Dynamic Programming of Innate Immunity in Health and Disease

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Abstract

Whether innate immune cells may be adapted into potential memory states has becoming an important question in the field of immunity. Although previous conceptual paradigm failed to acknowledge this important question, emerging clinical and basic observations have started to shed intriguing clues to shake the previous dogma regarding innate immunity of being "simple", "raw", "first-line defense with no memory". We have aimed to further address this fundamental issue in this dissertation work, under the close guidance of Dr. Liwu Li. We have chosen to use the model system of Toll-Like-Receptor (TLR) signaling networks within primary monocytes.

TLRs play fundamental roles in sensing pathogen-associated molecular patterns (PAMPs) and modulation of innate immunity. Lipopolysaccharide (LPS), an endotoxin found on the cell membrane of gram-negative bacteria, is the ligand of TLR4 and induces a range of inflammatory as well as anti-inflammatory responses. Higher dosages of LPS were known to cause robust yet transient expression of pro-inflammatory mediators. On the other hand, the effects of super-low dose LPS, commonly manifested in humans with adverse health conditions, have been largely ignored in the basic research field. Super-low dose LPS may skew host immune environment into a mild non-resolving pro-inflammatory state, which is a risk factor for inflammatory diseases such as atherosclerosis, compromised wound healing, and elevated risks for sepsis.

Our central hypothesize is that monocytes may be adapted by super-low dose LPS into a non-resolving low-grade inflammatory state conducive for the pathogenesis of inflammatory diseases. We have employed both *in vitro* cell culture system as well as *in vivo* disease models to test this hypothesis.

For the *in vitro* system, we have cultured primary murine monocytes with increasing signal strength of LPS. Monocyte phenotypes such as the expression of key inflammatory

mediators including cytokines, chemokines, and cellular surface markers were studied. Potential molecular and cellular mechanisms were examined. We revealed a novel low-grade inflammatory monocyte phenotype termed M_L adapted by super-low dose LPS, mediated through IRF5.

For the *in vivo* system, we have employed both acute and chronic models of inflammation. For the chronic model, we have tested the effects of super-low dose LPS on monocyte polarization *in vivo*, as well as its contribution to the pathogenesis of atherosclerosis. Furthermore, we have tested the effects of programmed monocytes on wound healing. For the acute model, we have tested the effects of pre-conditioning with super-low dose LPS on the subsequence risks of sepsis elicited by cecal ligation and puncture. We have demonstrated aggravated atherosclerosis, compromised wound healing, and increased sepsis mortality in mice pre-conditioned with super-low dose LPS.

Taken together, our findings reveal that monocytes can be differentially programmed into distinct states, depending on the signal strength of LPS. The differential programming and adaptation of monocytes can occur both *in vitro* and *in vivo*, and may bear profound pathological consequences.

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Attributions

Several Colleagues contributed to the projects, research and writing which presented as part of my dissertation.

Chapters 1, 3 and 4 of this dissertation are published research articles.

Chapter 1 ‘Dynamic modulation of innate immunity programming and memory’ is an article published in Science China Life Sciences (2016; 59(1):38-43)

Chapter 3 ‘Low-grade inflammatory polarization of monocytes impairs wound healing’ is an article published in Journal of Pathology (2016; 238: 571-583)

Chapter 4 ‘Super-low dose endotoxin pre-conditioning exacerbates sepsis mortality’ is an article published in EbioMedicine (2015; 2(4):324-333)

Chapter 1 through 5:

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Chapter 1. Literature Review

1.1 Introduction

Mediated by innate pattern recognition-receptors, innate immune cells may respond to broad molecular patterns and are responsible for the modulation of general immune environments, through the expression of diverse inflammatory mediators. Unlike the adaptive immune cells that are known to adopt distinct memory states toward highly specific antigens, innate immune cells were thought to be highly plastic and have no memory of prior challenges. To the contrary, data from recent studies increasingly suggest that innate immune cells may also be able to adopt "rudimentary" memory states under distinct challenge conditions. For example, some of the earlier studies suggest that unique combinations of cytokine environments may program several distinct macrophage phenotypes such as the pro-inflammatory M1, the anti-inflammatory M2, and various intermediate subsets (1,2). Innate macrophages and monocytes may also adopt highly dynamic "primed" or "tolerant" state that may correlate with the establishment of "non-resolving" vs "resolving" inflammation depending on the signal strength and history of prior challenges (3,4). Clinical observations support the existence of distinct innate leukocyte subsets *in vivo*, and render the pathophysiological significance of innate programming and memory. The non-resolving pro-inflammatory macrophages are often correlated with the pathogenesis of inflammatory diseases, and the resolving anti-inflammatory macrophages tend to associate with tissue repair (5). Here we review some of the key features and underlying mechanisms of innate leukocyte programming.

1.2 Distinct programming by different cytokine environments

Innate leukocytes such as macrophages have the potential to express a large array of cellular mediators including cytokines, chemokines, co-stimulatory molecules, and lipid mediators. However, depending upon the nature of the external stimulants, the gene expression profiles of macrophages can vary dramatically. Macrophages treated with interferon gamma (IFNγ) are skewed to selectively express pro-inflammatory mediators as represented by IL-12 or TNFα, and fail to express anti-inflammatory mediators such as IL-10 or TGFβ. IFNγ-conditioned macrophages are named as M1 macrophages (6). In

contrast, the gene expression profiles of macrophages treated with IL-4 are drastically different, and are represented by the selective expression of anti-inflammatory mediators such as arginase 1 (6). IL-4 pre-conditioned macrophages are also classified as the M2 macrophages. On the other hand, immune complexes coupled with LPS may program macrophages into various M2b subsets with selective expression of cell-surface co-stimulatory molecules (5). TGFβ or IL-10-treated macrophages tend to adopt a suppressive phenotype with selective expression of IL-10 (Table 1).

In terms of molecular mechanisms, epigenetic programming through IFN-activated STAT1 may be responsible for the global histone acetylation associated with the inflammatory gene loci (7). Likewise, IL-4 or TGFβ may induce distinct epigenetic programming events in macrophages, leading to the differential skewing of gene expression profiles (8).

Table 1. Programming dynamics of innate leukocyte

	Inducers	Effects
Programming through different challenge	IFNγ	M1
	IL-4, IL-13	M2a
	ICs + LPS	M2b
	TGFβ, IL-10	M2c
Sequential/ combinational programming	β-glucan + LPS	Priming
	IFNγ + LPS	Priming
Signal strength dependent programming	Super low dose LPS	Non-resolving inflammation
	High dose LPS	Resolving inflammation

Abbreviations: ICs, Immune complexes.

1.3 Combinatorial programming with multiple immune stimulants

Although well-defined and distinct culturing stimulus may offer clear differentiation phenotypes of macrophages/monocytes, it may not reflect the complex *in vivo* environment where monocytes may constantly face changing landscapes of multiple cellular mediators in a temporal and spatial fashion. To address whether monocytes may gain potential "memory" from previous challenges, experimental systems were designed to provide

simultaneous or sequential challenges to monocytes with multiple distinct stimulants. For example, monocytes with a prior challenge of beta-glucan were "trained" to respond with a more robust inflammatory response to a subsequent LPS challenge (9,10). We reported that retinoic acid may synergize with IL-4 in triggering the expression of M2-type cellular mediators such as arginase 1, while co-stimulation of monocytes with IL-4/retinoic acid with LPS annihilate the expression of arginase 1 (11).

These phenomenological studies suggest that intra-cellular pathways triggered by distinct innate stimulants may converge into inter-twined circuits, and responsible for the cross-talk within monocytes toward multiple stimulants. For example, we and others demonstrated that nuclear receptors (e.g. RARα, RORα) activated by retinoic acid can not only synergize with STAT4 activated by IL-4, but also can potently suppress the inflammatory NFκB pathway activated by LPS (12,13).

1.4 Signal strength-dependent programming of innate leukocytes

Recent studies from our group and other suggest another intriguing aspect of innate immunity programming and memory, in that innate leukocytes may not only be able to recognize different combinations of extra-cellular stimulants, but also discern their relative signal strength (4,13-17). This is reflected in the cardinal example of endotoxin priming and tolerance. A salient example of selective innate pre-conditioning is the paradigm of endotoxin priming and tolerance (18). Host monocytes/macrophages are the most potent responders to bacterial endotoxin, LPS (1). Most studies regarding cellular responses to LPS utilized higher tolerant dosages of LPS (although often referred as "low dose" in the literature) (>10 ng/mL *in vitro* culture, >1 μg/mouse or > 50 μg/kg body weight *in vivo* injection). Higher doses LPS cause robust induction of pro-inflammatory mediators in monocytes/macrophages through the Toll-Like-Receptor 4 (TLR4) pathway (19). Shortly after the initial wave of expression, host macrophages develop a state of "endotoxin tolerance", in which the expressions of pro-inflammatory mediators are suppressed (20-22). Endotoxin tolerance serves as a compensatory mechanism for the resolution of inflammation (23,24). In stark contrast, subclinical super-low levels of circulating LPS (~1-100 pg/mL) in experimental animals and humans with adverse health conditions do

not induce compensatory tolerance (21,25-27). Instead, we and others have shown that super-low dose LPS (~1-100 pg/mL *in vitro*, 100pg-10ng/mouse or 5 ng-0.5 μg/kg body weight *in vivo* injection) "primes" macrophages for a more robust response to a secondary LPS challenge *in vitro* and *in vivo*, a phenomenon known as the "Shwartzman reaction" (15,22,26,27) (Figure 1.1).

Through toll-like-receptor 4 (TLR4), Higher dosages of LPS induce transient and resolving inflammation through the activation of both classical and alternative NFκB pathways (28,29) The classical NFκB pathway contributes to the robust yet transient induction of pro-inflammatory mediators (19). As a compensatory mechanism, higher dose LPS also activates the anti-inflammatory PI3K pathway that leads to the activation and induction of several negative regulators such as MKP-1, RelB, p-AKT, p-ERK, RORα, and RARα, which serve as negative regulators of pro-inflammatory NFκB function (29-34). This underlies the phenotype of "endotoxin tolerance" (29).

In stark contrast, the mechanisms for endotoxin priming are less well studied and poorly understood. We first reported that super-low dose LPS fails to induce negative regulators such as nuclear receptors (RARα, RORα, etc), PI3K/Akt pathway, ERK, Tollip and RelB in innate leukocytes (13) . This may explain the lack of tolerance by super-low dose LPS. Instead, we observed that super-low dose LPS suppresses aforementioned negative regulators and primes monocytes/macrophages for more robust expression of pro-inflammatory mediators. Among intracellular components of TLR4 pathway, we reported that IRAK-1 is critical for the effect of super-low dose LPS and priming of innate leukocytes (13,16). Mechanistically, we documented that IRAK-1 is required for suppressor clearance induced by super-low dose LPS (16). In contrast, higher dose LPS activates PI3K and leads to IRAK-1 degradation (21,35) (Figure 1.1).

These studies may explain many previous clinical observations and bear far-reaching pathophysiological consequences. For example, varying dosages of endotoxin have been shown to differentially affect the pathogenesis of asthma, atherosclerosis, and sepsis in animal studies (16,36-39). Differential development of asthma and other inflammatory diseases associated with people from developing countries prompted the "hygiene theory"

that resonates with these dynamic programming of innate leukocytes (40). It may be an evolutionary survival advantage for human hosts to adopt a compensatory homeostatic tolerance when challenged with higher dosages of innate stimulants. On the other hand, chronic exposure to minute amount of innate stimulants may dispense the tonic homeostatic mechanism of tolerance, and develop low-grade wax and wane inflammation. As the chronic inflammation persists, innate leukocytes as well as their hematopoietic precursors may be exhausted, which may contribute to immune exhaustion and "inflammaging" during the aging process (41).

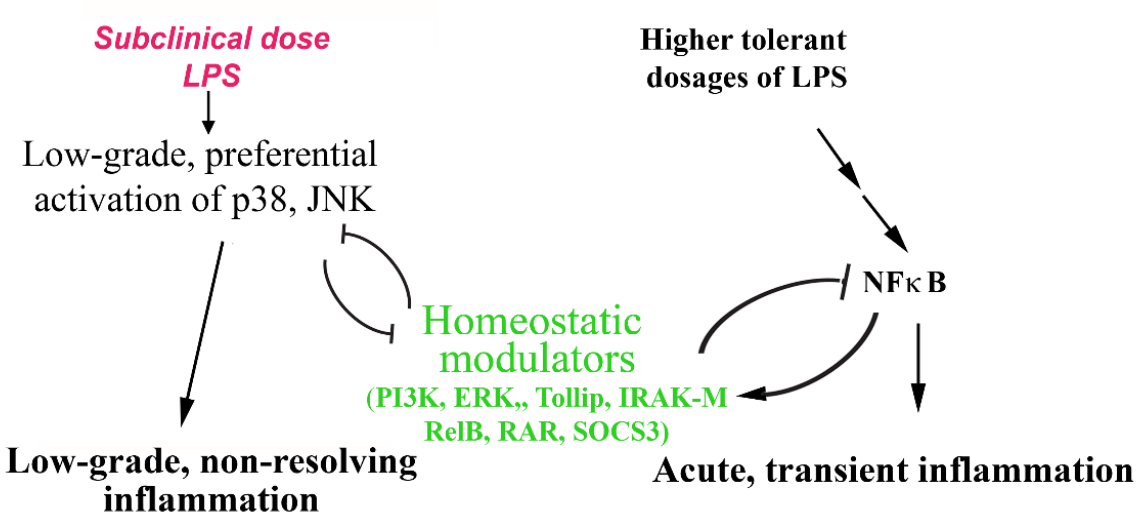

Figure 1.1 A schematic diagram illustrating the dynamic circuitry potentially responsible for the establishment of either low-grade non-resolving inflammation or acute resolving inflammation.

For the establishment of acute resolving inflammation, the occurrence of negative feedbacks due to the activation of homeostatic modulators such as PI3K/AKT, IRAK-M, Tollip, SOCS3, and ERK would be required. On the other hand, the clearance of these negative homeostatic modulators would favor the low-grade non-resolving inflammation.

The dynamic responses to varying dosages of innate stimulants are not limited to endotoxin, and are observed with other agonists such as TLR7/8 agonists (42,43). This may be a generalized phenomenon in innate leukocytes during their dynamic and tailored adaption to changing environments over time.

1.5 Systems biology of innate programing and memory

The highly complex and diverse profiles of leukocyte programming pose immense challenges for experimental characterization. Recent advances in computational systems analyses may offer significant assistance in deciphering complex leukocyte dynamics and memory. In collaboration with computational biologists, we have identified several salient functional motifs required for the establishment of priming and homeostatic tolerant states in innate leukocytes. For the homeostatic tolerance, the sustained presence of robust homeostatic negative regulator(s) would be required (44,45). This is consistent with experimental observations from our lab and others that reveal the induction of multiple negative regulators during the homeostatic tolerance of innate leukocytes such as Tollip, PI3K, ERK, IRAK-M, SOCS3, and IκBα (16,29,32,34). These homeostatic modulators may function at multiple levels during the signaling process. On the other hand, for the priming process to occur, the pathway switching from the induction to the clearance and disruption of negative suppressors must occur. Our laboratory has provided data confirming the clearance of suppressors such as Tollip and IRAK-M, as well as suppression of ERK and PI3K during the priming process of innate leukocytes (14-16,46).

Computational and experimental analyses also converge to suggest a dynamic circuit consisting of two mutually-inhibitory arms of positive and negative regulators, which may give rise to the bi-stable states of cellular priming and homeostatic tolerance (45). This salient motif may be critically involved in the sensing of rising signal strength of innate stimulants, and responsible for the fine-tuning of leukocyte functions. Intriguingly, systems analyses suggest that this motif may also be at work during the dynamic differentiation of T helper cells into distinct Th1/Th2/Th17/Treg states (47,48), and may hold general significance in the modulation immune cells.

The distinct programming of innate leukocytes may also be finely modulated by spatial coordination of signaling circuitries. For example, we observed that the Tollip molecule may serve dual roles as both a positive and a negative regulator during the activation process of innate leukocytes, depending upon its sub-cellular localization. Lysosomal Tollip is crucial for maintaining homeostasis by facilitating the fusion of lysosome and autophagosome, and the completion of autophagy (49). However, unknown modification may drive its translocation away from lysosome to mitochondria, and switch Tollip from a homeostatic factor to a stress-promoting molecule (16,49).

1.6 Innate leukocyte memory in resolving and non-resolving inflammation

Dynamic modulation and memory of innate immunity may have expanding roles in the pathogenesis and resolution of inflammatory diseases ranging from acute sepsis to chronic diseases such as atherosclerosis, diabetes, impaired wound healing, neurological disease and aging. During acute inflammatory disease such as acute injury, trauma, acute colitis and sepsis, dysregulated innate immune programming may lead to defective host defense toward invasive microbes, as well as dysregulated tissue inflammation. We recently reported that pre-conditioning with super-low dose endotoxin pre-disposes animals for increased mortality in subsequence sepsis (36). Mice pre-conditioned with subclinical super-low level of endotoxin exhibit severe tissue damage, inflammation, increased bacterial load in circulation, and elevated mortality when subjected to cecal-ligation and puncture (CLP). This is in contrast to the protective phenomenon with CLP mice pre-conditioned with higher tolerant level of LPS. Tolerant dosages of LPS skews an anti-inflammatory phenotype in both monocyte and neutrophils as reflected in reduced expression of pro-inflammatory mediators (36). In contrast, super-low dose LPS polarizes a low-grade pro-inflammatory state in monocytes and neutrophils, conducive of tissue damage (36). Furthermore, we demonstrated that varying dosages of LPS differentially modulate the formation of neutrophil extracellular trap (NET) in neutrophils. Super-low dose LPS suppresses the homeostatic circuits such as PI3K/AKT and ERK that are required for the generation of neutrophil NET (36,50). In contrast, higher tolerant dosages of LPS activates PI3K/ERK and facilitate NET formation (50). Balancing homeostasis in innate leukocytes may hold potential in treating acute injury and sepsis. During the chronic

inflammatory processes, ongoing studies suggest that polarization of the low-grade inflammatory state in innate leukocytes through the disruption of homeostatic pathways may similarly play a key role in sustaining the non-resolving inflammation that underlies the pathogenesis of atherosclerosis, diabetes, delayed wound repair, and ageing.

Thus, homeostatic resolution of innate leukocyte activation through the induction of homeostatic molecules, autophagy, and biogenesis of homeostatic organelles such as lysosomes may be a viable approach for the treatment of both acute and chronic inflammatory disease. To this regard, potential promising compounds such as Tauroursodeoxycholic Acid (TUDCA) and trehalose have been tested and shown to have beneficial effects in maintaining partial homeostasis in innate leukocytes and the treatment of inflammatory and infectious diseases (36,51,52).

1.7 Conclusion

In summary, we are witnessing the emerging concept of "innate memory", and what is known so far only may reflect a tip of an iceberg in terms of its complexity and translational potential. The continued integration of experimental and computational approaches, coupled with genomics and proteomics analyses of large datasets, may yield rich and exciting profiles of innate leukocyte activation. With particular urgency, epigenetic analyses should be performed to define key molecular markers that may assist the classification and characterization of novel innate leukocyte subsets in health and disease. Translational studies in humans will help to assign clinical relevance to these emerging "memory" innate leukocytes in the future.

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Chapter 2. Dynamic programming of innate immunity by bacterial endotoxin and its pathophysiological impacts in atherosclerosis

2.1 Introduction

Atherosclerosis and related cardiovascular complications are among the leading causes of morbidity and mortality in industrialized countries (1). Complex and intertwined alterations in non-resolving low-grade inflammation and lipid metabolism may collectively contribute to the initiation and progression of atherosclerosis. The lack of clear understanding with regard to the cause and contribution of low-grade inflammation during the complex pathogenesis of atherosclerosis hinders the development of effective cures.

At the cellular level, innate monocyte is one of the key linchpins that connect low-grade inflammation and altered lipid metabolism, through the expression of inflammatory mediators as well as the modulation of intra-cellular lipid accumulation (2). Inflammatory mediators are critically involved in the coordination of atherosclerotic plaque composition and stability, while lipid deposition inside infiltrating monocytes/macrophages may lead to the generation of foam cells within the atherosclerotic core (3). Differential activation states of monocytes may govern the expression profiles of inflammatory mediators and the surface levels of lipid transporters such as SR-B1. In the context of resolving inflammation, the expression of pro-inflammatory mediators by inflammatory monocytes is transient and subsequently suppressed due to the induction of homeostatic negative regulators (4). In contrast, under non-resolving inflammatory conditions, it has been speculated that monocytes might adopt a sustained inflammatory state conducive for the pathogenesis of atherosclerosis (5). Despite its significance, causes and molecular mechanisms responsible for non-resolving inflammatory monocyte polarization conducive for the pathogenesis of atherosclerosis are not well understood.

Clinical risk factors for non-resolving inflammation and atherosclerosis such as chronic infection, obesity, ageing, chronic smoking and drinking often lead to mucosal leakages and subclinical levels of circulating bacteria endotoxin liposaccharide (LPS) (6,7). Recent clinical studies suggest that subclinical endotoxemia may be closely correlated with the occurrence of chronic low-grade inflammatory diseases in humans and animal models such as atherosclerosis (8,9). Studies conducted *in vitro* suggest that subclinical super-low

levels of endotoxin are capable of inducing low-grade inflammatory responses from cultured monocytes/macrophages (10,11). However, *in vivo* study is lacking to examine the monocyte polarization by subclinical endotoxemia and its pathological consequences in atherosclerosis.

In this current study, we aim to test the pathological consequence of subclinical endotoxemia during the polarization of low-grade inflammatory monocytes and progression of atherosclerosis. We hypothesize that subclinical endotoxemia may set in motion a sustained program of inflammatory polarization and altered lipid deposition in monocytes, which may contribute to the aggravation of atherosclerosis.

2.2 Materials and methods

2.2.1. Experimental animals.

ApoE$^{-/-}$ mice were purchased from the Jackson laboratory. The mice were bred and maintained in the animal facility at Virginia Tech with the approved protocol from the Intuitional Animal Care and Use Committee (IACUC) in compliance with in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. All male mice were 7–10 weeks of age when experiments were initiated. Animal numbers were empirically determined to consider the need for statistical significance, and also adhere to IACUC policy on minimizing animal numbers. Breeding and handling limitations were also considered for determining the number of animals used for experiments. No randomization method was used due to the experimental setup. Animals that showed health concerns un-related to the experimental conditions (for example, fight wounds and dermatitis) were excluded.

2.2.2. High fat diet feeding and LPS injection.

Two experimental models were employed in this study. In the first model, Western Diet (Harlan Teklad 94059) fed-mice were i.p. injected with either PBS or LPS (5 ng/kg body weight) twice weekly for two month. In the second model, Western Diet (Harlan Teklad 94059) fed-mice were i.p. injected with either PBS or LPS (5 ng/kg body weight) twice weekly for one month. Following the termination of injection, mice were continuously fed

with Western Diet (Harlan Teklad 94059) for another month. Animals were monitored daily and body weights were taken weekly.

2.2.3. Histology and Immunofluorescence.

Histological analyses were performed on fresh-frozen, OCT-embedded proximal aortic sections (10 μm). Slides were fixed in 4% neutral buffered formalin for 5 min. Hematoxylin and eosin (H&E) as well as Oil-Red O staining were performed. The total lesion area and the percentage of vessel occlusion were measured. The percentage of vessel occlusion was measured as the ratio of the vessel intima area (without plaque) to the vessel lumen area (with plaque). All calculations analyzed used the mean of samples from 6 mice per group. Immunofluorescence analyses were performed on fresh-frozen, OCT-embedded proximal aortic sections (10 μm). Slides fixed in 4% neutral buffered formalin for 5 min were stained with anti-mouse primary antibody (anti mouse MOMA-2) followed by followed by a biotinylated anti-Ig secondary Ab (BD Biosciences) and Streptavidin-PE or FITC. DAPI was used to stain nucleus.

2.2.4. Lipid analyses

Plasma was collected from all mice at the time of sacrifice. The total cholesterol and free cholesterol in the plasma were measured by using Cholesterol Quantitation Kit (Sigma-aldrich). For the measurement of triglycerides, the Triglyceride Quantification Colorimetric/Fluorometric Kit (Biovision) was used.

2.2.5. ELISA of cytokines and chemokines

Plasma samples collected from peripheral blood were subjected to ELISA analyses for selected cytokines and chemokines. The kits for measurement of TNF-α, IL-10, IL-6, TGF-β and MCP-1 were from eBioscience (San Diego, CA).

2.2.6. Statistical analysis

All experiments were performed at least for 3 times. Representative and reproducible results were shown. Statistical analysis was performed with Prism software (GraphPad Software). Values were expressed as means ± SEM. The significance of the differences was assessed by Student's t test or one-way ANOVA where appropriate. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1. Aggravated pathogenesis of atherosclerosis with subclinical endotoxemia conditioning

We first tested the effect of subclinical super-low dose LPS on the pathogenesis of atherosclerosis in the ApoE deficient animal model. As determined by Oil-O-Red staining of aorta sections, chronic injection of super-low dose LPS significantly elevated the lipid content within the atherosclerotic plaques in ApoE deficient mice fed with the Western high fat diet (HFD) for 8 weeks, as compared to mice fed with high fat diet alone (Fig 2.1a, b). We further documented significantly elevated areas of the necrotic cores within the observed plaques (Fig 2.1c, d). Immunohistochemical staining revealed a reduction in collagen content of the plaques (Fig 2.1e, f). The overall plaque sizes were not increased following LPS injection (Fig 2.2a-b). Collectively, these data suggest that subclinical dose LPS exacerbates the pathogenesis of un-stable atherosclerotic plaques in mice.

Given the emerging interest in innate immune memory, we further tested whether a shorter period of super-low dose LPS pre-conditioning may have a lasting impact and be sufficient to aggravate HFD-associated atherosclerosis. To test that, we pre-conditioned HFD-fed ApoE mice with super-low dose LPS injection for 4 weeks, followed by continued HFD feeding for an additional 4 weeks without further LPS injection. We observed that a short-time LPS pre-conditioning caused similar impacts by elevating lipid content within atherosclerotic plaques, increasing sizes of necrotic cores, reducing plaque contents of collagens and smooth muscle actin (Fig 2.1g-l, Fig 2.2c-d). Given these observation, we focused our subsequent analyses with the short LPS pre-conditioning model.

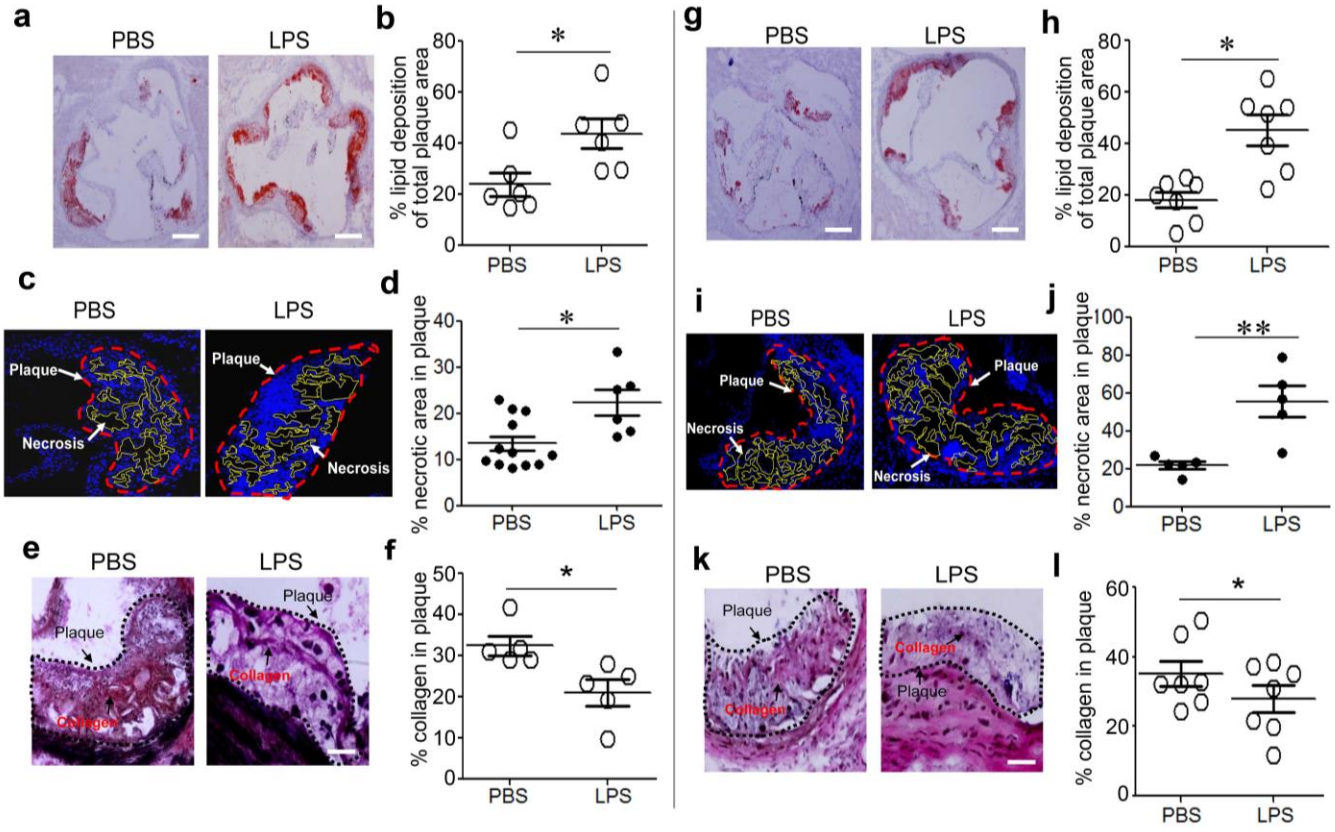

Figure 2.1 Subclinical endotoxemia aggravates the progression of atherosclerosis.

(**a-f**) ApoE$^{-/-}$ mice were conditioned with PBS or super-low dose LPS together with high fat diet for 8 weeks. (**a**) Representative images of atherosclerotic plaques within aortic root areas stained by Oil-Red-O. (**b**) Quantification of the lipid deposition area as a percentage of the total atherosclerotic plaque area within the aortic root. (**c**) Representative images of necrotic core areas within the aortic roots. (**d**) Quantification of the necrotic area as a percentage of the total atherosclerotic plaque area within the aortic root. (**e**) Representative images of collagen containing areas within aortic roots. (**f**) Quantification of the collagen containing area as a percentage of the total atherosclerotic plaque area within the aortic root. (**g-l**) ApoE$^{-/-}$ mice were pre-conditioned with PBS or super-low dose LPS for 4 weeks together with high fat diet, followed by high fat diet feeding only for an additional 4 weeks. (**g**) Representative images of atherosclerotic plaques within aortic root areas stained by Oil-Red-O. (**h**) Quantification of the lipid deposition area as a percentage of the total atherosclerotic plaque area within the aortic root. (**i**) Representative images of necrotic core areas within the aortic roots. (**j**) Quantification of the necrotic area as a percentage of the total atherosclerotic plaque area within the aortic root. (**k**) Representative images of collagen containing areas within aortic roots. (**l**) Quantification of the collagen containing area as a percentage of the total atherosclerotic plaque area within the aortic root. Error bars show means ± s.e.m.; * $P < 0.05$; ** $P < 0.01$; student t-test.

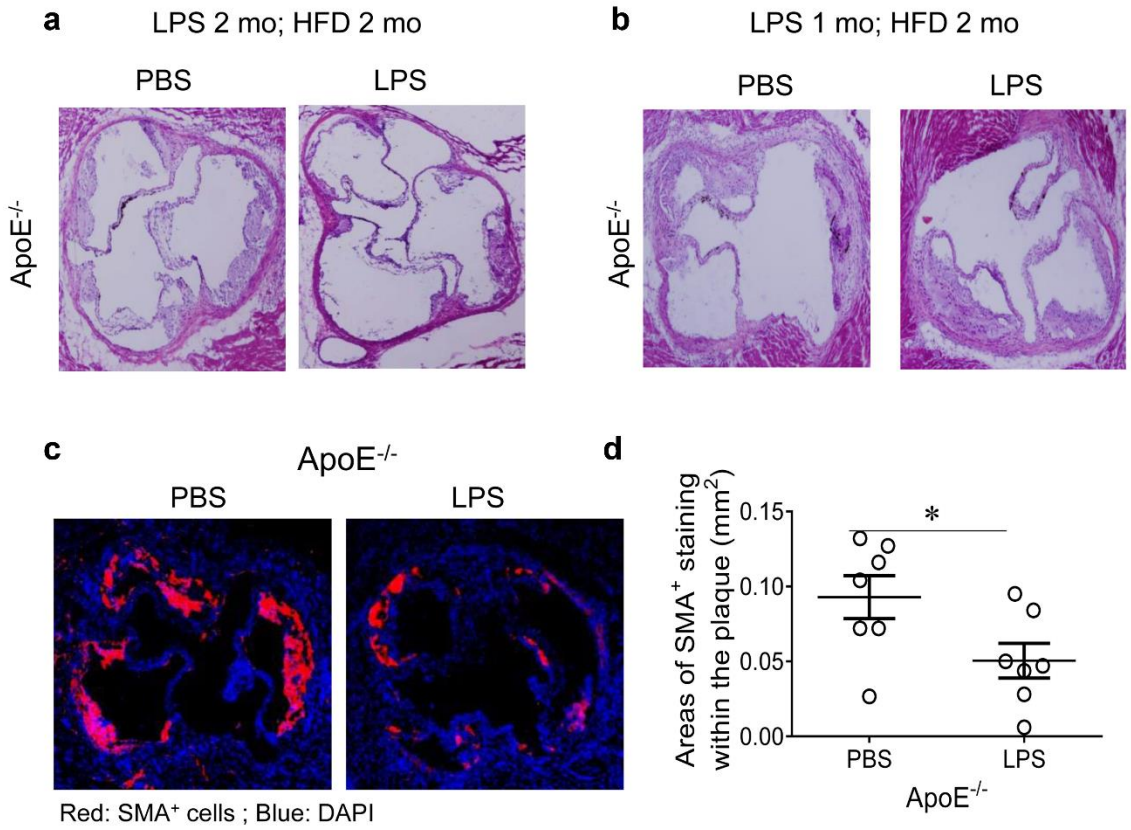

Figure 2.2 Subclinical endotoxemia exacerbates the pathogenesis of un-stable atherosclerotic plaques in mice.

(a) ApoE$^{-/-}$ mice were conditioned with PBS or super-low dose LPS together with high fat diet for 8 weeks. (**b**) ApoE$^{-/-}$ mice were pre-conditioned with PBS or super-low dose LPS for 4 weeks together with high fat diet, followed by high fat diet feeding only for an additional 4 weeks. Representative H&E staining images of atherosclerotic plaques within aortic root areas were shown. (c-d) ApoE$^{-/-}$ mice were pre-conditioned with PBS or super-low dose LPS for 4 weeks together with high fat diet, followed by high fat diet feeding only for an additional 4 weeks. (c) Representative images of SMA^{+} staining within atherosclerotic plaques of aortic root areas. (d) Quantification of SMA^{+} positive staining areas per mm^2 in the lesion area of aortic root. Data are shown for aortic plaque areas from PBS (n = 7) and super-low dose LPS conditioned (n = 7) mice. Error bars show means ± s.e.m.; * $P < 0.05$; student t-test.

2.3.2. Low-grade inflammation coupled with rising levels of plasma lipids in mice pre-conditioned with subclinical dose endotoxin

To test whether the aggravation of atherosclerosis was the result of elevated inflammation and lipid compositions, we examined the levels of plasma lipids as well as selected inflammatory mediators in ApoE deficient mice fed with HFD injected with either PBS or super-low dose LPS. Super-low dose LPS injection caused significant elevation of plasma levels of total cholesterol as well as free cholesterol, without affecting the levels of triglycerides (Fig 2.3a-c). In terms of inflammatory mediators, super-low dose LPS caused significant elevation of pro-inflammatory cytokines such as TNFα and IL-6, as well as chemokine MCP-1 (Fig 2.3d-f).

Given the elevated levels of MCP-1, we further tested whether there were increased levels of tissue macrophages within the atherosclerotic plaques. Indeed, immunohistochemical staining revealed significantly elevated levels of macrophages within the plaque areas of HFD-fed ApoE deficient mice injected with super-low dose LPS as compared to PBS controls (Fig 2.3g, h). Our data indicate that super-low dose LPS conditioning contributed to elevated inflammation, increased recruitment of macrophages as well as elevated plasma cholesterol contents.

2.3.3 Polarization of inflammatory monocytes by subclinical endotoxin in atherosclerotic mice.

Innate monocytes/macrophages are key players that contribute to the expression of inflammatory mediators as well as altered lipid metabolism, and play critical roles during the pathogenesis of atherosclerosis (5). To test whether aggravated atherosclerosis in mice conditioned with super-low dose LPS may be due to distinct alterations in monocytes/macrophages, we examined the activation status of circulating monocytes as well as macrophages within atherosclerotic plaques. We observed that HFD-fed ApoE deficient mice conditioned with super-low dose LPS had significantly higher levels of circulating $CD11b^{+}Ly6C^{Positive}$ pro-inflammatory monocytes, as compared to mice conditioned with PBS (Fig. 2.4a, b). As another indicator for monocyte activation, we observed elevated expression of CCR5 within peripheral blood and bone marrow

monocytes from super-low dose LPS conditioned mice as compared to PBS control mice (Fig. 2.4c, d).

2.4 Discussion

Non-resolving inflammation is considered as a major contributor for chronic disease such as atherosclerosis. Despite extensive interest, the mechanisms leading to the establishment of non-resolving inflammation, however, have drawn debates with less clarity (4). At first glance, the existence of persistent stimulatory signals coupled with prolonged and/or excessive responses seem to be the most intuitive answer, and may appear partially consistent with observed phenomena (12). However, there are ample instances that persistent inflammatory signals, when excessive, may trigger compensatory anti-inflammatory tolerance (13,14). Thus, the sole emphasis on the presence of excessive positive signals may be an overly simplistic view and a far cry from the complex dynamics that underlies non-resolving inflammation as evident during the pathogenesis of atherosclerosis.

To our knowledge, our study provide the first causal evidence that reveal the pathological significance of subclinical endotoxemia *in vivo*, a phenomenon in humans increasingly correlated with non-resolving inflammatory conditions (15,16). We demonstrate that mice exposed with subclinical dose LPS can be programmed with increased levels of circulating inflammatory monocytes. Previous studies on monocyte modulation by LPS almost exclusively utilized higher tolerant dosages of LPS (17). Higher dose LPS causes robust yet transient expression of pro-inflammatory cytokines, followed by a compensatory homeostatic "tolerant" state, through induction of multiple negative feedback regulators (18,19). In sharp contrast, we previously reported that innate monocytes/macrophages fail to develop tolerance when challenged with a subclinical super-low dose LPS *in vitro* (11,20). Extending these observations, our current study demonstrate that a prolonged stimulation with subclinical dose LPS can sustain the low-grade inflammatory polarization of monocytes both *in vitro* and *in vivo*. Together, our data explain the aggravated atherosclerosis due to subclinical endotoxemia, characterized by low-grade non-resolving inflammation, elevated cholesterol levels in plasma and atherosclerotic plaques.

Our data extend the emerging concept of innate immune memory in a pathologically relevant model of non-resolving inflammation and atherosclerosis. Recent studies suggest that innate immune leukocytes may be "trained" and adopt "memory" states with either reduced or increased inflammatory potential *in vitro* (18,21). The development of innate memory, if well defined, may have significant impacts on our understanding of chronic inflammatory disease, and may reconcile the varying disease severity in individuals with distinct innate immune environments. However, existing experimental systems regarding innate memory largely utilized *in vitro* cell culture systems with short incubation periods (17,21). Our data reveal that chronic conditioning of mice *in vivo* with subclinical dose LPS may cause a long-term polarization of inflammatory monocytes. Intriguingly, the polarization and memory effect can persist one-month after the stoppage of LPS injection. Mechanisms that contribute to the development of innate leukocyte memory are poorly studied. Based on systems analyses of cellular memory in general, the establishment of distinct memory states may require the existence of dynamic circuits with competitive feedbacks (22-24).

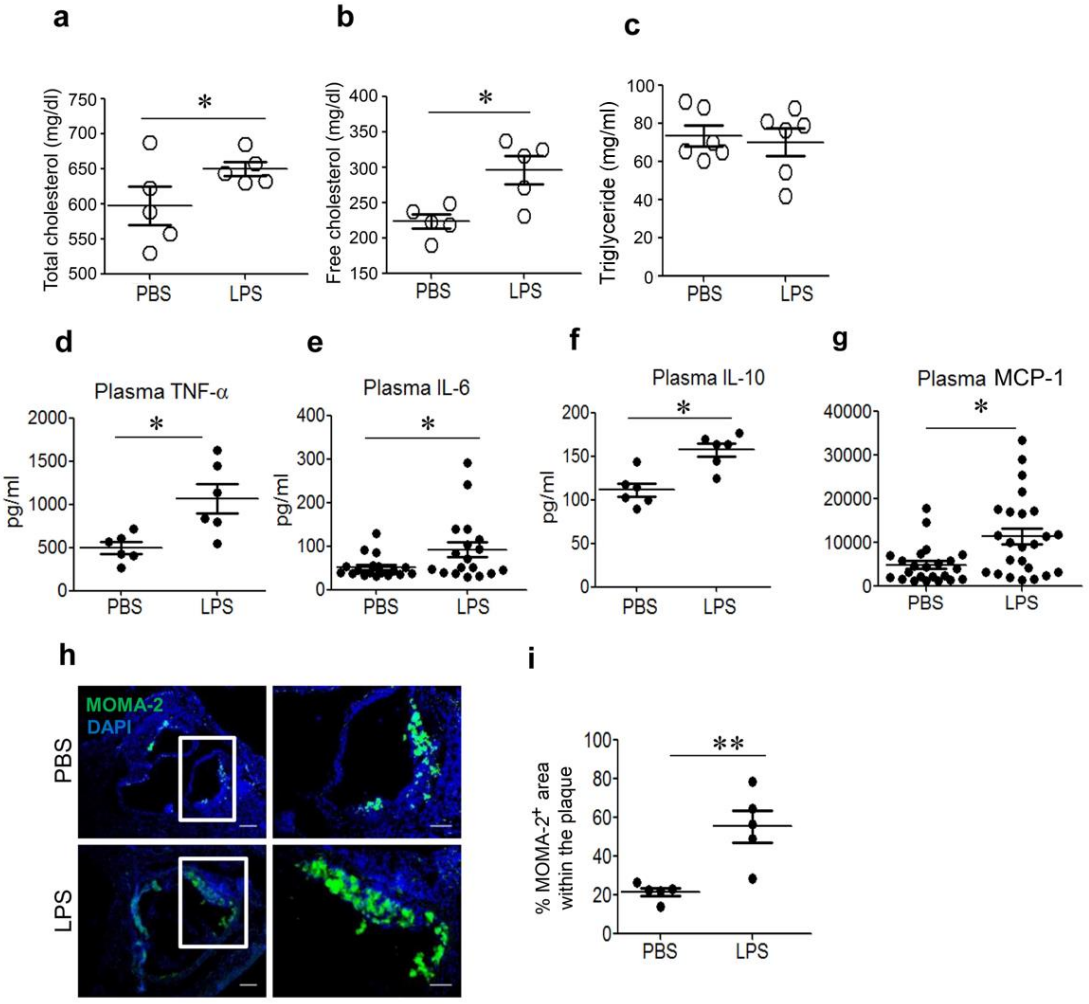

Figure 2.3 Subclinical endotoxemia induces low-grade inflammation together with elevating plasma lipid levels.

ApoE$^{-/-}$ mice were pre-conditioned with PBS or super-low dose LPS for 4 weeks together with high fat diet, followed by high fat diet feeding only for an additional 4 weeks. The levels of total (**a**) and free cholesterol (**b**) in the plasma were tested. Data are shown from PBS (n = 5) and super-low dose LPS conditioned (n = 5) mice. (**c**) Data are shown for triglyceride levels in the plasma from PBS (n = 6) and super-low dose LPS conditioned (n = 6) mice. (**d**) Data are shown for plasma TNF-α levels from PBS (n = 6) and super-low dose LPS conditioned (n = 6) mice. (**e**) Data are shown for plasma IL-6 levels from PBS (n = 18) and super-low dose LPS conditioned (n = 18) mice. (**f**) Data are shown for plasma IL-10 levels from PBS (n = 5) and super-low dose LPS conditioned (n = 6) mice (left panel), and plasma CCL2 levels from PBS (n = 23) and super-low dose LPS conditioned (n = 24) mice (right panel). (**g**) Representative images of MOMA-2^{+} macrophages in the atherosclerotic plaques of aortic root areas. (**h**) Quantification of MOMA-2^{+} area as a percentage of the total atherosclerotic plaque area within the aortic root. Data are shown for aortic plaque areas from PBS (n = 5) and super-low dose LPS conditioned (n = 5) mice. * $P < 0.05$; ** $P < 0.01$; student t-test.

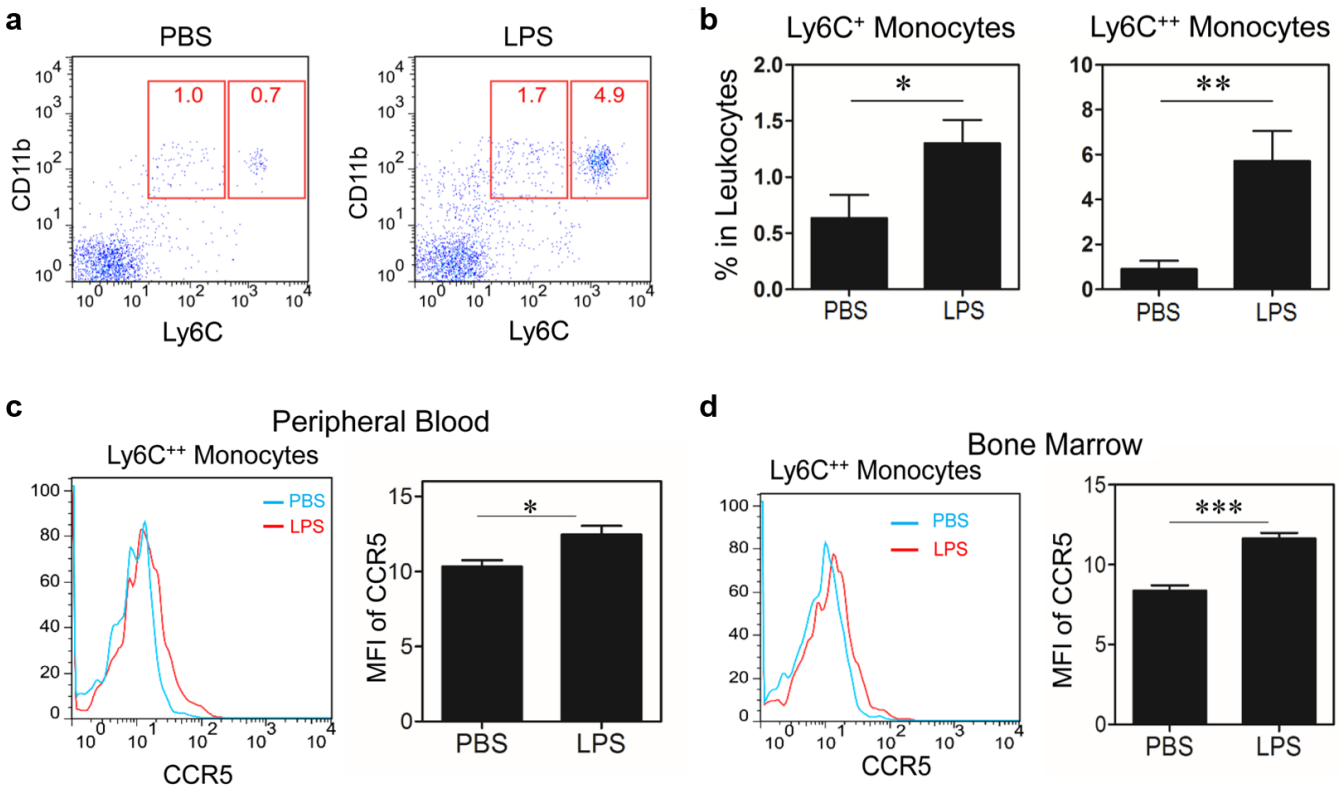

Figure 2.4 Polarization of inflammatory monocytes by subclinical endotoxin in atherosclerotic mice.

ApoE$^{-/-}$ mice were pre-conditioned with PBS or super-low dose LPS for 4 weeks together with high fat diet, followed by high fat diet feeding only for an additional 4 weeks. (**a**) Peripheral blood cells were collected, and CD11b^{+}Ly6C^{++} and CD11b^{+}Ly6C^{+} monocytes gated within the Ly6G^{-} population were examined by flow cytometry. (**b**) The frequency of inflammatory monocytes within total leukocytes was quantified. Data are shown from PBS (n = 7) and super-low dose LPS conditioned (n = 7) mice. Data are shown for aortic plaque areas from PBS (n = 5) and super-low dose LPS conditioned (n = 7) mice. Error bars show means ± s.e.m.; * $P < 0.05$; ** $P < 0.01$; student t-test. (c-d) The expression levels of CCR5 within Ly6G^{-}CD11b^{+}Ly6C^{++} monocytes in peripheral blood and BM were analyzed by flow cytometry. Quantified data are shown from PBS (n = 6) and super-low dose LPS conditioned (n = 6) mice. Error bars show means ± s.e.m.; *, $P < 0.05$; ***, $P < 0.001$; student t-test.

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Chapter 3. Dynamic programming of innate immunity by bacterial endotoxin and its pathophysiological impacts in wound healing

3.1 Introduction

Monocytes play key roles during both the progression and resolution of various inflammatory processes. Past studies reveal the dynamic existence of distinct subtypes of innate monocytes in mice and humans. Murine monocytes are represented by $CD11b^{+}$ $Ly6C^{high}$ inflammatory monocytes and $CD11b^{+}Ly6C^{-}$ anti-inflammatory monocytes (1). In humans, there are three subsets of circulating monocytes that include the classical $CD14^{++}CD16^{-}$, the non-classical $CD14^{+}CD16^{++}$, and the intermediate $CD14^{+}CD16^{+}$ monocytes (2). Current literature suggests that the intermediate human monocytes share most similarities with the murine $CD11b^{+}Ly6C^{high}$ inflammatory monocytes in their expression profiles of inflammatory mediators, and may be closely correlated with the severity of chronic inflammatory diseases (2-4). However, molecular mechanisms leading to the expansion of the inflammatory monocytes are not clearly understood.

In the context of the wound repair process in murine models, the initial influx of inflammatory $Ly6C^{high}$ monocytes into the cutaneous wound bed may be important for the early phase of vascular sprouting (5). Subsequent transition into the anti-inflammatory $Ly6C^{-}$ monocytes would enable proper wound closure and tissue regeneration during the resolving phase of wound repair (5,6). The bi-phasic process switching from an initial inflammatory state to a subsequent compensatory anti-inflammatory state is critical for the ordered wound repair. Persistent existence of inflammatory monocytes in wound bed may be one of the primary culprits for hard-to-heal wounds, as often seen in diabetic patients (7).

Subclinical super-low dose endotoxin lipopolysaccharide (LPS) in circulation has been increasingly recognized as a health concern (8). Chronic infection, obesity, aging, chronic smoking and drinking are common risk factors that contribute to mucosal leakage and elevated plasma levels of endotoxin (9,10). There remains an intriguing correlation that super-low dose endotoxemia may underlie diabetes and obesity-associated complications such as impaired wound healing (11-16).

Recent mechanistic studies may support the correlation between super-low dose endotoxemia and the low-grade non-resolving inflammation (8). Higher doses LPS cause robust yet transient induction of inflammatory mediators, followed by "endotoxin tolerance", a state of compensatory resolution to restore homeostasis through various processes such as autophagy completion (17-19). In sharp contrast, super-low levels of LPS (~1-100 pg ml^{-1}) fail to induce homeostatic tolerance (20,21). Our *in vitro* studies suggest that the disruption of compensatory feedback mechanisms such as the completion of autophagy and the resolution of cellular stress may be the potential culprit (20,22).

One key limitation to the existing studies of the dynamic monocyte priming and tolerance paradigm is the short time course being examined. Most studies involve only one LPS treatment to induce priming or tolerance within a 24 hour time period. To better examine the sustained polarization of monocytes, we aim to test the hypothesis that sustained challenges with super-low dose LPS may polarize monocytes into a low-grade inflammatory state, not conducive for effective wound healing.

To test this hypothesis, we examined the monocyte behavior challenged with sustained super-low dose LPS *in vitro* and *in vivo*, as well as in human monocytes. Using a cutaneous wound healing animal model, we examined the pathological consequence of subclinical dose LPS *in vivo*. We also test the therapeutic potential of Tauroursodeoxycholic acid (TUDCA), a potent inhibitor of cellular stress, in restoring lysosome function, monocyte homeostasis and effective wound repair affected by subclinical dose LPS.

3.2 Materials and Methods

3.2.1. Animals

C57BL/6 were maintained and bred under standard pathogen-free conditions. 8-12-week-old male mice were used for the experiments. All animal experiments were approved, prior to the initiation of this study, by the Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State University.

3.2.2. Reagents

LPS (*Escherichia coli* 0111:B4) was purchased from Sigma. TUDCA was purchased from Prodotti Chimici E Alimentari S.p.A. Murine macrophage colony stimulating factor (M-CSF), CCL3 and CCL5 were obtained from PeproTech. Anti-mouse monocyte/macrophage marker (MOMA-2) antibody and anti-phospho-JNK were purchased from Santa Cruz, anti-mouse CD 16/32 antibody, anti-mouse Ly-6G (Gr-1) antibody, biotin-anti-mouse IgG, biotin-anti-rat IgG and streptavidin-PE, streptavidin-FITC were from eBioscience. PE-rat anti-mouse CD31 antibody, biotin-goat anti-rabbit Ig antibody were from BD Pharmingen. PE-anti-mouse TGF-β antibody, streptavidin-HRP were from Biolegend. Anti-neutrophil antibody (7/4) was from Abcam. Dab substrate kit for peroxidase was from Vector Laboratories. Anti-SAPK/JNK antibody was obtained from Cell Signaling Technology.

3.2.3. Wounding procedure and LPS treatment protocol

The wound repair model was as previously described (23-25). Briefly, mice under proper anesthetization were partially shaved at the back, and sterilized with betadine solution followed by 70% ethanol. 4 full-thickness punch biopsies (Acu.Punch, 6 mm, Acuderm, FL) were created. The biopsy sites were covered with a form-fitting bandage. Mice were injected IP with either PBS or LPS (5 ng/kg body weight) once every three days for 10 days (total three times) before biopsy and one time every three days after biopsy (Fig. 3.1a). Wounds were monitored daily and photographed using a Nikon 9000D digital camera (Nikon, Japan). Changes in wound contraction over time were calculated using the NIH ImageJ software. For histologic analysis, wounds were excised at different times after injury, and the tissue was either fixed overnight in 10 % formaldehyde or embedded in optimal cutting temperature compound (OCT), (Tissue-Tek, Zoeterwoude, NL).

3.2.4. Histopathology and Immunohistochemistry

Skin tissues embedded in OCT were sectioned (4 μm) and stained with H&E. Collagen staining was performed with elastic stain kit (Sigma Aldrich). 10-μm cryosections were immunostained for the macrophage marker MOMA-2 and for the neutrophil marker Ly-

6G. The secondary antibody was biotinylated rabbit anti-rat IgG antibody. The slides were developed using streptavidin–horseradish peroxidase, followed by diaminobenzidine, after which they were counterstained with Mayer's hematoxylin. For immunofluorescence, frozen sections (10 µm) were stained with antibodies as indicated on figure legends.

3.2.5. Cytokine Assay from Plasma

Whole blood samples were collected from Naïve, PBS-treated, LPS-treated mice, and plasma samples were obtained by centrifugation. Serum cytokine levels were examined by ELISA according to the manufacturer's instructions (eBioscience).

3.2.6. Protein extraction and analyses

Cells were washed with cold PBS after specified treatments and harvested in SDS lysis buffer containing protease and phosphatase inhibitors as previously described (26). Protein concentration was assessed by Bradford assay. Following SDS-PAGE, protein bands were transferred to an immunoblot PVDF membrane (Bio-Rad) and subjected to immunoblot analysis with indicated antibodies.

3.2.7. Real-time RT-PCR analyses

Total RNA was extracted using TRIzol (Thermo Fisher Scientific), according to the manufacturer's protocol. RNA was reverse-transcribed using the High-Capacity cDNA Reverse transcription kit (Thermo Fisher Scientific). Real-time PCR was performed on a Bio-Rad CFX96 machine using SYBR Green mix (Bio-Rad). The relative levels of different transcripts were calculated using the ΔΔCt method and results were normalized based on the expression of GAPDH.

3.2.8. *In vitro* culture of murine monocytes and flow cytometry analyses

Crude BM cells isolated from C57BL/6 mice were cultured in complete RPMI medium supplemented with 5% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and with M-CSF (10 ng ml^{-1}) in the presence of different doses of LPS (from 100 pg ml^{-1} to 1 µg ml^{-1}). TUDCA (500 µM) was also added to the cell cultures in some experiments. Fresh LPS and TUDCA was added to the cell cultures every 2 days. After 5 days, cells were harvested and

stained with anti-Ly6C, anti-CD11b and anti-CCR5 antibodies (BioLegend). The samples were then analyzed by FACSCanto II (BD Biosciences). The data were processed by FACSDiva (BD Biosciences), or Flow Jo (Tree Star).

3.2.9. Confocal Microscopy

Cells were fixed with 4% paraformaldehyde and permeabilization with methanol, stained with Cy3-conjugated anti-mouse LAMP1 antibody (Abcam) together with Alexa Fluor 488-conjugated anti-mouse LCIII antibody (Novus Biologicals). The samples were analyzed under the Zeiss LSM 510 confocal microscope.

3.2.10. Chemotaxis Assays

Chemotaxis of monocytes in response to CCL3 (100ng ml^{-1}) and CCL5 (100ng ml^{-1}) was performed using a 48-well Micro Chemotaxis Chamber (NeuroProbe) in which an 8-µm-pore-size polycarbonate filter separated the upper and the lower chamber. The cells were incubated in complete RPMI medium supplemented with 5% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and allowed to migrate towards CCL3 or CCL5 for 2 hours. After the migration period, the filters were fixed and stained with giemsa, and the cells migrated across the filters were counted under light microscope. The results were expressed as the means ± S.E. of the chemotaxis index (CI), representing the increase in the number of migrated cells in response to chemoattractants compared with spontaneous cell migration (to control medium).

3.2.11. Statistical analysis Statistical analyses were performed using Prism Version 5 software (GraphPad). Significance of difference was analyzed with a Student *t* test. When more than two groups were compared, one-way ANOVA was performed. Data were presented as means ± SEM. P values less than 0.05 were considered significant.

3.3 Results

3.3.1. Impaired cutaneous wound healing in mice pre-conditioned with subclinical super-low dose LPS

Although past evidence suggests a connection between subclinical super-low dose endotoxemia and chronic disease, no causative data is available to confirm this connection. In this study, we used the cutaneous wound healing model to test whether injection of super-low dose endotoxin may impact the proper course of wound repair. Mice were pre-conditioned *i.p.* with either PBS or super-low dose LPS (5 ng/kg body weight) as shown in the Fig. 3.1A. Mice were then subjected to a procedure that yields full skin-deep cutaneous wounds, and were closely observed for recovery. As shown in Fig. 3.1B and 3.1C, the wound closure was significantly impaired in LPS pre-conditioned mice, especially at the later stage of wound healing (10-11 days post wounding, Fig 3.1C).

In addition to the superficial evaluation of the overall wound size being affected by LPS pre-conditioning, we further evaluated the quality of tissue repair through histological examination. Specifically, we studied the course of blood vessel sprouting and regression in wound bed, as well as collagen deposition. As shown in Fig. 3.1D-E, blood vessel sprouting occurs well in the wound tissues of PBS and LPS pre-conditioned mice 6 days post-wounding. However, the pruning and regression of blood vessels at the late stage of wound repair were significantly compromised in LPS pre-conditioned mice as compared to PBS pre-conditioned mice. The deposition of collagen was also significantly reduced in the wound bed of LPS pre-conditioned mice (Fig. 3.1F-G).

3.3.2. Sustained presence of pro-inflammatory monocytes in wound tissues of mice conditioned with super-low dose LPS

Sustained polarization of pro-inflammatory monocytes play a key role responsible for the delayed wound repair (6,27). With particular relevance, sustained presence of inflammatory monocytes were shown to negatively impact the effective resolution of tissue repair through prolonged blood vessel sprouting and impaired pruning (5,28). However, agents and mechanisms responsible for the sustained polarization of low-grade inflammatory monocytes were not well understood. Thus, we tested whether super-low

dose LPS may be capable of sustained polarization of low-grade inflammatory monocytes *in vivo*. As measured by immuno-histochemical staining analyses, tissue levels of monocytes/macrophages underwent an initial elevation at day 1 post-wounding, and subsequently subsided at day 11 post-wounding in PBS pre-conditioned mice (Fig. 3.2A). In contrast, the tissue levels of monocytes/macrophages as determined by immuno-histochemical staining remained at a significantly higher level at day 11 post-wounding in LPS pre-conditioned mice. Furthermore, flow cytometry analyses of elutriated suspension cells from the wound tissues demonstrated a significantly elevated population of the $CD11b^{+}Ly6C^{high}$ pro-inflammatory monocytes in mice pre-conditioned with LPS as compared to PBS treated mice, both at day 1 and day 11 post-wounding (Fig. 3.2B). Circulating levels of the $CD11b^{+}Ly6C^{high}$ pro-inflammatory monocytes were also significantly higher in LPS treated mice as compared to PBS treated mice (Fig. 3.2C). Plasma levels of selected cytokines were also significantly elevated in LPS-treated mice (Fig. 3.3).

3.3.3. Polarization of low-grade inflammatory monocytes *in vitro* by sustained challenges with super-low dose LPS

Given our *in vivo* observation of elevated inflammatory monocytes in mice injected with super-low dose LPS, we next examined whether super-low dose LPS may directly modulate the expansion of $CD11b^{+}Ly6C^{high}$ inflammatory monocytes *in vitro*. To this regard, we cultured bone marrow-derived monocytes with M-CSF together with varying dosages of LPS for 5 days. As shown in Fig. 3.4A, continuous incubation with super-low dose LPS significantly expanded the population of the $CD11b^{+}Ly6C^{high}$ inflammatory monocytes *in vitro*. The expansion of the $CD11b^{+}Ly6C^{high}$ inflammatory monocytes reached a plateau upon challenge with the intermediate levels of LPS, and declined upon challenge with higher dosages of LPS (Fig. 3.4B). We further examined the expression levels of a key chemokine receptor CCR5, representative of inflammatory monocytes in both murine and human systems (3). Likewise, both the percentages of $CCR5^{+}$ monocytes as well as the mean fluorescent intensities of CCR5 were significantly elevated in cells incubated with super-low dose LPS, and reduced in cells cultured with higher dosages of LPS (Fig. 3.4B). We further performed real-time RT-PCR analyses of representative

inflammatory mediators, and observed that monocytes continually incubated with super-low dose LPS exhibits a unique expression profile distinct from the traditionally defined M1 or M2 subtypes of monocytes (Fig. 3.5). We therefore use the term of low grade inflammatory monocytes (M_L) to define this phenotype.

Although there are still controversies with regard to the human equivalence of the murine inflammatory monocytes, recent studies indicate that the intermediate $CD14^+CD16^+$ monocytes are the preferential inflammatory subsets in humans (2,3). This particular subset also express high levels of CCR5. Thus, we tested whether LPS may preferentially expand the intermediate inflammatory monocytes in human blood. Since human monocytes are much more sensitive to LPS and likely respond to much lower dosages of LPS, we used a much lower concentration range for the human study. As shown in Fig. 3.4C, super-low dose LPS selectively and significantly expanded the population of intermediate $CD14^+CD16^+$ $CCR5^+$ monocytes, while higher dosages of LPS drastically reduced this population *in vitro*.

3.3.4. Increased migratory ability of monocytes programmed by super-low dose LPS *in vitro*

Based on our above observation that super-low dose LPS elevates the tissue levels of inflammatory monocytes, we further studied the functional regulation of monocytes in terms of their migratory behavior programmed by super-low dose LPS *in vitro*. We utilized the *in vitro* chemotaxis assay in transwell chambers with selective CCR5 agonist CCL3. As shown in Fig. 3.6A, murine monocytes cultured with super-low dose LPS demonstrated significantly elevated ability to migrate toward CCL3. We further confirmed the relative expression of *Ccr5* mRNA through real-time RT-PCR, and observed a significant induction of *Ccr5* mRNA levels in cells programmed by sustained challenges with super-low dose LPS (Fig. 3.6B).

3.3.5. Super-low dose LPS polarizes monocytes via cellular stress and IRF5 accumulation

We previously reported that super-low dose LPS selectively induces cellular stress and related kinases such as the c-Jun N-terminal kinase (JNK) in macrophages (29). Next, we studied whether prolonged challenges of monocytes with super-low dose LPS may sustain JNK activation. Furthermore, we aim to test whether the alleviation of cellular stress through the application of TUDCA may reduce the migratory behavior of the inflammatory monocytes. The rationale of applying TUDCA in this study is the potential therapeutic usage in translational applications, given its traditional medicinal value in the treatment of tissue injury (30). On a molecular basis, TUDCA has also been shown to be a potent inhibitor of cellular stress and JNK activation (31). As shown in Fig. 3.6C, prolonged incubation of monocytes with super-low dose LPS for 5 days led to the activation of JNK, and addition of TUDCA ablated the JNK activation mediated by LPS.

TUDCA may exert its inhibitory effect on JNK through restoring cellular homeostasis (31). We previously reported that super-low dose LPS disrupts cellular homeostasis through disrupting the orderly fusion of lysosome with autophagosome (22). Next, we further tested whether TUDCA may restore the completion of autophagy through facilitating the fusion of autophagosome with lysosome. Indeed, as shown in Fig. 3.6D, monocytes with prolonged super-low dose LPS challenges experienced a disruption of lysosome fusion with autophagosome, as represented with the separation of LCIII staining and LAMP1 staining observed under confocal microscopy. In contrast, application of TUDCA restored this process and the co-localization of LCIII and LAMP1 (Fig. 3.6D).

IRF-5 was shown to be a signature transcription factor within inflammatory monocytes (32). We further tested the IRF-5 status in monocytes programmed by varying dosages of LPS. We observed that IRF-5 levels were elevated in monocytes programmed by super-low dose LPS, and dramatically reduced in monocytes programmed by higher dose LPS (Fig. 3.7A). Our data further supports the notion that super-low dose LPS programs monocytes into an IRF-5^{+} low-grade inflammatory state. Since IRF-5 protein is known to be subjected to modulation by degradation (33), and lysosome function is among key

mechanisms modulating protein degradation (34), we asked whether super-low dose LPS may enhance IRF-5 stability through disrupting lysosome function. To test this, we first employed lysosome inhibitor chloroquine. Through Western blot analyses, we observed that chloroquine treatment effectively increased IRF-5 protein levels in cultured monocytes, suggesting that disruption of lysosome function may account for the enhanced IRF-5 protein stability (Fig. 3.7A). On the other hand, we observed that TUDCA treatment blocked the induction of IRF-5 by super-low dose LPS (Fig. 3.7A). To further test the modulation of IRF-5 within the $Ly6C^{high}$ monocyte population, we performed flow cytometry analyses with cultured monocytes. We observed that sustained super-low dose LPS challenges significantly increased the IRF-5 levels within the $Ly6C^{high}$ monocytes (Fig. 3.7B, C). Application of chloroquine alone significantly elevated the levels of IRF-5 levels within the $Ly6C^{high}$ monocytes, mimicking the effect of super-low dose LPS (Fig. 3.7B). In contrast, application of TUDCA significantly reduced the levels of IRF-5 within the $Ly6c^{high}$ monocytes challenged with super-low dose LPS (Fig. 3.7B, C).

Functionally, we tested whether TUDCA may inhibit CCR5 expression and suppress monocyte migration toward CCL3 in LPS-conditioned monocytes. As shown in Fig. 3.7D, the application of TUDCA significantly reduced the CCR5 expression induced by super-low dose LPS. Likewise, TUDCA also drastically prevented the migration of LPS-incubated monocytes toward CCL3 and CCL5 (Fig. 3.7E).

3.3.6. TUDCA facilitates wound healing *in vivo*

We next tested whether TUDCA may facilitate wound repair in mice pre-conditioned with super-low dose LPS. Indeed, *i.p*. injection of TUDCA dramatically improved the wound repair (Fig. 3.8A, B) and facilitated the pruning and regression of blood vessels at the later stage of wound healing (Fig. 3.8C, D).

Flow cytometry analyses of inflammatory monocytes *in vivo* showed that mice treated with TUDCA together with super-low dose LPS exhibited significantly reduced levels of inflammatory $CD11b^{+}Ly6C^{high}$ monocytes than mice challenged with super-low dose LPS. (Fig. 3.8E)

3.4 Discussion

Our data demonstrates that sustained challenges with super-low dose endotoxin can polarize the low-grade inflammatory monocytes both *in vitro* and *in vivo*. Inflammatory polarization of monocytes as reflected in the sustained expansion and recruitment of inflammatory monocytes could be critically involved in the altered wound healing dynamics. Mechanistically, a disruption of lysosome function in monocytes may contribute to the accumulation of IRF5, a key transcription factor representative of inflammatory monocytes. Our intervention study with TUDCA may hold potential promise in re-balancing monocyte homeostasis that is necessary for effective wound healing.

Our data complement and extend the emerging concept of innate immunity programming (8). Innate immune cells may be differentially programmed by simultaneous or sequential challenges of distinct innate agonists, as measured by the expression levels of selected cytokines (35). For example, as compared to naïve monocytes, monocytes previously "trained" with beta-glucan may express much higher levels of IL-6 when subsequently challenged with LPS (36). In terms of innate programming by LPS, we and others reported that monocytes/macrophages with an initial challenge of varying dosages of LPS may adopt either a "primed" or "tolerant" state, in terms of their subsequent response to a second dose of LPS challenge, with regard to the expression of selected pro-inflammatory cytokines (20,21,26,37). In contrast to these existing reports, this current study is designed to examine the programming of monocytes under continuous and prolonged incubation with LPS, a question not previously studied in the field. This may better reflect *in vivo* pathophysiological situations in humans with subclinical endotoxemia due to mucosal leakage. Our data reveal that monocytes under prolonged incubation with higher dose LPS exhibit reduced expression of selected inflammatory mediators such as CCR5, reminiscent of the endotoxin tolerant phenotype as observed in cells challenged with two consecutive doses of LPS. In contrast, monocytes under prolonged and continued incubation with super-low dose LPS exhibit sustained expression of inflammatory cytokines, elevated surface levels of Ly6C as well as chemokine receptor CCR5.

Our data with primary human cells cultured with LPS further confirm this intriguing dynamics. We demonstrate that super-low dose LPS selectively expand the inflammatory intermediate monocyte population. In contrast, higher dose LPS dramatically reduced the population of the inflammatory intermediate monocytes. It is interesting to point out that human cells are much more sensitive to LPS, and the LPS concentration required for inflammatory monocyte expansion is ~10 fold lower as compared to the murine monocytes. At clinical settings, the intermediate human monocytes have been closely correlated with chronic inflammatory diseases (3,38). However, the origin of this monocyte subset is poorly understood or studied. Our data regarding the dynamic modulation of this monocyte subset by super-low and higher dosages of LPS provides a unique system for future mechanistic studies with regard to the ontology and maintenance of human intermediate inflammatory monocytes.

In the context of wound healing, sustained presence of inflammatory monocytes within wound tissues would be detrimental and may contribute to delayed wound healing, as often seen in chronic diseases such as diabetes (7). Our data support this notion and provide evidence that super-low dose LPS can sustain the population of inflammatory monocytes *in vivo*, as well as delay blood vessel maturation and effective wound repair.

IRF-5 is a key polarizing transcription factor involved in the activation of inflammatory monocytes/macrophages (32,39). However, the cause and mechanism for the accumulation of IRF-5 in monocyte polarization have not been previously clarified. Our study provides first evidence that super-low dose LPS potently programs the low-grade inflammatory monocytes by inducing the signature transcription factor IRF-5. Our data also reveal that the disruption of lysosome function through the activation of cellular stress kinases JNK by super-low dose LPS is responsible for the accumulation of IRF-5 within polarizing inflammatory monocytes. The sustained JNK activation is consistent with *in vivo* findings in chronic animal disease models (31). Our finding may suggest a potential intervention strategy in the future treatment of chronic diseases such as defective wound repair. Indeed, our data suggest that, TUDCA, a traditional medicine effective in the blockage of cellular stress and JNK activation, has a potential in facilitating wound repair.

Taken together, our current study reveals the intriguing dynamics of monocyte polarization, and its pathological consequence in compromised wound repair. Intervention of such dynamics by TUDCA may hold promise for the treatment of chronic wounds and other inflammatory diseases.

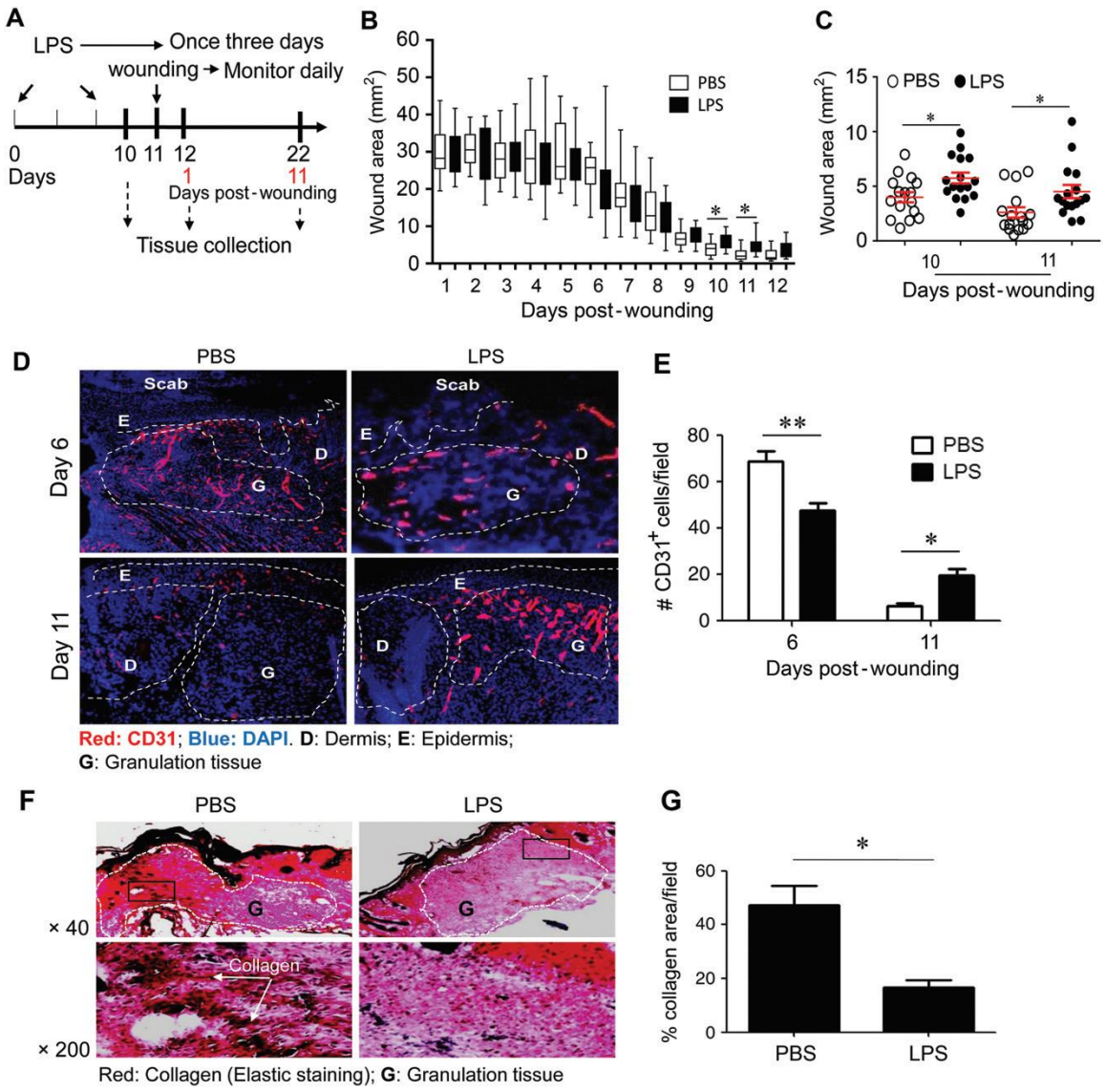

Figure 3.1 Super-low dose LPS pre-conditioning impairs cutaneous wound healing

(A) LPS treatment regimen and the wounding procedure. (B, C) The wounds were monitored and photographed daily. Wound sizes at different time points after wounding are represented in a box and whisker plot; the box regions represent data from the first to third quartiles and the whisker lines represent the remaining outlying data range. Significant differences were apparent on days 10 and 11 after wounding. (C) A separate dot-plot representing days 10 and 11 is shown with mean and SE bars; Student's *t*-test, **p* <0.05. (D, E) Immunohistochemical analyses of: (D) CD31-positive endothelial cells as measurements of blood vessel sprouting in wound tissues; (E) the number of CD31-positive cells in the granulation tissues/viewing field at days 6 and 11 after puncture (six fields/slide, six or seven slide samples/group collected from at least five different animals); error bars show mean±SE; Student's *t*-test, **p*<0.05, ***p*<0.01. (F, G) Super-low-dose LPS reduced collagen content in the granulation tissues at day 11 after puncture: (F) elastic staining; magnifications=(upper panel) × 40, (lower panel) × 200: (G) percentages of collagen in the granulation tissues/viewing field at day 11 after puncture (six fields/slide, six or seven slide samples/group from at least five animals); error bars show mean±SE; Student's *t*-test, **p*<0.05, ***p*<0.01

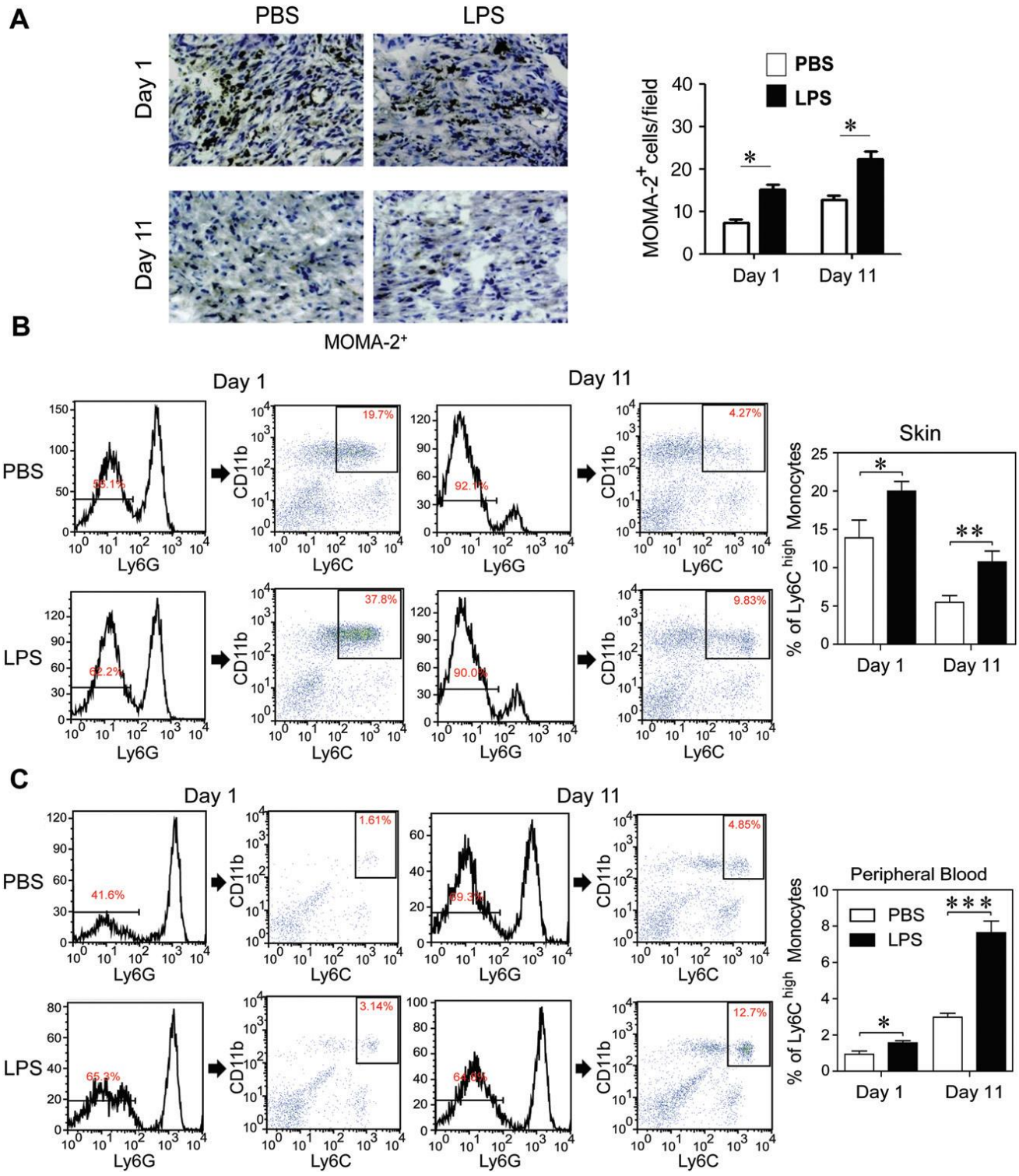

Figure 3.2 Super-low dose LPS increases recruitment of pro-inflammatory monocytes into wound tissues

Figure 3.2 Super-low dose LPS increases recruitment of pro-inflammatory monocytes into wound tissues

(A) Immunohisto-chemical staining of MOMA-2^+ cells in the skin around the wound at day 1 and day 11 post puncture, Brown color: MOMA-2 positive cells. Quantification of MOMA-2^+ cells in the skin around the wound at the indicated time points post puncture (n = 7). **(B)** Flow cytometry analyses of $Ly6G^-/CD11b^+/Ly6C^{high}$ inflammatory monocytes in the wound tissues at the indicated time points post puncture were measured. The frequency of inflammatory monocytes within total leukocytes was quantified. Data are shown from PBS and super-low dose LPS conditioned mice (Day 1, n = 6; Day 11, n = 7). **(C)** Flow cytometry analyses of $Ly6G^-/CD11b^+/Ly6C^{high}$ inflammatory monocytes in circulation. The frequency of inflammatory monocytes within total leukocytes was quantified. Data are shown from PBS and super-low dose LPS conditioned mice (Day 1, n = 6; Day 11, n = 7). Error bars show means ± s.e.m.; * P < 0.05; ** P < 0.01; *** P < 0.001; student t-test.

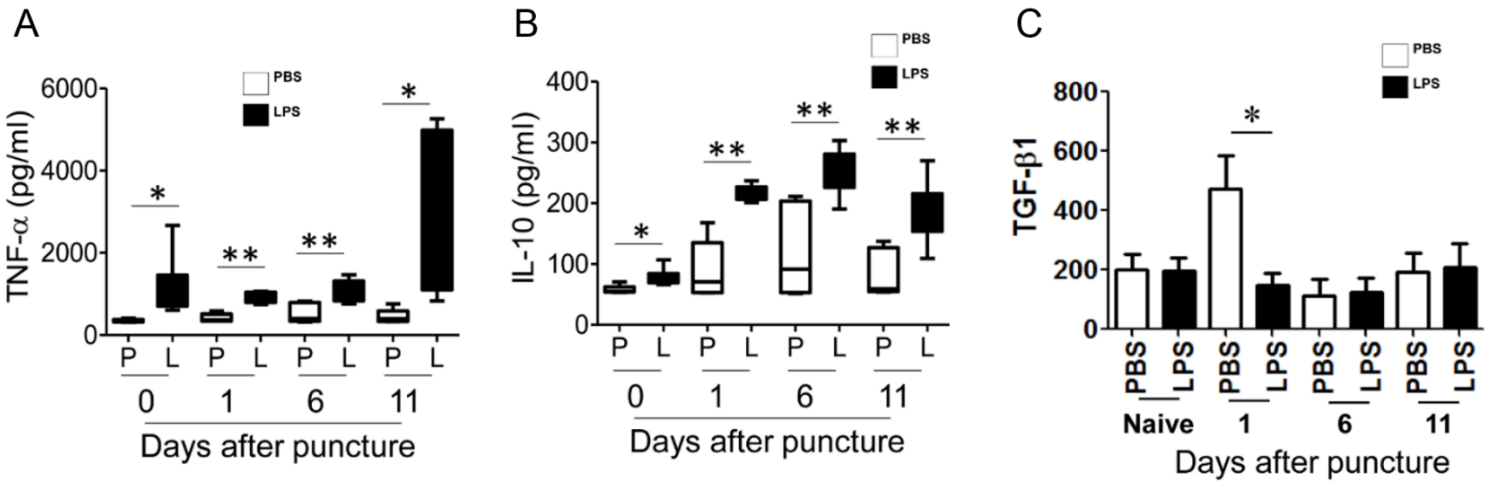

Figure 3.3 Plasma levels of selected cytokines were measured by ELISA analyses.

Blood plasmas were prepared from wounded mice at indicated days post wounding. *p<0.05; ** p<0.01. Data represent at least five animals in each group.

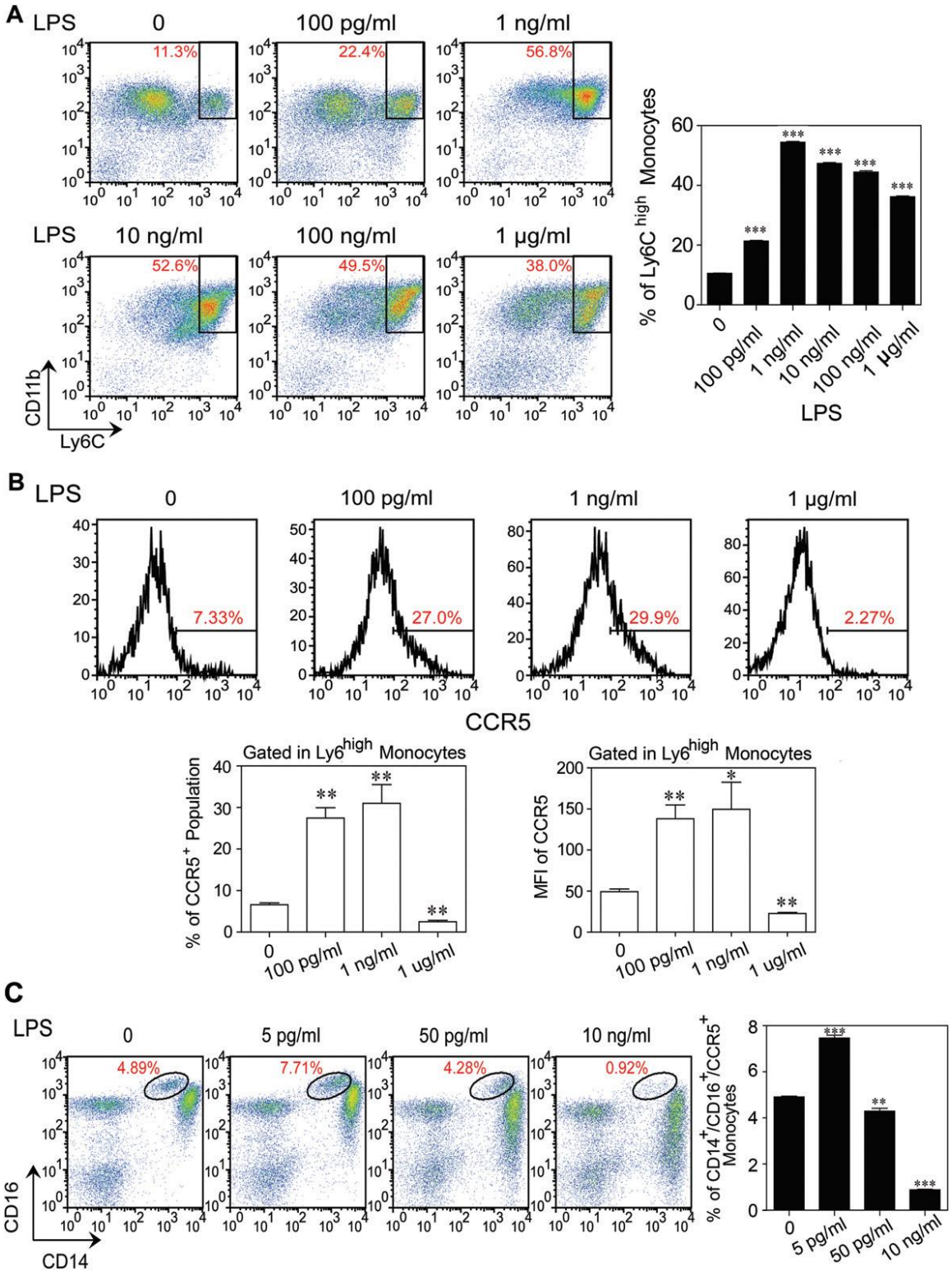

Figure 3.4 Polarization of inflammatory monocytes by sustained challenges with super-low dose LPS

Figure 3.4 Polarization of inflammatory monocytes by sustained challenges with super-low dose LPS

(A) Expansion of $Ly6G^{-}/CD11b^{+}/Ly6C^{high}$ inflammatory monocytes by super-low dose LPS. BM cells from C57 BL/6 mice were cultured with M-CSF (10 ng ml^{-1}) in the presence of different doses of LPS for 5 days, and fresh LPS was added to the cell cultures every 2 days. Representative flow cytometry plots of the frequencies of $Ly6G^{-}/CD11b^{+}/Ly6C^{high}$ inflammatory monocytes were shown (n = 3). **(B)** Induction of CCR5 by super-low dose LPS. The expression levels of CCR5 within $Ly6G^{-}/CD11b^{+}/Ly6C^{high}$ inflammatory monocytes were analyzed by flow cytometry. The frequencies of $CCR5^{+}$ population and the MFI of CCR5 within $Ly6C^{high}$ inflammatory monocytes were quantified (n = 3). **(C)** Super-low dose LPS sustains CCR5 expression in human intermediate inflammatory monocytes. Peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals were culture with M-CSF (100 ng ml^{-1}) in the presence of different doses of LPS for 2 days. Three monocyte sub-populations were detected by flow cytometry based on differential expressions of CD14 and CD16. Frequency of $CD14^{+}/CD16^{+}$ intermediate inflammatory monocytes is displayed. Frequency of $CCR5^{+}$ intermediate inflammatory monocytes were quantified (n = 3). Error bars show means ± s.e.m.; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to controls; one-way ANOVA. Data are representative of three experiments.

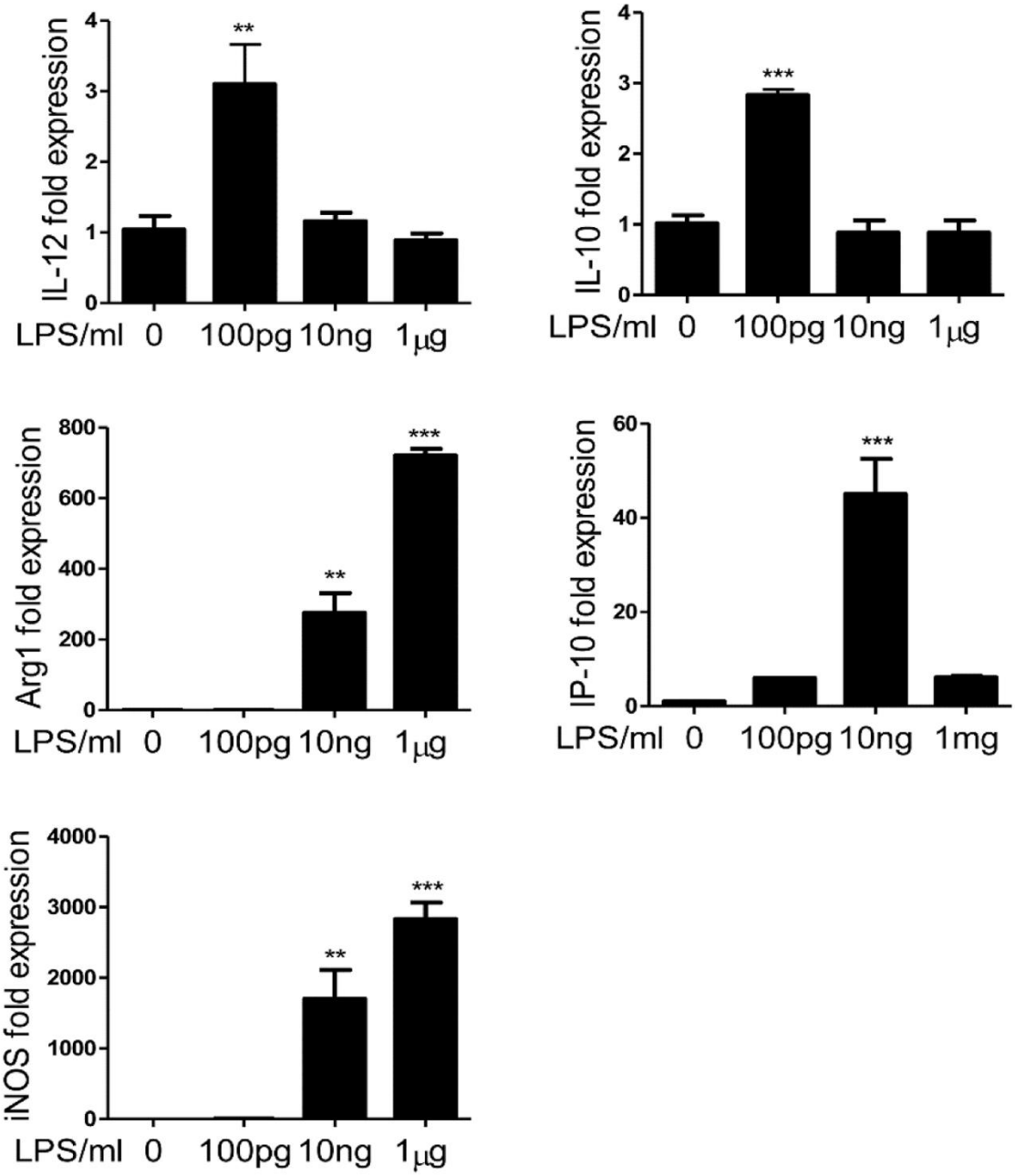

Figure 3.5 Real-time RT-PCR analyses of selected inflammatory mediators from bone marrow-derived monocytes continuously cultured in the presence of varying dosages of LPS as indicated for 5 days.

*p<0.05; ** p<0.01; *** p<0.001 as compared to controls. Data represent at least three independent experiments.

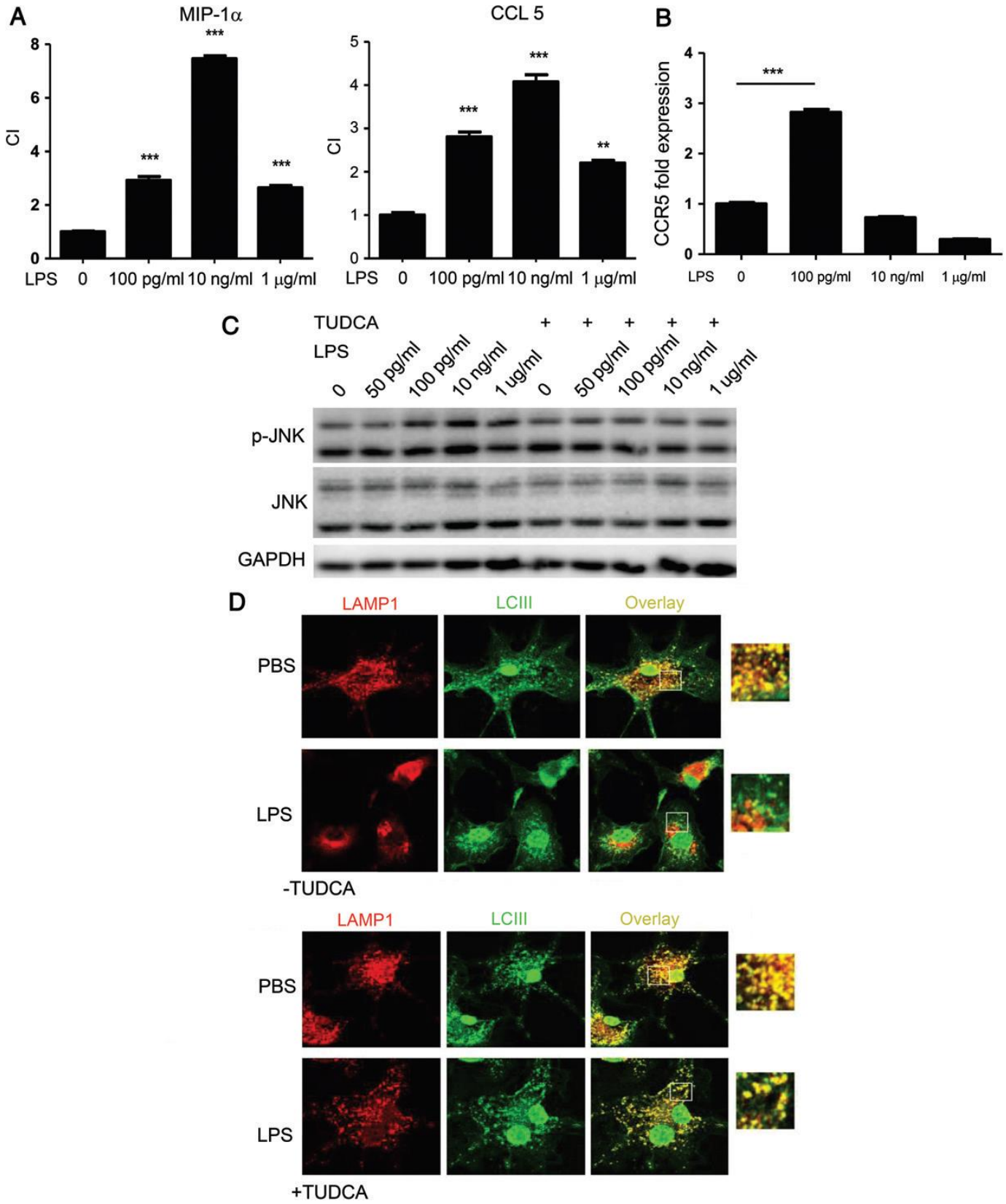
A
MIP-1α
CCL 5
CI
LPS 0 100 pg/ml 10 ng/ml 1 μg/ml
B
CCR5 fold expression
C
TUDCA
LPS
0 50 pg/ml 100 pg/ml 10 ng/ml 1 ug/ml
p-JNK
JNK
GAPDH
D
LAMP1
LCIII
Overlay
PBS
LPS
-TUDCA
+TUDCA

Figure 3.6

Figure 3.6 Super-low dose LPS increases migratory ability of monocytes, and polarizes monocytes via sustained cellular stress

(A) *In vitro* chemotaxis assays of monocytes toward CCL3 and CCL5 following continuous incubation with various concentrations of LPS. The chemotaxis index (CI) were quantified (n = 3). **(B)** real-time RT-PCR analyses of chemokine receptor CCR5 (n = 3). ***p<0.001, as compared to controls. Error bars show means ± s.e.m.; ** P < 0.01; *** P < 0.001 as compared to controls; one-way ANOVA. Data are representative of three experiments. **(C)** Western blot analysis of phosphorylated JNK in cells treated with LPS or LPS plus TUDCA. Total JNK levels served as controls. **(D)** Restoration of autophagy completion by TUDCA. Cultured monocytes were seeded on coverslips and treated with or without super-low dose LPS (100 pg ml^{-1}) for 24 h. TUDCA (500 μM) was added to some cultures. Cells were then stained with anti-LAMP1 antibody and anti-LCIII antibody after starvation. The fusion of lysosomes with autophagosomes was visualized under confocal microscope. Data are representative of three experiments.

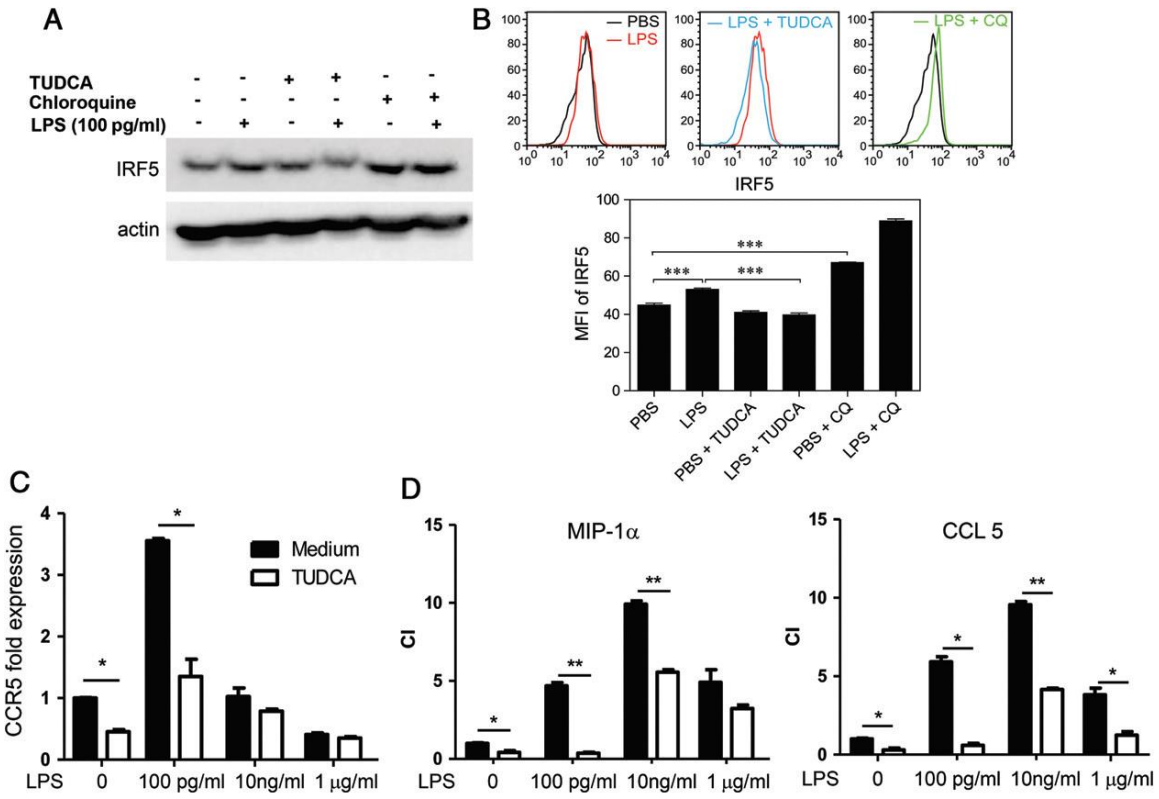

Figure 3.7 Super-low dose LPS polarizes monocytes via IRF5 accumulation

Monocytes from C57 BL/6 mice were cultured with M-CSF (10 ng/ml) in the presence super low dose LPS (100 pg ml^{-1}) for 5 days, and TUDCA (10 nM) or chloroquine (CQ, 1 μM) was also added to some cultures. Fresh LPS, TUDCA and CQ was added to the cell cultures every 2 days. **(A)** The expression levels of IRF5 in monocytes were analyzed by Western blot. **(B)** The expression levels of IRF5 within $CD11b^{+}/Ly6C^{high}$ inflammatory monocytes were analyzed by flow cytometry. The MFI of IRF5 within $CD11b^{+}/Ly6C^{high}$ inflammatory monocytes was quantified (n = 3). **(C)** Real-time RT-PCR analyses of *Ccr5* levels in monocytes treated with either LPS alone or LPS plus TUDCA (n = 3). **(D)** Chemotaxis assays of cultured monocytes toward CCR5 ligands, CCL3 and CCL5. The chemotaxis index (CI) were quantified (n = 3). Error bars show means ± s.e.m.; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA. Data are representative of three experiments.

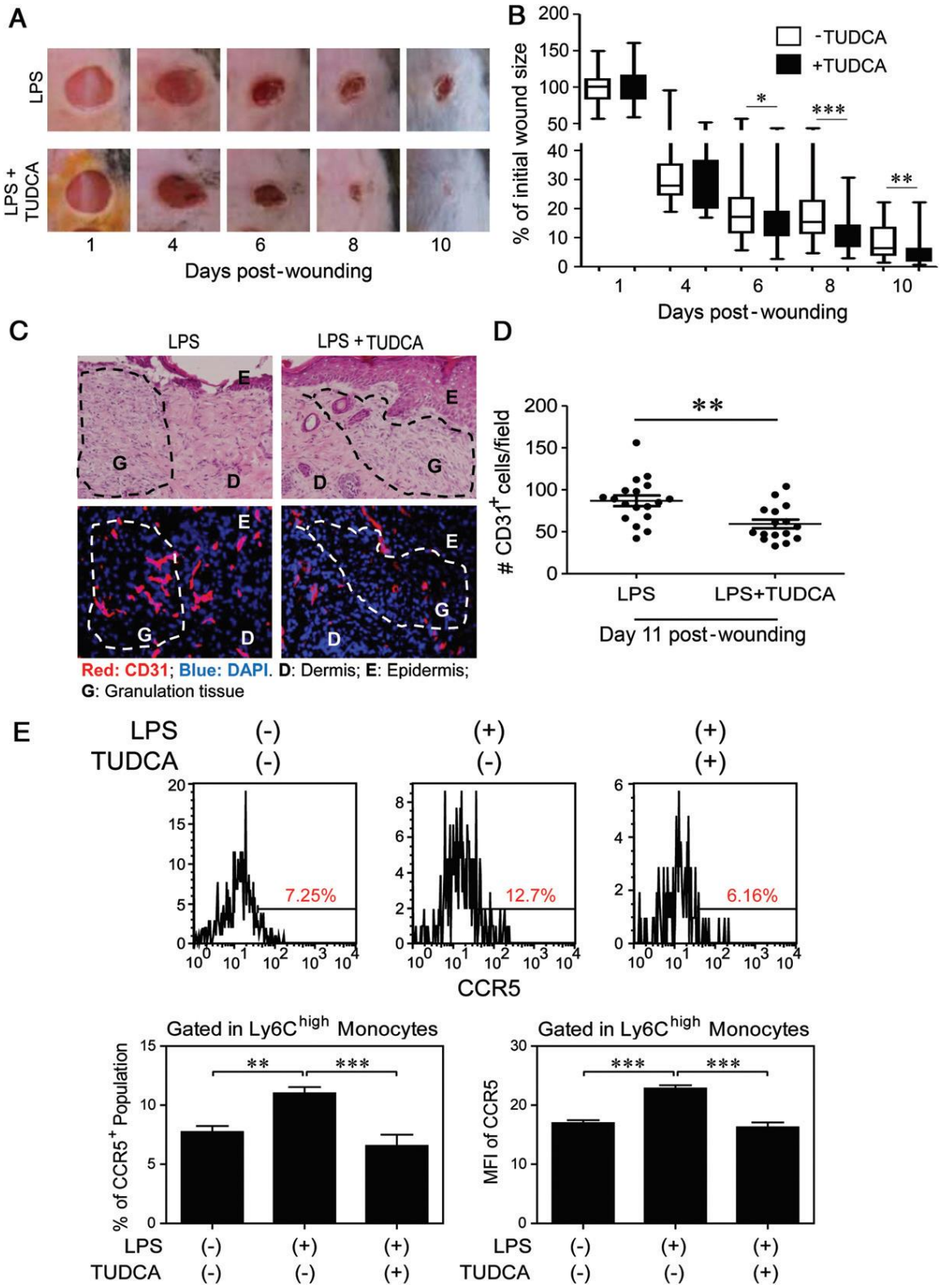

Figure 3.8 TUDCA facilitates wound healing in vivo

Figure 3.8 TUDCA facilitates wound healing *in vivo*

Two groups of mice were injected *i.p.* with super low dose LPS (5 ng/kg body weight) three times for 10 days before skin puncture and once every three days after puncture. After puncture, one group of mice were injected *i.p.* with TUDCA (5 mg/kg) every day for 10 days. **(A)** Wounds were monitored daily, and the changes in wound sizes were calculated. Wound area (Initial wound size, %, n > 8) over the total observation periods were plotted in a box and whisker plot. Student t test, P < 0.05; **, P < 0.01; ***, P < 0.001. **(B)** Immuno-histochemical analyses of CD31 positive endothelial cells as measurements of blood vessel sprouting within the wound tissues (n = 6 fields/slide, 6-7 slides per group). D: Dermis; E: Epidermis; G: Granulation tissue; M: Muscle. Error bars shown on dot plot represent means ± s.e.m. student t test, **, P < 0.01. **(C)** Peripheral blood cells were collected and the expression levels of CCR5 within $Ly6G^{-}/CD11b^{+}/Ly6C^{high}$ inflammatory monocytes were analyzed by flow cytometry. The frequency of $CCR5^{+}$ population and MFI of CCR5 within $Ly6C^{high}$ inflammatory monocytes were quantified (n > 5). Error bars show means ± s.e.m.; ns, not significant; * P < 0.05; **, P < 0.01; ***, P < 0.001; one-way ANOVA.

3.5 References

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Chapter 4. Dynamic programming of innate immunity by bacterial endotoxin and its pathophysiological impacts in sepsis

4.1 Introduction

Sepsis is a life-threatening syndrome commonly experienced by patients in critical care units. Sepsis syndrome poses a particular concern for people with prior health conditions, a phenomenon often referred as "second-hit" or "pre-conditioning" (1,2). Prior immune condition may have a critical impact on the clinical outcome of sepsis. Despite extensive past studies, mechanisms contributing to the alteration of sepsis risks remain poorly understood. A classic example of innate immunity pre-conditioning is manifested in the endotoxin tolerance of innate leukocytes (3). Most studies regarding cellular responses to endotoxin lipopolysaccharide (LPS) utilized tolerant dosages of LPS (often referred as "low dose" in the literature) (>10 ng/mL *in vitro* culture, >1 μg/mouse or >50 μg/kg body weight *in vivo* injection). Low dose LPS (L-LPS) causes robust induction of pro-inflammatory mediators in monocytes/macrophages through the Toll-Like-Receptor 4 (TLR4) pathway (4). Shortly after the initial wave of expression, host leukocytes develop a state of "endotoxin tolerance", in which the expressions of pro-inflammatory mediators are suppressed (5-7). Endotoxin tolerance serves as a compensatory mechanism for the resolution of inflammation (8,9). Based on the tolerance concept, endotoxin has been employed experimentally to elicit a protective mechanism for subsequent inflammatory conditions that include ischemia injury and sepsis (10,11). Mechanistically, the prior conditioning with L-LPS not only reduces tissue inflammation through inducing tolerance, but also boosts bacterial killing activities of neutrophils partly through increasing the formation of neutrophil extra-cellular trap (NET) (12). NETs released by neutrophils not only traps and clears bacteria, but may also degrade inflammatory mediators and therefore limit further exacerbation of inflammation (13). Indeed, individuals with impaired NETosis demonstrate excessive production of inflammatory mediators from neutrophils and persistent inflammation (14). NET-deficient mice also develop exacerbated and chronic inflammation that can be reduced by adoptive transfer of aggregated NETs (14). In the context of sepsis, depletion of neutrophil NETs *in vivo* results in hyper susceptibility to polymicrobial sepsis (15). Mechanistically, NET formation in neutrophils may depend

upon the activation of the extracellular signal-regulated kinase (ERK) (16,17), and selective inhibition of ERK has been demonstrated to be a potent suppressor of NETosis (16). ERK is known to be activated by LPS and may serve as a compensatory anti-inflammatory signal and contribute to endotoxin tolerance in other innate immune cells (18). Although LPS tolerance may explain the beneficial and protective effect of L-LPS pre-conditioning in the context of sepsis, it may not reconcile increased sepsis mortality often observed in patients with prior health conditions. In sharp contrast, emerging studies hint at an intriguing connection between subclinical super-low levels of circulating bacterial endotoxin lipopolysaccharide (SL-LPS) (<100 pg/mL) in human with adverse health conditions and/or life styles such as chronic infections, aging, chronic smoking and drinking(19-25). Compromised mucosal barriers, altered commensal microbiota, and vasculature leakage collectively contribute to the mild elevation of plasma endotoxin in these individuals. We and others have previously shown that super-low dose LPS (SL-LPS, ~1-100 pg/mL *in vitro*, 100 pg-10 ng/mouse or 5 ng-0.5 μg/kg body weight *in vivo* injection) "primes" monocytes/macrophages for a more robust response to a secondary LPS challenge, a phenomenon known as the "Shwartzman reaction" (5,26-28). At the molecular levels, we demonstrate that SL-LPS preferentially clears away the compensatory anti-inflammatory signals in monocytes/macrophages, in sharp contrast to the effect of low dose LPS (18,26). The dynamic path way switching in monocytes/macrophages induced by varying dosages of LPS may underlie the "pre-conditioning" of innate immunity and "rudimentary" innate memory (29). Despite this intriguing connection, pre-conditioning with SL-LPS in the context of sepsis mortality has never been studied. Thus, we tested the hypothesis that SL-LPS pre-conditioning may exacerbate, instead of alleviate sepsis mortality *in vivo*. We employed the classical cecal ligation and puncture (CLP) model of polymicrobial sepsis, and examined mortality, tissue inflammation, and neutrophil bacterial killing in mice pre-conditioned with either low dose (50 μg/kg body weight) or super-low dose (5 ng/kg body weight) LPS. Another feature of this study involves the emerging concept of innate leukocyte programming. Although emerging studies reported the differential programming of monocytes/macrophages by SL-LPS and L-LPS (30), no data is available with regard to whether neutrophil, a key innate leukocyte in the context of sepsis, could be dynamically programmed by SL-LPS and L-LPS. Our study reveals that

pre-conditioning with super-low dose LPS significantly increased CLP sepsis mortality and tissue inflammation, through reducing neutrophil NETosis and bacterial killing. Counter-intuitively, we observed that SL-LPS significantly suppressed ERK activation in neutrophils, instead of activating ERK. Taken together, our study reveals programming dynamics of innate immunity *in vivo* by SL-LPS.

4.2 Materials and Methods

4.2.1. Mice

Inbred C57 BL/6mice between the ages of 8 and 12weekswere used for experimentation. The mice were bred and housed in our pathogen free, limited access, ultra-barrier facility with a 12-h light-dark cycle. All experiments described were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals, and approval was obtained from the Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State.

4.2.2. Reagents

Fluorescein isothiocyanate (FITC) conjugated anti-mouse Ly6G/Ly6C (Gr-1) antibody and Streptavidin conjugated anti-mouse CXCR2 antibody were from Biolegend. Anti-citrullinated histone H3 antibody was from Abcam and the anti-citrullinated Histone H4 antibody was from Millipore.

4.2.3. Cecal Ligation and Puncture (CLP)

CLP were performed as previously described (31). Briefly, anesthetized mice (male, 8 weeks old) were operated at the abdomen, with the cecum being identified, exposed, and ligated with a 3-0 silk suture at its base below the ileocecal junction. The antimesenteric border of the cecum was punctured once with a 21-gauge needle, and the cecum was gently squeezed to extrude a small amount of stool. The cecum was returned to the abdomen, which was closed in two layers with 3-0 silk by means of running sutures. Sham controls were performed with ligation but without puncture. After surgery, mice were resuscitated

with 1.0 mL of saline including Buprenorphine (0.05mg/kg in 1 mL saline) given by subcutaneous injection. The mice were monitored every 4 hours.

4.2.4. Bacterial counts in blood and peritoneal exudates

The bacterial count was determined as previously described (32). In brief, mice were sacrificed at various days post CLP. Aliquots of serial dilutions were placed on agar dishes (*Difco Laboratories*) and incubated at 37°C. CFU were analyzed after 24 h. The results were expressed as log of CFU per mL or CFU $\times 10^3$/mL of blood.

4.2.5. Pathology

For routine histologic analysis, frozen livers (1 mm × 5 mm), lungs (left lobe) and kidneys (whole kidney) were sectioned, stained with H&E. PMN were counted in a blinded and standardized fashion by light microscopy (Axiovert 40). Briefly, a micrometer ocular (x20) was used to count PMN in 6 different visual fields of each section. The histologic grading of liver injury was evaluated by the following score scale of values: 1). Infiltration of inflammatory cells (Numbers/Field): **1:** 0-9; **2:** 10-19; **3:** 20-29; **4:** 30-39; **5:** 40 and Over; 2). Hepatocyte degeneration (%): **1:** 0-9; **2:** 10-29; **3:** 30-49; **4:** 50-69; **5:** 70 and Over; 3). Hepatocyte necrosis (%): **1:** 0-9; **2:** 10-19; **3:** 20-29; **4:** 30 and Over. For the scoring of lung damage, infiltration of inflammatory cells, vascular congestion and interstitial edema were evaluated. For kidney damage, infiltration of inflammatory cells, necrosis, degeneration of tubular cells and glomerulus damage were evaluated. All parameters were evaluated by the following score scale of values: 0, absent; 1, mild; 2, moderate; and 3, severe (33). All histopathological evaluations were done in a blinded fashion by an independent pathologist.

4.2.6. NET formation through flow cytometry and immunofluorescence

For *in vivo* analyses, mice were administrated with super-low dose (5 ng/kg body weight) or low dose (40 μg/mouse) LPS through *i.p*. injection. After 24 h, splenocytes were harvested and stimulated with PMA (50 nM) for 3 h. Cells were fixed and permeabilized with BD Phosflow^{TM} buffer (BD Biosciences). Anti-citrullinated Histone H4 antibody (Millipore) was labeled with Alexa Fluor 647 using Zenon^{TM} labeling kit (Thermo Fisher

Scientific), and then was used to stain splenocytes together with anti-Ly6G and anti-CD11b antibodies (BioLegend). For *in vitro* studies, bone marrow cells from C57 BL/6 mice were cultured with G-CSF (100 ng/mL) in the presence of super-low dose (100 pg/mL) or higher dose (1 μg/mL) LPS for 3 days, with fresh LPS added to the cell cultures every 24 h. In some experiments, TUDCA (0.5 mM) was also added to the cell culture. After stimulation with PMA (50 nM) for 3 h, cells were fixed, permeabilized and stained with Alexa Fluor 647 labeled anti-Histone H4 (citrulline 3) antibody together with anti-Ly6G and anti-CD11b antibodies. The samples were then analyzed by FACSCanto II (BD Biosciences). The data were processed by FACSDiva (BD Biosciences), or Flow Jo (Tree Star). For *immunofluorescence analyses*, peritoneal cells were harvested from PBS or LPS conditioned-mice at indicated time point post CLP, and spun on glass slides through cytospin. Neutrophils were co-stained with antibodies against Ly6G and citrullinated Histone H3. DAPI was used to stain cell nucleus. Samples were analyzed with fluorescence microscopy.

4.2.7. ELISA of cytokines

Plasma samples were collected from peripheral blood. TNF-α and CXCL1/KC/GROα levels were measured by ELISA using ELISA Kit from eBioscience and RayBiotec, respectively.

4.2.8. Chemotaxis assays

Chemotaxis of neutrophils was measured with 48-well microchambers and polycarbonate filters (5 μm pore size) (NeuroProbe, Cabin John, MD) as described (34). The results were expressed as the mean ± SEM of the chemotaxis index (CI), representing the fold increase in the number of migrated cells in response to chemoattractants over spontaneous cell migration (to control medium).

4.2.9. Statistical analysis

Statistical analysis was performed with Prism software (GraphPad Software, La Jolla, CA). Values were expressed as means ± SEM. The significance of the differences was assessed by Student's t test or one-way ANOVA where appropriate. $P < 0.05$ was considered

statistically significant. The mortality studies were assessed with log rank test with $p<0.05$ considered as significant.

4.3 Results

4.3.1 Opposing sepsis outcomes in mice pre-conditioned with super-low and low doses of LPS

To compare the impacts of low and super-low LPS pre-conditioning on sepsis mortality, we used the murine model of cecal ligation and puncture (CLP). In consistent with previous reports (10,12), mice injected *i.p.* with low dose LPS (50 μg/kg body weight) 24 h before CLP exhibited significantly improved survival as compared to sham mice or mice injected with PBS controls (Fig. 4.1A). In sharp contrast, mice injected with super-low dose LPS (5 ng/kg body weight) displayed significantly reduced survival as compared to PBS controls (Fig. 4.1A).

Next, we examined bacterial loads in blood after CLP. As shown in Fig 1B, bacterial counts were significantly increased in peripheral blood of CLP mice pre-conditioned with 5 ng/kg body weight LPS. In sharp contrast, bacterial counts were significantly reduced in peripheral blood of CLP mice pre-conditioned with 1 50 μg/kg body weight LPS (Fig. 4.1C).

We further examined selected inflammatory markers in circulation. In consistent with previous findings (10,12), CLP mice pre-conditioned with tolerant dose (1 μg/mouse) LPS had significantly reduced levels of plasma TNFα and KC as compared to CLP mice pre-conditioned with either PBS or priming-dose (5 ng/kg body weight) LPS (Fig. 4.1D). In contrast, CLP mice pre-conditioned with priming dose (5 ng/kg body weight) LPS had a significant and persistent elevation of plasma levels of TNFα and KC (CXCL2) as compared to other two groups. Collectively, these data reveal that the mice pre-conditioned with super-low dose (5 ng/kg body weight) LPS displayed significantly reduced survival, increased blood bacteria counts and increased systemic inflammatory responses upon

septic challenge. This is sharp contrast to the protective effects observed in mice pre-conditioned with tolerant low dose LPS.

4.3.2 Differential modulation of tissue injuries in CLP mice pre-conditioned with super-low or low dose LPS

Given the opposite survival outcomes, we further examined tissue damage and inflammation in vital organs. Similar to previous reports, the disease scores and neutrophil infiltrations in kidney, liver, and lung were significantly lower in CLP mice pre-conditioned with tolerant low-dose LPS (Fig. 4.2A-C). In contrast, we observed significantly elevated disease scores and neutrophil infiltrations in kidney, liver and lung from CLP mice pre-conditioned with super-low dose LPS (Fig. 4.2A-C). The elevated neutrophil levels in vital organs are consistent with our above data that the circulating KC levels are highest in the CLP mice pre-conditioned with super-low dose LPS. On the other hand, the reduced neutrophil levels and reduced tissue damages in CLP mice pre-conditioned with tolerant low dose LPS are correlated with reduced KC levels, and in line with previous studies (10,12).

4.3.3 Differential modulation of neutrophil CRXCR2 expression by super-low and low dose LPS

Neutrophil infiltrations in vital organs are not only affected by the circulating levels of inflammatory chemokines such as KC, but also by the surface expression levels of chemokine receptors such as CRXR2. Indeed, CXCR2 was shown to play a critical role in CLP-related organ injury and mortality, and that tolerant dose LPS potently leads to a dramatic reduction in cellular levels of CXCR2 in neutrophils, correlating with reduced neutrophil infiltration in vital organs (35,36). We confirmed this finding as shown in Fig. 4.3. The levels of CXCR2 expression on peritoneal neutrophils harvested from CLP mice pre-conditioned with low dose LPS were significantly reduced as compared to CLP mice pre-conditioned with PBS (Fig. 4.3A-B). In contrast, we observed that super-low dose LPS failed to significantly reduce the expression levels of CXCR2 (Fig. 4.3A-B). We further tested the *in vitro* modulation of CXCR2 by LPS. As shown in Fig. 4.3C-D, only higher dose LPS led to significant reduction of CXCR2 expression in cultured neutrophils as

measured by flow cytometry. In contrast, super-low dose LPS failed to significantly alter the expression levels of CXCR2. Coupled with the fact the super-low dose LPS pre-conditioning leads to a primed induction of KC, the CXCR2 ligand, these results may explain the increased infiltration of neutrophils in vital tissues such as liver and kidney in CLP mice pre-conditioned with super-low dose LPS.

4.3.4 Differential modulation of neutrophil NET formation by super-low and low dose LPS

In addition to the differential modulation of inflammatory responses, we also observed differential bacterial loads in circulating blood of CLP mice pre-conditioned with super-low and low dose LPS. Our data suggest that the bacterial killing ability of neutrophils may be altered in mice pre-conditioned with varying dosages of LPS. Indeed, it has been reported that pre-conditioning with low dose LPS facilitates neutrophil NET formation, thus favoring bacteria clearance (12). NET is also implicated in the clearance of inflammatory mediators and the reduction of inflammation (13). Thus, we focused our current study to examine the NET formation. First, we stained the citrullinated Histones in peritoneal neutrophils as an indication of NET formation. Consistent with previous findings, we observed elevated NET formation in peritoneal neutrophils from CLP mice pre-conditioned with low dose LPS (Fig. 4.4A). In contrast, we observed that peritoneal neutrophils from CLP mice pre-conditioned with super-low dose LPS failed to have noticeable elevation in NET formation, as observed under microscopy.

To better examine and quantitate the neutrophil potential of generating NET, we used flow cytometry to measure neutrophils containing citrullinated Histones. As shown in Fig. 4.4B, significantly higher percentages of neutrophils (54.7%) harvested from mice pre-conditioned with low dose LPS were positive for citrullinated Histones upon *ex vivo* PMA challenge, as compared to neutrophils from PBS pre-conditioned mice (45.2%). In contrast, significantly lower percentages of neutrophils (26.5%) from mice pre-conditioned with super-low dose LPS were positive for citrullinated Histones upon *ex vivo* PMA challenge.

We further examined NET formation in cultured neutrophils conditioned with varying dosages of LPS *in vitro*. Consistent with our *in vivo* and *ex vivo* data, we observed that G-

CSF cultured neutrophils together with super-low dose LPS exhibited reduced NET formation triggered by PMA (Fig. 4.4C, D). In sharp contrast, cultured neutrophils with low dose LPS had significantly elevated NET formation upon further PMA challenge. Taken together, our data reveal that super-low dose LPS pre-conditioning dampens, while high dose LPS pre-conditioning potentiates the NET forming potential of neutrophils both *in vitro* and *in vivo*.

4.3.5 Differential modulations of JNK and ERK by super-low and low dose LPS contribute to dynamic modulation of neutrophil function

Next, we focused on examining the molecular mechanisms responsible for the opposite regulation of NET formation in neutrophils pre-conditioned by super-low and low dose LPS. Studies from other systems suggest that ERK is responsible for NET formation (16,17), while recent studies suggest that JNK and ERK may form a mutually competitive signaling circuit (37-40). However, no published study is available with regard to the potential regulation and involvement of ERK/JNK in NET formation in neutrophils challenged with varying dosages of LPS. Based on these clues, we first tested the activation status of JNK and ERK in neutrophils via flow cytometry. As shown in Fig. 4.5A and consistent with previous studies, we observed that neutrophils cultured with 1 μg/mL tolerant dose LPS had significantly elevated levels of p-ERK. In contrast, we observed that super-low dose LPS selectively activated p-JNK accompanied with significantly reduced the cellular levels of p-ERK. This further supports the hypothesis that super-low and low dose LPS differentially skew the dynamic signaling circuit within neutrophils.

We further explored a potential intervention strategy in re-balancing neutrophil responses. Among published studies with existing small chemical compounds, we noticed the intriguing effects of TUDCA, a bile acid derivative from the Chinese traditional medicine (41). TUDCA was shown to have remarkable beneficial effects in treating wound and injury, as well as other inflammatory conditions (41,42). At the biochemical level, TUDCA was shown to suppress JNK and activate ERK in other cellular systems (41,43). We hypothesize that TUDCA may be able to intervene the harmful effects of super-low dose LPS in neutrophils. Indeed, we observed that co-stimulation of cultured neutrophils

with TUDCA and super-low dose LPS restored ERK activation and suppressed JNK activation as compared to neutrophils treated with super-low dose LPS alone (Fig. 4.6A).

Finally, given the promising effects of TUDCA on JNK and ERK in neutrophils, we further tested its effects on neutrophil NET formation. As shown in Fig. 4.6B, co-stimulation of neutrophils with TUDCA and super-low dose LPS restored the NET-forming ability of neutrophils as assessed by flow cytometry. Taken together, our data suggest that super-low dose LPS reduces neutrophil NET formation through suppressing ERK activation. Further, TUDCA may restore neutrophil NET formation by augmenting ERK activation in neutrophils challenged with super-low dose LPS.

4.4 Discussion

Collectively, our current study provides one of the first systematic analyses of *in vivo* relevance of dynamic innate pre-conditioning in the context of sepsis (Fig. 4.7). Mortality risks for sepsis vary dramatically in humans, and patient conditions prior to septic insult may be a critical contributing factor. To this regard, previous studies solely examined the potential beneficial effects of "tolerant" dose LPS in animal models of sepsis (10,12). These studies are consistent with *in vitro* mechanistic studies that tolerant dose LPS tempers the pro-inflammatory cytokine storm (3). Intriguingly, recent studies in humans and mice reveal the presence of subclinical super-low dose LPS in circulation, potentially responsible for non-resolving chronic inflammation (44,45). We and others reported that innate leukocytes can be dynamically pre-conditioned to opposite functional states in *vitro*, with super-low dose LPS selectively "primes" the expression of pro-inflammatory cytokines (26,46). Despite the emerging significance of dynamic innate immunity preconditioning or "training", no existing study is available to systematically reconcile the diverse effects on sepsis. We have aimed at an integrated study that compares and reconciles the pre-conditioning effects of both super-low and low dose LPS in septic mice. Consistent with previous studies, we found that CLP-mice pretreated with low dose LPS showed reduced mortality, reduced inflammatory responses, diminished injury of vital organs (liver, lung and kidney), and increased NET formation as well as effective bacterial

clearance. In sharp contrast, CLP-mice pre-conditioned with super-low dose LPS displayed increased mortality, elevated plasma levels of TNF-α and KC, increased neutrophil infiltration in vital tissues, attenuated ability of neutrophil NET formation and increased circulating levels of bacteria. Our analyses reveal the pathological relevance of super-low dose endotoxemia, and provide a glimpse of potential mechanisms responsible for the dynamic programming of innate environment.

Our data extend the intriguing dynamics of innate priming and tolerance to an *in vivo* model of sepsis. Emerging *in vitro* studies reveal that innate monocytes may adopt distinct phenotypes by prior conditioning or "training" with varying dosages of innate stimulants (26,47,48). For example, pre-conditioning with super-low dose LPS "primes" the expression of selected pro-inflammatory mediators such as TNFα and IL-12, while suppressing the expression of iNOS (27). In contrast, pre-conditioning with elevated low dose LPS "tolerizes" the expression of selected genes (27). In consistent with these *in vitro* studies, we confirmed the "priming" and "tolerance" effects *in vivo*, and demonstrated that pre-conditioning with super-low dose LPS exacerbates the levels of TNFα and KC in CLP-mice. Studies also suggest that the innate responses to varying dosages of LPS may not fit into the simple paradigm of priming and tolerance, and hint at more complex adaptation profiles. For example, FPR1 expression is not tolerizable with LPS (49), and our current functional study is consistent with this phenomenon. On a separate note, the adaptation phenomenon to varying dosages of LPS is different as compared to the traditional M1/M2 concept, in which distinct agonists are required for the differential activation of M1 (by IFNγ) or M2 (through IL-4) (50). Similar adaptation paradigm to varying signal strength may also exists in other immune cell types. For instance, although T helper cells are well known to adopt different functional states (Th1, Th2, Th17, Treg) when challenged with distinct combination of cytokines (51), emerging recent study reported that T helper cell differentiation can also be achieved through varying the signal strength of the TCR signal (52,53). Collectively, these studies draw serious attention to the issue of dynamic responses of immune cells when challenged with varying dosages of the same stimulant.

Our study also reveal additional novel aspects of innate pre-conditioning and adaptation in neutrophils. Neutrophil is a key innate leukocyte, and its modulation bears critical

relevance to the outcome of sepsis (54). Neutrophils have multiple functions that include cytokine expression, degranulation, suppression of T cells, and NET formation (55). Although the dynamic pre-conditioning of monocytes by varying dosages of LPS is well noticed, no information is available regarding the dynamic responses of neutrophils in this context. Our current study fills this critical void and reveals intriguing dynamics of neutrophils when challenged with varying dosages of LPS. We particularly focused on neutrophil NET and revealed opposing behaviors of NET generation in neutrophils pre-conditioned with super-low and low dose LPS. Our current work offers a first glimpse of neutrophil dynamics pre-conditioned with varying dosages of LPS, and begets future systems studies about the complex programming of neutrophils in health and disease. Furthermore, or data suggest that differential pre-conditioning of neutrophils may be harnessed for the effective treatment of sepsis.

In term of underlying molecular mechanisms, our study eludes to the functional significance of mutually inhibitory circuits in fine tuning leukocyte responses. Traditional studies overly simplified the signaling systems into cascades of events that by no means can reconcile the complex dynamics of living systems. Instead, emerging studies from other systems emphasize the necessity of mutually inhibitory circuits required for complex cellular outcomes (56). This may enable organisms highly coordinated adaptation to changing environments. We reported dynamic circuits that are responsible for the priming and tolerance phenotypes in monocytes/macrophages challenged with varying dosages of LPS (18,29). Similarly-wired circuits also exist in T Helper cells, responsible for the dynamic differentiation into a plethora of T helper cells (51). JNK and ERK may constitute a mutually inhibitory circuits based on previous studies (37-40). In this current study, we first observed that super-low dose LPS selectively suppresses ERK and favors JNK activation in neutrophils. Furthermore, suppression of ERK by super-low dose LPS correlates with suppressed neutrophil NET formation and reduced bacterial killing activity. In contrast, higher dose LPS may flip this circuit, induces ERK and NET formation. Mechanisms responsible for the competitive regulation of ERK and JNK are not clear, and warrant future studies. One possible scenario may be the competitive regulation of opposing MAP kinase phosphatases (MKPs). A recent study indicates that the varying signal strength of CD40 ligand may differentially activate either MKP1 (a selective JNK

and p38 phosphatase) or MKP3 (a selective EKR phosphatase) (57). Further comprehension of the dynamic circuits within innate leukocytes is clearly warranted. Capitalizing on this initial finding, we attempted at re-balancing the skewed circuit in neutrophils with a promising drug TUDCA. We demonstrate that TUDCA can restore ERK activation in the presence of super-low dose LPS, as well as the NET formation in neutrophils. This attempt raises an intriguing potential for the future treatment of sepsis through re-balancing network dynamics in leukocytes. Supporting this concept, a recent study suggest that pre-conditioning with an un-conventional TLR2 agonist may render a broader protection toward viral infection (58).

Taken together, our data reveal the dynamic effects of innate pre-conditioning in the outcomes of sepsis, and provide a focused mechanism for differential modulation of neutrophil NET formation by super-low and low dose LPS. Clearly much is needed to further study this important question. Our study suggest that strategies aimed at re-balancing the innate immunity dynamics may hold promise in the treatment of severe sepsis.

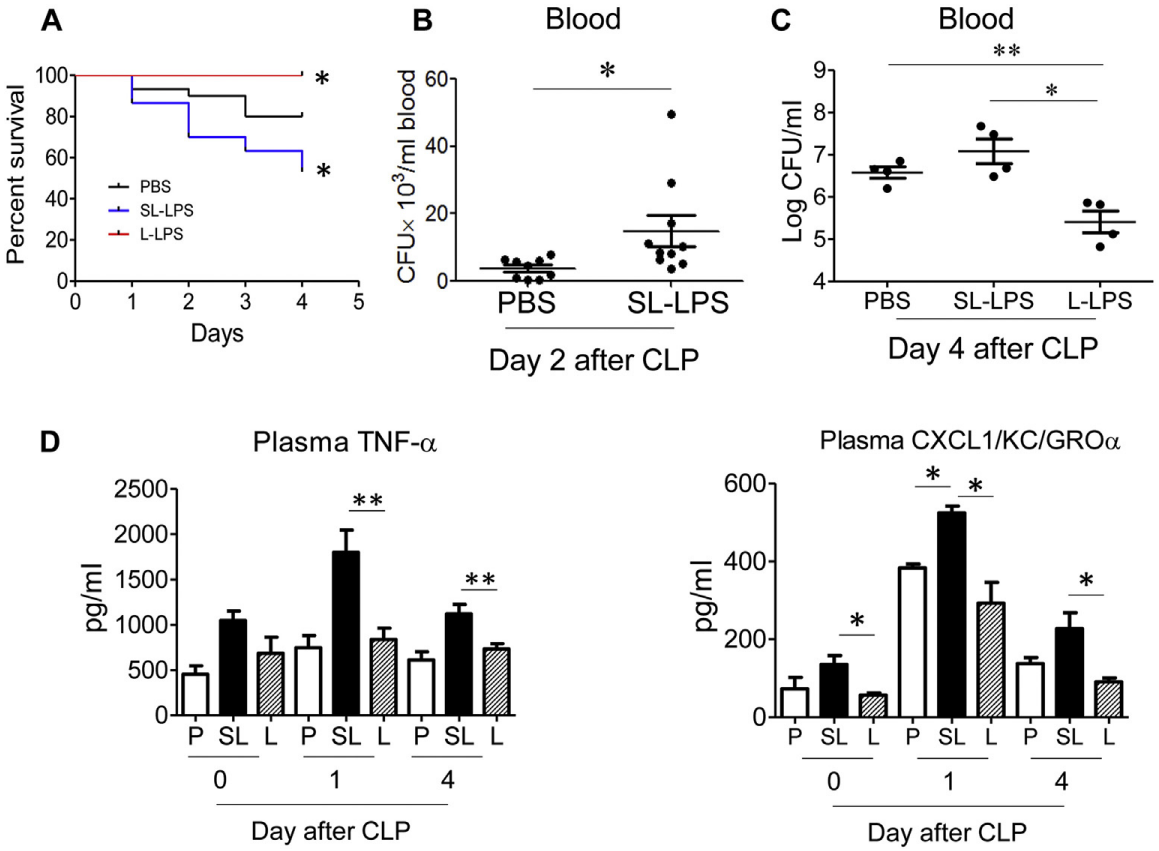

Figure 4.1 Opposite sepsis outcomes in mice pre-conditioned with super-low and low dose LPS.

(A). WT mice (male, 8 weeks old) were pre-conditioned through *i.p.* injection of either PBS (30 mice), super-low dose (SL-LPS, 5 ng/kg) LPS (30 mice), or low-dose (L-LPS, 50 μg/kg) LPS (30 mice). CLP were performed 1 d post pre-conditioning, and mortality was closely monitored every 4 h for 4 d. *p<0.05. (B). Blood were collected from mice d 2 post CLP. Bacteria were cultured, counted and plotted. The results were expressed as colony-forming units (*CFU*)/mL. *$P < 0.05$, N= 11 mice per group. (C). Blood were collected from mice d 4 post CLP. Bacteria were cultured, counted and plotted. The results were expressed as log of colony-forming units (*CFU*)/mL. *$P < 0.05$, **$P < 0.01$. N= 4 mice per group, the data were representative from 3 similar results. (D). Blood collected at d 0, 1, 4 post CLP were used to measured plasma levels of TNFα and KC through ELISA. P: PBS; SL: SL-LPS; L: L-LPS *p<0.05; **p<0.01.

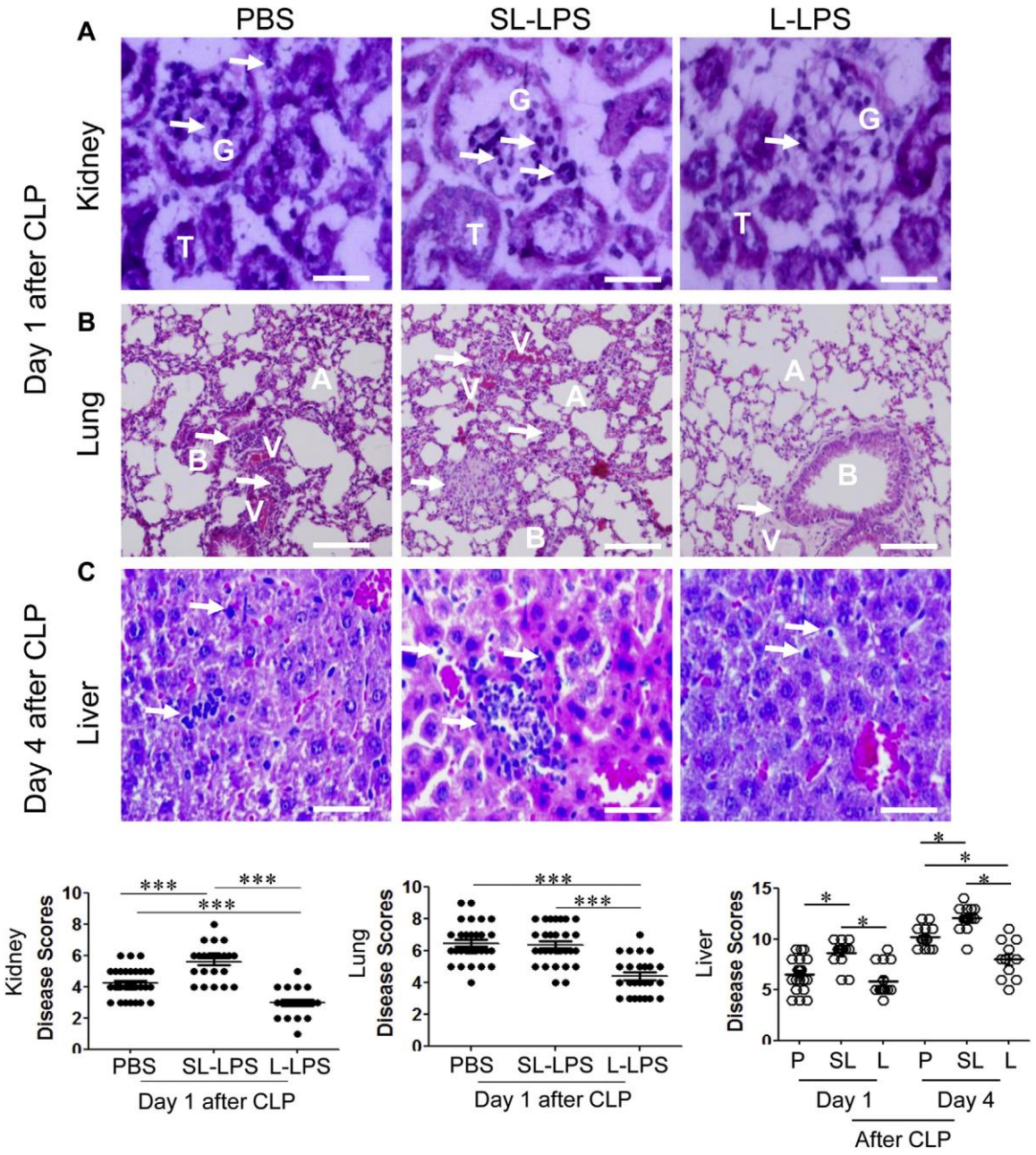

Figure 4.2 Differential modulation of tissue injuries in CLP mice pre-conditioned with super-low or low dose LPS.

Vital organs were collected from CLP mice pre-conditioned with either PBS, 5 ng/kg LPS, or low dose (50 μg/kg) LPS. (**A**) Frozen kidney sections were stained with H & E. G: glomerulus; T: tubular cells; Arrow: PMN. Scale bar=50 μm. (**B**) Frozen lung sections (5 μm) were stained with H & E. A: Alveolus; B: Bronchiole; V: Blood vessels; Arrow: PMN. Scale bar=50 μm. (**C**) Frozen liver sections (5 μm) were stained with H & E. Arrow: PMN. Scale bar=50 μm. Disease scores were evaluated and plotted. *p<0.05. ***p<0.001.

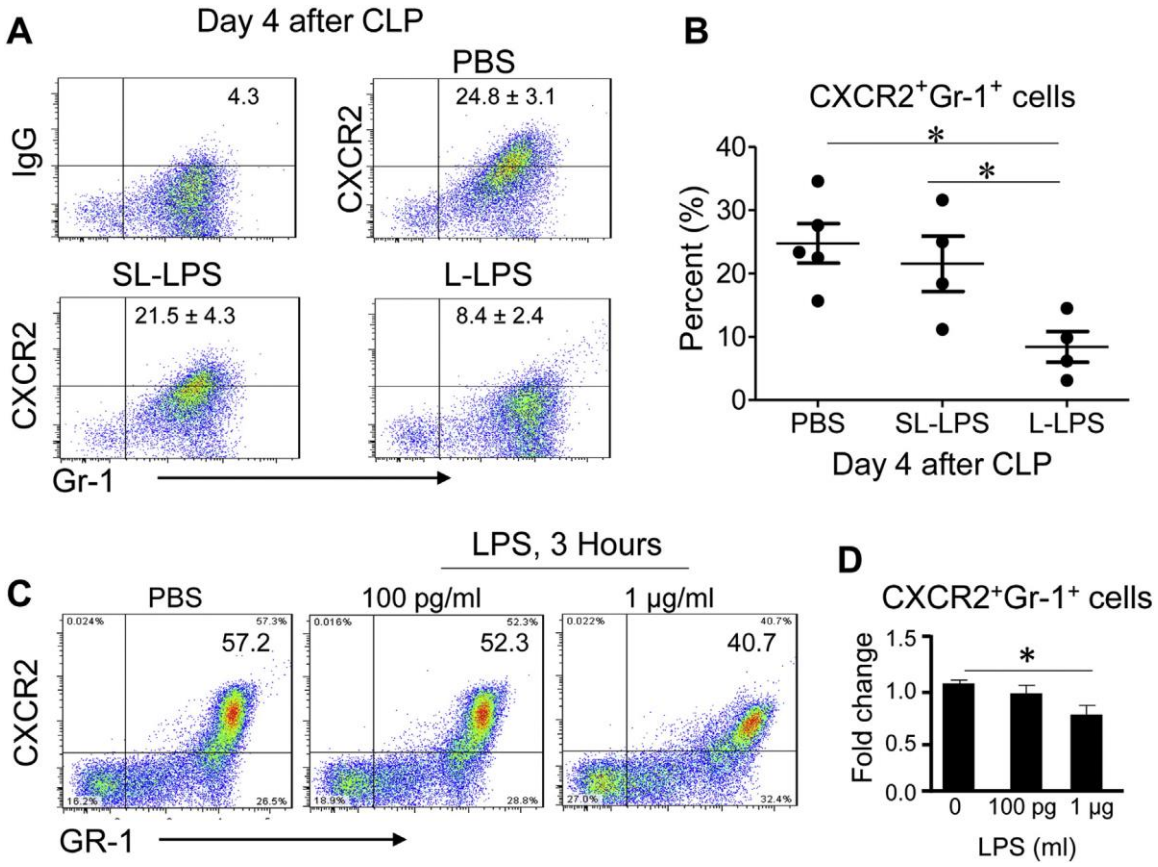

Figure 4.3 Differential modulation of neutrophil migration by super-low and low dose LPS.

(A) CXCR2 expression on the Gr-1$^+$ cell population isolated from the peritoneal lavages of mice d 4 post CLP was analyzed by flow cytometry. Mice were pre-conditioned with either 5 ng/kg (SL-LPS) or 50 µg/kg (L-LPS) 1 d prior to the CLP procedure. (B) Cumulative percentages of CXCR2$^+$Gr-1$^+$ cells from the peritoneal lavage d 4 post CLP. Data from a representative experiment out of 3 performed was shown. Results were expressed as the mean ± SEM, N = 4-5 mice per group. *P < 0.05. All mice used were male and 8-10 weeks old. (C) CXCR2 expression on neutrophils *in vitro* was analyzed by flow cytometry after stimulation of indicated LPS concentration. Bone marrow cells were stimulated with either PBS, 100 pg/mL or 1 µg/mL LPS for 3 h. The expression levels of CXCR2 on neutrophils were quantified through flow cytometry. *p<0.05. (D) Cumulative fold change of CXCR2$^+$Gr-1$^+$ cells *in vitro* after stimulation with LPS. Data from a representative experiment out of 3 performed was shown. Results were expressed as the mean ± SEM, N = triplicate per group. *p<0.05.

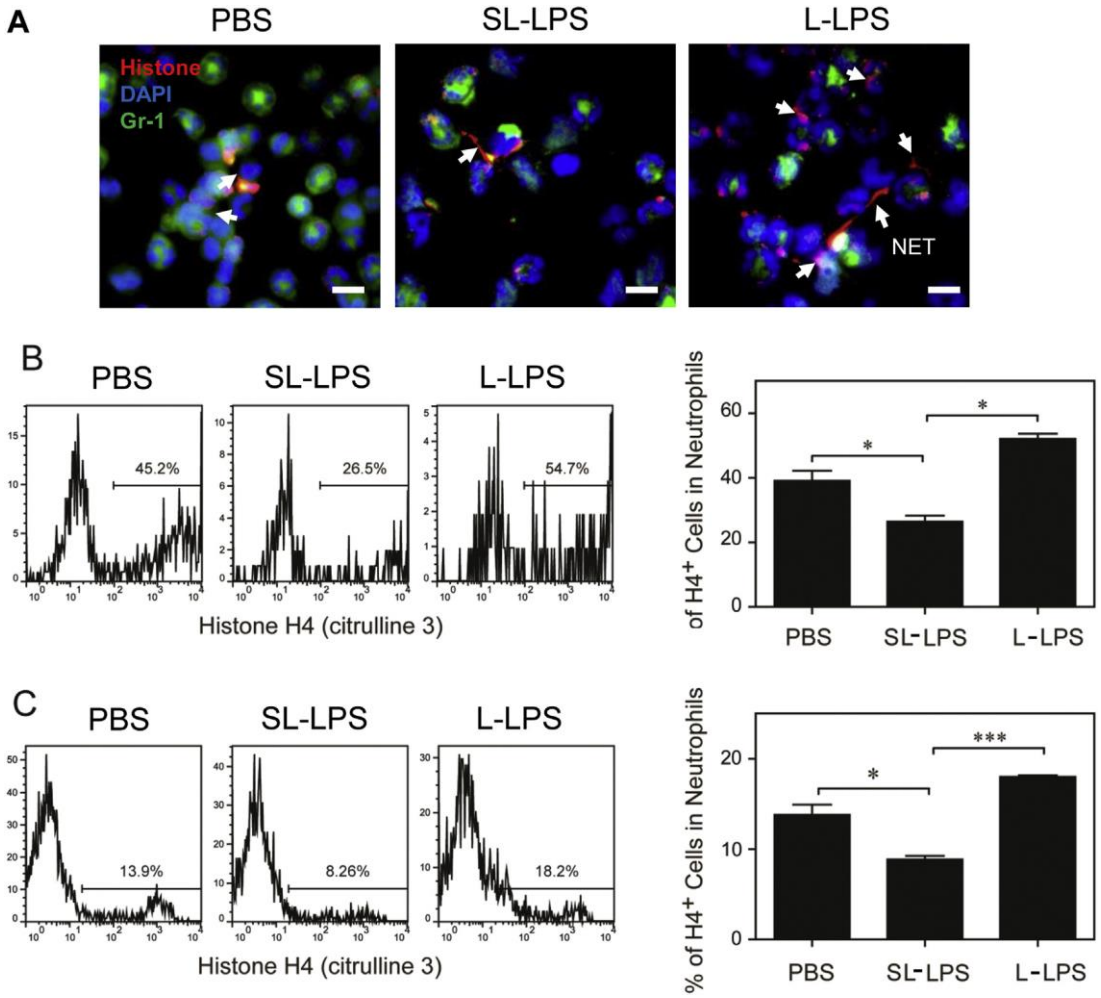

Figure 4.4 Differential regulation of NET formation by super-low and low dose LPS.

(A) The PMN isolated from the peritoneal lavage at the day 1 after CLP from the mice pre-conditioned with either PBS, low dose LPS (L-LPS), or super low dose LPS (L-LPS) were spun on the slides. Neutrophils were stained with the anti-mouse Gr-1 antibody followed by biotinylated anti-Rat Ig antibody and Streptavidin-FITC (green). NETs were stained with the anti-citrullinated Histone H3 antibody followed by the biotinylated goat anti-rabbit Ig antibody and Streptavidin-PE (Red). DNA was stained with DAPI (blue). White arrow: NET. Scale bar = 30 μm. (B) C57 BL/6 mice were *i.p.* injected with super-low dose (5 ng/kg) or low dose (50 μg/kg) LPS. After 24 h, splenocytes were harvested and re-stimulated with PMA (50 nM) for 3 h. The levels of histone H4 (citrulline 3) within $CD11b^+/Ly6G^+$ neutrophils were analyzed by flow cytometry. (C) BM cells from C57 BL/6 mice were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS for 3 days, and fresh LPS was added to the cell cultures every 24 h. After stimulation with PMA (50 nM) for 3 h, the levels of histone H4 (citrulline 3) within $CD11b^+/Ly6G^+$ neutrophils were analyzed by flow cytometry. Quantitative data were shown as means ± SEM from triplicated samples. Asterisks indicate statistically significant differences compared between indicated groups (*, $p < 0.05$; ***, $p < 0.001$).

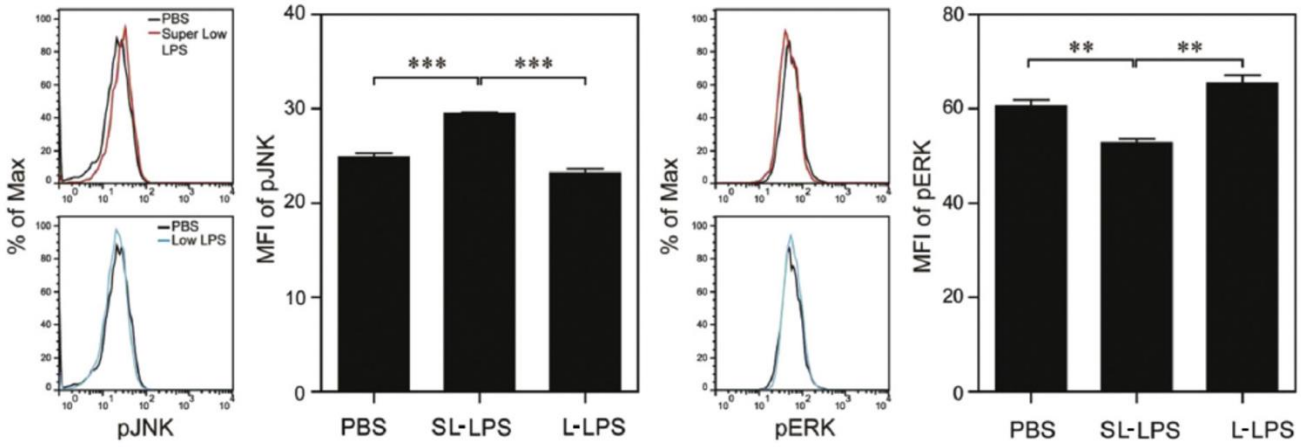

Figure 4.5 Differential modulations of JNK and ERK phosphorylation by super-low and low dose LPS contribute to dynamic regulation of NET formation.

(A) Bone marrow (BM) cells from C57 BL/6 mice were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS. The phosphorylation levels of JNK (T183/Y185) or ERK1/2 (T204/Y202) within $CD11b^{+}/Ly6G^{+}$ neutrophils were analyzed by flow cytometry after 3 days or 3 h respectively. (B) BM cells were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS. Some cells were also treated with TUDCA (0.5 mM). The phosphorylation levels of JNK (T183/Y185) or ERK1/2 (T204/Y202) within $CD11b^{+}/Ly6G^{+}$ neutrophils were analyzed by flow cytometry after 3 days or 3 h respectively. (C) BM cells were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS for 3 days, and some cells were also treated with TUDCA (0.5 mM). After stimulation with PMA (50 nM) for 3 h, the levels of histone H4 (citrulline 3) within $CD11b^{+}/Ly6G^{+}$ neutrophils were analyzed by flow cytometry. Quantitative data were shown as means ± SEM from triplicated samples. Asterisks indicate statistically significant differences compared between the indicated groups (**, $p < 0.01$; ***, $p < 0.001$).

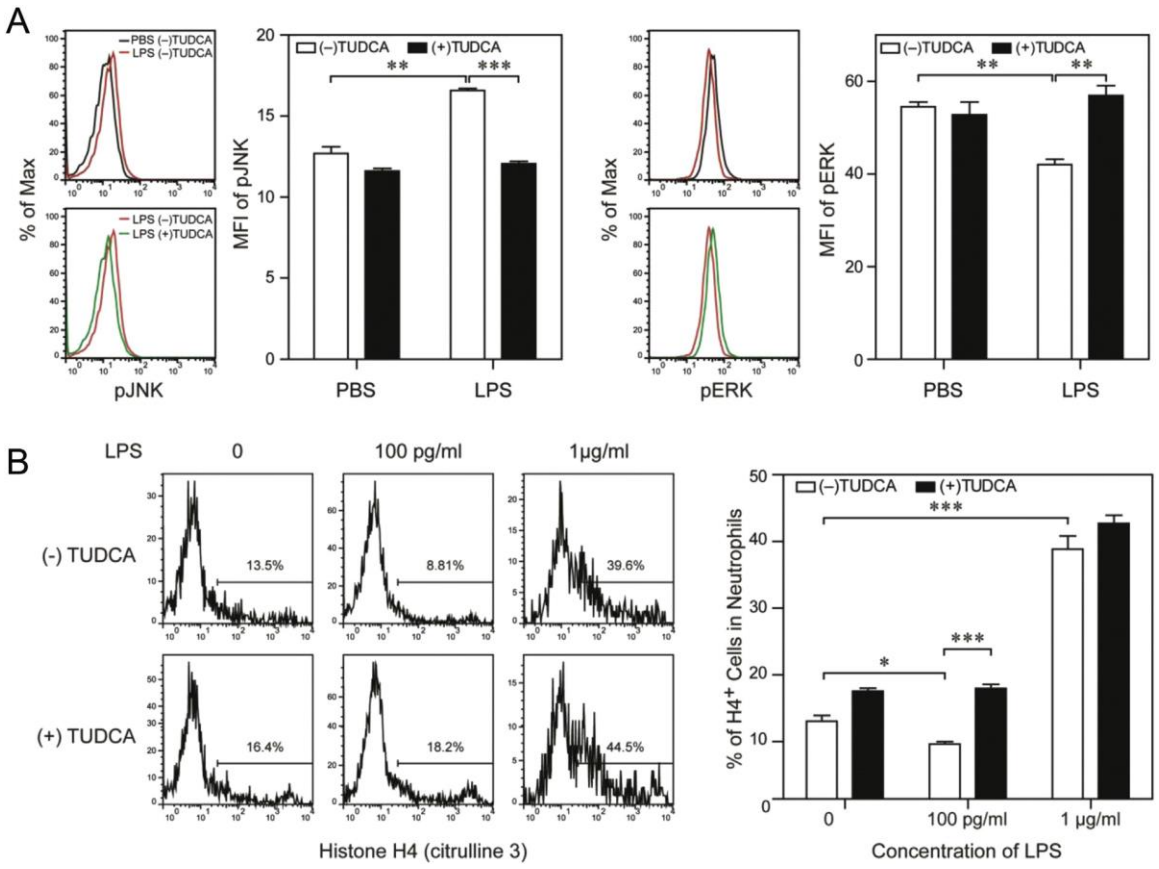

Figure 4.6 TUDCA restores ERK phosphorylation, NET formation in neutrophils treated with super-low dose LPS.

(A) BM cells were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS. Some cells were also treated with TUDCA (0.5mM). The phosphorylation levels of JNK (T183/Y185) or ERK1/2 (T204/Y202) within $CD11b^+/Ly6G^+$ neutrophils were analyzed by flow cytometry after 3 d or 3 h respectively. (B) BM cells were cultured with G-CSF (100 ng/mL) in the presence of either super-low dose (100 pg/mL) or low dose (1 μg/mL) LPS for 3 days, and some cells were also treated with TUDCA (0.5mM). After stimulation with PMA (50 nM) for 3 h, the levels of histone H4 (citrulline 3) within CD11b+/Ly6G+ neutrophils were analyzed by flow cytometry. Quantitative data were shown as means ± SEM from three independently treated samples. Asterisks indicate statistically significant differences compared between the indicated groups (*, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$).

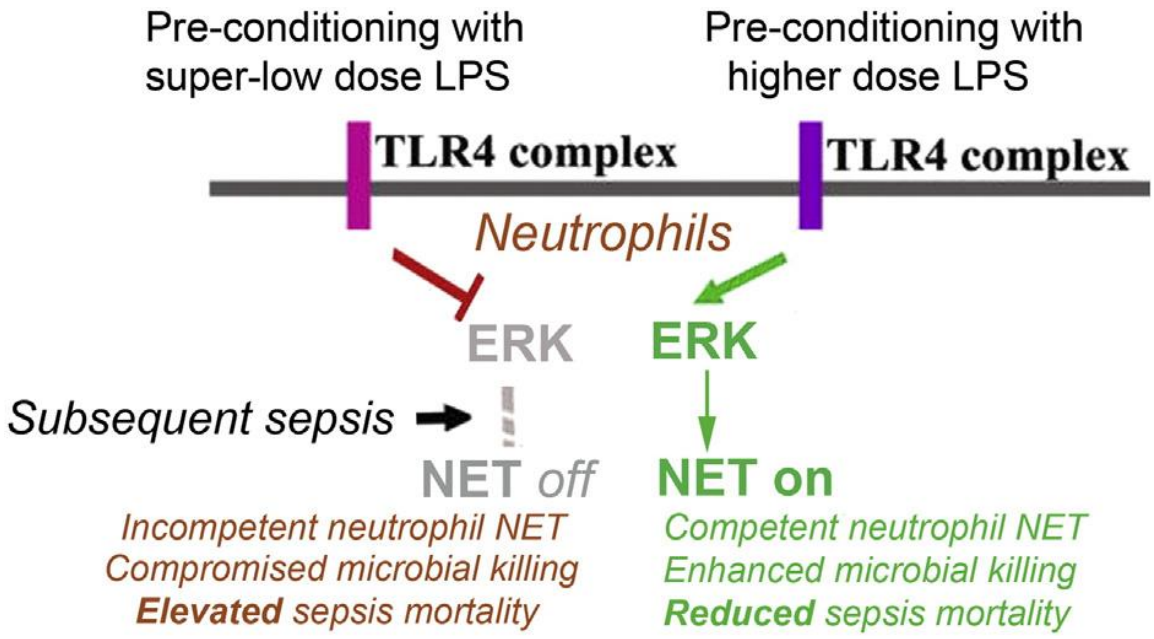

Figure 4.7 An illustrative diagram summarizing the dynamic programming of neutrophils by super-low dose LPS.

Low dose LPS activates ERK and neutrophil NET formation. Together with other tolerant effects in other cells such as monocytes/macrophages, low dose LPS pre-conditioning offers protection to subsequent sepsis challenge. In contrast, this work reveals that super-low dose LPS significantly suppresses ERK and NET formation in neutrophils. This dynamic switch in signaling may exacerbate sepsis mortality. Restoration of this switch may serve as a potential future therapeutic target for the prevention or treatment of sepsis.

4.5 References

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Chapter 5. Molecular mechanisms that underlie the dynamic adaptation of innate monocyte memory to varying stimulant strength of TLR ligands

5.1 Introduction

Emerging studies suggest that innate leukocytes may adopt "rudimentary" memory states depending on challenge history and strength, as reflected in the priming and tolerance paradigm of monocytes challenged with varying dosages of bacterial endotoxin Lipopolysaccharide (LPS) (1). LPS is a membrane component of common mucosa Gram-negative bacteria. Upon leakage into host systemic circulation, higher dosages of LPS may elicit a strong yet transient inflammatory cytokine storm followed by suppression of inflammation and tolerance (2). Both the dramatic upswing of inflammatory cytokines as well as the late-phase refractory tolerance contribute to severe morbidity and mortality associated with sepsis (3). In sharp contrast, subclinical leakage of super-low dose LPS may occur in humans with chronic low-grade inflammatory disease (4,5). Monocytes with prolonged adaptation to super-low dose LPS fail to develop tolerance, and give rise to a non-resolving low-grade inflammatory phenotype conducive for chronic inflammatory disease (6). However, differential monocyte adaptations to challenges by other microbial products have not been well studied.

Upon microbial challenges, Toll-Like-Receptors (TLRs) modulate complex plethora of signaling molecules that eventually activate both transcriptional activators and suppressors of inflammatory mediators. Of particular significance, interferon regulatory factor 5 (IRF5) has been a recognized master transcription factor of pro-inflammatory monocytes and was shown to induce expression of pro-inflammatory genes, such as IL-12 and IL-23, in both murine and human inflammatory monocytes (7). On the other hand, B lymphocyte-induced maturation protein-1 (Blimp-1) has been reported to be a critical transcriptional repressor of inflammatory genes. Blimp-1 may also be involved in the induction of anti-inflammatory mediators (8). In addition, Blimp-1 is essential for modulating homeostasis of NK cell (9), T cell (10) and dendritic cell (11). Blimp-1 also contributes to the homeostatic regulation of bone (12) and intestinal tissues (13). However, the modulations

of IRF5 and Blimp-1 by varying dosage of LPS and their connection to monocyte adaptation have not been well examined.

Emerging data and computational analyses suggest that the potential competition among multiple signaling pathways within monocytes may be responsible for the dynamic adaptation of monocytes (14). In the context of TLR signaling processes, there are at least two potentially competitive pathways, namely MyD88 dependent and MyD88 independent pathways (15). MyD88 is a critical adaptor molecule that directs signaling traffic within innate monocytes. Other key adaptors include TRIF and TRAM (16). Previous studies suggest that MyD88 and TRIF were involved in high dose LPS induced endotoxin tolerance effects (17). However, little is known about the roles of other adaptor molecules, especially TRAM, which was shown to be pro-inflammatory and pro-atherogenic during the pathogenesis of atherosclerosis (18).

To fill the critical void in this intriguing area of innate monocyte adaptation and memory, we examined the differential adaptation of monocytes by varying dosages of LPS and potential underlying mechanisms. We characterized the expression profiles of selected pro- and anti-inflammatory mediators in murine monocytes challenged with varying dosages of LPS as well as TLR3/7 agonists. The dynamic expression profiles of inflammatory mediators were cross-examined with key transcriptional modulators such as IRF5 and Blimp-1. The potential involvement of MyD88 and TRAM/TRIF during the dynamic adaptation of monocytes were studied by employing primary bone-marrow monocytes (BMM) collected from wild type (WT), MyD88$^{-/-}$, TRIF$^{-/-}$ and TRAM$^{-/-}$ mice.

5.2 Materials and Methods

5.2.1 Animals

C57BL/6 were maintained and bred under standard pathogen-free conditions. MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were kindly provided by Dr. Michael Fessler at National Institute of Environmental Health Sciences. TRAM$^{-/-}$ mice were generously provided by Dr. Holger Eltzschig at University of Colorado. 8-12-week-old male mice were used for the

experiments. All animal experiments were approved, prior to the initiation of this study, by the Institutional Animal Care and Use Committee (IACUC) of Virginia Polytechnic Institute and State University.

5.2.2 Reagents

LPS (*Escherichia coli* 0111:B4) was purchased from Sigma. CL264 and Poly I:C were purchased from InvivoGen. Murine macrophage colony stimulating factor (M-CSF) was obtained from PeproTech. Anti-Blimp-1 antibody, anti-IRF5, anti-TRAF2 antibodies were obtained from Cell Signaling Technology. Anti-β-actin antibody was obtained from Santa Cruz.

5.2.3 Protein extraction and analyses

Cells were washed with cold PBS after specified treatments and harvested in SDS lysis buffer containing protease and phosphatase inhibitors as previously described (19,20). Protein concentration was assessed by Bradford assay. Following SDS-PAGE, protein bands were transferred to an immunoblot PVDF membrane (Bio-Rad) and subjected to immunoblot analysis with indicated antibodies. Intensity of each band was quantified using the Multi Gauge software (Fujifilm).

5.2.4 Real-time RT-PCR analyses

Total RNA was extracted using TRIzol (Thermo Fisher Scientific), according to the manufacturer's protocol. RNA was reverse-transcribed using the High-Capacity cDNA Reverse transcription kit (Thermo Fisher Scientific). Real-time PCR was performed on a Bio-Rad CFX96 machine using SYBR Green mix (Bio-Rad). The relative levels of different transcripts were calculated using the ΔΔCt method and results were normalized based on the expression of β-actin.

5.2.5 *In vitro* culture of murine monocytes

Crude BM cells isolated from C57 BL/6 mice were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 1% penicillin/streptomycin (Thermo Fisher Scientific), and with M-CSF (10 ng mL^{-1}) in the

presence of different doses of LPS (from 100 pg mL^{-1} to 1 µg mL^{-1}). Fresh LPS and M-CSF was added to the cell cultures every 2 days. After 5 days, cells were harvested.

5.2.6 Statistical analysis

Statistical analyses were performed using Prism Version 5 software (GraphPad). Significance of difference was analyzed with a Student *t* test. When more than two groups were compared, one-way ANOVA was performed. Data were presented as means ± SEM. P values less than 0.05 were considered significant.

5.3 Results

5.3.1 Monocytes adapted to higher dose LPS exhibit inflammatory tolerance while preferentially express homeostatic mediators. In contrast, monocytes adapted to super-low dose LPS develop a low-grade inflammatory profile without the expression of homeostatic mediators

Although the phenomena of endotoxin tolerance and priming triggered by a short-period of single dose LPS challenge have been well documented (19,21,22), limited studies are performed to characterize the adaptation effects of prolonged LPS exposures on monocytes. In this context, we assessed the gene expression profiles of murine monocytes continuously challenged with varying dosages of LPS for a 5-day period. As shown in Fig 1, monocytes were adapted into an anti-inflammatory "tolerant" phenotype after a prolonged challenge with higher dose LPS (1 µg/ml), as reflected in a significant reduction of selected pro-inflammatory genes such as IL-12 and CCR5 (Fig. 5.1A-B, resembling the phenotype of endotoxin tolerance. Higher dose LPS-adapted monocytes become potent producers of homeostatic genes involved in tissue repair such as ARG1 and iNOS (Fig 1C-D). In sharp contrast, monocytes challenged with super-low dose LPS (100 pg/ml) were adapted to express significantly higher levels of inflammatory mediators (IL-12 and CCR5) as compared to either non-adapted monocytes or high dose LPS-adapted monocytes (Fig. 5.1A-B). The super-low dose LPS-adapted monocytes did not express homeostatic tissue-repair genes ARG1 or iNOS. These data reveal distinct adaptation of monocytes into either

a non-resolving inflammatory state or resolving tolerant state, dependent upon the relative signal strengths of prolonged LPS challenges.

5.3.2 The signature transcription factor of inflammatory monocyte IRF5 is induced in monocytes adapted by super-low dose LPS, and drastically reduced in monocytes adapted by higher dose LPS. On the other hand, the transcriptional modulator Blimp-1 is reduced in monocytes adapted by super-low dose LPS, and elevated in higher dose LPS-adapted monocytes.

IRF5 is a key signature transcription factor within inflammatory monocytes (7,23), while Blimp-1 serves as an important homeostatic modulator in myeloid cells (24). Elevation of IRF5 or reduction of Blimp-1 were associated with the pathogenesis of chronic inflammatory diseases (8,25,26). Together with other transcriptional activators, IRF5 activates the expression of inflammatory mediators. On the other hand, Blimp-1 may serve as an anti-inflammatory mediator through competing and inhibiting target sequences of IRFs (27). To determine the molecular mechanisms responsible for the dynamic adaptation of monocytes, we examined the cellular levels of IRF5 and Blimp-1. Consistent with the tolerant phenotype, monocytes adapted with higher dose LPS (1 μg/ml) had markedly reduced levels of IRF5 and restored Blimp-1 levels as compared to control monocytes (Fig. 5.2A). In sharp contrast, the non-resolving inflammatory monocytes adapted with 100pg/ml LPS had elevated levels of IRF5 and reduced levels of Blimp-1 (Fig. 5.2A).

The reduction of inflammatory suppressor Blimp-1 may allow for the development of non-resolving inflammatory monocyte. We further tested whether reduced gene expression or protein stability may account for Blimp-1 reduction in non-resolving inflammatory monocytes. As shown in Fig. 5.2B, the mRNA levels of PRDM1, the gene encoding Blimp-1, were not altered comparing control monocytes and monocytes adapted by 100 pg/ml LPS. This suggests that Blimp-1 reduction in adapted inflammatory monocytes may be caused by reduced protein stability. To confirm this, we applied proteasome inhibitor MG-132. As shown in Fig. 5.2C, Application of MG-132 abolished the reduction of Blimp-1 in monocytes adapted by lower dose LPS. The degradation of Blimp-1 has not been studied in monocytes, although a previous report suggest that TNF receptor associated

factor 2 (TRAF2) mediated JNK activation may be critically involved in Blimp-1 down-regulation in B cells (28). Previous reports revealed that TRAF2 overexpression was correlated with upregulated expression of inflammatory genes (29,30). In addition, TRAF2 also plays central role in signaling pathway induced by ER stress (31,32). Thus, we further tested the levels of TRAF2 in differentially adapted monocytes. We observed that TRAF2 levels were induced in non-resolving inflammatory monocytes adapted by super-low dose LPS (Fig. 5.2A). In contrast, TRAF2 levels returned to resting levels in tolerant monocytes adapted by 1 μg/ml LPS (Fig. 5.2A).

5.3.3 The non-resolving inflammatory monocyte adaptation is independent of MyD88, but dependent on TRAM and TRIF

Next we tested the roles of TLR adaptor molecules during the dynamic adaptation of monocytes. Bone marrow monocytes from $MyD88^{-/-}$ and $TRIF^{-/-}$ mice were adapted for 5 days with super-low and higher dosages of LPS. As shown in Figure. 5.3A-B, the induction of inflammatory mediators such as IL-12 and CCR5 was not affected by MyD88 deficiency, suggesting that MyD88 is not involved in the inflammatory adaptation of monocytes. In contrast, the induction of inflammatory IL-12 and CCR5 was abolished in TRIF or TRAM deficient monocytes (Fig. 5.3E-F, I-J). On the other hand, the homeostatic monocyte adaption to higher dose LPS as reflected in the expression of ARG1 was dependent upon MyD88, but not TRIF or TRAM (Fig. 5.3C, G, K). Our data further support the emerging concept that the adaptation processes of monocytes are highly dynamic, complex, and may differentially involve unique signaling pathways. In terms of the non-resolving inflammatory monocyte adaptation, TRAM and TRIF, instead of MyD88 are critically required.

Downstream of TRAM/TRIF, we further tested whether TRAM or TRIF may be involved in the activation of IRF5, TRAF2 and reduction of Blimp-1 in monocytes adapted by super-low dose LPS. As shown in Fig. 5.4A, the induction of TRAF2, IRF5 and the reduction of Blimp-1 in inflammatory monocytes adapted by super-low dose LPS (100 pg/ml) were abolished in TRAM deficient BMDM. On the other hand, MyD88 deficient BMM adapted by 100 pg/ml LPS experienced similar induction of TRAF2, IRF5, and reduction of Blimp-

1 as compared to WT BMM (Fig. 5.4B). Compared with WT monocytes, the induction of TRAF2 in TRIF deficient monocytes was attenuated. Taken together, our data suggest that the non-resolving inflammatory adaptation of monocytes to super-low dose LPS is not MyD88 dependent, and dependent on TRAM/TRIF pathway. Our results are consistent with previous findings of pro-inflammatory role of TRAM/TRIF in the chronic pathogenesis of atherosclerosis (18).

5.3.4 TLR7 agonist induces both inflammatory and tolerant adaptation of monocyte, while TLR3 agonist selectively induces inflammatory adaptation without inducing monocyte tolerance

TLR4 is unique among TLRs for its usage of both MyD88 and TRAM/TRIF-dependent pathways. Other TLRs may preferentially use one of these pathways. This led us to explore whether the inflammatory adaptation of monocytes is limited to TLR4 and LPS signaling pathway, or may be similarly programmed by other TLR agonists. To this regard, we specifically tested the effects of TLR3 and TLR7 agonists, given the fact that TLR3 selectively uses TRIF-dependent pathway and that TLR7 may use both the MyD88 and TRAM pathways (16,33). Indeed, monocytes adapted by varying dosages of TLR7 agonist CL264 exhibited similar dynamics as compared to monocytes adapted by LPS. Monocytes with prolonged challenges with lower dose of TLR7 agonist CL264 (100 nM) were selectively adapted into an inflammatory state with elevated expressions of IL-12 and CCR5, and no expression of Arg1 and iNOS (Fig. 5.5A, B, C, D). In contrast, monocytes adapted with higher dose CL264 (100 nM, 1 μM) were tolerant with reduced expression of IL-12 and CCR5, and elevated induction of homeostatic genes such as ARG1 and iNOS.

In terms of the molecular mechanisms, we observed partially similar patterns of IRF5 and Blimp-1 modulation by CL-264 as compared to LPS. Lower dose CL-264 induced IRF5 and reduced Blimp-1. In contrast, although failed to reduce IRF5 level, higher dose CL-264 restored Blimp-1 in adapted monocytes (Fig. 5.5E).

In contrast to TLR7/4 agonists, monocytes failed to develop tolerant adaptation when challenged with TLR3 agonist Poly I:C (Fig. 5.6). The expression of pro-inflammatory mediators IL-12 and CCR5 kept rising with increasing amount of Poly I:C challenges, and

ARG1 was not induced by higher dosages of Poly I:C (Fig. 5.6A-C). At the mechanistic level, the inductions of IRF5 and TRAF2 were unabated by rising concentrations of Poly I:C (Fig. 5.6E). Rising concentrations of Poly I:C also led to a persistent reduction of Blimp-1 without restoration as compared to adapted monocytes by higher dose of LPS challenge (Fig. 5.6E). Taken together, our data reveal that TLR7 agonist induces similar inflammatory monocyte adaptation and tolerance, as elicited by LPS. In contrast, TLR3 agonist preferentially induces inflammatory adaptation without tolerance.

5.4 Discussion

Our data collected from this study offer novel insight with regard to the adaptation dynamics of innate monocytes. We observed that TLR4 agonist LPS may adapt monocytes into either a non-resolving inflammatory state or a tolerance anti-inflammatory state depending upon LPS signal strength. Prolonged monocyte adaptation to higher dosages of LPS led to reduced expression of pro-inflammatory mediators such as IL-12 and CCR5, and elevated expression of anti-inflammatory mediators such as ARG1 and iNOS. In contrast, prolonged adaptation to super-low dose LPS gave rise to an opposite phenotype with elevated expression of IL-12/CCR5. Lower dose LPS may favor the inflammatory monocyte adaptation via the activation of IRF5 and reduction of Blimp-1 through TRAM/TRIF, instead of MyD88, mediated pathway. Resembling TLR4, TLR7 agonist may similarly modulate the dynamic monocyte adaptation, with lower dosages of CL264 inducing, while higher dosages of CL264 reducing the expression of inflammatory mediators IL-12/CCR5. On the other hand, TLR3 agonist Poly I:C exhibited uni-phasic adaptation of pro-inflammatory monocytes. Together, our data demonstrated the potential presence of competing circuitries that can fine tune monocyte memory dynamics in adaptation to varying TLR signal strength.

Monocyte adaptation reflects a unique facet of the emerging innate memory concept (19,34). Previous attempts at defining innate memory have largely limited to the leukocyte phenotypic modifications due to challenges with distinct stimulants, as reflected in the distinct macrophage phenotypes programmed by either IFN-γ or IL-4 (35). Limited

attention has been given to signal strength-dependent programming on innate leukocytes. Reports from us and others indicated that LPS can dose-dependently induce either monocyte/macrophage priming or tolerance to a subsequent higher dose LPS challenge (6,19,36). This current study expands these previous findings and demonstrate that prolonged stimulation of innate monocyte with lower dose LPS drives an inflammatory phenotype, while prolonged stimulation with higher dose LPS causes an anti-inflammatory adaptation. We further observed that monocytes may differentially adapt to prolonged challenges of unique TLR agonists such as TLR7 and TLR3 agonists. Our data may bear significant conceptual and translational implications. In contrast to the simple paradigm of one signal accounting for one phenotype that is a far-cry from the complex dynamics of monocyte memory, our data reveal dynamic adaptation of innate monocytes dependent upon not only the chemical nature of corresponding stimulant, but also the signal strength and history of stimulation. In line with our finding, a recent report demonstrates opposing effects of acute and chronic administration of TGF-β on immune cell activation (37). Our current study provides a conceptual clue which warrants future systems analyses of complex dynamics of monocyte adaptation.

At the mechanistic level, our data clarify an important issue with regard to the differential usages of MyD88 and TRAM/TRIF pathways during monocyte adaptation and memory. Although initial studies with cell lines and over-expression analyses suggested that MyD88 may account for the inflammatory signaling processes down-stream of TLR4 (38), *in vivo* analyses with MyD88 knockout mice suggested a much more complex scenario (39,40). With the chronic atherosclerosis model, TRAM or TRIF deficiency, instead of MyD88 deficiency, was found to have reduced inflammation and atherosclerosis progression (18). Recent molecular analyses reveal that MyD88 pathway not only induces NF-κB activation, but also multiple negative feedback loops that cause tolerant adaptation (41,42). In contrast, our current study was the first report revealing TRAM/TRIF path as the key circuit involved in the low-grade inflammatory adaptation of monocytes. Instead of inducing negative feedback modulators, our data suggest that TRAM/TRIF pathway may be responsible for the removal of negative modulators such as Blimp-1, thus dis-allowing the

development of anti-inflammatory tolerance and favoring the inflammatory monocyte adaptation.

Our observation with regard to inflammatory and anti-inflammatory adaptation of monocytes to distinct TLR agonists with varying stimulant strength may hold potential translational values in therapies against chronic disease. For example, anti-inflammatory monocyte adaptation by higher TLR7 agonist may hold potential value in the treatment of chronic inflammatory disease such as atherosclerosis. Re-enforcing this consideration, a previous animal study with TLR7 agonist has shown promise in alleviating the progression of atherosclerotic plaques (43). It is intriguing to note that TLR3 agonist fails to illicit anti-inflammatory monocyte adaptation, potentially due to its sole usage of TRIF pathway instead of the MyD88 pathway. This is in consistent with some previous reports that demonstrated the lack of tolerance in monocytes/macrophages treated with TLR3 agonist. For example, a previous study with RAW264.7 cells revealed that the pretreatment with Poly I:C led to increased IL-12 production following a subsequent LPS stimulation (20,44). At the translational level, low-grade inflammatory monocyte adaptation by TLR3 agonist may serve as a potential strategy to boost immune surveillance of tumor. Our work also re-enforces the significance of carefully considering the effects of drug dosages in the prevention and treatment of chronic inflammatory diseases. Extensive future studies are warranted to perform fine-mapping of monocyte phenotypes in adaptation to diverse TLR agonists and antagonists, in order to harness the full potential of monocyte memory in the treatment and prevention of human disease.

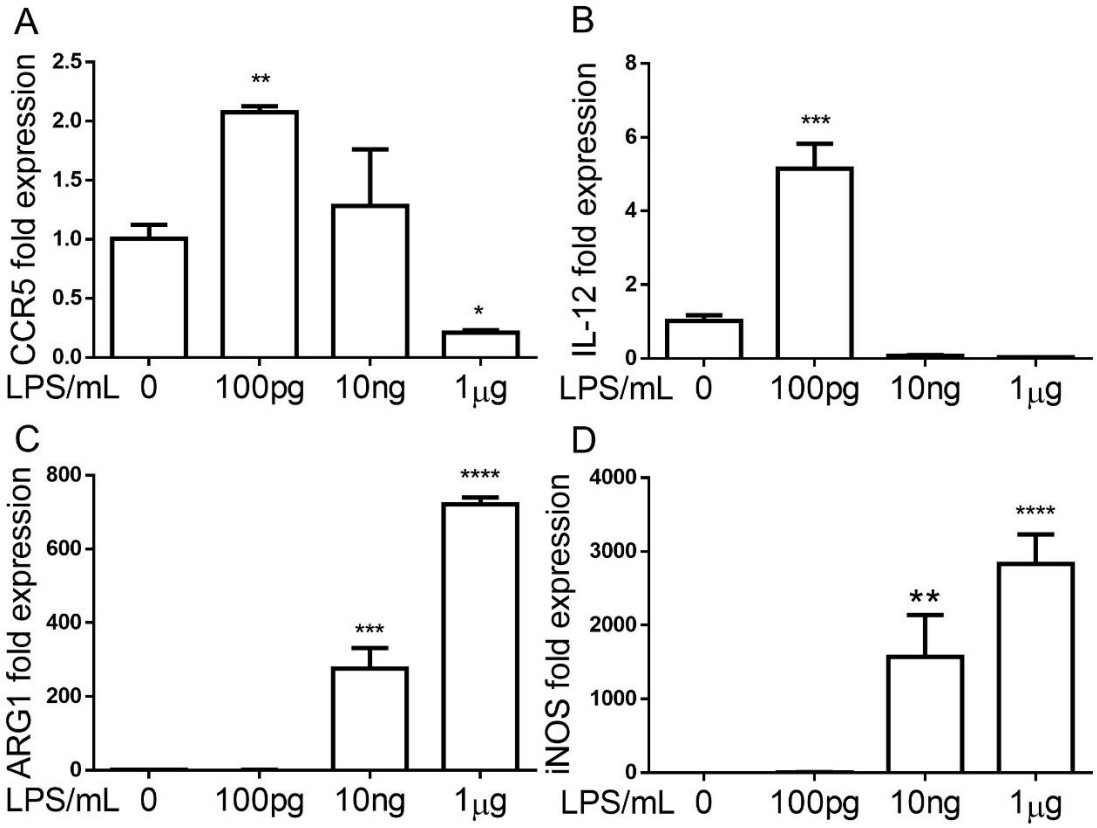

Figure 5.1 Differential priming and tolerance by TLR agonists

Total RNA was isolated from monocytes treated with different dosages of LPS for 5 days. Real-time PCR was performed to determine the expression levels of CCR5 (A), IL-12 (B), ARG1 (C) and iNOS (D). Data are representative of three separate experiments (**, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$; One-way ANOVA).

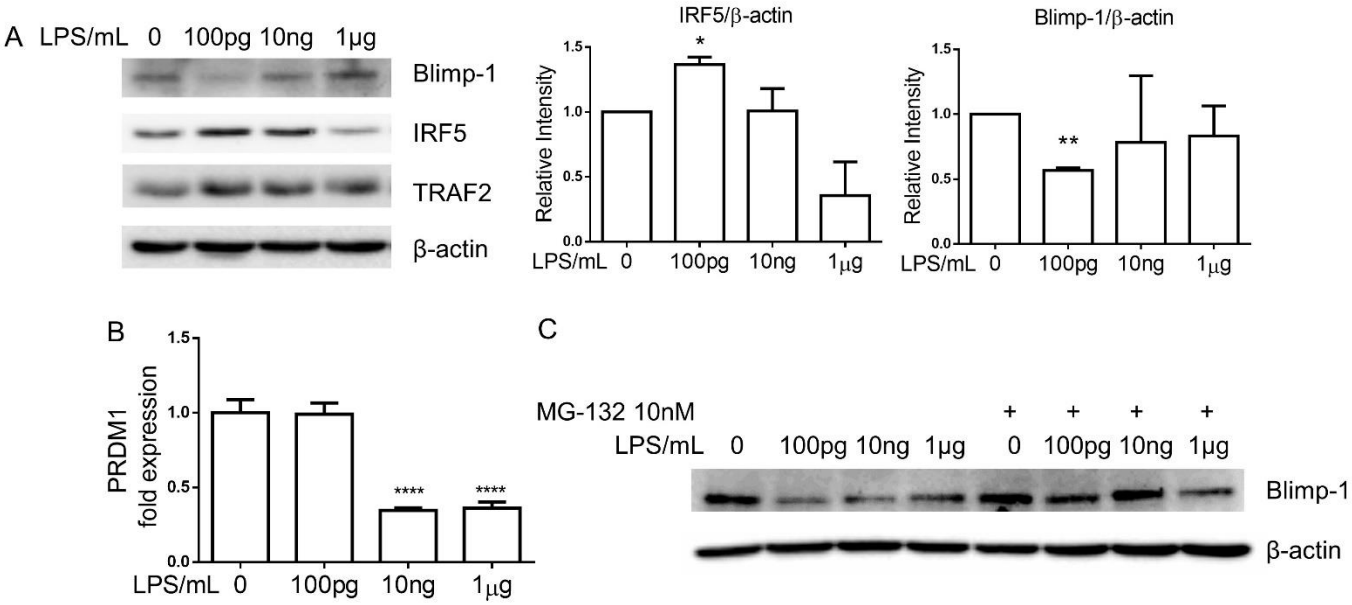

Figure 5.2 Differential regulation of IRF5 and PRDM1 by different dosages of LPS

Monocytes were treated with different dosages of LPS w/o MG132 as indicated, total RNA was isolated for RT-PCR (B) and whole cell lysates were obtained for western blot. Proteins were separated on SDS-PAGE and levels of Blimp-1, IRF5, TRAF2 and β-actin were detected (A, C). Data are representative of three separate experiments.

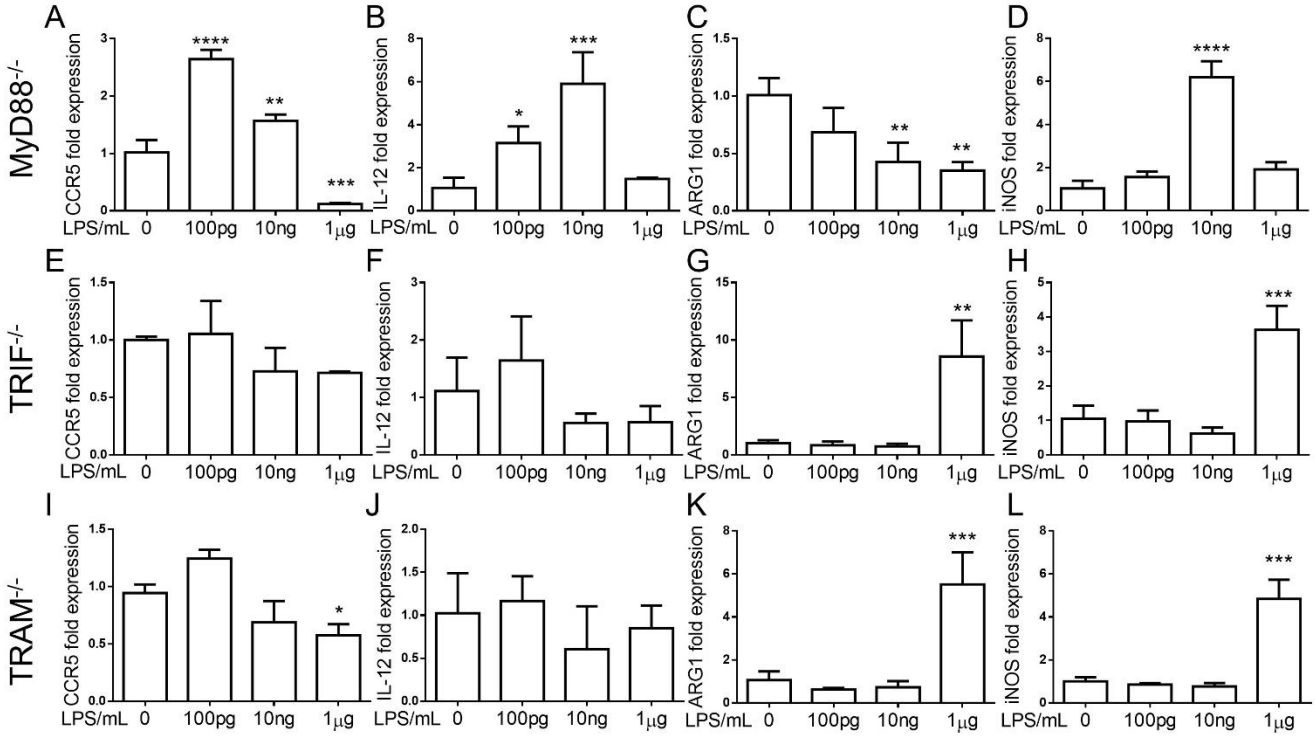

Figure 5.3 The non-resolving inflammatory monocyte adaptation is independent of MyD88, but dependent on TRAM and TRIF

Total RNAs were harvested from LPS-treated monocytes with MyD88, TRIF and TRAM deficiency. Real-time PCR was performed to determine the expression levels of CCR5, IL-12, ARG1 and iNOS. Data are representative of three separate experiments (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$; One-way ANOVA).

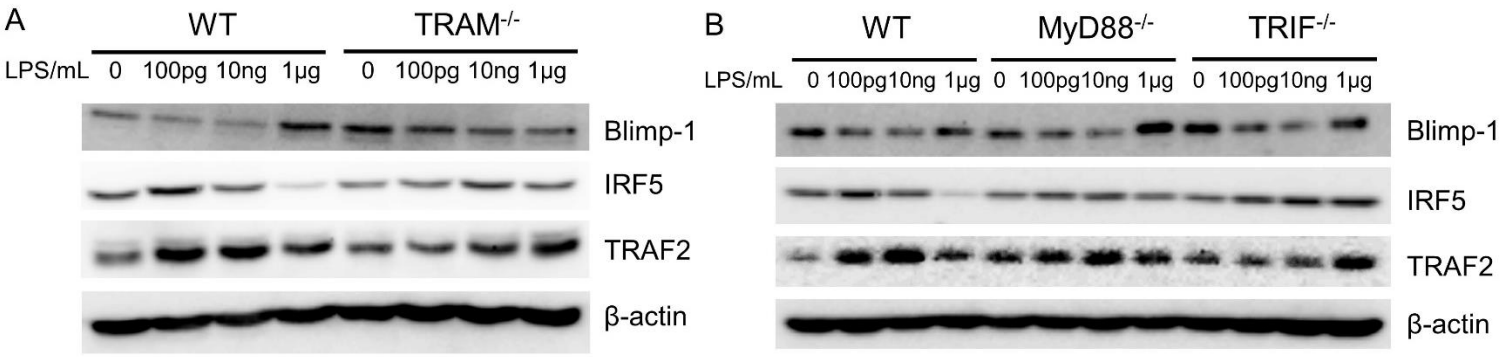

Figure 5.4 The non-resolving inflammatory monocyte adaptation is independent of MyD88, but dependent on TRAM and TRIF (con.)

Whole cell lysates were harvested from LPS-treated monocytes with MyD88, TRIF and TRAM deficiency. Protein levels of Blimp-1, IRF5, TRAF2 and β-actin were detected (A, B). Data are representative of three separate experiments.

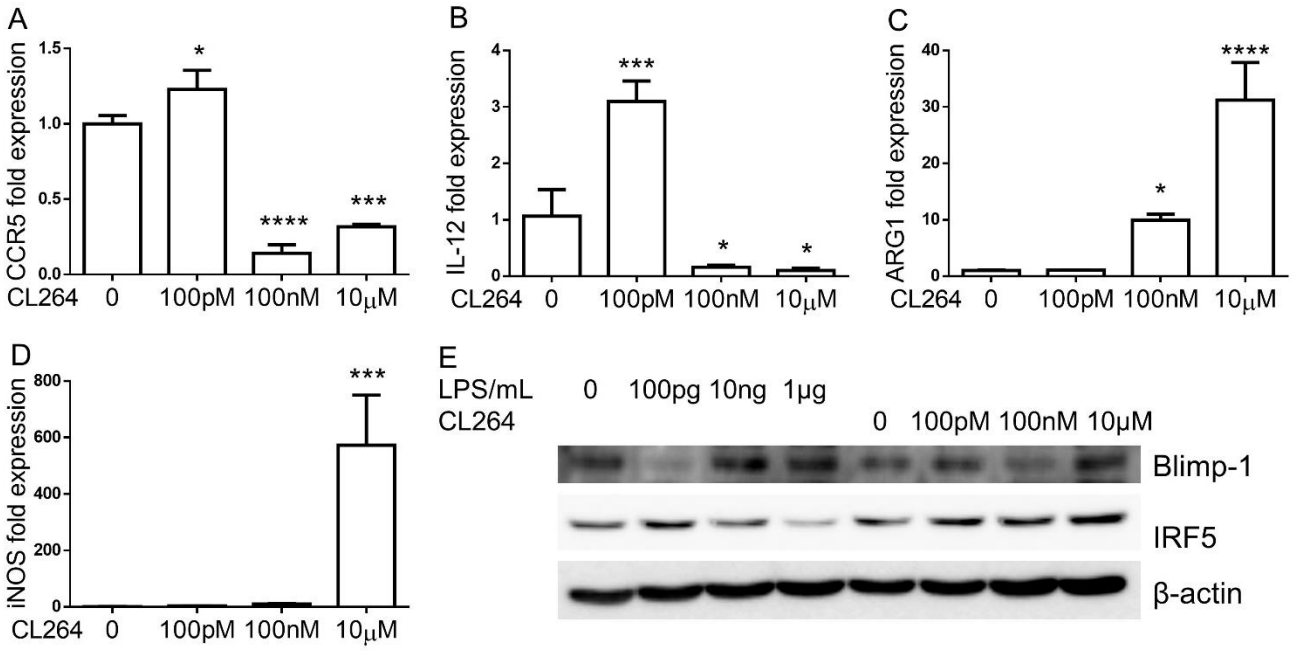

Figure 5.5 Differential regulation of monocytes responses by TLR7 ligand CL264.

Total RNA was isolated from monocytes treated with different dosages of CL264 for 5 days. Real-time PCR was performed to determine the expression levels of CCR5 (A), IL-12 (B), ARG1 (C) and iNOS (D). Protein levels of Blimp-1, IRF5 and β-actin were determinate by western blot (E). Data are representative of three separate experiments (*, $p<0.05$, ***, $p<0.001$, ****, $p<0.0001$; One-way ANOVA).

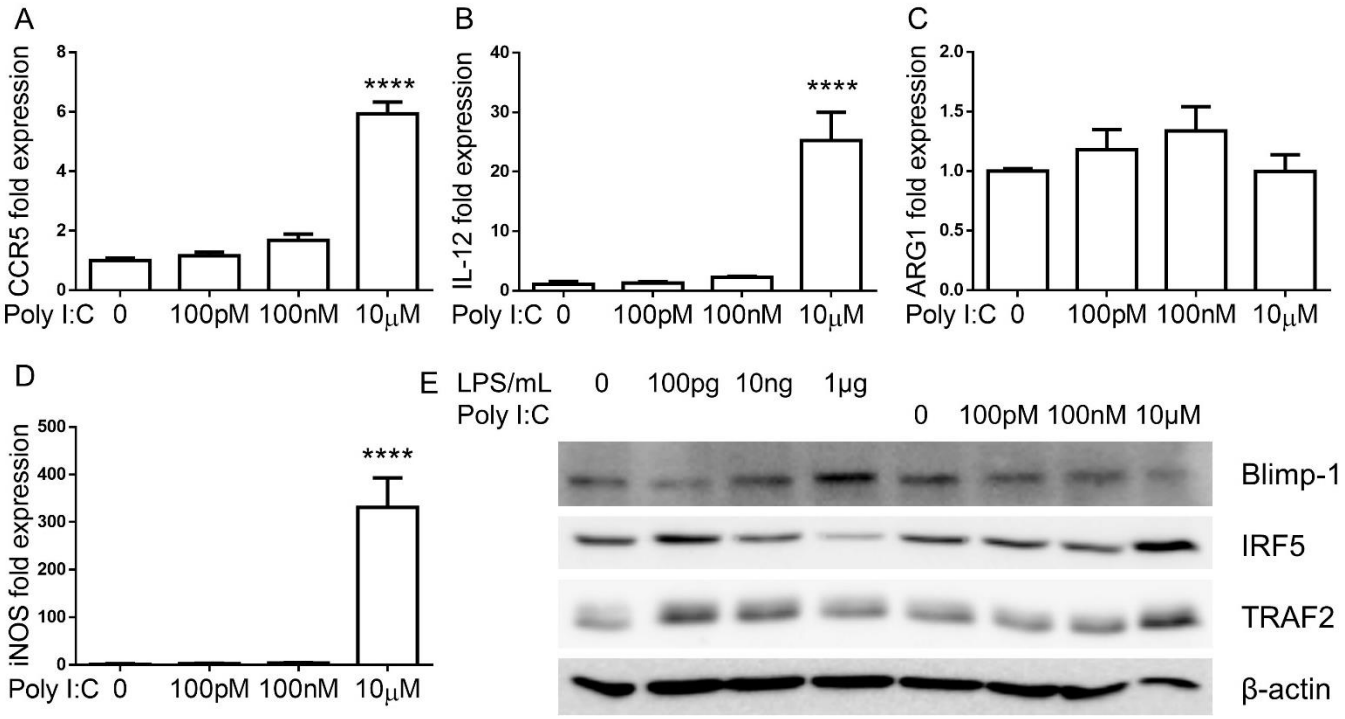

Figure 5.6 Differential regulation of monocytes responses by TLR3 ligand Poly I:C.

Total RNA was isolated from monocytes treated with different dosages of Poly I:C for 5 days. Real-time PCR was performed to determine the expression levels of CCR5 (A), IL-12 (B), ARG1 (C) and iNOS (D). Protein levels of Blimp-1, IRF5, TRAF2 and β-actin were determinate by western blot (E). Data are representative of three separate experiments (****, $p<0.0001$; One-way ANOVA).

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Chapter 6. Conclusions and Future Directions

In the past decade, the concept of innate immune memory has been appreciated and numerous studies regarding to this topic have been published. The initiation and progression of chronic inflammatory disease has been linked to low grade inflammation in which innate programming plays a key role. To the authors knowledge this is the first systemic analysis that using *both in vivo* and *in vitro* model to study the effects of low-grade inflammation and its potential mechanisms in disease development. We investigated the outcomes of non-resolving inflammation in mice and revealed that super-low dose endotoxin pre-conditioning accelerated pathogenesis of atherosclerosis and sepsis while lead to delayed wound healing by skewing the phenotype of innate immune cells. During atherosclerosis progression and wound healing process, we observed that super-low dose LPS selectively induces and sustains the polarization of pro-inflammatory monocyte which characterized by $Ly6C^{high}$ and elevated CCR5 expression. On the other hand, in acute disease, for instance, sepsis, our findings suggest additional aspects of innate pre-conditioning and adaption in neutrophil, as reflected by elevated migration and decreased NET formation abilities.

At the mechanistic level, the adaptors of TLR4 may be important for the adaptation of inflammatory state. Our data clarify an important issue with regard to the differential usages of MyD88 and TRAM/TRIF pathways during monocyte adaptation and memory. Signaling through TRAM or TRIF, instead of MyD88 was important for SL-LPS mediated low-grade inflammation, which confirms previous reports that TRAM or TRIF deficiency was found to have reduced inflammation and atherosclerosis progression. We found higher levels of pro-inflammatory mediators (IL-12 and CCR5) in monocytes challenged with super-low dose LPS as compared to non-adapted or high dose LPS-adapted monocytes. Mechanistically, the differential modulation of inflammatory genes by super low and high dose LPS may be mediated through divergent regulation of transcription factors degradation, monocytes adapted by super-low dose LPS exhibited higher levels of transcription factor IRF5 and reduced levels of transcriptional modulator Blimp-1. We also expanded and updated our understanding of monocyte adaptation and memory toward other TLR ligands such as TLR3 and TLR7 ligands. Therapies targeting the unique cellular

network of low-grade inflammation and restoring the anti-inflammatory mediators may hold promise in prevention and intervention of the pathogenesis of chronic diseases.

In conclusion, our studies revealed the polarization of low-grade inflammatory monocytes by subclinical dose LPS and its pathological outcomes in diseases. We also identified the essential adaptor molecule and fundamental transcription factors that may be responsible for the dynamic balance between pro- and anti-inflammatory responses in monocytes.

Although more genetic and epigenetic studies have been conducted to elucidate pathways and modulators that are involved in this dynamic process, the range of duration and dosage that lead to these opposite phenomenon remains obscure. Systemic analysis of the underlying mechanisms will no doubt be the hot topic of research in this field to identify new targets as well as methods to artificially regulate the direction of immune responses. Moreover, most of the studies are limited with monocytes and macrophages. Since other white blood cells also stand in the first line of host defense against invading microorganisms, it is of interest to consider these dynamic modulations in other leukocytes, especially neutrophils.