Molecular mechanisms responsible for the dynamic modulation of macrophage responses to varying dosages of lipopolysaccharide

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ABSTRACT

The innate immune system depends for its effectiveness on the function of specialized pattern recognition receptors which enable it to target pathogens for destruction on the basis of conserved molecular patterns such as flagellin or lipopolysaccharide (LPS). Specifically, LPS is recognized by the Toll-like receptor 4 (TLR4), activating a signaling pathway which triggers the production of both pro- and anti-inflammatory mediators. Very low doses of LPS, however, preferentially induce pro-inflammatory cytokines, which can lead to persistent low-grade inflammation, a contributing factor in a host of chronic diseases. The mild pro-inflammatory skewing induced by super-low-dose LPS also potentiates the inflammatory response to later challenge with a higher dose of LPS in a phenomenon known as the "Shwartzman reaction" or "endotoxin priming". We investigated the mechanisms involved in pro-inflammatory skewing by super-low-dose LPS in THP-1 cells and found it to be governed by a regulatory circuit of competitive inhibition between glycogen synthase kinase 3 (GSK3) and Akt, which promote the activity of the transcription factors FoxO1 and CREB, respectively. Super-low-dose LPS mildly activated FoxO1 and pro-inflammatory gene transcription without inducing anti-inflammatory genes or activating CREB, and this pro-inflammatory skewing could be abolished by inhibition of GSK3 or direct activation of CREB. We then examined the dynamics of the LPS response at various different dosages in murine bone-marrow-derived macrophages (BMDM). The pro-inflammatory cytokine IL-12 was most strongly induced by intermediate LPS dosages, with very low or high doses inducing less robust IL-12 production. Knockout of the inhibitory TLR4 pathway molecules Lyn or IRAK-M resulted in sustained induction of IL-12 by high doses of LPS. By activating CREB, we were able to reduce inflammation in WT BMDM, and saw that this corresponded with increased phosphorylation of CREB. Overall, we are confident that this subnetwork is an important switch regulating the resolution of inflammation in response to TLR4 stimulation. Furthermore, we propose that endotoxin priming is an example of the generalized capacity of all signaling networks to recall prior states, and that an appreciation for the history and context of exposure to stimuli is critical for the understanding of signaling behavior.

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Chapter 1. Introduction

The most important development in immunology over the last thirty years has been the discovery of innate immunity. Pattern recognition theory, first posited by Charles Janeway in 1989 (1), fundamentally changed the direction of immunology research. Previously, immunity was thought to depend almost exclusively on the specific, adaptive response of T and B cells, relying on the recombination of T cell receptors and antibodies both to distinguish self from non-self antigens and to target pathogens for destruction by effector mechanisms such as phagocytosis and complement. Janeway's hypotheses about non-clonally distributed pattern recognition receptors have since been borne out, and form one of the pillars of modern immunology.

The innate immune system is highly effective at discriminating self from non-self molecules on the basis of highly conserved motifs characteristic of broad classes of pathogens. These pathogen-associated molecular patterns (PAMPs) bind to specialized pattern-recognition receptors (PRRs), triggering cytokine production and other effector responses by innate immune cells such as macrophages and neutrophils. This system works in tandem with the specialized, directed response of T and B cells. Many innate immune cells have antigen-presenting function, by which they process pathogens and alert T and B cells to their presence, driving the deployment of a targeted adaptive immune response. Clonal expansion and antibody production by specific subpopulations of T and B cells, in turn, potentiate the microbicidal functions of innate immune cells, enabling them to target and destroy pathogens more effectively.

The inception of pattern recognition theory led rapidly to the identification of specific PRRs, such as the NOD-like, RIG-I-like, and Toll-like receptors (TLR) (2-4). In humans, there are ten TLRs, recognizing a menagerie of PAMPs including peptidoglycan, double-stranded

RNA, flagellin, and lipopolysaccharide (LPS), among others (5). The initial discovery of these PRR families precipitated an explosion of research into their signaling networks. The last fifteen years have seen extensive identification of the important molecules in PRR signaling, and focus is now shifting towards their interactions and regulation.

LPS is a cell wall component of Gram-negative bacteria, and is thus ubiquitous in the environment. It is a powerful inflammatory stimulus (5). Acute challenge with LPS can lead to septic shock, characterized by massive systemic expression of pro-inflammatory cytokines, often culminating in death. Septic shock is one of the leading causes of death in the United States, with a mortality rate of approximately 30% worldwide. The rate of incidence is increasing, but therapeutic protocols have remained essentially unaltered for decades (6). Exposure to LPS over a long period has been associated with a number of chronic conditions, including atherosclerosis (7), arthritis (8, 9), and diabetes (10). A fuller understanding of the mechanisms governing the inflammatory response to LPS is therefore relevant to improved treatment and prognosis for a number of the most significant acute and chronic illnesses in the United States.

Innate immune cells respond to LPS through TLR4, resulting in activation of the NFκB, PI3K (11), and MAPK (12) pathways, with ensuing induction of pro- and anti-inflammatory genes. The difference between an acute, resolving response and a chronic, unresolved response depends on the stimulatory dose and the duration of exposure. Higher doses trigger robust inflammation followed by compensatory induction of anti-inflammatory mediators (13). Low dose LPS, on the other hand, leads to mild induction of pro-inflammatory cytokines without concomitant activation of anti-inflammatory genes, skewing cells towards pro-inflammatory phenotypes (14). The networks responsible for this skewing are complex, involving numerous molecules and their interactions.

There are major outstanding questions regarding the signaling events triggered by LPS. The means by which macrophages distinguish between low and high doses are poorly understood, as are the downstream consequences of different challenges and the signaling pathways they engage. We hypothesize that the character of the innate immune response to LPS is in large part determined by the activation of a novel network involving competition between the transcription factors FoxO1 and CREB and the signaling kinases GSK3 and Akt, with GSK3-FoxO1 preferentially activating pro-inflammatory genes such as IL-12, IL-6, and TNFα, while Akt-CREB signaling plays a modulatory role, promoting the induction of IL-10, IL-33, and iNOS in opposition to the pro-inflammatory cytokines. Our aims are 1) to show that the response to LPS is dynamic, with different dosages preferentially activating different arms of the TLR signaling cascade, 2) to identify the mechanisms governing this differential activation, and 3) to recalibrate the response to different LPS dosages by pharmacological intervention. The following chapters present a survey of current literature in the field and experimental findings regarding the LPS response in different cell types.

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Chapter 2. Dynamic programing of innate leukocytes by bacterial endotoxin and its pathophysiological consequences

Abstract

Innate leukocytes such as monocytes and macrophages play pivotal roles in the modulation of inflammation and homeostasis. Recent studies suggest dynamic programming of macrophages and monocytes may give rise to distinct expression profiles of pro- and anti- inflammatory mediators. Both epigenetic modifications and competing molecular circuits may be responsible for the dynamic programming and potential memory of innate leukocytes. Lipopolysaccharide (LPS) is a classical pathogen-associated molecular pattern that may dynamically program innate immune responses. Emerging studies reveal complex dynamics of cellular responses to LPS, with lower doses of LPS associated with low-grade and chronic non-resolving inflammation, and higher doses of LPS associated with acute and resolving inflammation. In this review, we examine pathological effects of varying LPS doses, differential programming of innate leukocytes, and key molecular regulatory circuits within innate leukocytes responsible for these effects.

2.1. Pathological effects of varying dosages of endotoxin

Chronic diseases currently affect large proportions of the US population where currently 1 in 3 adults and almost 1 in 5 children are considered obese (1-4). In 2005, the CDC estimated that 1 in 2 Americans suffered from at least one chronic disease, such as arthritis, greatly decreasing their quality of life and participation in daily activities (5).

Lipopolysaccharide (LPS) is a ubiquitous molecule found on the surface of Gram-negative bacteria and is recognized by innate immune cells in humans. Slightly elevated levels of LPS persist in humans with chronic diseases and lifestyles that involve chronic smoking and

drinking (6-12). Through a better understanding of how inflammation plays a role in the development of chronic disease it may be possible to devise better treatments to prevent or mitigate their debilitating effects. Currently, it is believed that low grade inflammation plays a significant role in slowing and preventing normal healing processes from occurring leading to chronic diseases including heart disease, diabetes, reduced wound healing and even Parkinson's disease and rheumatoid arthritis (7, 13-18).

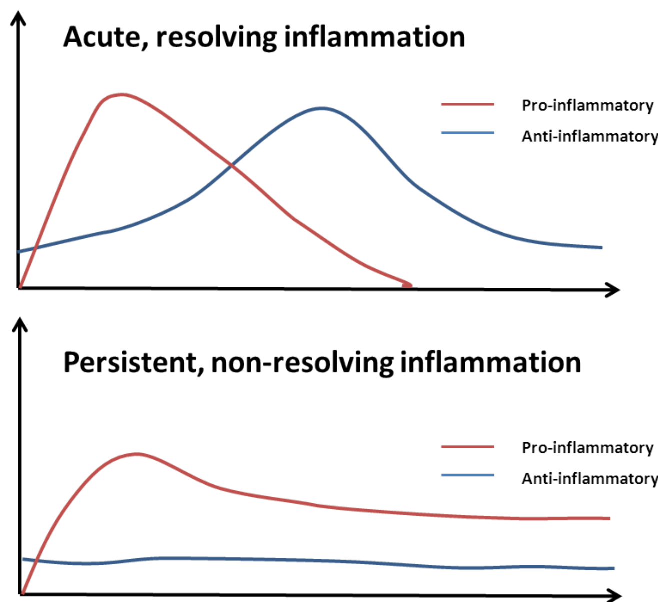

Figure 2.1. The course of acute and persistent inflammatory responses. The upper panel depicts the trajectory of a typical inflammatory response. An initial pro-inflammatory phase characterized by recruitment of neutrophils and production of cytokines such as IL-6 and TNFα is followed by resolution, with compensatory induction of anti-inflammatory cytokines (e.g. IL-10, TGFβ). Non-resolving and persistent inflammation is mild but unopposed by anti-inflammatory mechanisms.

A normal inflammatory response comprises an early, pro-inflammatory phase, in which microbicidal functions predominate, and a secondary, anti-inflammatory phase, where wound healing occurs and inflammatory cells leave the area of damage (19-21). An overview of the contrasting profiles of acute and persistent inflammation is presented in Figure 1. In chronic disease, the pro-inflammatory phase fails to resolve, leading to a persistent state of low-grade inflammation. This leads to changes in mucosal barriers and commensal bacteria that line the

gastrointestinal tract. As a result, these individuals tend to have slightly elevated levels of LPS (1-100 pg/mL) circulating in their blood (6, 7, 22-27). However, while inflammatory processes in response to high doses of LPS (> 10 ng/mL) have been intensively studied for their role in septic shock (28, 29), the immunological response to low doses of LPS is inconsistent with our current understanding of the molecular mechanisms governing sepsis. Typically, the activation of the Toll-like receptor-4 (TLR4) complex leads to the activation of nuclear factor κB (NFκB), which subsequently initiates the transcription of genes encoding inflammatory cytokines (30-35). These inflammatory cytokines are responsible for the recruitment of neutrophils, NK cells and antigen-presenting cells (APCs) to the site of the infection (30). Once the infection is cleared, other cytokines such as IL-10 and TGFβ combined with apoptosis of pro-inflammatory cells such as neutrophils lead to resolution of inflammation (30). Additionally, macrophages can enter a state of endotoxin tolerance wherein they suppress their expression of pro-inflammatory mediators to prevent excessive inflammation (36, 37). Suppression at multiple levels including the negative NFκB regulator IκBα, SOCS1, PI3K, MKP-1, and the inactivation of IRAK-1 helps to prevent inflammation in the absence of danger signals (38-40).

Much of the current work regarding the effects and mechanisms of exposure to high and low doses of LPS has been done in mice. Interestingly, exposure to low doses of LPS does not activate the classical NFκB pathway, but instead follows an alternative pathway that results in inhibition of the PI3K pathway, resulting in a persistent, low-grade inflammatory response that never reaches the threshold for negative feedback regulation (6-12, 41). For example, in type 2 diabetic mice it has been found that there are decreased numbers of endothelial progenitor cells (EPCs) and decreased expression and activity of manganese superoxide dismutase, leading to impaired wound healing in these mice (42). Additionally, guanylate binding protein-1 (GBP-1)

has similar effects in rheumatoid arthritis (RA). For example, expression of GBP-1 is higher in RA patients, and GBP-1 induces early differentiation of EPCs while inhibiting the proliferation and migration of EPCs *in vitro* in a rat model (43).

There are few published studies regarding the underlying molecular mechanisms of these effects, but it has been observed that low-dose LPS selectively counteracts repression of some transcription factors and activates C/EBPδ in an IRAK-1 dependent fashion (41). C/EBPδ itself does not efficiently induce pro-inflammatory mediation, but it can induce its own expression further, forming a positive feedback circuit. This enables low-level expression of pro-inflammatory mediators by macrophages and other innate immune cells. This "leaky" expression is allowed to persist by the LPS-effected absence of transcriptional repressors (41, 44, 45), leading to chronically elevated systemic levels of inflammatory cytokines.

2.2. Mechanisms of LPS recognition

LPS challenge is known to induce a refractory state in cells, whereupon subsequent challenge, even with a high dose of LPS, is characterized by less robust induction of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines, a state known as endotoxin tolerance (46-48). The duration of exposure has also been implicated in different immune responses (49). Pretreatment with a very low dose of endotoxin (in the pg/mL range), in contrast, has an opposite effect, potentiating or "priming" the pro-inflammatory response to subsequent endotoxin challenge. This phenomenon is referred to as the Shwartzman-like reaction (50). We and others have documented the priming response to very low-dose LPS *in vitro*, where it results in augmented expression of pro-inflammatory cytokines such as IL-6 and TNFα, and *in vivo*, where mice pretreated with super-low-dose LPS exhibit increased mortality in response to

challenge with a higher dose (51, 52). Endotoxin priming and tolerance have both been well documented, though the molecular mechanisms governing the decision between one response and the other have not been well defined. Regardless, the "decision" must be made at the time of the primary challenge: because the secondary stimulus can be delivered at precisely the same dosage and for the same duration, the differences in the response cannot originate with the secondary challenge. The difference between priming and tolerance must therefore be in the response to the preparatory dose, and it is here that a detailed examination of the dynamics of the macrophage response to LPS would be most fruitful.

The first events in the immune response to LPS occur outside of the cell. LPS must first come into contact with the LPS-binding protein (LBP). The LPS-LBP complex can then be recognized by TLR4, acting in conjunction with MD-2 and CD14 (53). Once this recognition has occurred, the TLR4 signaling cascade can commence.

Upon ligation of TLR4 and its co-receptors by the LPS-LBP complex, signaling can proceed through either a MyD88-dependent or MyD88-independent pathway. The intracellular portion of TLR4 contains a Toll/IL-1R homology (TIR) domain, by which it is enabled to interact with a family of related proteins and adaptor molecules, most prominently MyD88 and TIR-domain-containing adaptor protein inducing interferon-β (TRIF) (54). TLR4 is unique for its ability to signal through both MyD88 and TRIF, as the other TLRs are limited exclusively to either MyD88-dependent or TRIF-dependent signaling (55). Recruitment of MyD88 to TLR4 is followed by a signaling cascade involving the interleukin-1-receptor-associated kinases, or IRAKs. There are currently four known IRAKs, among which IRAK-1, -2, and -4 play positive roles in signal transduction, while IRAK-M (also known as IRAK-3) acts to suppress TLR signaling (56). The MyD88-dependent pathway of TLR signaling culminates in the activation of

MAPK and NFκB, with subsequent induction of pro-inflammatory genes (57). Figure 2 presents an overview of prominent mediators of TLR4 signaling.

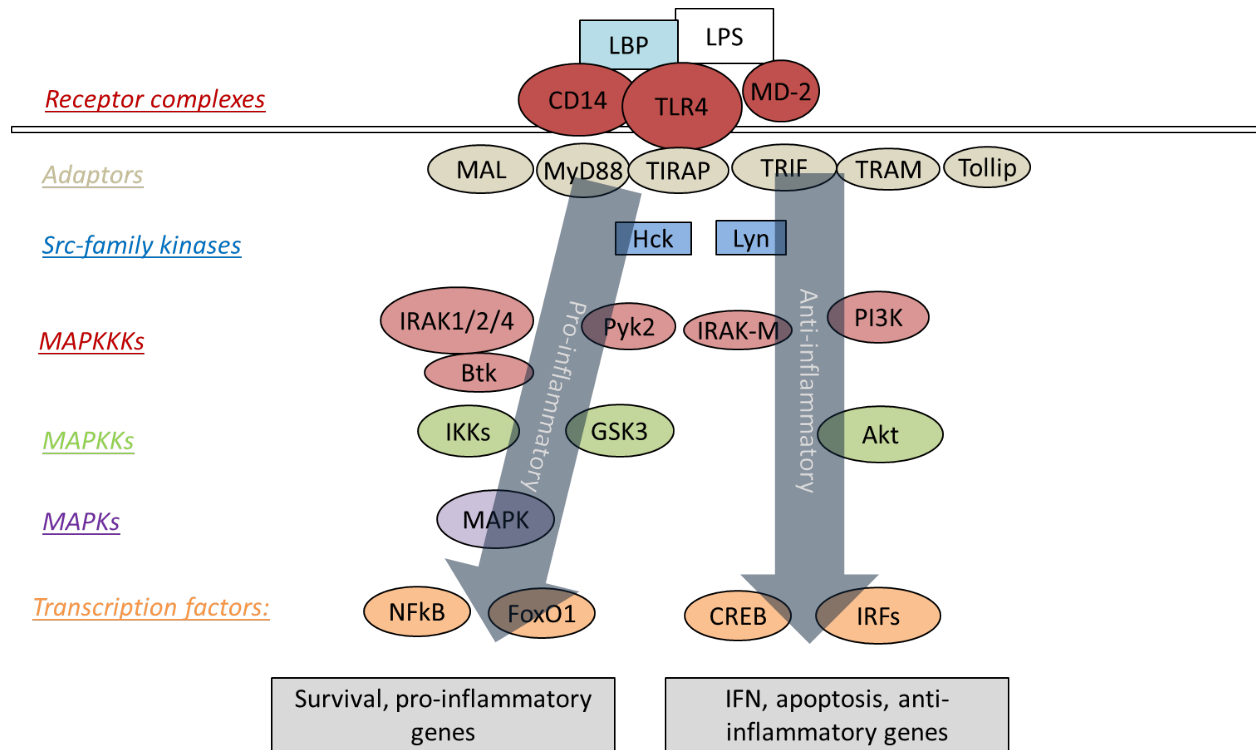

Figure 2.2. Overview of signaling cascades engaged by TLR4. LPS triggers recruitment of MyD88 to the cytoplasmic domain of membrane-bound TLR4, resulting in predominantly pro-inflammatory gene expression. LPS binding leads to endocytosis of TLR4, and intracellular TLR4 signals through TRIF to promote IFN and anti-inflammatory gene expression.

Signaling through TRIF usually requires endocytosis of TLR4 (58). This endocytosis in turn requires CD14, as interference with CD14-TLR4-LBP interactions prevents effective TLR4 internalization (59, 60). Interestingly, Watanabe et al. demonstrated that TRIF-dependent signaling can be activated in the absence of CD14 (61), but this required the direct delivery of LPS to the interior of the cell, suggesting even more strongly that TRIF-dependent signaling requires signaling by TLR4 within the confines of the cytoplasmic membrane. TRIF and MyD88 are closely related, with a single amino acid mutation in TLR3 sufficing to induce signaling through MyD88, rather than TRIF (62). Once recruited, TRIF in turn recruits interferon

regulatory factor (IRF) 3, and signaling proceeds through phosphoinositide-3-kinase (PI3K), ultimately resulting in the activation of interferon-β (IFNβ) and related genes (46). LPS preconditioning has TRIF-dependent protective effects with respect to the ischemic injury associated with stroke (63), and deletion of TRIF exacerbates allergic dermatitis in mice (64). Taken together, these results point to a broadly anti-inflammatory role for TRIF-dependent signaling in the context of TLR4 activation.

2.3. Regulation of LPS response

Currently, the MyD88-dependent and TRIF-dependent pathways are treated in isolation, with little attention paid to the possibility of cross-talk between them. There are intriguing indications that such cross-talk does occur. IRAK-1, a pivotal actor in MyD88-dependent signaling, plays a suppressive role in TRIF-dependent TLR3 signaling (65), and MyD88 is important for the suppression of TRIF-mediated apoptosis (66). The Src-family tyrosine kinases (SFK) also play a role in the differential regulation of pro- and anti-inflammatory effects downstream of TLR stimulation (67), including direct involvement in MyD88-dependent activation of NFκB (68). The phenomena of endotoxin priming and tolerance indicate that the pro- and anti-inflammatory responses to TLR4 stimulation are not wholly independent; rather, the activation of one must affect the other somehow. This cross-talk therefore bears closer investigation.

The major mediators of cross-talk between MyD88- and TRIF-dependent TLR4 signaling appear to be the SFKs and phosphatidylinositol-3-kinase (PI3K), which act at different “layers” downstream of the receptors to integrate signals from the different pathways. The SFKs are engaged by tumor-necrosis-factor-receptor associated factor 6 (TRAF6) (69) and activated

within minutes of LPS stimulation, along with the Syk kinase Pyk2 and the Tec-family kinase Btk (70). The SFK Lyn has been studied chiefly in B cells, due to the spontaneous appearance of a lupus-like B-cell mediated autoimmune disease in Lyn-deficient mice (71). Lyn activates PI3K through B cell adaptor for PI3K (BCAP) in B cells (72), but dendritic-cell (DC)-specific deletion of Lyn still causes hyperactive MyD88 signaling and B cell-mediated autoimmunity (73), pointing to a role for Lyn in myeloid cells. Knockout of MyD88 either globally or conditionally in B cells or dendritic cells counteracts the autoimmune symptoms characteristic of Lyn deficiency (74, 75). BCAP itself is active in myeloid cells as well (76), indicating that the network is not limited to lymphoid cells. In mast cells, Lyn is necessary for TLR4-dependent NFκB and MAPK activation (77), and further contributes to the activation of Bruton's tyrosine kinase (Btk) (78), a TIR-domain-containing molecule which promotes LPS-induced NFκB activation in macrophages (79). Btk also drives MAPK-dependent TNFα production in response to TLR2 and TLR4 stimulation of myeloid cells (80). While the role of Lyn in Btk activation points to pro-inflammatory effects in the context of TLR4 stimulation, it appears that overall, Lyn is a suppressor of pro-inflammatory signaling, enabling effective activation of PI3K and ensuing anti-inflammatory signaling in multiple different cell types. The interactions uncovered by these studies are depicted in figure 3.

Inhibition of Pyk2 ameliorates the symptoms of LPS-induced lung injury (81), and it also promotes MyD88-dependent signaling and NFκB activation (68). Together, these findings point toward a generally pro-inflammatory role for Pyk2, but the discovery that PI3K inhibitors suppress Pyk2 activity (82) indicates that Pyk2 is involved in both pathways. Downstream of PI3K, Pyk2 may act in part to modulate the inflammatory suppression driven by PI3K/Akt signaling.

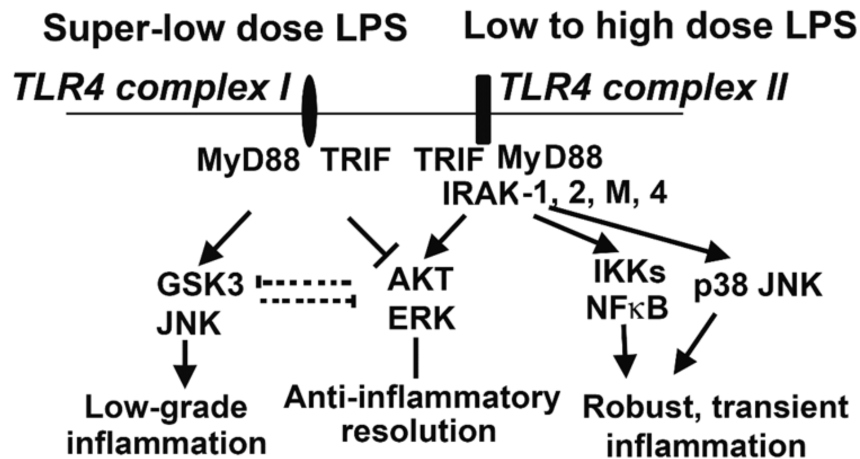

Figure 2.3. Competitive molecular circuits potentially responsible for the dynamic programming of innate leukocytes. Varying doses LPS may engage unique TLR4 complexes that may cause intra-cellular pathway switching, leading to either low-grade non-resolving inflammation, or acute resolving inflammation. Super-low dose LPS selectively activates GSK3 and JNK, while suppressing AKT and ERK. These two branches may also have mutually inhibitory interactions, further fine-tuning cellular inflammatory states. Low to high dose LPS potently induces NFκB and MAP kinases, leading to robust inflammatory reactions. On the other hand, low to high dose LPS could also trigger the activation of AKT and ERK, which may serve to dampen inflammatory responses. IRAK-1 appears to be critical for both the low-grade inflammation triggered by super-low dose LPS and the anti-inflammatory responses (e.g. IL-10 expression through ERK activation) activated by high dose LPS.

The need for further study of the regulation of pro- and anti-inflammatory responses to TLR4 stimulation is clear. Since the “switch” between endotoxin priming and tolerance appears to depend on the dosage of the first challenge, the dynamics of that response must be investigated, with the aim of determining what conditions lead to the activation of one pathway or another. The network motifs active here are key to the understanding of the LPS response. There exists a role for epigenetics in the broad reprogramming of macrophages and endotoxin tolerance (83-85), but the response to single dosages over a matter of hours is likely to be regulated by faster dynamic molecular mechanisms, as discussed below in further detail.

2.4. Competitive circuitry governing pro- and anti-inflammatory responses to LPS

A growing body of literature suggests that a competitive network may be responsible for the decision between a predominantly pro- or anti-inflammatory response to LPS, with PI3K, Akt, and the cAMP-response-element-binding-protein (CREB) acting in opposition to glycogen synthase kinase 3 (GSK3) and forkhead box O1 (FoxO1). BCAP, as mentioned above, is crucial for TLR-dependent PI3K activation in myeloid cells and the ensuing suppression of inflammation (76). PI3K dampens NFκB activation by means of phosphoinositide-dependent kinase-1 (PDK-1), which suppresses TRAF-6 activity and is necessary for the LPS-induced activation of Akt and ERK (40). PI3K also activates Akt in response to mTOR, competing with MAPK/p38/JNK signaling (86). Inhibition of PI3K leads to increased production of IL-6 and TNFα in response to TLR2 stimulation of macrophages (39), and activation of PI3K results in deactivation of FoxO1, preventing it from promoting TLR4 signaling (87). Overall, PI3K is important for negative feedback and control of TLR signaling, acting to counteract both NFκB and MAPK, two of the main transcription factors responsible for pro-inflammatory gene transcription in response to LPS.

Akt exerts its anti-inflammatory effects through NFκB and MAPK signaling, as well as its activation of CREB. In non-canonical NFκB signaling, the processing of p100 to p52 requires Akt, paving the way for increased activity of RelB (88), a suppressive NFκB family member. Activation of Akt through the mTOR-PI3K pathway both counteracts MAPK signaling and activates CREB (89), at the same time directly inactivating FoxO1 (86). Quercetin treatment activates Akt in multiple cell types, leading to decreased activity of FoxO1 in pancreatic islets (90), and ameliorating the inflammatory response of adipocytes to TNFα (91). Akt activation has also been shown to correlate with suppression of FoxO1 in HEK293 cells (92). Inhibition of

JAK3 leads to decreased activity of both Akt and CREB, and this loss of activity correlates with an augmented pro-inflammatory response to LPS (93). The role of Akt, then, seems to be to mediate the anti-inflammatory effects of PI3K, in large part by suppressing FoxO1 and activating CREB.

GSK3 has been implicated in many inflammatory signaling pathways. It has been identified as a suppressor of CREB based on its responsibility for the downregulation of genes with CREB binding sites (94). Inhibition of GSK3 increases IL-10 production and decreases IL-12 in response to LPS in monocytes (95), which, in light of the importance of IL-10 to the anti-inflammatory effects of PI3K (96), points strongly to GSK3 as an actor in opposition to PI3K. GSK3 suppresses IFNβ induction by LPS (97), indicating a suppressive effect on TRIF-dependent signaling. GSK3 inactivates CREB directly (98, 99), and IFNγ activates GSK3 and suppresses CREB (100), indicating that the pro-inflammatory effects of IFNγ may be due in large part to its effects on this sub-network.

The opposing effects of PI3K activation and GSK3 activation have been described in multiple cell types. They have opposing effects on the LPS response in DC (96), and in H_2O_2-induced apoptosis in neurons (101). Inactivation of GSK3β is important in inflammatory resolution, and is associated with a blunted pro-inflammatory response to LPS (20). In macrophages, PI3K-Akt signaling directly opposes GSK3 activity during the LPS response, with GSK3α knockdown potentiating the effects of IL-10, while CREB knockdown reduces them. Furthermore, the pro-inflammatory effects of PI3K inhibition can be counteracted by treatment with IL-10 (102), another indicator that IL-10 is a downstream effector of PI3K. The PI3K-dependent increase in IL-10 production is due to its inactivation of GSK3 (97, 103), and direct activation of PI3K-Akt results in inhibitory phosphorylation of GSK3 (104). Knockout of the

mTOR signaling molecule rictor prevents Akt from inactivating GSK3 upon TLR4 stimulation, correlating with increased FoxO1 activity and pro-inflammatory gene expression (105). Taken together, these findings constitute a strong body of evidence that the anti-inflammatory PI3K/Akt/CREB signaling axis acts by suppressing GSK3/FoxO1, and that this competition is the lynchpin of the primarily pro- or anti-inflammatory characteristics of the dynamic LPS response (Figure 3). We recently reported that super-low-dose LPS selectively activates GSK3 and JNK, while suppressing Akt and ERK (106). This may explain the mild skewing of pro-inflammatory responses by low-grade endotoxemia in mice and humans. In contrast, high dose LPS can induce robust activation of all MAPKs, including p38, JNK, and ERK, as well as PI3K/Akt (106), which may lead to the robust yet transient resolving inflammation followed by anti-inflammatory tolerance associated with high-dose endotoxin challenge. With regard to upstream signaling networks, IRAK-1 is responsible for the effects of super-low-dose and high-dose LPS (Figure 3) (106, 107).

2.5. Conclusions and future directions

Remaining questions include the plasticity of this network, and the persistence of the programming for which it is responsible. Can the character of the LPS response be easily altered by pharmacological intervention aimed at activating or inhibiting different components of this signaling nexus? Once the character of the response has been established, will the system reset itself? Answers to these questions will significantly advance the understanding of TLR signaling in particular and the behavior of innate immune cells in general. Some efforts have been made to apply large-scale systematic methods to the study of this system (108, 109), but a great deal of work remains to be done, particularly with respect to the network herein described. There is a

growing appreciation for plasticity and memory in macrophages, with a movement away from strict classifications of macrophage populations along M1/M2 lines to more flexible schemes of classification based on dedication to a variety of different functions (110, 111). It is likely that further examination of this and other myeloid signaling networks will accelerate this. Innate immune "memory" is not a function of dedicated cell types as in lymphoid cells but rather a characteristic intrinsic to individual cells, whereby signals percolating through a network change its state in such a way as to influence its responses to subsequent stimuli. Such "memory" is therefore likely to be an important characteristic of many different cell types, particularly those responding to many different stimuli through interlocking networks of receptors and signaling cascades (neurons, in particular, come readily to mind). Innate immune cell populations may come to be seen as temporary workers, dedicated to their functions less strongly than has hitherto been supposed. Increasing appreciation for this plasticity will open broad new vistas for both the theoretical understanding of innate immunity and the treatment of associated diseases. Further studies aimed at the unique characteristics of innate memory and the underlying mechanisms are urgently needed.

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Chapter 3. Dynamic modulation of innate immune response by varying dosages of LPS in human monocytic cells

Abstract

Innate monocytes and macrophages can be dynamically programmed into distinct states depending upon the strength of external stimuli. Innate programming may bear significant relevance to the pathogenesis and resolution of human inflammatory diseases. However, systems analyses with regard to the dynamic programming of innate leukocytes are lacking. In this study, we focused on the dynamic responses of human promonocytic THP-1 cells to lipopolysaccharide (LPS). We observed that varying dosages of LPS differentially modulate the expression of selected pro- and anti- inflammatory mediators such as IL-6 and IL-33. Super-low dosages of LPS preferentially induced the pro-inflammatory mediator IL-6, while higher dosages of LPS induced both IL-6 and IL-33. Mechanistically, we demonstrated that super-low and high doses of LPS cause differential activation of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. Inhibition of GSK3 enabled THP-1 cells to express IL-33 when challenged with super-low dose LPS. On the other hand, activation of CREB with adenosine suppressed IL-6 expression. Taken together, our study reveals a dynamic modulation of monocytic cells in response to varying dosages of endotoxin, and may shed light on our understanding of the dynamic balance that controls pathogenesis and resolution of inflammatory diseases.

3.1 Introduction

Increasing evidence from both clinical and laboratory studies indicate that innate immune cells can be programmed into diverse states with varying degrees of pro- and anti-inflammatory phenotypes (1, 2), with consequences for host defense and inflammation (3). Despites its clinical

relevance, mechanistic studies with regard to innate cell programming are scant. To fill this critical void, we have examined the dynamic responses of human THP-1 monocytic cells challenged with a model stimulant, bacterial endotoxin lipopolysaccharide (LPS), a major component of the cell walls of Gram-negative bacteria. It is a ubiquitous environmental toxin (4, 5). High doses of LPS are responsible most prominently for septic shock (6, 7). On the other hand, low doses of circulating LPS are common in chronic disease settings, and may contribute to the development of persistent, low-grade, non-resolving inflammation (8-10). The distinct pathological effects of varying dosages of LPS may reflect differential programming of innate leukocytes.

At the biochemical level, LPS is recognized by the Toll-like receptor (TLR) 4. High dosages of LPS can activate multiple pathways capable of inducing both pro- and anti-inflammatory genes (11, 12). Of particular note, the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway induced by high-dose LPS serves as a negative mechanism to down-regulate inflammatory processes, and is also responsible for the expression of anti-inflammatory mediators, partly through the activation of CREB (13). In contrast, super-low dose LPS fails to induce anti-inflammatory mediators, and preferentially induces low-grade inflammatory mediators (14). Studies from other groups in other cellular systems indicate that GSK3 and Akt may form a mutually inhibitory circuit (12, 15). Akt was shown to inhibit the function of GSK3 (16), while GSK3 may in turn inhibit Akt.

Other studies also suggest that GSK3 is a critical molecule involved in various inflammatory processes *in vitro* and *in vivo*. Inhibition, knockdown, or knockout of GSK3 has been shown to inhibit the expression of pro-inflammatory mediators in response to LPS (17, 18). Inhibitory phosphorylation of GSK3 mediated by PI3K/Akt is necessary for the protective

induction of anti-inflammatory IL-10 following ischemia/reperfusion injury (19), which triggers inflammation through TLR4. Pharmacological activation of PI3K also suppresses toxicity-induced apoptosis in neurons in a GSK3-dependent fashion (20). These findings suggest that the overall role of GSK3 in the LPS response consists of the promotion of pro-inflammatory cytokine production and suppression of anti-inflammatory mediators (21, 22).

Downstream, GSK3 appears to play a role in the regulation of the transcription factors forkhead box O1 (FoxO1) and cAMP response-element-binding protein (CREB). The anti-inflammatory effects of Akt on TLR4 signaling appear to be mediated through FoxO1 (23), suggesting that GSK3 may be important for the activation of FoxO1. GSK3 can potently suppress CREB (24). Recently, it was shown that the anti-inflammatory effects of JAK3 in the context of TLR4 stimulation are exerted by suppressing GSK3, which enables increased CREB activity (13). In cells stimulated by the TLR2 ligand zymosan, the activation status of CREB corresponds closely with heightened production of IL-10 (25). Suppression of CREB by GSK3 may result in the pro-inflammatory skewing of immune responses. Circumstantially, genes suppressed by GSK3 tend to be regulated by CREB (26), and the suppression of IL-10 production by interferon-γ is due to its activation of GSK3 and ensuing suppression of CREB (15). CREB seems to oppose the pro-inflammatory effects of GSK3 by augmenting anti-inflammatory gene expression.

Akt (also known as protein kinase B) is regulated by the PI3K and mammalian target of rapamycin (mTOR) pathways (12, 27). Activation of Akt by rapamycin blunts the sensitivity of pro-inflammatory genes to LPS and increases CREB activity (28), and mTOR/Akt signaling additionally suppresses inflammation by inactivating the transcription factor forkhead box O1 (FoxO1) (23). The role of FoxO1 in TLR4 signaling is predominantly pro-inflammatory.

Overexpression of FoxO1 results in increased expression of TLR4 and pro-inflammatory cytokine genes in response to LPS, and knockdown or removal blunts the ability of TLR4 stimulation to induce these genes (29). FoxO1 is also necessary for pro-inflammatory cytokine production by memory T cells (30), pointing to a generalized role for FoxO1 in the regulation of inflammatory gene transcription. Blocking of inhibitory phosphorylation of FoxO1 results in increased pro-inflammatory cytokine production by macrophages upon challenge with a TLR2 ligand (31). Defective Akt activation results in increased FoxO1 activity and pro-inflammatory cytokine production (23). The broadly anti-inflammatory effects of PI3K signaling in TLR4 stimulation (32) can thus be ascribed to the tandem activation of CREB and suppression of FoxO1 by Akt, but the competition between GSK3 and Akt has not been well characterized in the context of LPS challenge.

In this study, we tested the hypothesis that monocytes may be dynamically programmed by varying dosages of LPS through the competing circuits of GSK3 and Akt. To test this, we examined the expression profiles of selected pro- and anti- inflammatory mediators in human monocytic THP-1 cells challenged with varying dosages of LPS. We observed distinct expression patterns of IL-6 and IL-33 in THP-1 cells treated with super-low or high dose LPS, and this pattern correlated with distinct activation statuses of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. We further demonstrated that pharmacological intervention by either GSK3 inhibition or CREB activation is sufficient to reverse the pro-inflammatory skewing characteristic of cells stimulated by super-low-dose LPS. Our study reveals a unique network responsible for the dynamic programming of innate leukocytes by varying dosages of bacterial endotoxin.

3.2 Materials and Methods

3.2.1. Cell Culture and Reagents

THP-1 cells were grown in RPMI-1640 (Life Technologies 11875-119) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies 15140-122), and 1% fetal bovine serum (FBS) (Seradigm 1600-500). Before performing experiments, cells were seeded overnight at a density of 10^6 cells/mL in RPMI containing 2 mM L-glutamine, 1% penicillin/streptomycin, and 1% FBS. LPS from *E. coli* O114:B4 (Sigma L2630) was dissolved in PBS. The GSK3 inhibitors SB216763 (Sigma S3442) and indirubin-3'-oxime (Sigma I0404) were dissolved in DMSO. Adenosine (Sigma A4036) was prepared in ddH_2O.

3.2.2. Real-time PCR

RNA was extracted using TRIzol (Life Technologies 15596-026). Samples were then treated with 20% chloroform by volume at room temperature for 3 minutes, followed by centrifugation at 12,000 RPM for 15 minutes at 4°C. Aqueous phase was transferred to new tubes and isopropanol (50% initial volume) was added, then samples were incubated for 10 minutes at room temperature. Samples were then centrifuged at 12,000 RPM for 10 minutes at 4°C and the supernatant was discarded. The RNA pellet was then washed with 75% ethanol in DEPC-treated water (100% initial volume), and centrifuged for an additional 10 minutes at 12,000 RPM, 4°C. Supernatant was discarded and RNA pellets were resuspended in 30 µL DEPC-treated water. DNA digestion was performed at 37°C for 30 minutes (Life Technologies AM2222), followed by 5 minutes treatment with 85°C to degrade DNase. 1500 ng RNA was reverse-transcribed using a high-capacity kit (Life Technologies 4368813) at 37°C for 2 hours, followed by enzyme inactivation for 5 minutes at 85°C. Real-time PCR was performed on a BioRad CFX96 machine using a 2x SYBR Green mix (Bio-Rad 172-5271); the PCR protocol was denaturation at 95°C

for 3 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Primers for human IL-6 (F 5'-AGCCACTCACCTCTTCAGAACGAA, R 5'-AGTGCCTCTTTGCTGCTTTCACAC), TNFα (F 5'-TCAATCGGCCCGACTATCTC, R 5'-CAGGGCAATGATCCCAAAGT), IL-10 (F 5'-TCCTTGCTGGAGGACTTTAAGGGT, R 5'-TGTCTGGGTCTTGGTTCTCAGCTT), and IL-33 (F 5'-GGAAGAACACAGCAAGCAAAGCCT, R 5'-TAAGGCCAGAGCGGAGCTTCATAA) were purchased from IDT. Readouts were analyzed by the ΔΔCQ method.

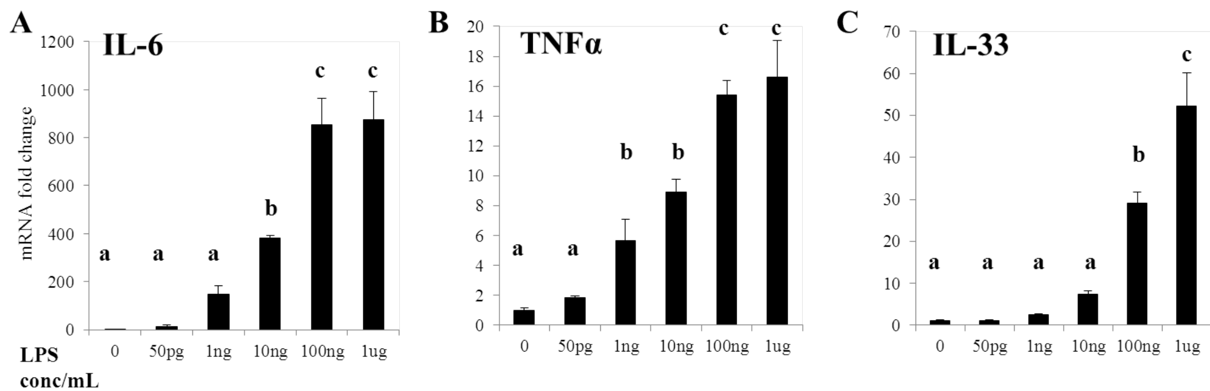

Figure 3.1. Relative induction of pro- and anti-inflammatory genes in THP-1 cells by 4 h stimulation with different concentrations of LPS. Total RNAs were harvested from THP-1 cells challenged as indicated. Real-time RT-PCR was performed to determine the expression levels of **A)** IL-6; **B)** TNFα; and **C)** IL-33. Different letters denote statistically significant differences between groups ($p < 0.05$, Holm-Sidak).

3.2.3. Western blots

Whole-cell lysates were harvested using lysis buffer consisting of 2% SDS, 5% Tris-HCl pH 6.8, and 10% glycerol, placed on ice for 20 minutes, boiled for 5 minutes, then centrifuged at 12,000 RPM for 4 minutes at room temperature for removal of intracellular debris. Protein concentration was assessed by Bradford assay. Protein samples were run on 10% acrylamide gels at 100 V, followed by transfer at 110 V before blocking in 5% milk in TBS-T. Antibodies against pCREB-S133 (Cell Signaling 9191) and pGSK3β-S9 (Cell Signaling 5558) were used at a concentration of 1:4000 in 5% milk in TBS-T, and CREB (Cell Signaling 9197S), pFoxO1-S256 (Cell

Signaling 9461S), FoxO1 (Cell Signaling 9454S), Akt (Santa Cruz sc-8312), GSK3β (Santa Cruz sc-9166), and GAPDH (Santa Cruz sc-25778) at 1:1000. pAkt-S473 (Cell Signaling 9271S) and pGSK3β-Y216 (Santa Cruz sc-135653) antibodies were diluted 1:4000 in 5% BSA in TBS-T.

3.2.4. *Statistics*

Statistical analysis by Holm-Sidak pairwise comparison or Student's *t* test was performed using SigmaPlot 11 software (SigmaPlot) as detailed in figure legends. Results were considered to be statistically significant at $p < 0.05$.

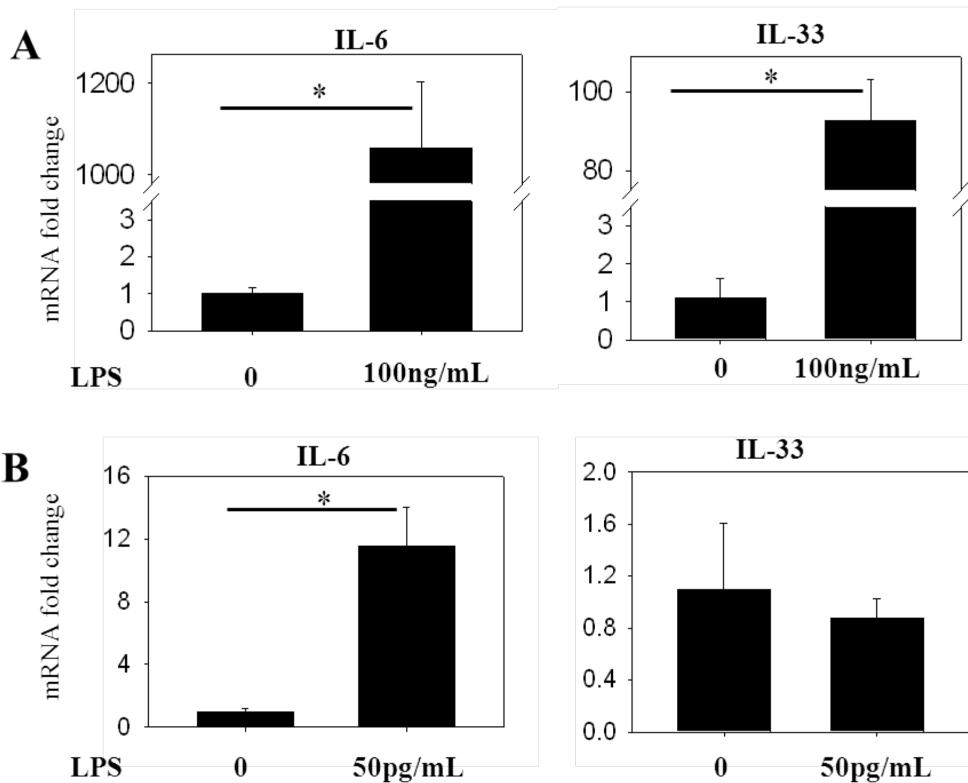

Figure 3.2. Relative induction of pro-and anti-inflammatory genes in human THP-1 cells by low- and high-dose LPS. Total RNA was isolated from THP-1 cells treated with either **A)** 100 ng/mL LPS or **B)** 50 pg/mL LPS for 4 h. Real-time RT-PCR was performed to determine the expression levels of both IL-6 and IL-33. Data were representative of three separate experiments (* $p < 0.05$, Student's *t* test).

3.3. Results

3.3.1 Differential regulation of inflammatory genes by LPS

The phenomena of endotoxin tolerance and priming, in which stimulation with LPS alters the nature of the inflammatory response to a subsequent challenge, have been extensively documented (33-36). We therefore sought to characterize the differences between the inflammatory response to super-low (<0.1 ng/mL) and high (>10 ng/mL) doses of LPS. Stimulation of THP-1 cells with varying dosages of LPS for 4 hours reveals that the pro-inflammatory genes IL-6 and tumor necrosis factor alpha (TNFα) are more sensitive to LPS than IL-33, as transcription of the latter is significantly upregulated only by high doses of LPS (Fig. 1). In a separate experiment, closer analysis employing different statistical methods reveals that IL-6 transcription is mildly but significantly upregulated by LPS doses as low as 50 pg/mL, while IL-33 is not (Fig. 2), suggesting that stimulation with super-low-dose LPS results in skewing of the TLR4 response towards pro-inflammatory gene expression.

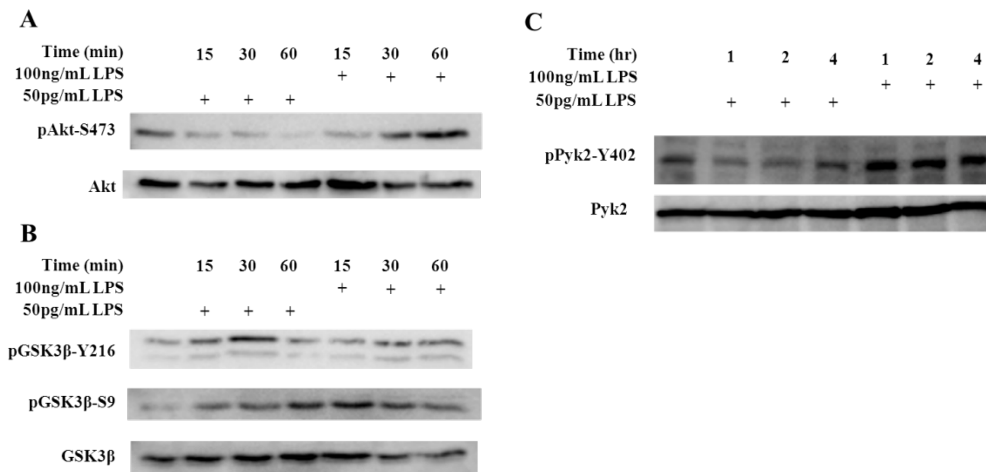

Figure 3.3. Differential regulation of GSK3 and Akt in THP-1 cells by LPS. THP-1 cells were treated with either 50 pg/mL or 100 ng/mL LPS for various time periods as indicated. Whole cell lysates were separated on SDS-PAGE. The levels of **A)** pAkt-S473 and total Akt; **B)** pGSK3β-Y216 and total GSK3β; and **C)** pPyk2-Y402 and total Pyk2 were determined by western blot with specific antibodies. Data were representative of three separate experiments.

3.3.2 Mechanistic effects of different LPS dosages

The signaling kinases GSK3 and Akt are known to compete in the regulation of inflammation (22, 37). This led us to explore whether the differential regulation of pro- and anti-inflammatory genes by varying dosages of LPS could be explained by differential effects on signal transduction through GSK3 and Akt. Stimulation of THP-1 cells with 100 ng/mL LPS for 60 minutes resulted in robust phosphorylation of Akt at S473, a marker of its activation (38), and inhibitory phosphorylation of GSK3β at S9. In contrast, super-low-dose LPS failed to increase, but rather decreased Akt-S473 phosphorylation. Correspondingly, we observed that super-low dose LPS failed to elevate GSK3β-S9 phosphorylation (Fig. 3A).

Our data suggest that GSK3 may be differentially modulated by super-low and high doses LPS. Given the fact that GSK3 activation is facilitated by tyrosine 216 phosphorylation, we further probed the pY216 GSK3 levels in cells treated with super-low dose LPS. As shown in Fig. 3B, 50 pg/mL LPS caused a rapid increase of pY216-GSK3 in THP-1 cells. Pyk2 is the upstream kinase responsible for the activating Y216 phosphorylation of GSK3 (39). We then tested the Pyk2 activation status through Western blot. The phosphorylation of Pyk2 at Y402 inhibits its tyrosine kinase activity. We found that 50 pg/mL LPS caused a rapid and dramatic drop in the levels of inhibitory Y402 phosphorylation of Pyk2, indicating an increase in its activity (Fig. 3C). In contrast, higher dose LPS (100 ng/mL) caused a robust elevation of Y402 phosphorylation, indicating its inhibition (Fig. 3C). This correlates with our above finding that super-low dose LPS activates GSK3 through increasing its Y216 phosphorylation.

Downstream of GSK3 and Akt lie two competing transcription factors: FoxO1 and CREB (13, 29). While FoxO1 contributes to the expression of pro-inflammatory mediators (23, 29), CREB is largely involved in the expression of anti-inflammatory mediators (25, 40). Pharmacological activation of Akt corresponds with suppression of FoxO1 (41), which, in light

of GSK3 suppression by Akt (26), points to a connection between GSK3 and FoxO1 activity. Akt is also important for the activation of CREB (13, 15).

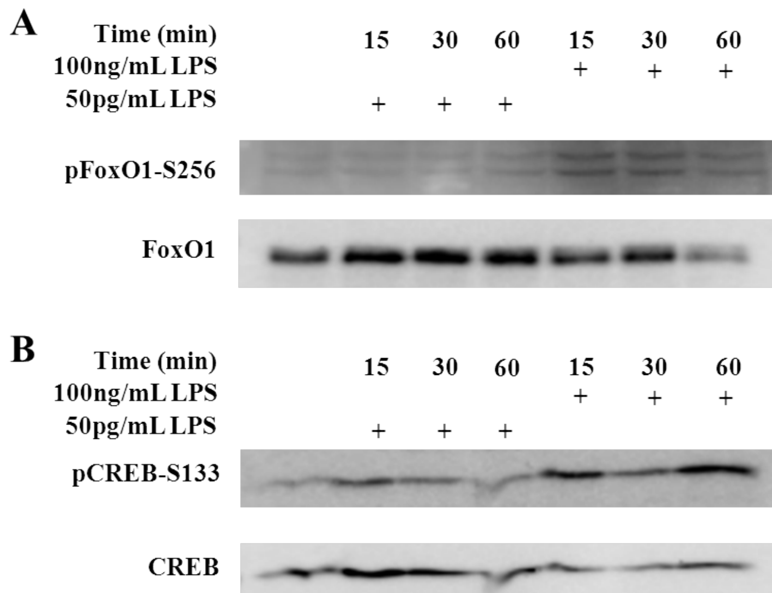

Figure 3.4. Differential regulation of FoxO1 and CREB by LPS. THP-1 cells were treated with either 50 pg/mL or 100 ng/mL LPS for various time periods as indicated. Whole cell lysates were separated on SDS-PAGE. The levels of **A)** pFoxO1 and total FoxO1; and **B)** pCREB-S133 and total CREB were determined by western blot with specific antibodies. Data were representative of three separate experiments.

Since we observed opposing effects of super-low and high dose LPS on GSK3 and Akt, we further tested the activation status of FoxO1 and CREB. We found that treatment with 50 pg/mL LPS caused an increase in total FoxO1 proteins levels, while treatment with 100 ng/mL LPS led to a decrease in the total FoxO1 level (Fig. 4A). Since the decrease in FoxO1 may be attributed to its inhibitory phosphorylation by Akt at S256 (42), we also tested the level of S256 phosphorylation of FoxO1. As shown in Fig 4, only the high dose LPS triggered FOXO1 phosphorylation at S256, while super-low dose LPS had no effect. The effects of LPS on the activation of CREB followed an opposite pattern. Phosphorylation of CREB at S133 results in its

activation (24), and this phosphorylation was only induced by high-dose LPS, with low-dose LPS not triggering appreciable S133 phosphorylation of CREB (Fig. 4B).

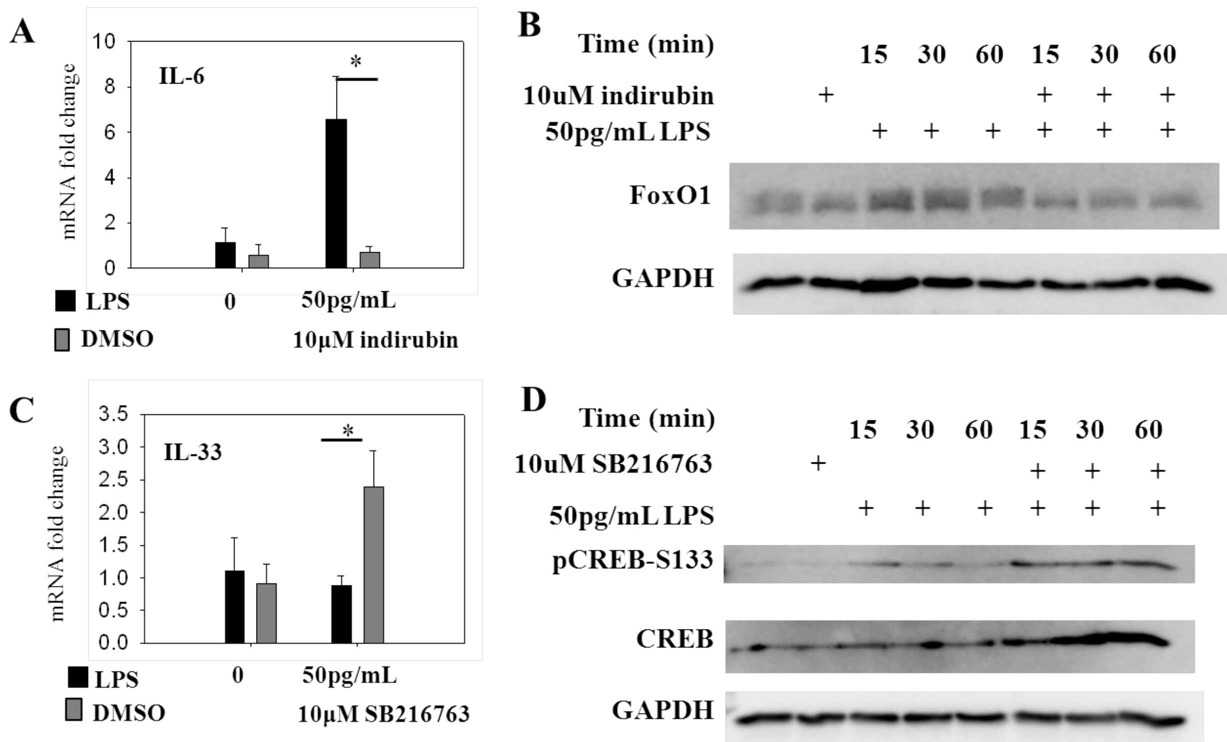

Figure 3.5. Effect of GSK3 inhibition on cellular responses to LPS stimulation. **A)** THP-1 cells were treated with the combination of 50 pg/mL LPS alone or together with 10 μM indirubin for 4 h. Total RNA was harvested and used for real-time RT-PCR analysis for IL-6. **B)** Total protein lysates were harvested from THP-1 cells treated as indicated, and used for western blot analyses for FoxO1 and GAPDH. **C)** THP-1 cells were treated with the combination of 50 pg/mL LPS alone or together with 10 μM SB216763 for 4 h. Total RNA was harvested and used for real-time RT-PCR analysis for IL-33. **D)** Total protein lysates were harvested from THP-1 cells treated as indicated, and used for western blot analyses for CREB and GAPDH. Data were representative of three separate experiments (*, $p < 0.05$, Student's *t* test).

3.3.4. Inhibition of GSK3 reverses pro-inflammatory skewing

In vivo studies indicate that GSK3 is a key kinase controlling chronic inflammation (12, 13, 43). Given our above findings, we tested whether selective inhibition of GSK3 may alter the inflammatory skewing of innate monocytes by super-low dose LPS. We first tested indirubin, a GSK3 inhibitor derived from natural compounds (18), and found that costimulation of THP-1 cells with 50 pg/mL LPS and 10 μM indirubin abolished the preferential IL-6 induction typically triggered by this dosage (Fig. 5A). Mechanistically, we observed that the suppression of IL-6 by

indirubin corresponds with prevention of FoxO1 upregulation (Fig. 5B). We observed that SB216763, a synthetic compound which potently and selectively inhibits GSK3 (44), sensitized IL-33 to super-low dose LPS, resulting in significant IL-33 induction by 50 pg/mL LPS (Fig. 5C). The effect of SB216763 on IL-33 correlated with increased CREB activation (Fig. 5D).

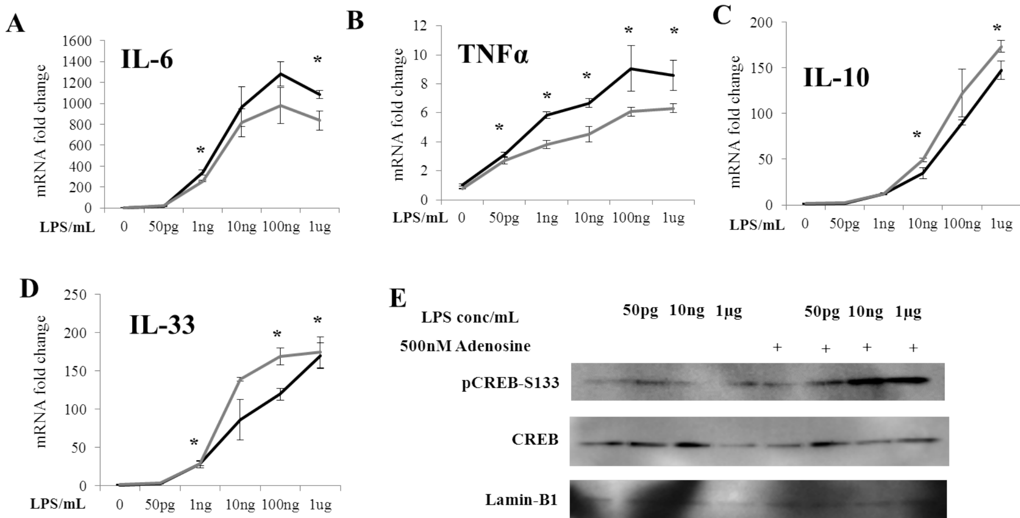

Figure 3.6. Activation of CREB suppresses pro-inflammatory gene induction and promotes anti-inflammatory gene induction by LPS in THP-1 cells. THP-1 cells were treated with various dosages of LPS with 500 nM adenosine for 4 h. The levels of **A)** IL-6; **B)** TNFα; **C)** IL-10; **D)** IL-33 mRNA were measured by real-time RT-PCR (*, $p < 0.05$, Student's *t* test). **D)** THP-1 cells were treated with various dosages of LPS with 500 nM adenosine for 2 h. Nuclear protein lysates were separated on SDS-PAGE and probed with specific antibodies against pCREB-S133, CREB or Lamin-B1. Data were representative of three separate experiments.

3.3.5. Activation of CREB reverses pro-inflammatory skewing

We hypothesized that the pro-inflammatory effects of GSK3 may owe to its inhibition of CREB. We tested this with adenosine, which exerts anti-inflammatory effects in macrophages by protein kinase A-mediated increase of CREB phosphorylation (45-47). Stimulation of THP-1 cells for 4 hours with a range of LPS concentrations in the presence of 500 nM adenosine significantly suppressed the transcription of IL-6 and TNFα while promoting the transcription of IL-10 and IL-33 (Fig. 6A-D). We further tested the levels of CREB in cell nuclei. Co-stimulation of cells with 50 pg/mL LPS and adenosine dramatically elevated the nuclear levels of pCREB-

S133 (Fig. 6E). Taken together, our data reveal a potential competitive circuit responsible for the dynamic sensing of varying dosages of endotoxin by THP-1 cells (Fig. 7).

3.4. Discussion

We investigated the pro-inflammatory skewing effects of super-low-dose LPS and found that the mild and preferential induction of pro-inflammatory genes by 50 pg/mL LPS. This phenomenon was correlated with preferential activation of GSK3 and inhibition of Akt. This was in contrast to the effect of high dose LPS, which induced both pro- and anti-inflammatory mediators. The proper regulation and resolution of inflammation requires activation of both pro- and anti-inflammatory mediators (48, 49), and the non-responsiveness of these resolving circuits to very low doses of LPS may be key to the pro-inflammatory skewing effects of these dosages, contributing to chronically dysregulated low-grade inflammation. By inhibiting GSK3, we were able to abolish the mild, preferential induction of pro-inflammatory IL-6 usually caused by super-low-dose LPS, and confer sensitivity to IL-33, which is not ordinarily induced by super-low dose LPS. The balanced expression of IL-6 and IL-33 could also be achieved by the addition of adenosine, an agonist capable of augmenting Akt and CREB. In the context of TLR4 activation, IL-6 and TNFα are both activated by FoxO1 (29), while CREB drives the transcription of IL-10 and IL-33 (15, 40). Our finding that adenosine potentiated the response of IL-10 and IL-33 to LPS while blunting induction of IL-6 and TNFα is therefore strongly suggestive of a competitive link between FoxO1 and CREB.

There is increasing appreciation for the role of GSK3 in inflammation (17, 43, 50). Inhibition of GSK3, in particular, is becoming an appealing strategy for the alleviation of inflammatory symptoms (50-52). Our finding that pharmacological inhibition of GSK3 reverses

the pro-inflammatory skewing effects of super-low-dose LPS is thus in line with recent developments in the field. The inhibitors we employed are not necessarily selective for the β or α isoform of GSK3 (44). In particular, indirubin may have inhibitory effects on other kinases such as cyclin-dependent kinases (18). These pleiotropic effects may explain the failure of indirubin to significantly alter the expression of IL-33 (data not shown). Likewise, SB216763 may have differential effects on various isoforms of GSK3, and this may explain its selective effects on IL-33 instead of IL-6. Further study is necessary to determine the precise contribution of the different GSK3 isoforms to the pro-inflammatory skewing of monocytes. The anti-inflammatory effects of GSK3 inhibition appear to be mediated through differential activation of FoxO1 and CREB. This relationship is further buttressed by our observation that CREB activation by adenosine recapitulates the suppression of pro-inflammatory skewing in monocytes brought about by GSK3 inhibition.

We posit that super-low-dose LPS preferentially activates pro-inflammatory genes through selective activation of GSK3, leading to heightened activation of FoxO1 at the expense of CREB (Fig. 7). Competitive inhibition between these kinases may be a compelling explanation for the phenomena herein described, as we were able to reverse it either by inhibiting GSK3 or activating Akt/CREB. This is consistent with previous observations that reveal an anti-inflammatory role of Akt in the context of TLR4 stimulation (12, 53).

We realize that further biochemical studies are needed to tease out the detailed mechanisms responsible for the competitive circuitry between GSK3 and Akt, as well as the consequences of this relationship. Missing links include membrane receptor combinations, distinct adaptor molecules (e.g. MyD88, TRIF, etc), and a myriad of kinases and phosphatases, as well as intracellular trafficking of signaling molecules responsible for the sensing of varying doses of

LPS. Nevertheless, this study is among the first to elucidate a fundamental principle and a key functional motif that may be responsible for the dynamic balance of pro- and anti-inflammatory responses in innate leukocytes.

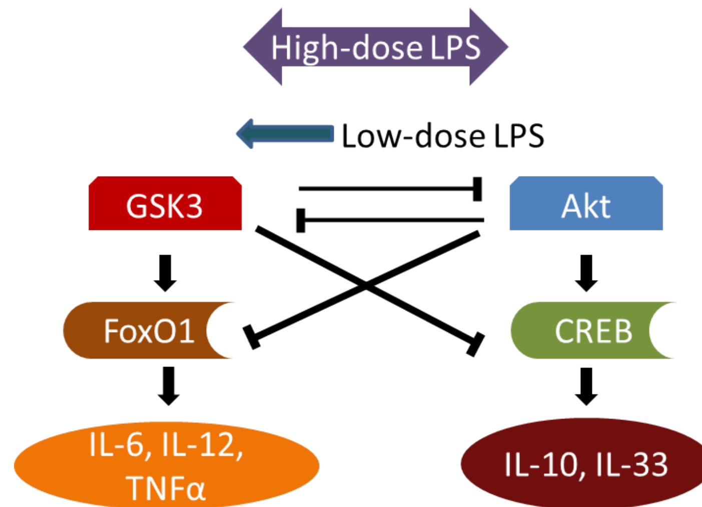

Figure 3.7. Proposed signaling network contributing to the pro-inflammatory skewing of innate immunity by super-low dose LPS. GSK3 and Akt are engaged in competitive inhibition while simultaneously driving the activity of downstream transcription factors, ultimately promoting the expression of pro- or anti-inflammatory genes. High-dose LPS robustly activates both arms of this response, while super-low dose LPS preferentially causes mild activation of GSK3.

3.5. References

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Chapter 4. Signaling networks responsible for the modulation of IL-12 in response to varying dosages of LPS in macrophages

Abstract

The innate immune response to lipopolysaccharide (LPS) has many possible outcomes depending on the dose, from acute endotoxic shock to broad immunomodulation. High doses (>10 ng/mL) evoke both pro- and anti-inflammatory mediators, while super-low doses (<0.1 ng/mL) result in preferential induction of pro-inflammatory genes. The mechanisms governing the switch between a preferentially pro-inflammatory or a balanced, resolving response are poorly understood. We show that in murine macrophages, the pro-inflammatory cytokine interleukin 12 (IL-12) is induced most robustly by intermediate LPS doses (1-10 ng/mL), while higher doses evoke a diminishing response. In the absence of the suppressive Toll-like receptor 4 signaling cascade members Lyn and IRAK-M, macrophages sustain robust IL-12 production in response to high doses of LPS. The transcription factor cAMP response element-binding protein (CREB) appears to play an important role in the regulation of the inflammatory response to LPS, with Lyn and IRAK-M deficient macrophages failing to activate CREB effectively in response to LPS. On the other hand, co-stimulation of these cells with LPS and the CREB agonist adenosine suppresses pro-inflammatory responses to LPS in wild-type cells, but does not robustly suppress IL-12 production in either Lyn- or IRAK-M-deficient cells. Our study reveals potential mechanisms for the dynamic modulation of innate responses by varying dosages of LPS.

4.1. Introduction

Inflammation is the first step in the natural response of the body to infection or injury. It broadly consists of an initial phase, in which damaged cells, pathogens, and debris are destroyed

and cleared away, and a resolving phase, in which anti-inflammatory mediators and cell types allow for the regeneration and restoration of damaged tissue (1). Unresolved inflammation may become persistent, and is implicated in a wide range of chronic conditions including atherosclerosis and diabetes (2-4).

Low-grade inflammation is more likely to become persistent, since the inflammatory signals involved tend not to cross the thresholds necessary to trigger resolution. We have shown that very low doses (<0.1 ng/mL) of lipopolysaccharide (LPS), which signals through the Toll-like receptor 4 (TLR4), can induce low-grade inflammation (5). The signaling pathways downstream of TLR4 are complex, with different adaptor proteins triggering divergent responses (6, 7). Prominently, cell-surface TLR4 preferentially activates pro-inflammatory signaling through MyD88 and NFκB, while CD14-dependent TLR4 endocytosis leads to signaling through TRIF, resulting in the activation of phosphoinositide-3-kinase (PI3K) and anti-inflammatory compensating mechanisms (8, 9). Most existing studies of the inflammatory response to LPS have been conducted with doses between 100-1000 ng/mL, leaving the specific response to stimulation by LPS at doses lower than 1 ng/mL largely unexplored.

PI3K appears to be an important means of resolving TLR-induced inflammation, playing a predominantly anti-inflammatory role in the LPS response (10, 11). Interference with signaling molecules upstream of PI3K such as Lyn or B-cell adaptor for PI3K (BCAP) inhibits PI3K activation upon challenge with inflammatory stimuli including LPS, leading to pro-inflammatory skewing of the immune reaction due to a lack of modulatory influence (12).

The Src-family kinase Lyn is implicated in TLR signaling and B cell function, but its precise role is unclear. $Lyn^{-/-}$ dendritic cells (DC) have heightened sensitivity to LPS, showing increased NFκB and MAPK activity upon LPS stimulation (13), and DC-specific knockout of

Lyn leads to B cell hyperactivity. B cells lacking Lyn show reduced Akt activity in response to CD19 stimulation (12). HEK293 cells transfected with TLR4 and MD-2 display decreased association between Lyn and TLR4 after tolerization with LPS (14), and Lyn knockdown in THP-1 cells increases TRIF-dependent signaling in response to cytomegalovirus infection (15). The autoimmunity associated with Lyn deficiency can be reversed by additional knockout of MyD88 (16, 17), suggesting that Lyn is important for the suppression of MyD88-dependent TLR4 signaling, and that it acts by engaging the PI3K/Akt pathway in response to LPS.

The interleukin-receptor associated kinase M (IRAK-M) is a well-known suppressive modulator of TLR4 signaling, dampening signal transduction through the MyD88-dependent pathway (18), although the precise mechanisms of its activity are unclear. It suppresses p38 MAPK activation by stabilizing MKP-1, and IRAK-M$^{-/-}$ cells show altered distribution of the suppressive NFκB family member RelB upon TLR stimulation (19). As such, it represents a promising target for study of the dynamics of the inflammatory response to LPS.

In this study, we tested the effects of varying LPS doses on the expression of IL-12 from primary murine macrophages harvested from wild type, IRAK-M deficient and Lyn deficient mice. We demonstrated differential expression profiles of IL-12 in macrophages challenged with varying LPS doses. Deletion of either Lyn or IRAK-M skews macrophages toward heightened expression of IL-12, but addition of adenosine can nudge the macrophages toward reduced expression of IL-12. The dynamic balance of IL-12 expression may be controlled by a molecular circuit that requires Lyn- and IRAK-M.

4.2. Materials and Methods

4.2.1. Cell culture

Wild type (WT) mice were purchased from the Charles River laboratory. IRAK-M-deficient mice were initially supplied by Dr. Richard Flavell of Yale. All animals were housed and bred in the animal care facility of Life Sciences I in accordance with protocols approved by the Virginia Tech IACUC. Lyn-deficient mouse bones were generously provided by the laboratory of Dr. Clifford Lowell from the University of California, San Francisco. Murine BMDM were cultured as described previously (5).

4.2.2. Western blots

BMDM were cultured overnight in DMEM containing 1% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine before performing experiments. Whole-cell lysates were harvested using lysis buffer consisting of 2% SDS, 5% Tris-HCl pH 6.8, and 10% glycerol, placed on ice for twenty minutes, boiled for five minutes, then centrifuged at 12,000 RPM for four minutes at room temperature for removal of intracellular debris. Protein concentration was assessed by Bradford assay. Nuclear lysates were obtained using a lysis buffer containing10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.05% NP40. Lysates were placed on ice for ten minutes, and then centrifuged at 3,000 RPM for ten minutes at four degrees C. The resulting pellet was then resuspended in 5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol by volume, and 300mM NaCl and vortexed before being placed on ice for thirty minutes, then centrifuged at 12,000 RPM for twenty minutes at four degrees C. The supernatant was recovered and concentration was determined by Bradford assay. Protein samples were run on 10% acrylamide gels at 100 V, followed by transfer at 110 V before blocking in 5% milk in TBS-T. Antibody against pCREB-S133 (Cell Signaling 9191) was used at a concentration of 1:4000 in 5% milk in TBS-T, and CREB (Cell Signaling 9197S), GAPDH

(Santa Cruz sc-25778), and lamin-B1 (AbCam ab16048) at 1:1000. Densitometric analysis was performed using Fujifilm MultiGauge v. 3.0.

4.2.3. Flow cytometry

Cells were starved overnight as described above, and then stimulated with different concentrations of LPS. After two hours, 3 μg/mL of brefeldin A (eBioscience 00-4506) was added, followed by 1 μg/mL ionomycin (Sigma I0634-1MG), 20 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma P8139-1MG), and additional brefeldin eighteen hours later. At twenty-four hours, cells were harvested and processed using a fixation and permeabilization kit (BD Biosciences 554715). Flow cytometry was performed using antibodies against IL-12 (BD Biosciences 554480) and analyzed on BD FacsDiva software.

4.2.4. Statistics

Statistical analysis by Holm-Sidak pairwise comparison or Student's *t* test was performed using SigmaPlot 11 software (SigmaPlot) as detailed in the figure legends. Results were considered to be statistically significant at $p < 0.05$.

4.3. Results

4.3.1. Dynamic modulation of IL-12 expression by increasing doses of LPS

IL-12 is a classic pro-inflammatory cytokine induced by stimulation of macrophages through TLR4 (8). Thus, we chose to investigate the effects of different LPS doses on the expression of IL-12 by murine BMDM. As shown in Figure 1, rising doses of LPS (from 100 pg/mL to 10 ng/mL) induced elevated expression of IL-12 in wild type bone marrow-derived macrophages (BMDM) as shown by flow cytometry. Intriguingly, as the LPS concentration rose above 10 ng/mL, the expression of IL-12 failed to increase further and was reduced instead.

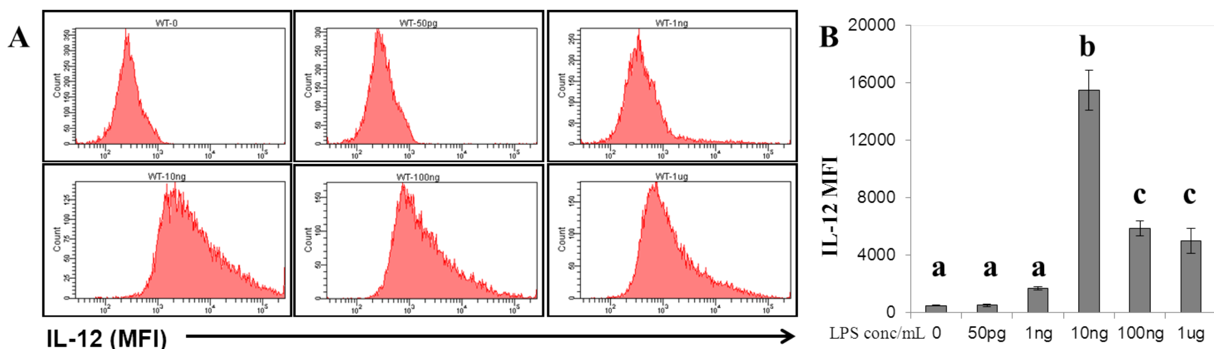

Figure 4.1. Effects of different LPS doses on IL-12 production by WT BMDM. Cells were stimulated for 24h with the indicated concentration of LPS, with addition of brefeldin at 2h and PMA and ionomycin at 20h, at the conclusion of which cytokine production was analyzed by flow cytometry. **A)** Representative MFI plots. **B)** IL-12 production by WT BMDM peaks at 10 ng/mL LPS, while higher doses induce less IL-12. Letters denote statistically significant differences between groups ($p < 0.05$, Holm-Sidak).

4.3.2. The biphasic expression pattern of IL-12 depends on the presence of negative regulators Lyn or IRAK-M

The biphasic responses of IL-12 expression to low and high doses LPS may be due to competing cellular circuits in macrophages. Previous studies suggest that both pro- and anti-inflammatory processes may be induced by the Toll-like-receptor pathways (20). We hypothesized that higher doses of LPS may be more efficient in engaging negative regulators of inflammation, and thus causing compensatory reduction of IL-12 expression. To test this

approach, we examined the involvement of two key negative regulators Lyn and IRAK-M. IRAK-M is a known negative regulator of TLR signaling (18), and Lyn has been implied to be a negative regulator proximal to TLR complex (13, 14).

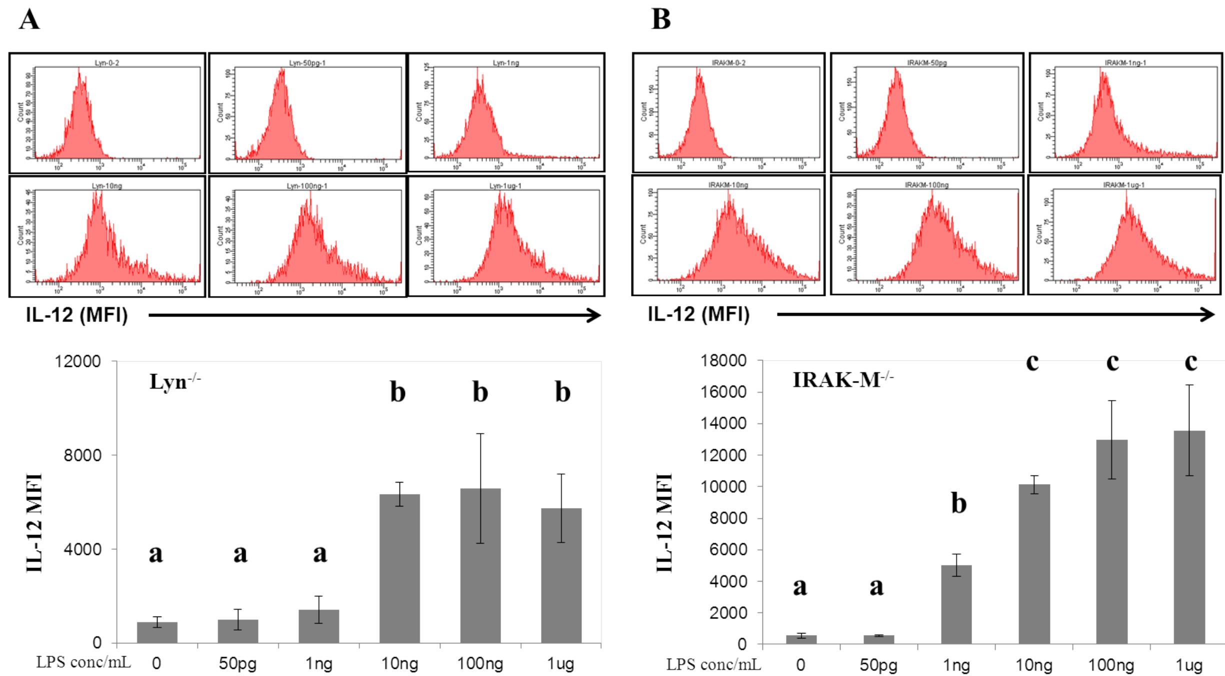

Figure 4.2. Effects of different LPS doses on IL-12 production by Lyn$^{-/-}$ and IRAK-M$^{-/-}$ BMDM. Cells were stimulated for 24h with the indicated concentration of LPS, with addition of brefeldin at 2h and PMA and ionomycin at 20h, at the conclusion of which cytokine production was analyzed by flow cytometry. Upper panel comprises representative dot plots, lower panel analyzes production of IL-12 by **A)** Lyn$^{-/-}$ and **B)** IRAKM$^{-/-}$ BMDM in response to varying dosages of LPS. Letters denote statistically significant differences between groups ($p < 0.05$, Holm-Sidak).

As shown in Fig 2A, the biphasic response pattern of IL-12 induction by LPS was not present in Lyn deficient macrophages. Instead, IL-12 expression levels steadily rose in Lyn deficient cells with increasing amount of LPS. Likewise, the expression levels of IL-12 were not ablated with rising LPS concentrations (Fig 2B).

4.3.3. CREB activity corresponds with reduced IL-12 production in BMDM

The potential molecular mechanisms for the compensatory suppression of inflammatory mediators may include the transcriptional modulator CREB, which promotes the production of

IL-10 (21) and has also been linked to the suppression of IL-12 (22). CREB is activated by phosphorylation at Serine 133, which results in increased transcription of anti-inflammatory genes and skews macrophages towards an alternatively-activated phenotype (22-24). In particular, Lyn has been shown to play a role in CREB activation through ERK in the context of brain ischemia (25). In light of this, we hypothesized that higher doses LPS may induce CREB phosphorylation through Lyn or IRAK-M, and investigated the phosphorylation status of CREB in cells treated with varying doses LPS. As shown in Fig 3A, only higher doses LPS induced robust phosphorylation of CREB. In contrast, the levels of CREB phosphorylation were reduced in Lyn deficient and IRAK-M deficient macrophages, particularly with respect to nuclear translocation of activated CREB (Fig 3B).

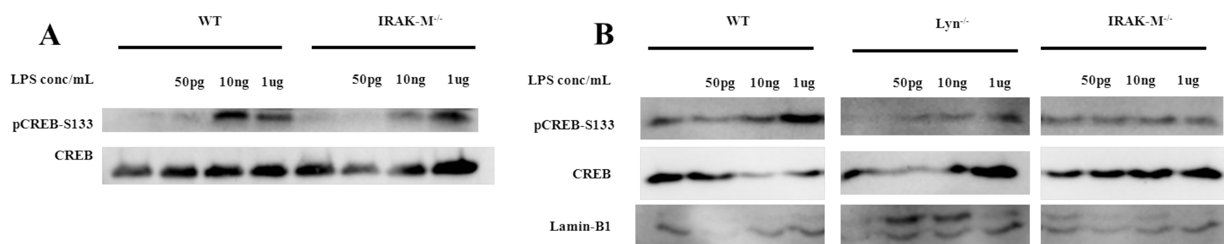

Figure 4.3. Effects of Lyn and IRAK-M knockout on CREB activation in response to LPS. WT, Lyn$^{-/-}$, and IRAK-M$^{-/-}$ BMDM were stimulated for two hours with the indicated concentration of LPS. **A)** Whole cell lysate of IRAKM$^{-/-}$ BMDM shows diminished activative phosphorylation of CREB in response to high concentrations of LPS. **B**) Nuclear levels of active CREB are increased by 1ug/mL LPS in WT BMDM. Lyn$^{-/-}$ BMDM have low levels of nuclear pCREB-S133 throughout, responding only slightly to high concentrations of LPS, and nuclear pCREB-S133 in IRAK-M$^{-/-}$ BMDM is unresponsive to LPS at any concentration. Lamin is included as a loading control.

4.3.4. Reversal of pro-inflammatory phenotype by adenosine

To further test that CREB phosphorylation may be associated with the compensatory inhibition of IL-12 expression, we treated cells with adenosine, an established CREB agonist with anti-inflammatory effects in neutrophils (26, 27). We observed that co-stimulation of BMDM with 500 nM adenosine and varying dosages of LPS suppressed the expression of IL-12

in wild type BMDM (Fig. 4A). As expected, adenosine failed to exert its inhibitory effect in IL-12 expression in either Lyn or IRAK-M deficient cells (Fig. 4B, C).

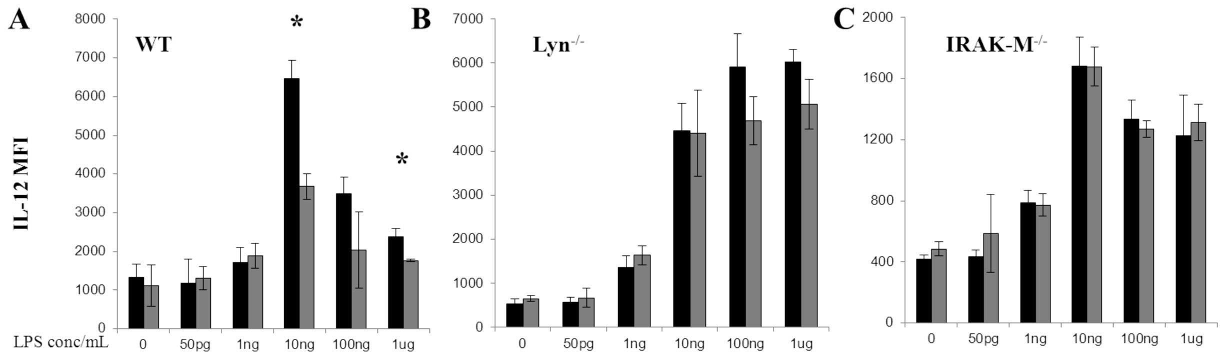

Figure 4.4. Effects of CREB activation on IL-12 response to LPS stimulation of WT BMDM. Cells were stimulated for 24h with the indicated concentration of LPS +/- 500nM adenosine, with addition of brefeldin at 2h and PMA and ionomycin at 20h, at the conclusion of which cytokine production was analyzed by flow cytometry. **A-C)** Production of IL-12 by **A)** WT, **B)** Lyn$^{-/-}$, and **C)** IRAK-M$^{-/-}$ BMDM in response to LPS and adenosine (Black = LPS alone, grey = LPS+500nM adenosine, * $p < 0.05$, Student's *t* test).

4.4. Discussion

The patterns of IL-12 production in response to LPS stimulation of macrophages show complex dynamics. The phenomena of LPS priming and tolerance are defined as different responses to secondary LPS challenge after a primary stimulation (11), but it is clear that even the primary response can vary widely depending on the LPS dosage. In a typical dose-dependent response, production of a cytokine or activity of a gene increases or decreases in a manner correlating with the concentration of the stimulant. However, the IL-12 response to LPS is more complex. Rather than a simple increase or plateauing of IL-12 production, the patterns of the LPS response in WT cells peak at an intermediate dose, while further increase in the stimulatory dose actually results in decreased IL-12 production. Removal of Lyn or IRAK-M changes the dynamics of this response, resulting in undiminished or continually increasing production of IL-12 as the stimulatory dose of LPS increases.

The dynamic modulation of IL-12 is critically important for host physiology during microbial infection. Robust induction of IL-12 is essential to mount an effective Th1 type immune defense (28, 29). On the other hand, down-regulation of IL-12 may serve as a compensatory mechanism to avoid excessive inflammation. Indeed, as shown in this report and other previous studies, immune macrophages dampen their IL-12 expression when challenged with higher dosages of endotoxin, as dysregulation of IL-12 suppression increases mortality of mice in the context of endotoxic shock (30).

Mechanistically, our study reveals that higher doses LPS may engage negative regulators such as IRAK-M and Lyn in the compensatory suppression of IL-12. This is consistent with previous findings that IRAK-M and Lyn may serve as negative regulators for the TLR signaling circuit (13, 19, 31, 32). Our work further extends this concept and reveals a differential engagement of these negative regulators by varying dosages of LPS.

The importance of CREB in the modulation of IL-12 is further buttressed by the observation that treatment of BMDM with adenosine consistently suppresses IL-12 expression in response to LPS, especially at higher LPS doses. In WT cells, co-stimulation with LPS and adenosine resulted in clear suppression of IL-12, and promoted the increased activation of CREB. Lyn- and IRAK-M-deficient BMDM did not respond in the same way to adenosine, retaining a heightened pro-inflammatory response to high-dose LPS and activating CREB only slightly if at all. The suppression of IL-12 in response to higher doses of LPS is therefore likely governed in part by the activation of CREB in a Lyn- and IRAK-M-dependent fashion. Taken together, our study shed some intriguing light on the dynamics of IL-12 expression in macrophages modulated by IRAK-M and Lyn-mediated CREB intervention.

It is not clear whether Lyn or IRAK-M work in concert or parallel to each other during the dynamic regulation of IL-12 expression. Based on previous studies and limited data from this report, it appears that both Lyn and IRAK-M may modulate IL-12 expression through increasing CREB phosphorylation. Lyn- and IRAK-M-deficient cells fail to up-regulate CREB phosphorylation when treated with higher doses LPS, correlating with sustained expression of IL-12 in these cells when challenged with higher doses of LPS. However, it is likely that Lyn and/or IRAK-M may also affect other critical molecules involved in the dynamic modulation of IL-12 in macrophages challenged with varying dosages of LPS. The potential involvement of CREB in the modulation of IL-12 is further supported by the observation that treatment of BMDM with adenosine consistently suppresses IL-12 expression in response to LPS, especially at higher LPS doses. Future studies are needed better to define the complex molecular circuits involved in the dynamic modulation of IL-12 in innate leukocytes by varying dosages of LPS. Taken together, our study shed some intriguing light on the involvement of IRAK-M and Lyn during the dynamic expression of IL-12 in macrophages.

4.5. References

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Chapter 5. Conclusions and Future Directions

We have presented evidence for a novel signaling network operating downstream of TLR4, responsible for the pro-inflammatory skewing associated with super-low-dose LPS stimulation. Low-dose LPS preferentially activates pro-inflammatory genes in a GSK3-dependent fashion, while higher dosages of LPS simultaneously activate Akt/CREB, counteracting GSK3 signaling by suppressing pro-inflammatory and inducing anti-inflammatory genes, enabling resolution of the inflammatory response.

The endocytosis of TLR4 may be important for this process. Directly downstream of TLR4, the adaptor molecule TRIF is engaged. TRIF-dependent signaling has broadly anti-inflammatory effects in the context of LPS stimulation (1) mediated through PI3K (2, 3). Before TRIF can be engaged, TLR4 must undergo endocytosis (4, 5). MyD88-dependent TLR4 signaling is under no such constraint, and is more selectively pro-inflammatory. Stimulation with LPS induces homodimerization of TLR4 (6), along with clustering of the receptor on lipid rafts in the membrane (7), perhaps facilitating endocytosis of the LPS-bound receptors. It is thus easy to imagine that different dosages of LPS result in different degrees of clustering on the cell membrane such that low-dose LPS results in only limited endocytosis while high-dose LPS causes robust clustering and efficient uptake of TLR4-LPS complexes. This may present an obstacle specifically to TRIF-dependent signaling, whereby low-dose LPS is able to activate the MyD88-dependent pathway and its pro-inflammatory downstream effectors but TRIF-PI3K signaling is only engaged upon binding of large quantities of LPS by membrane-bound TLR4. If this is indeed the case, the selectivity of the pro-inflammatory response to LPS should be augmented by either reduction of surface TLR4 expression or inhibiting endocytosis of the receptor, while overexpression of TLR4 specifically on the cell surface may abolish the pro-

inflammatory skewing effects of low-dose LPS. However, this might only hold true in the context of sterile LPS stimulation, as whole bacteria are rich with PAMPs and antigens, and never stimulate one PRR exclusively. Nevertheless, such a scenario suggests several possible strategies for the treatment of chronic disease. Low circulating levels of LPS are well known to be important in the development of atherosclerotic plaques (8, 9). The pro-inflammatory skewing associated with this mild endotoxemia might be reversible by transiently increasing the circulating dosage of LPS, so as to activate anti-inflammatory mechanisms and trigger resolution.

The precise mechanistic connections governing this network have also been explored. The involvement of Lyn in TLR4 signaling has been well established in the setting of B cells and the development of a lupus-like autoimmune disease (10, 11), and is likely to be important in other chronic disease settings. We found that Lyn suppressed IL-12 production in response to different LPS dosages. This anti-inflammatory effect is likely to be mediated by BCAP-dependent activation of PI3K (12), a pathway which is active in macrophages as well as B cells (13). Lyn, then, may suppress inflammation in part by linking CREB activation to TLR4 stimulation by means of PI3K. Though we also found evidence linking GSK3 to FoxO1 activation and IRAK-M to CREB, the exact nature of these connections remains to be elucidated. Mathematical modeling has pointed to IRAK-M as being upregulated by PI3K (14), raising the possibility that IRAK-M may indicate CREB activity by means of itself being likewise activated by PI3K, rather than acting directly upstream of CREB, although the failure of adenosine to increase CREB activity in IRAK-M$^{-/-}$ cells points to a more direct role. In the context of LPS stimulation, Lyn and IRAK-M both appear to intervene between TLR4 and CREB. Since adenosine is able to activate CREB in Lyn$^{-/-}$ but not IRAK-M$^{-/-}$ cells, it follows that IRAK-M fits

into the PI3K/Akt/CREB pathway downstream of both TLR4 and the adenosine receptor, while Lyn acts downstream of TLR4 but can be circumvented by adenosine.

Directions for further study include the mechanisms of signal transduction between GSK3 and FoxO1. While these molecules, along with Akt and CREB, appear to be the key players in the regulatory network explored above, the pro-inflammatory effects of super-low-dose LPS appeared to require upregulation of FoxO1 protein levels, an effect for which GSK3 is not likely to be directly responsible. It is more probable that a substrate of GSK3 is activated in the context of super-low-dose LPS stimulation, and then proceeds either directly or by means of further downstream effectors to increase FoxO1 levels. This increase is likely to depend on a suppression of ubiquitylation and degradation of FoxO1, as this is the primary means by which Akt suppresses FoxO1 activity (15). The precise locations of Lyn and IRAK-M in the signaling networks downstream of TLR4 also remain to be determined. The apparent requirement for IRAK-M in adenosine receptor signaling is particularly intriguing.

This work has broader implications for what has recently been described as “innate memory”. Rather than being a function of dedicated cell types, the capacity of innate immune cells to recall previous states is a generalized characteristic intrinsic to signaling networks. History and context are tremendously important to the functioning of signal transduction pathways: Faulkner's observation that “the past is never dead” is every bit as true of macrophages.

The entanglement of many different signaling pathways can create an almost infinitely subtle gradient of possible outputs based on the sum of responses to different inputs. Feedback loops and other network motifs are critical (16), but they operate simultaneously with and in direct response to stimuli. Signaling memory is rather the means by which a network can recall

the past. In innate immunity, perhaps the clearest example is the phenomenon of endotoxin priming (17, 18), where exposure to super-low-dose LPS alters the TLR4 signaling cascade in such a way as to prepare it for a far stronger inflammatory response to later challenge. The use of adjuvants with vaccines similarly illustrates this concept, as the presence of a costimulatory factor alters the immune response to an antigen. Any system through which signals propagate is altered by those signals, and the imprint of this history influences its response to future stimulation—in computers, this may manifest as electronic components heating, and in cells the individual kinases and transcription factors which are activated or inactivated will remain so until further altered in response to another signal. The image of the cell as a computational unit has become popular, but the interior machinery of each cell is more like an abacus which must be actively reset by subsequent signals. An appreciation for the history of cellular states is absolutely crucial if our understanding of innate immunity is to progress further.

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