minutes to detach cells after 3 hours adherence. Cells were then gently scraped off and resuspended in desired medium volume.

**Macrophage stimulation.** BMDMs (1 x 10\(^5\) cells/250 µL) were plated in a 96-well plate and incubated overnight at 37°C, 5% CO\(_2\). The next day, BMDMs or peritoneal
macrophages (2 x10^6 or 2.5 x10^5) were pretreated with 0, 10 or 100 ng/mL Escherichia coli O55:B5 LPS (Sigma-Aldrich; St. Louis, MO) for the indicated hours according to experiments followed by 1 hours 2 mM adenosine triphosphate (ATP) (InvivoGen; San Diego, CA) stimulation for inflammasome activation. Supernatant and mRNA were collected and analyzed for cytokine induction.

**ELISA.** IL-1β, IL-6, and TNFα level were measured in the supernatant after cell culture using the ELISA Duo-Set kit according to the manufacturer’s instructions (R&D System; Minneapolis, Minn).

**FACS analysis.** Single-cell suspensions were washed with FACS buffer (PBS/ 1% BSA) and incubated for 30 minutes at room temperature with combinations of the following Abs: PE anti-mouse F4/80 (clone Cl:A3-1; AbD Serotec; Raleigh, NC), PE-Cy7 anti-mouse CD11b (clone M1/70; BD Pharmingen; San Jose, CA), Alexafluor-647 anti-mouse Ly6C (clone ER-MP20; AbD Serotec; Raleigh, NC), and FITC anti-mouse Ly6G (clone 1A8; BD Pharmingen; San Jose, CA). Cells were washed once with FACS buffer and analyzed on a FACSCanto II (BD Immunocytometry System; San Jose, CA), and analysis was performed using Flow Jo software (Tree Star; Ashland, OR). For experiment conducted to determine immune cell population in peritoneal cavity, the following Abs were used: APC anti-mouse CD19 (clone 1D3), PE anti-mouse CD23 (clone B3B4), PE-Cy7 anti-mouse IgM (clone R6-60.2) or CD3 (clone145-2C1) or FcεRⅠα (clone MAR-1), FITC anti-mouse B220 (clone RA3-6B2), PerCp-Cy5.5 anti-mouse CD11b (clone M1/70) or CD11c (clone HL3), AlexaFluor750 anti-mouse c-kit
(clone 2B8) or CD4 (clone GK1.5, BD Pharmingen; San Jose, CA) and PE anti-mouse MHC class II (clone NIMR-4; eBioscience; San Diego, CA). All antibodies were used in a 1:200 dilution in FACS buffer.

**Western blot.** Bone marrow-derived macrophages or peritoneal macrophages after stimulation were lysed using M-PER mammalian protein extraction reagent (Life Technologies; Carlsbad, CA). Approximately 40 µg proteins were separated on 4%-12% Bis-Tris gel and transferred to a nitrocellulose membrane (Life Technologies; Carlsbad, CA). The following antibodies were used: rabbit anti-mouse caspase-1 p10 (M20) (Santa Cruz; Santa Cruz, CA) and goat anti-mouse IL-1β/IL-1F2 (R&D System; Minneapolis, Minn) followed by goat anti-rabbit (Calbiochem; Darmstadt, Germany) or sheep anti-goat (Jackson ImmunoResearch Laboratories; West Grove, PA) peroxidase-conjugated antibodies and ECL-plus detection reagent (Life Technologies; Carlsbad, CA). Rabbit anti-mouse β-actin (Sigma; St. Louis, MO) was used as loading control.

**Real-time PCR analysis.** The RNA samples (1 µg) were subjected to reverse transcription analysis using SuperScript II reverse transcriptase (Life Technologies; Carlsbad, CA) according to manufacturer’s instructions. Mouse *Il1b, Caspase-1, Ifnb, Ccl12, and Cxcl10* were quantified by real-time PCR using the iQ5 multicolor real-time PCR detection system (Bio-Rad Laboratories; Hercule, CA) with iQ5 software V2.0 and LightCycler FastStart DNA Master SYBR Green I. Primer sets used were as follows: mouse *Il1b* (76 bp), TTGACGGACCCCAAAAAGAT and GAAGCTGGATGCTCTCATCTG; mouse *Caspase-1* (144 bp),
CTTCAATCAGCTCCATCAGC and AGGGCAAAACTTGAGGGTC; mouse hprt (153 bp), GTAATGATCAGTCAACGGGGGAC and CCAGCAAGCTTGCAACCTTAACCA; mouse Ifnb (267 bp) ATAAAGCAGCTCCAGCTCAA and CTGTCTGCTGGTGGAGTTCA; mouse Ccl12 (95 bp) CCATCAGTCCTCAGGTATTGG and CTCTCCGGACGTGAATCTTCT; mouse Cxcl10 (111 bp) GCTGCCGTCATTTTCTGC and TCTCACTGGCCCGTCATC. Gene expression was determined as relative expression on a linear curve based on a gel-extracted standard and was normalized to Hprt amplified from the same cDNA mix. Results were expressed as the gene of interest/Hprt ratio that had been normalized each group to un-stimulated p65^mye group.

**MTT assay.** Cells (1x10^6 or 5x10^4) were plated in a 96-wells plate and stimulated with indicated concentrations of LPS and time according to experiments. Supernatant was poured off after stimulation and 100 uL of 4 mg/mL MTT (Sigma-Aldrich; St. Louis, MO) was added followed by 1 hour incubation at 37°C. The formazan crystals were dissolved using 100 µL of MTT lysis buffer (10% SDS in isopropanol) and absorbance was measured at 570 nm subtracted by OD 630 nm. Results are presented as percentage of survival taking the control (untreated cells) as 100% survival.

**IFNβ bioassay.** 5x10^4 / 100 µL L929 cells were plated in a 96-wells plate and incubated overnight at 37°C, 5% CO₂. The next day, cells were stimulated with either IFNβ standard, mice serum, or supernatants from U-LPS stimulated peritoneal MΦs. Reporter lysis buffer was added following 5 hours incubation and cells were frozen at -80°C for 10 mins. Cells were thawed at RT and luciferase bioluminescence was quickly
measured at OD 570 after applying luciferase assay reagent into the wells. All the reagents needed were provided in Luciferase Assay System (Promega; Madison, WI)

**Statistical analysis.** Data are expressed as mean ± SEM. Statistical significance comparing two groups was determined by Mann-Whitney U test. In experiments comparing multiple experimental groups, one-way ANOVA was performed across groups with a post hoc comparison analysis (Tukey’s test) to determine significance. $p < 0.05$ was considered significant.
Chapter 3. Results

1. Ultra pure LPS challenge decreases survival rate in p65Δmye mice

To determine whether myeloid-specific p65 deletion plays a role in the regulation of innate immune responses, we examined the consequence of myeloid-specific p65 deletion in a murine model of endotoxic shock by using p65Δmye mice. Mice were generated by backcrossing p65fl/fl mice (C57BL/6) [125] onto LysM-Cre mice (C57BL/6) [126] as described in our previous study to specifically delete p65 in myeloid cells [127].

p65Δmye mice and littermate WT (LysM-Cre p65WT/WT) control mice were injected i.p. with ultra pure LPS (U-LPS) (40 mg/kg), and survival were monitored. More than 90% p65Δmye mice died within 18 hours after U-LPS injection while WT littermates remained alive (Fig 1), suggesting that myeloid p65 deletion renders mice more susceptible to U-LPS challenge.

2. Ultra pure LPS challenge increases pro-inflammation cytokines and alters cell recruitment in p65Δmye mice

As endotoxin-induced shock is linked with innate immune activation, we examined pro-inflammatory cytokine levels in the sera and inflammatory cell populations in peritoneal cavity in U-LPS challenged WT and p65Δmye mice. U-LPS induced significant high level of IL-1β by 6 hours in p65Δmye mice compared to WT (Fig 2A). U-LPS challenge-induced TNFα response in p65Δmye mice was significantly attenuated at 1 hour compared to WT. In contrast, U-LPS induced IL-1β and IL-6 secretion in p65Δmye mice at
1 and 2 hours post challenge which was significantly greater than that observed in WT mice. Notably, the levels of these cytokine while tapering down at 6 and 7 hours remained significantly elevated compared to WT. To investigate whether the cytokine response was reflective of altered cellular recruitment, we examined peritoneal cavity cell populations at steady state and following U-LPS challenge by flow cytometry analyses (Fig 2B and C). At steady state, p65Δmye mice exhibited significantly lower numbers of total MΦs (CD11b+F4/80+), resident MΦs (CD11b+F4/80+Ly6C+Ly6G−), and mast cells (c-kit+FcεRI+) compared to WT. However, numbers of neutrophils (CD11b+F4/80med-Ly6G+) and CD11b+F4/80med cells, which are possibly cells undergoing differentiation, were significantly higher in p65Δmye mice (Fig 2B). There was no difference in the numbers of inflammatory MΦs (CD11b+F4/80Ly6C+Ly6G+) and B cells (CD19+B220+). We observed increased cell numbers in both WT and p65Δmye mice peritoneal cavity 1 hour following U-LPS challenge. The profile of cell type distribution between the two groups remained the same; meaning a decrease in numbers of total MΦs, resident MΦs, and mast cells with an increase in that of neutrophils and CD11b+F4/80med cells, and comparable number of inflammatory MΦs and B cells in p65Δmye mice, compared to WT (Fig 2C). Notably, the cell numbers of total MΦs and resident MΦs increased significantly in WT mice while these remained the same magnitude in p65Δmye mice (Fig 2C). The number of neutrophils increased following U-LPS challenge in both groups, however the fold change was 3 times higher in p65Δmye mice than that in WT. These data suggest that myeloid p65 deletion in mice results in
increased IL-1β and IL-6 production and neutrophil recruitment, but decreases the recruitment of MΦs upon U-LPS challenge.

3. Decreased inflammation and viability in p65Δmye BMDMs upon ultra pure LPS stimulation

To determine if the altered phenotype in p65Δmye mice was attributed to an inherent defect in myeloid cells following p65 deletion, and as MΦ activity has long been associated with mortality in sepsis [128], we in vitro stimulated BMDMs from WT and p65Δmye mice with U-LPS in vitro. Firstly we examined the importance of p65 in the development of BMDMs generated from BM cells stimulated with M-CSF for 5 days in vitro. Flow cytometry profile of WT BMDMs revealed that majority of the cultured cells were large in size with granular morphology (SSC^hiFSC^hi) (Fig 3A, gate a). This population was identified as F4/80^+CD11b^+ cells that consisted of both Ly6C^lo and Ly6C^hi cell subsets, representing both mature and immature MΦs, respectively. In the parent gate (SSC vs. FSC), we also observed a population that was small in size with low granularity (SSC^loFSC^lo) and expressed medium or low level of F4/80 but were CD11b^+Ly6C^hi (Fig 3A, gate b and c), indicative of being immature MΦs. In addition, a small pool of cultured cells within this gate did not express surface F4/80 and therefore were non-MΦ (Fig 3A, gate d). Flow cytometry analyses showed BM cells from p65Δmye mice were capable of giving rise to a similar profile and percentage of BMDMs (Fig 3B), suggesting that p65 is dispensable for the development of BMDMs in vitro. To assess the efficiency of LysM-Cre-mediated deletion of p65 in myeloid cells, we examined the
presence and activation (phosphorylation) of p65 in p65Δmye BMDMs following U-LPS stimulation. Western blot analyses revealed a reduced protein expression of total p65 in p65Δmye BMDMs (Fig 3C). Further, p65 activation was ablated in p65Δmye BMDMs following U-LPS (1 µg/mL) stimulation as we failed to detect the phosphorylation at Ser536 position of p65 protein (Fig 3C). To confirm that p65 deletion did not impact signaling components upstream of p65, we examined IκBα protein levels. We showed comparable levels of IκBα between WT and p65Δmye BMDMs at steady state, indicating that signaling components upstream of p65 remained intact. Notably, U-LPS stimulation induced a rapid decline in IκBα protein levels in WT BMDMs which restored to steady state levels at 120 minutes post U-LPS stimulation. Previous studies showed that LPS induces a rapid IκBα degradation via K48 ubiquitin and proteasome degradation and p65 induces IκBα expression and replenishment in cells [129]. Consistent with previous findings, IκBα degradation induced by U-LPS stimulation could not be restored in myeloid cells deficient of p65 at 120 minutes. WT BMDMs pretreated with U-LPS for 3 or 6 hours produced significant amounts of pro-inflammatory cytokines IL-1β, IL-6, and TNFα (Fig 3D). In contrast, U-LPS pretreatment of p65Δmye BMDMs did not induce IL-1β and IL-6 secretion but only minimal amount of TNFα, suggesting the loss of p65 impaired BMDM ability to produce pro-inflammatory cytokines. To determine the effect of p65 deletion on myeloid cell viability, we conducted MTT assay. Viability of p65Δmye BMDMs was significantly decreased at 6 and 24 hours following high U-LPS (10 ng/mL or higher) stimulation compared to that of WT BMDMs (Fig 3E). These data suggest that myeloid
p65 deletion decreases cells’ capability to produce pro-inflammatory cytokine production and cellular longevity in BMDMs.

4. Dirty LPS decreases pro-inflammatory cytokine secretion and viability in p65Δmye peritoneal cells

We next examined the effect of p65 deletion in myeloid cells on the function of inflammatory MΦs, we therefore assessed the responsiveness of thioglycollate elicited MΦs to dirty LPS (D-LPS). We used D-LPS that contains multiple TLR ligands to induce synergistic signaling in peritoneal MΦs [130]. Peritoneal cells were acquired from WT and p65Δmye mice 3 days after i.p. thioglycollate injection and purity after 4 hours adherence was analyzed by flow cytometry (Fig 4A and B). Flow cytometry analyses revealed 4 cell populations in WT peritoneal cells based on their SSC/FSC and surface marker profile. 1) Small and granular (SSC\text{hi}FSC\text{lo}) F4/80\text{med}CD11b\text{med}Ly6C\text{+} cells (Fig 4A, gate a) were likely to consist of mast cells and eosinophils (F4/80\text{lo}CD11b\text{+}Ly6C\text{lo-med}). 2) Larger sized and granular (SSC\text{med}FSC\text{med}) F4/80\text{+}CD11b\text{+}Ly6C\text{med} MΦs (Fig 4A, gate b). 3). Small cells that were predominantly F4/80\text{-}CD11b\text{-} cells with some F4/80\text{med}CD11b\text{med}Ly6C\text{lo} cells were possibly lymphocytes (Fig 4A, gate c). 4) Large and granular cells (SSC\text{med}FSC\text{hi}) that were CD11b\text{+}Ly6C\text{med}Ly6G\text{+}, suggesting they were possibly neutrophils. Compared to peritoneal cells form WT mice, p65Δmye peritoneal cells exhibited similar profile with a decreased occurrence of mast cells and eosinophils population but increased frequency of F4/80\text{-}CD11b\text{-}MΦ (Fig 4B, gate a and b). Collectively, our data indicated that myeloid deletion of p65 does not alter the response
of mice to thioglycollate and cell recruitment into peritoneal cavity. Upon D-LPS stimulation, WT peritoneal cells secreted significant level of IL-1β, IL-6, and TNFα (Fig 4C) and express high level of pro-IL-1β protein (Fig 4D). Consistent with IL-1β secretion, caspase-1 expression in WT peritoneal cells was slightly increased following D-LPS stimulation as caspase-1 is required for cleaving pro-IL-1β into mature IL-1β. Although not statistically significant, there was a trend that D-LPS induced Il-1b and Caspase-1 gene expression (Fig 4E) and viability in WT peritoneal cells (Fig 4F). p65 deletion decreased IL-1β, IL-6, and TNFα secretion (Fig 4C) and pro-IL-1β protein expression and resulted in an undetectable intracellular IL-1β expression (Fig 4D) in peritoneal cells. p65 deletion did not alter caspase-1 protein expression (Fig 4D) nor the expression of Il-1b and Caspase-1 genes (Fig 4E). The viability of p65Δmye peritoneal cells was decreased (Fig 4F), however there was no significant difference when compared to WT. These data indicate that myeloid p65 deletion results in decreased D-LPS induced cytokine production. This decreased IL-1β was not associated with decreased Il-1b and Caspase-1 mRNA, in fact, D-LPS induced normal expression level of both genes in p65 deficient cells.

5. Ultra pure LPS stimulation results in increased pro-inflammatory cytokine production in p65Δmye peritoneal MΦs

Our data indicated that elicited p65Δmye peritoneal cell activation with D-LPS was associated with decreased cytokine production (Fig 4). However, we noted that these elicited MΦs consisted of heterogeneous population including mast cells, eosinophils,
lymphocytes, and a small amount of neutrophils as shown in Fig 4A and 4B. As contamination of MΦs by other cell population has been shown to skewed the functional readout of MΦs [131] and p65 is deleted from myeloid cells, we set out to examine myeloid enriched population. We modified our protocol to generate cleaner MΦ population. Flow cytometry analyses of WT and p65Δmye peritoneal cells showed that more than or equal to 60% of the cells were F4/80+CD11b+, and importantly, no contaminating granulocytes population (Fig 5A). We then stimulated peritoneal MΦs with U-LPS to specifically activate TLR4-NFκB signal axis as it was in our previous in vivo U-LPS challenged murine model. U-LPS stimulation resulted in significantly elevated IL-1β and IL-6 secretion by p65Δmye peritoneal MΦs compared to WT (Fig 5B). A previous study recently demonstrated NFκB function as a negative regulator of IL-1β production and that loss of NFκB enhances caspase-1 activation and IL-1β processing [132]. Our data showed that the loss of p65 in peritoneal MΦs resulted in decreased pro-IL-1β and increased caspase-1 expression (Fig 5C), which in turn, led to increased IL-1β secretion examined by ELISA. Surprisingly, p65Δmye peritoneal MΦs expressed lower protein level of intracellular IL-1β compared to WT peritoneal MΦs (Fig 5C), suggesting a discordance between IL-1β processing and secretion in our model. IL-1β can be secreted through exosomes and IL-1β secretion from ATP-stimulated BMDMs is accompanied by the release of MHC II molecules [133], a characteristic hallmark of exosomes. A recent study has shown that IL-1β secretion and MHC class II molecules export are inhibited in the absence of inflammasome component, apoptosis-associated
speck-like protein (ASC) [73]. Given the differential caspase-1 expression observed in WT and p65Δmye peritoneal MΦs, it is possible that IL-1β secretion through exosomes was differentially regulated between groups. qRT-PCR analyses revealed a significantly decrease of Caspase-1 gene expression in p65Δmye peritoneal MΦs compared to that in WT, but a comparable Il-1b expression was observed regardless of the loss of p65 (Fig 5D). These data suggest that p65 deletion in MΦs increases IL-6 and IL-1β production and caspase-1 expression in response to signaling through TLR4.

6. p65 deletion does not induce surface TLR4 expression in MΦs upon thioglycollate challenge

To test the possibility of differential TLR expression conferring to differential signaling magnitude and cytokine levels in our model, expression of TLR4 was examined by FACS and qRT-PCR in steady state and thioglycollate elicited peritoneal cells. FACS analyses revealed 2 populations, CD11b+F4/80+ MΦ population (Fig 6A, gate a) and granulocytes population (Fig 6A, gate b), in elicited peritoneal cells prior to adherence. The proportion of MΦs and granulocytes decreased following thioglycollate activation in both WT and p65Δmye mice. Histogram of TLR4 indicated that TLR4 expression in MΦs was slightly higher after thioglycollate activation in WT mice while the expression of TLR4 was not altered in p65Δmye mice (Fig 6B). We did not observe a statistically significant difference of the mean fluorescence intensity differences (ΔMFI) of TLR4 expression and Tlr4 mRNA expression by peritoneal MΦs at steady state or post thioglycollate activation between WT and p65Δmye mice (Fig 6B and C). In addition, Tlr2
mRNA expression in MΦs was comparable between groups (Fig 6C). Granulocytes showed similar TLR4 expression pattern, however, TLR4 was significantly lower in p65Δmye granulocytes after thioglycollate activation compared to WT (Fig 6B). These data suggest that p65 deletion in MΦs does not alter TLR4 expression by MΦs in response to thioglycollate activation at both the mRNA and protein level.

7. MyD88-independent TLR4 signaling pathway is intact in p65Δmye peritoneal MΦs

In addition to NFκB activation, signaling through TLR4 also activates another axis of transcription factor, IRF3, through a MyD88-independent pathway. Activation of IRF3 leads to the induction of Ifnb and other LPS-induced IFNβ-dependent gene expression including Cxcl10 and Ccl12 [134]. We found that the induction of Ccl12 and Cxcl10 expression were not altered by p65 deletion in peritoneal MΦs as demonstrated by qRT-PCR analyses showing a comparable increment level of gene expression after U-LPS stimulation in WT and p65Δmye peritoneal MΦs, suggesting that MyD88-independent signaling pathway in p65Δmye MΦs remains intact (Fig 7A). Interestingly, we observed significantly decreased Cxcl10 expression in p65Δmye MΦs after U-LPS stimulation compare to WT, while IFNβ secretion was significantly increased (Fig 7B). Though induction of Cxcl10 by type I IFN in MΦs is well accepted in the field, studies with type I IFN-activated STAT3 inhibiting Cxcl10 gene expression had also been reported [135]. Our observation of increased U-LPS-induced IFNβ production and decreased Cxcl10 in p65Δmye MΦs is consistent with this concept and this increment of IFNβ might result from increased IL-1β, which is known to stimulate IFNβ [136].
Recent study indicates that endotoxic shock response is regulated by counter-inflammatory signaling that may dominate the pro-inflammatory mediators such that an immunosuppressive state exists where inflammatory effects may be inadequate [137]. These anti-inflammatory signals include IL-10 and TGFβ; in fact, IL-10 has been shown to reduce TNFα release and prevent lethality in experimental endotoxemia [138]. Consistent with this concept, we found increased IL-10 secretion in both WT and p65Δmye peritoneal MΦs following U-LPS stimulation but the level of IL-10 between WT and p65Δmye MΦs were not different (Fig 7B), indicating that the elevated inflammatory state in p65Δmye mice was not due to decreased anti-inflammatory responses.
Figures
Figure 1. *In vivo* ultra pure LPS (U-LPS) challenge results in increased mortality in p65Δmye mice. U-LPS challenged survival of p65Δmye and littermate WT mice. Mice were injected i.p. with U-LPS (40 mg/kg). Data represent the mean ± SEM of 2 independent experiments with n = 7-11 mice per group. Data was generated by Amanda Waddell and Richard Ahrens.
Figure 2. *In vivo* ultra pure LPS (U-LPS) challenge results in increased pro-inflammatory cytokine production and altered cell recruitment in p65\(^{Δmye}\) mice.

A, Serum cytokine (IL-1\(β\), IL-6 and TNF\(α\)) levels at indicated time points in WT and p65\(^{Δmye}\) mice following U-LPS challenge (1 mg/kg). B and C, Peritoneal cell types before and 1 hour post U-LPS challenge analyzed by flow cytometry. Data represent the mean ± SEM of 2 independent experiments with n = 3-11 mice per group. Significant differences (*p < 0.05; **p < 0.01) between groups. *Data in panel 2A and 2C were generated by Jared Klarquist.
Figure 3.  *In vitro* ultra pure LPS (U-LPS) stimulation decreases pro-inflammatory cytokine expression and viability in p65Δmye BMDMs. A and B, Flow cytometry analyses of F4/80, CD11b and Ly6C expression in WT and p65Δmye BMDMs respectively.
Figure a, b, c and d represent indicated gatings. C, Representative western blot of phospho-p65 phosphorylation at Ser276 and Ser536, total p65, IκBα, and actin expression in BMDMs stimulated with U-LPS (1 µg/mL) for indicated times (minutes). D, Pro-inflammatory cytokine IL-1β, IL-6, and TNFα secretion from BMDMs pretreated for 0, 3, or 6 hours with 100 ng/mL U-LPS followed by 1 hour 2mM ATP stimulation. E, Viability of BMDMs determined by MTT assay. Cells were treated with 0, 1, 10 100, 1000 ng/mL U-LPS for 6 or 24 hours prior to MTT assay. Data represent the mean ± SEM of n = 1-2 mice per group. Significant differences (*p < 0.05; **p < 0.01; ***p < 0.005) between groups. N.D. – not detectable. *Data in panel 3E were generated by Jared Klarquist.
Figure 4. *In vitro* dirty LPS (D-LPS) challenge decreases pro-inflammatory cytokine expression and viability in p65\textsuperscript{Δmye} peritoneal cells. A and B, Flow cytometry analyses of F4/80, CD11b, Ly6C and Ly6G expression in WT and p65\textsuperscript{Δmye}
peritoneal cells respectively. Figure a, b, c and d represent indicated gatings. Grey line in histogram in figure d represents unstained cells. C, Pro-inflammatory cytokine IL-1β, IL-6, and TNFα secretion and D, Western blot of pro-IL-1β, IL-1β, pro-caspase-1, and caspase-1 expression and E, Il-1b and Caspase-1 gene expression analyzed by qRT-PCR in WT and p65Δmye peritoneal cells pretreated for 6 hours with 0, 10 or 100 ng/mL D-LPS followed by 1 hour 2mM ATP stimulation. F, Viability of peritoneal MΦs determined by MTT assay. Cells were treated with 0, 10 or 100 ng/mL U-LPS for 6 hours prior to MTT assay. Data represent the mean ± SEM of 1-3 independent experiments with n = 2-3 mice per group. Significant differences (*p < 0.05; **p < 0.01; ***p < 0.005) between groups. N.D. – not detectable.
Figure 5. *In vitro* ultra pure LPS (U-LPS) challenge increases pro-inflammatory cytokine expression in p65\(^{Δ/mye}\) peritoneal MΦs. A, Flow cytometry analyses of F4/80, CD11b, and Ly6C expression in WT and p65\(^{Δ/mye}\) peritoneal MΦs. B, Pro-inflammatory cytokine IL-1β, IL-6, and TNF\(α\) secretion and C, Western blot of pro-IL-1β, IL-1β, pro-caspase-1, and caspase-1 expression and D, *Il-1b* and *Caspase-1* gene expression analyzed by qRT-PCR in peritoneal MΦs pretreated for 6 hours with 0, 10 or 100 ng/mL U-LPS.
U-LPS followed by 1 hour 2mM ATP stimulation. Data represent the mean ± SEM of 2 independent experiments with n = 2-3 mice per group. Significant differences (*p < 0.05; **p < 0.01; ***p < 0.005) between groups. N.D. – not detectable.
Figure 6. TLR4 expression is not altered in p65Δmye MΦs at steady state or post thioglycollate activation. A, Flow cytometry analyses of steady state and thioglycollate activated peritoneal cells in WT and p65Δmye mice. a and b represent indicating gatings. B, Histograms and quantitative bar graphs of TLR4 expression represented by mean fluorescence intensity (thioglycollate activated subtracts steady state) in steady state and thioglycollate activated WT and p65Δmye peritoneal cells. Grey-isotype control
determined by lymphocyte affinity of PE-conjugated anti-mouse F4/80 IgG2a antibody, blue-steady state, red-thioglycollate activated. C, Tlr4 and Tlr2 gene expressions analyzed by qRT-PCR in peritoneal MΦs from thioglycollate injected WT and p65Δmye mice. Data represent the mean ± SEM of 2 independent experiments with n = 2-4 mice per group. Significant differences (**p < 0.01) between groups.
Figure 7. MyD88-independent signaling pathway is intact in p65<sup>Δmye</sup> peritoneal MΦs following in vitro ultra pure LPS (U-LPS) challenge. A, Ifnb, Cxcl10, and Ccl12 gene expressions analyzed by qRT-PCR and B, IFNβ and IL-10 secretion in peritoneal MΦs pretreated for 6 hours with 0, 10 or 100 ng/mL U-LPS followed by 1 hour 2mM ATP stimulation. Data represent the mean ± SEM of 1-2 independent experiments with n = 2-3 mice per group. Significant differences (*p < 0.05; **p < 0.01; ***p < 0.005) between groups.
Chapter 4. Discussion

In the present study, we investigated the contribution of NFκB RelA/p65 subunit in myeloid cells in the regulation of endotoxic shock response. We have demonstrated that p65Δmye mice had increased susceptibility to LPS-induced cell death and this was associated with an enhanced inflammatory response, as evidenced by increased pro-inflammatory cytokines (IL-1β and IL-6).

We showed:

1. **Differential cytokine production in p65Δmye BMDMs and peritoneal MΦs**

We demonstrated that LPS-induced pro-inflammatory cytokine production was ablated in p65Δmye BMDMs, while enhanced in thioglycollate-elicited p65Δmye peritoneal MΦs (Fig 3 & 5). BMDMs and peritoneal MΦs respond differently to stimuli has been addressed in several aspects. Infection of BMDMs with herpes simplex virus type 1 resulted in marked cytopathic effect and increased viral DNA within cells compared to that of resident peritoneal MΦs [139]. As thioglycollate results in the local accumulation of MΦs that appear activated by morphological and certain functional criteria including spreading, FcR-mediated phagocytosis, and release of plasminogen activator, thioglycollate-elicited MΦs are considered to be activated [140]. It is reported that thioglycollate-elicited MΦs synthesize different level of glycosaminoglycan compared to BMDMs in response to LPS [141]. Direct functional comparisons of BM and peritoneal exudate polymorphonuclear leukocytes (PMNs) has demonstrated that peritoneal PMNs
have enhanced generation of reactive oxygen species and they are fully primed upon isolation, while BM PMNs are in a resting state and have the capacity to become primed [142]. Though MΦs we show here, it is possible that p65 deletion renders MΦs more actively primed in response to thioglycollate activation and leads to increased IL-1β and IL-6 production.

2. p65 deletion increases cell death in BMDMs following U-LPS stimulation.

NFκB has long been implicated in both pro- and anti-apoptotic signaling. For example, NFκB inhibits PMA/ionomycin mediated induction of Fas and FasL-induced apoptosis but stimulates glucocorticoid-mediated apoptosis [143]. Despite the dual role of NFκB activation in apoptosis, less pro-apoptotic role of p65 was reported but rather its anti-apoptotic significance has been emphasized. p65 is important in regulating developmental processes and survival as mice lacking p65 die in utero and have extensive liver damage via enhanced apoptosis [144]. In MΦs, p65 phosphorylation at Ser276 is critical in M-CSF induced cell survival and inhibition of p65 Ser276 phosphorylation increased apoptosis through downregulating anti-apoptotic gene Bcl-xl [145]. p65 phosphorylation is clearly involved in cell survival as accumulating evidences have emphasized its importance not just in MΦs but also other cell types [146]. Consistent with the above concept, we show decreased viability in p65Δmye BMDMs and reduced level of p65 Ser276 phosphorylation in p65Δmye BM cells, indicating that p65 is required for maintaining cell viability of MΦs in LPS-induced endotoxic shock possibly through downregulating anti-apoptotic genes.
3. Increased IL-1β and IL-6 in p65Δmye peritoneal MΦs in response to U-LPS.

Our present data showing elevated IL-1β and IL-6 in serum and in p65Δmye enriched peritoneal MΦs following U-LPS stimulation (Fig 5B) suggest that p65 deletion increases pro-inflammatory cytokine levels. NFκB signaling is not the only factor inducing IL-6 and IL-1β production, MAPK pathways, which can be activated by LPS, contributes equally to both cytokines transcription activation [147]. For example, LPS-induced p38 MAPK activation results in C/EBP binding to IL-6 promoter region and increased IL-6 transcription [147]. Indeed, in some cases that MAPK pathway might overrule IL-6 production as shown in cardiac fibroblasts that ERK and p38 MAPK and not NFκB are critical for angiotensin-II induced IL-6 production [148]. A recent study pointed out diminished p38 MAPK activation and pro-inflammatory cytokines production in response to LPS stimulation in in vitro cultures of thioglycollate-elicited peritoneal MΦs with the presence of eosinophil contamination. It is likely that other signaling pathway dominates cytokine production in p65Δmye peritoneal MΦs and was diminished by contamination of other cell types and skewed the readout (Fig 4C). Consistent with this, enrichment of MΦs revealed a significantly higher IL-6 production in p65Δmye peritoneal MΦs (Fig 5B). Together, these data suggest that other signaling pathways are involved and may play a dominant role in IL-6 production in p65Δmye mice in response to LPS challenge.

We also observed increased IL-1β in U-LPS challenged p65Δmye mice sera and peritoneal MΦs, suggesting that NFκB is not required for IL-1β production and in fact may inhibit it. Several mechanisms might contribute to the up-regulation of IL-1β in
p65$^{Δmye}$ MΦs. Experimental evidence indicate 1) MΦ apoptosis enhances caspase-1 activation and 2) increased MΦ apoptosis which has been linked to defective expression of anti-apoptotic gene plasminogen activator inhibitor (PAI-2) in IKKβ deficient myeloid cells, suggesting that loss of NFκB enhances caspase-1 activity [149]. Consistent with this, we show that p65$^{Δmye}$ MΦs expressed more caspase-1 and secrete higher level of IL-1β after LPS stimulation (Fig 5C). Furthermore, Greten et al. showed that IL-1β secretion and caspase-1 activity are negatively regulated by NFκB, as inhibition of IKKβ, which interferes with NFκB activation, increased LPS-induced plasma IL-1β [132]. Our data further support that the impaired NFκB signaling due to deletion of p65 in MΦs enhances IL-1β secretion as lower intracellular IL-1β expression and higher IL-1β in supernatant were detected in p65$^{Δmye}$ MΦs.

4. Increased IFNβ production in p65$^{Δmye}$ peritoneal MΦs upon U-LPS stimulation.

IL-1β and IL-6 have both been linked to cell death and septic shock [125], [49]. IL-6 induces phosphorylation and redistribution of VE-cadherin that leads to vascular leakage [53], which is associated with increased mortality in patients with endotoxic shock. On the other hand, IL-1β contributes to endotoxic shock by further augmenting critical cytokine production such as IL-6, TNFα, and IL-1β through activating NFκB and AP-1 [151]. In addition to IL-6 and IL-1β, type I IFN has also been shown to play an important role in endotoxic shock as IFNβ$^{-/-}$ mice are resistant to LPS-induced endotoxic shock [152]. Furthermore, by using IRF3-deficient mice, Sakaguchi et al. were able to demonstrate endotoxic shock resistance in these mice and determined IRF3 as an
essential molecule for LPS-mediated *Ifnb* gene induction [153]. Although NFκB is one of the known components of IFNβ transcription enhanceosome, impaired activation of NFκB dose not influence IFNβ induction [154]. Consistent with this, we observed an increased IFNβ secretion by p65Δmye peritoneal MΦs, suggesting a possible role of p65 in regulating endotoxic shock response through IFNβ. Alternatively, increased IL-1β production in p65Δmye mice may result in increased IFNβ expression [136] and together with increased IL-6, contributing to severe endotoxic shock in these mice.

One limitation of these analyses is that deletion of p65 in myeloid cells using LysM-Cre system leads to deletion of p65 in common myeloid progenitors derived cells, i.e. MΦs, neutrophils, and DCs. Therefore, we can not exclude the contribution of p65 signaling in neutrophils and DCs to the increased sera pro-inflammatory cytokines and mortality *in vivo*. However, in the present study, we have demonstrated that IL-1β and IL-6 in serum and in p65Δmye peritoneal MΦs were elevated upon U-LPS stimulation (Fig 5B), indicating that MΦs and the cytokines they produced are likely the important factors contribute to endotoxic shock–induced death in p65Δmye mice. In addition, we showed that neutrophil recruitment following U-LPS challenge was increased in p65Δmye mice. This finding may allow us to further elaborate the role of neutrophils in our model by examining phagocytic activity and production of oxidants and neutrophil extracellular traps (NETs), which have all been reported to be subjected to changes in septic patients [155]. Another limitation of this study is the use of i.p. LPS injection. Endotoxic shock induced by i.p. LPS injection does resemble human sepsis syndromes such as
increased coronary blood flow and splanchnic perfusion and relates more closely to clinical picture that sepsis is usually caused by abdominal infection in patients [156]. However, it may not represent the infection time course of the disease since most septic patients have an infectious focus from which bacteria continuously disseminate over time. Alternatively, local infection models can be employed such as peritonitis models, in which sepsis is induced by bacteria containing-fibrin clot implanted in abdominal cavity or by cecal ligation and puncture [157]. On the other hand, peritonitis induced by intraperitoneal administration of virulent E. coli is often associated with dissemination of bacteria from the peritoneal cavity to distant organs and multiple organ damage and is highly suitable to study the mechanisms of local anti-bacterial host defense as well as the development of systemic inflammatory response in sepsis [158].

p65 is required for basal transcription machinery through interaction with transcription factor IIB (TFIIB) and facilitates NFκB-induced transcription in cells [159]. p65 has also been shown to stabilize steady state IκBβ expression, which causes a severe growth defect associated with apoptosis when dysregulated, suggesting p65 is necessary for the maintenance of cellular homeostasis [160]. In addition, T cells homeostasis is reported to be regulated by autophagy through p65 interaction with Becn1 autophagic gene [161]. Furthermore, other studies have demonstrated the involvement of p65 in preserving the functional integrity of the cells through the regulation of the constitutive levels of glutathione. Indeed, p65 deficiency renders the fibroblasts extremely susceptible to toxicity caused by environmental and
pharmacological agents [162]. In addition to its expected role in activating inflammatory genes expression, p65 may contribute to the inhibitory machinery in cells as well. It is reported that certain serine protease inhibitor (Serpin) such as α1-antitrypsin (αAT) and SerpinE2 have NFκB binding site on their distal enhancer or promoter respectively [163], [164]. During LPS-mediated inflammation, NFκB plays a crucial role in the transcriptional induction of the inhibitory αAT gene, which has been linked with increased inflammation when being of deficiency [163]. αAT has been found to increase anti-inflammatory cytokine IL-10 release in LPS-stimulated human monocytes [165], although contradictory roles of αAT as an inhibitor and enhancer of the LPS response in monocytes have both been reported [166]. Moreover, in an animal model of endotoxin-induced lung injury, αAT attenuated lung injury in part due to the reduction of inflammatory mediators (IL-8 and TNFα) and neutrophil chemotaxis [167]. Another possible inhibitory effect of p65 may be elicited through nucleotide-binding oligomerization domain 2 (NOD2), which is a well-known upstream activator of NFκB [168]. Long-term NOD2 signaling down-regulates IL-1β and TNFα production upon TLR4 restimulation [169] while defective NOD2 signaling results in decreased IL-10 production in mononuclear cells from patients with Crohn’s disease [170]. Therefore, it is possible that myeloid p65 deletion interrupts the inhibitory machinery of either Serpin or NOD2, resulting in increased pro-inflammatory cytokines production, inflammation, and mortality.

In conclusion, our study shows that myeloid deletion of p65 aggravates LPS-induced endotoxic shock via the augmentation of IL-1β and IL-6 production from
LPS-stimulated MΦs. Although additional studies are required for confirming the lethal effect of IL-1β and IL-6 produced by MΦs in our model, our finding highlight an importance concept that one should be extra cautious to employ NFκB inhibitors as the therapeutic targets for endotoxic shock.
References


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