Dose-dependent effects of endotoxin on monocyte and the underlying mechanisms

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Abstract:

Monocytes are dynamic innate immune cells that respond differently based upon the dose and duration of an infection. While super low dose endotoxin is found in chronic inflammatory diseases such as atherosclerosis, exposure to high dose endotoxin leads to sepsis. However, clear characterization of monocytes and the underlying mechanisms in these disease conditions is lacking. To elucidate the missing information, we conducted two different projects.

In the first project, we investigated the role of super low dose endotoxin in polarizing monocytes to a prolonged low-grade inflammatory state with no resolution, disrupting homeostasis. This low grade inflammatory phenotype was confirmed by sustained induction of inflammatory mediators CD40 and CD11a. In addition, low grade inflammatory monocytes influence neighboring T cells by suppressing T cell regulatory functions. Mechanistically, we showed that the non-resolving inflammatory phenotypes in monocytes is dependent on non-traditional TLR4 adaptor called TRAM.

In the second project, we focused on the effects of high dose endotoxin on monocyte phenotypes. We reported that high dose endotoxin give rise to a mix of both immunosuppressive and pathogenic inflammatory monocytes, leading to monocyte exhaustion. While thorough research is conducted to study the immunosuppressive monocytes and underlying long term effects, role of pathogenic inflammatory monocytes is not well addressed. Monocyte exhaustion leads to elevated levels of CD38, an inflammatory mediator, elevated ROS levels, depleted NAD+ and mitochondrial respiration. STAT1 and KLF4 are critical transcription factors in sustaining exhausted phenotypes. Indeed, TRAM adaptor molecule also mediates this exhaustion as TRAM deletion restores monocyte health.

Taken together, our work defines novel monocyte phenotypes and mechanism in super-low dose or high dose endotoxin environments.

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General audience abstract:

Healthy inflammatory response is represented by initial induction of inflammatory cells in the site of infection and pathogen clearance, followed by resolution of inflammation and damage repair. This balance between inflammation and resolution maintains immune homeostasis. Imbalances in this homeostasis can be a cause or effect of various disease conditions such as atherosclerosis and sepsis, for example. Despite rigorous research, these diseases are still prevalent and treatments are still lacking. It is essential to investigate inflammatory responses at a cellular level and understand how an immune cell responds to a given pathogen. Depending upon the intensity, dose and duration of a pathogen can dictate immune cell functions.

Recent discoveries, including the research in our lab have reported that super low dose bacterial endotoxin exacerbates atherosclerosis. Mouse monocytes (innate immune cells) treated with super low dose endotoxin continuously induce mild but sustained inflammatory molecules but are unable to exhibit resolving mediators to dampen the inflammation and hence, monocyte homeostasis is disrupted.

Homeostatic imbalance is also in seen in sepsis, when monocytes exposed to high dose bacterial endotoxin. Due to a repetitive exposure to high dose endotoxin, monocytes are unable to respond accurately, where they simultaneously exhibit inflammatory and anti-inflammatory mediators but in a dysregulated manner.

Dedication:

This work is dedicated to my sister, Tisha, my constant motivation.

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Overview:

Inflammatory diseases have an adverse effect on human health and are considered most significant cause of deaths worldwide. More now than ever, deadly diseases like COVID-19 have highlighted the importance to understand how the immune system functions in a variety of disease conditions. Broadly, inflammation occurs when immune cells recognize and respond to bacteria, viruses, toxins and infections to eliminate the pathogen and assist in tissue repair and remodeling (1). Depending upon the dose, duration and type of infection, immune cells can respond differently (1, 2).

Acute inflammation is represented by a robust response to clear a foreign pathogen, followed by resolution of the inflammation and tissue repair (1). This homeostatic balance between inflammatory and tolerant immune cells is a hallmark for healthy functioning immune system (3). Chronic inflammation lasts longer, with elevated and prolonged inflammatory responses. Resolution of inflammation is compromised, leading to long-lasting non-resolving inflammation that further leads to alterations to tissues, organs and normal physiology (1, 4).

Innate immune cells (monocytes, neutrophils, dendritic cells, etc.) are the first line of defense that robustly act upon a foreign body. Adaptive immune cells (mediated by lymphoid cells such as T and B cells) are the second line of defense and respond in an antigen-specific way (5). Recognition of pathogens by the receptors of innate immune cells such as Toll-like receptors trigger the initial response (6). Even though inflammation and immune cells have been thoroughly studied for decades, it is challenging to understand the defined mechanisms and completely unravel the complexity that regulate immune system. In the efforts to further clarify the underlying mechanisms, we explore the role of innate immune cells such as monocytes in atherosclerosis and sepsis.

In recent years, interests in the role of monocytes in short term or long lasting inflammation has greatly increased. Lipopolysaccharides (LPS) also known as endotoxin are the components of the outermost membrane of gram-negative bacteria and are a commonly used activator of monocytes. Monocytes with their pattern recognition receptors (PRRs) such as TLRs are able to recognize LPS as a foreign body and relay a chain of signaling molecules, as a result (7). TLR4 on monocytes recognize LPS as a foreign body and activates downstream molecules mediated by adaptor molecule MyD88 or TRAM. It is essential to acknowledge and appreciate the complexities as there is no one simple way that these monocytes respond to any foreign stimuli. Hence, throughout my PhD journey, I studied the dose-dependent effects of endotoxin on monocytes and define key principles involved in atherosclerosis and sepsis as disease models.

In Chapter 1, we provide an overview of what is known about the innate memory in atherosclerosis and sepsis. We also highlight that dose-dependent signaling plays a critical role in remodeling the monocyte behaviors and discuss the known mechanisms that lead to priming, tolerance and exhaustion of these monocytes.

Previous work from our lab reported that chronic exposure to super low dose endotoxin exacerbates atherosclerosis with increase in plaque size, necrosis and monocyte infiltration (8). Moving forward, in Chapter 2, we focus on understanding the effects of super low doses of endotoxin on polarizing monocytes to a non-resolving prolonged inflammatory state with no resolution. I found that the bone marrow derived monocytes (BMDMs) repeatedly challenged with super low dose of LPS exhibit sustained inflammatory response as shown by increase in transcript and protein expressions of signature inflammatory markers CD40 (co-stimulatory molecule) and CD11a (adhesion molecule). These markers are critical in remodeling inflammatory responses in humans with coronary artery disease as well as atherosclerotic animal

models. In addition, immune-enhanced monocytes can communicate with neighboring T cells by suppressing the generation of regulatory $CD8^+$ $CD122^+$ T cells. Mechanistically, downstream of TLR4, I focus on the involvement of TRAM, an adaptor molecule and IRAK-M, a negative regulator of inflammation in polarizing monocytes to a non-resolving inflammatory state. Super low dose endotoxin activates TRAM-mediated pathway. We report that TRAM is responsible in sustaining prolonged inflammatory phenotypes in monocytes, IRAK-M is responsible in dampening the same phenotype as seen by the effects of TRAM deletion or IRAK-M deletion on CD40 and CD11a expressions.

In chapter 3, we characterize exhausted phenotypes in monocytes and the underlying mechanisms. In septic patients and in sepsis murine models, simultaneous expression of immunosuppression and pathogenic inflammation leads to long term dysfunctional immune response. We repetitively challenged BMDMs with high dose endotoxin in vitro and observed a complete shift towards inflammatory Ly6C ^{pos} population with negligible Ly6C ^{neg} population. Furthermore, we identified CD38, as an inflammatory mediator of exhausted monocytes. Increase in ROS, depletion of cellular NAD+, and depletion of mitochondrial respiration also corroborated with the exhausted phenotype. Mechanistically, exhausted monocytes depict robust elevation of STAT1 and KLF4. We also report a critical role of TRAM because TRAM deficiency significantly dampened exhausted phenotypes and improved monocyte health.

Conclusions

Taken together, we show that monocytes phenotypes are strongly affected by the dose and duration of endotoxin challenge. While repetitive challenge to super low dose LPS polarizes monocytes to a non-resolving inflammatory state as seen in atherosclerotic disease model, repetitive challenge to high dose LPS leads to monocyte exhaustion with simultaneous

pathogenic inflammation and immunosuppression as seen in sepsis disease model. We also highlight the significance of TLR4 adaptor molecule TRAM, as low grade inflammation as well as exhaustion in monocytes are TRAM-dependent.

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Chapter 1: Signal-strength and history-dependent innate immune memory dynamics in health and disease

1.1. Abstract:

Innate immunity exhibit memory characteristics, reflected not only in selective recognition of external microbial or internal damage signals, but more importantly in history and signal-strength dependent reprogramming of innate leukocytes characterized by priming, tolerance and exhaustion. Key innate immune cells such as monocytes and neutrophils can finely discern and attune to the duration and intensity of external signals through rewiring of internal signaling circuitries, giving rise to a vast array of discreet memory phenotypes critically relevant to managing tissue homeostasis as well as diverse repertoires of inflammatory conditions. This review will highlight recent advances in this rapidly expanding field of innate immune programming and memory, as well as its translational implication in the pathophysiology of selected inflammatory diseases. Keywords: Innate memory, priming, tolerance, exhaustion, inflammatory diseases.

1.2. Introduction

The establishment of "memory" is the cardinal and classical features of adaptive immunity, and has served as the guiding principle of empirical vaccine generation for millennium. Adaptive immunity develops lasting memory responses toward highly specific antigens through somatic recombination-mediated generation of T cell Receptors (TCR) and/or B cell Receptors (BCR), followed by clonal expansion via interaction with selective antigen presenting cells. In contrast, innate immune cells can only respond to general molecular patterns associated with pathogens through innate receptors [1]. Given limited repertoire of innate receptors, innate immune cells were not historically considered to be memory generating entities. However, emerging data from the last decade reveal fascinating, complex and dynamic "memory"-like behaviors of innate immune cells that transcend beyond the classical adaptive immune memory phenotypes. The distinct features of innate memory are reflected in signal-strength and history dependent behaviors such as priming, tolerance and exhaustion [2]. The generation of innate memory may have profound consequence related to pathophysiology of both acute and chronic inflammatory diseases [3].

1.3. Mechanisms for the generation of innate immune memory

In the classical sense of immune memory, adaptive immune cells such as T cells and B cells gain the capability to uniquely recognize and memorize highly distinct antigens through somatic VDJ recombination. In sharp contrast, innate immune cells do not have the machinery for VDJ recombination and thus relies upon limited innately-encoded receptors to recognize general molecular patterns (e.g.

PAMPs-pathogen associated molecular patterns; DAMPs-damage associated molecular patterns). Despite its limited specificity, innate immune cells can differentiate the signal strength and history of challenges, exhibiting "memory-like" behavior of priming, tolerance and exhaustion [4-8]. The establishment of such memory-like behavior is clearly distinct from the acquisition of adaptive memory and does not require genetic recombination. Instead, closely inter-twined intra-cellular circuitries involving redox signaling, sub-cellular trafficking, metabolic and epigenetic processes are likely involved to establish transient memory states with limited stability and plasticity [9-15] (Table 1).

A cardinal example of innate memory can be seen with monocyte/macrophage responses to rising dosages of bacterial endotoxin [9,16]. While a prolonged challenge with higher dosages of lipopolysaccharide (LPS) can lead to reduced expression of pro-inflammatory cytokines, commonly known as endotoxin tolerance [3], prolonged stimulation with a subclinical super-low dose LPS can polarize monocyte/macrophage into a "primed" low-grade inflammatory state with sustained expression of inflammatory mediators [5,9]. The mechanisms of endotoxin tolerance likely involve the activation and induction of molecular suppressors at multiple levels such as cytoplasmic signaling suppressors interleukin-1R-associated-kinase (IRAK)-M, and Phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K/AKT) [6,17], as well as nuclear transcriptional suppressor RelB [13,14]. On the other hand, the generation of primed low-grade inflammatory monocyte/macrophage requires the clearance of suppressors such as IRAK-M and PI3K/AKT [4,13]. At the sub-cellular level, subclinical super-low dose LPS preferentially disrupt the homeostatic processes of autophagic flux as well as pexophagy, leading to the accumulation of reactive oxygen species involved in the establishment of low-grade inflammation [5,18]. Innate leukocytes may sense the signal strength and duration of LPS via distinct usage and assembly of intra-cellular adaptor molecules such as myeloid differentiating factor 88 (MyD88) and TRIF-related adaptor molecule (TRAM), with TRAM preferentially directing the cellular response to sustained stimulation of super-low dose LPS [9,19]. On the other hand, MyD88 is preferentially involved in the response to higher dose LPS during both the acute response phase as well as the compensatory phase of tolerance [20,21]. The intra-cellular processes responsible for priming and tolerance may likely compete with each other forming multi-tiered competitive circuitries, assisting the decision-making processes of innate leukocytes in adopting dynamic activation behaviors [3,22,23] (Figure 1). The generation of mutually competitive circuitries is also a fundamental principle for the clear differentiation and activation of other immune cells such as T helper cells [24,25].

Sustained challenges with higher dose endotoxin not only lead to endotoxin tolerance, but also an exhausted state characterized by pathogenic inflammation and immuno-suppression often seen during the progression of sepsis [26,27]. "Endotoxin tolerant" cells are not inert and can still robustly respond to endotoxin stimulation, with a significantly altered landscape of gene expression potentially contributing to pathogenic

inflammation and immune exhaustion [7,16]. For example, monocyte/macrophage with prolonged LPS stimulation exhibit robust induction of iNOS and PD-L1 [7,16]. Persistent iNOS expression may contribute to pathogenic inflammation, and PD-L1 is a major contributor mediating immune-suppression. Recently, we demonstrate that endotoxin exhaustion is not limited to monocyte/macrophage, and can also be seen in neutrophils with prolonged challenge of higher dose LPS [8]. Exhausted neutrophils with prolonged LPS treatment manifest enhanced expression of pathogenic inflammatory mediators such as LTB4 and ICAM1, contributing to altered migratory and swarming behaviors reminiscing septic neutrophils [8]. Exhausted neutrophils similarly express elevated PD-L1, potentially contributing to immune-suppression [8].

1.4. Innate immune memory during the pathogenesis of acute and chronic diseases

Low-grade inflammatory memory monocyte in atherosclerosis

Atherosclerosis and related cardiovascular complications are among the leading causes of morbidity and mortality in the world [28]. Previously considered as a lipid storage disease, atherosclerosis is nowadays well recognized as a chronic low-grade inflammatory disease that occurs within the arterial wall [29]. The programming of low-grade inflammatory monocytes is crucially involved in the pathogenesis of atherosclerosis. Non-resolving low-grade inflammatory monocytes and monocyte-derived macrophages are the key mediators for the formation and progression of atherosclerotic plaques [30,31]. Monocytes can be primed by risk factors present in the circulation and in the vessel wall, such as pathogen-associated molecular patterns, oxidized lipoproteins, shear stress, and oxidative stress. Excessive inflammatory signals tend to trigger compensatory anti-inflammatory tolerance and therefore the expression of proinflammatory mediators in monocytes is transient and subsequently suppressed due to the induction of homeostatic negative regulators [32-34]. In contrast, under non-resolving low-grade inflammatory conditions, monocytes may fail to develop tolerance and are programmed into a sustained inflammatory state that favors the development of atherosclerosis [12,13,35].

LPS, also known as endotoxin, is the major stimulant to prime monocytes, which are the primary immune cells responding to LPS given their relatively high expression of TLR4. Trace amount of gut microbiota-derived LPS may leak into circulation via increased gut permeability, leading to subclinical endotoxemia [36,37]. According to epidemiological studies endotoxemia levels as low as 50 pg/mL may serve as a strong risk factor for the development of atherosclerosis [38]. Indeed, atherosclerosis patients have low but significantly elevated serum LPS level as compared with healthy individuals (79.0 ± 10.7 vs. 43.5 ± 11.9 pg/ml, $p < 0.001$). This concentration of LPS is sufficient to up-regulate Nox2 expression and elevate oxidative stress in human monocytes [39]. In the murine model of atherosclerosis, $ApoE^{-/-}$ mice fed with high-fat diet exhibit significantly higher level of serum LPS as compared to the counterparts fed with regular

diet. Oral administration of *Akkermansia muciniphila* decreases the circulating LPS level, alleviates atherosclerosis progression, as well as reduces monocyte/macrophage accumulation in the plaques [40]. These findings indicate that low-grade inflammatory monocytes primed by low-dose LPS are critically involved in the pathogenesis of atherosclerosis.

Chronic injection of subclinical dose LPS to high-fat diet fed $ApoE^{-/-}$ mice (a murine model of atherosclerosis) significantly exacerbates the pathogenesis of atherosclerosis accompanied by higher levels of circulating $Ly6C^{Positive}$ low grade inflammatory monocytes as well as increased number of macrophages within the plaque areas. The surface level of inflammatory chemotaxis receptor CCR5 is significantly elevated while the surface expression of SR-B1, a modulator for anti-inflammation and lipid metabolism, is reduced on circulating monocytes from the high-fat diet-fed $ApoE^{-/-}$ mice conditioned with super-low-dose LPS. The monocytes that are primed with subclinical dose LPS for a long-term exhibit similar phenotype, as characterized by enhanced levels of CCR5 and reduced levels of SR-B1. Adoptive transfer of these LPS primed monocytes to high-fat diet fed $ApoE^{-/-}$ mice results in significant elevation of plaque size and lipid deposition, suggesting that these low-grade inflammatory monocytes programed by subclinical dose LPS can directly contribute to atherosclerosis progression. Mechanistically, super-low-dose LPS treatment induces increased level of miR-24, which mediates the suppression of SR-B1, and reduction of IRAK-M, which is a critical negative-feedback regulator. IRAK-M deficiency in turn leads to elevated miR-24 levels, forming a positive feedback loop sustaining the low-grade inflammatory state conducive to atherosclerosis [4]. There are two competitive pathways transducing signals following LPS stimulation, namely the MyD88-dependent pathway and the MyD88-independent pathway mediated by TRIF and TRAM [41]. Intriguingly, the low-grade inflammatory monocyte primed by super-low dose LPS is dependent upon TRAM/TRIF but not MyD88 [9]. By employing a bone-marrow transplantation strategy, Lundberg et al. have shown that hematopoietic deficiency of TRAM and TRIF but not MyD88 adaptor-like (MAL) significantly reduces atherosclerosis in $Ldlr^{-/-}$ mice (another murine model of atherosclerosis). TRAM deficiency also leads to down-regulated level of pro-inflammatory mediators, such as TNF-α, IL-6, IL-12, CCL2, CCL5 and CXCL10, in the aorta of atherosclerotic mice [42]. These data suggest that the priming of low-grade inflammatory monocytes by subclinical dose LPS during atherosclerosis is mainly mediated by TRAM, and targeting TRAM may promote effective generation of resolving monocytes for the prevention and treatment of atherosclerosis.

In addition to low-dose LPS, low concentrations of oxidized low-density lipoprotein (oxLDL) can also induce epigenetic reprogramming of monocytes into a pro-inflammatory state. Primary human monocytes trained with low doses of oxLDL (below 10 μg/mL) for 24 h exhibit an enhanced response to secondary stimulation 6 days later by expressing a series of pro-inflammatory mediators, including IL-6, TNFα, IL-8,

MCP-1, MMP-2 and MMP-9. These trained monocytes have enhanced capacity to generate foam cells, elevated expressions of scavenger receptors (CD36 and SR-A), and reduced expression of cholesterol efflux transporters (ABCA1 and ABCG1). Therefore, these pro-inflammatory monocytes may contribute to the pathogenesis of atherosclerotic. The oxLDL-induced long-lasting proatherogenic profile can be significantly attenuated if the monocytes are pre-treated with histone methyltransferase inhibitor, suggesting that epigenetic histone modification is crucial for this innate immune memory of monocytes [43]. It has been found that oxLDL treatment can cooperatively boost the activation of macrophages induced by low dose LPS. Co-stimulation with oxLDL and low-dose LPS significantly up-regulates the genes transcribed by promoters containing an AP-1 binding site as well as induces the activation of ERK1/2. The combined effects of subclinical endotoxemia and oxLDL result in the establishment of pro-inflammatory state of macrophages and production a series of inflammatory cytokines within atherosclerotic lesions [44].

Exhausted memory innate leukocytes during the pathogenesis of sepsis

Sepsis is a systemic inflammatory response to severe infection and injury leading to multi-organ failure and remains one of the primary causes of death in hospitalized patients [45,46]. In 2017, global incidence of sepsis was around 48.9 million cases and sepsis-related deaths were estimated at 11.0 million cases [47]. The new coronavirus (SARS-CoV-2) in the ongoing outbreak and its associated disease COVID-19 pose tremendous threats to public health and drastically affect worldwide economies and societies [48,49]. Particularly, sepsis is the leading cause of death by COVID-19, which has been observed in nearly all deceased patients in numerous cohorts [50,51]. The immune response of sepsis patients consists of a hyperinflammatory phase featured by “cytokine storm” and an immunosuppressive phase exemplified by immune cell exhaustion and dysfunction [52]. Many clinical trials have been conducted to attenuate the hyperinflammatory effects by using anti-cytokine or anti-inflammatory agents, such as anti-IL-1β, anti-TNF-α, anti-LPS, and TLR inhibitors. Unfortunately, none of these approaches produces robust curative outcomes, and in some cases, the survival rate was even reduced [53-55]. A hallmark of sepsis is diminished clearance of primary pathogens and increased risk of secondary infection due to pathogenic inflammation and immune suppression [26]. Over 70% of deaths occur after the first 3 days of sepsis, many of which occur weeks after sepsis onset [56]. Thus, immunosuppression caused by leukocyte exhaustion has been increasingly recognized as a major factor for sepsis-induced mortality. A recent single cell study revealed that moribund COVID patients tend to have higher numbers of exhausted classical monocytes [57].

T cell exhaustion driven by persistent exposure to infections during sepsis has been well documented in the literature. The exhausted T cells are defined by a progressive loss of T cell effector function, a state of vigilant transcription distinct from functional effector or memory T cells. A typical alteration of exhausted T cells is the overexpression of a series of inhibitory molecules, such as PD-1, CTLA-4, LAG-3

and TIM-3 [58]. PD-1 is a critical negative regulator involved in suppressing lymphocyte responses. PD-1/PD-L1 pathway plays an important role in the initiation and promotion of immunosuppression [59]. Multiple studies using mouse model of cecal ligation and puncture (CLP) have unveiled elevated PD-1 expression on splenic $CD4^+$ and $CD8^+$ T cells. There is a continuously increased PD-1 expression on $CD4^+$ and $CD8^+$ T cells with the progression of sepsis, associated with a drastic reduction of total T cell population. Similarly, sepsis patients also have significantly increased PD-1 expression on T cells in the peripheral blood, spleen as well as injured organs [60]. These exhausted T cells from sepsis patients fail to efficiently produce inflammatory cytokines and their secretory profiles are potently compromised [61-63]. The ploy-functionality of $CD8^+$ cells is also significantly impaired in severe sepsis patients, and PD1 expression is inversely correlated with the number of poly-functional $CD8^+$ T cells [64]. PD-1 is considered as one of the most promising targets for immunomodulatory therapy to resume T cell function. However, anti-PD-1 treatment alone does not yield expected outcomes because multiple negative costimulatory molecules are expressed on the surface of exhausted T cells. For example, a recent study demonstrates that T cells co-expressing LAG3 and PD-1 are more significantly exhausted as compared to LAG3 or PD-1 single positive T cells in patients with acute sepsis. Furthermore, the frequency of co-expressing T cells is positively associated with the mortality and the length of hospital stay [65]. Thus, therapies targeting these suppressor molecule may maximize the recovery of T cells.

Correspondingly, monocytes in sepsis patients tend to express higher levels of immune receptors including CD63, CD163, CD206, TLR2 and TLR4, presumably rending them with elevated responses to infections [66,67]. However, studies reveal that monocytes from sepsis patients are less responsive than those from healthy individuals. They cannot efficiently produce TNF-α, IL-1β and IL-8 when challenged with LPS *ex vivo*, and the reduced TNF-α production by monocytes is employed as an index to evaluate the immune suppression of patients with sepsis [68]. The diminished capacity to produce pro-inflammatory cytokines may be due to the elevated expression of IRAK-M, an inhibitory Toll receptor signaling molecule, in the monocytes from sepsis patients. The patients with higher IRAK-M levels on admission have a higher mortality rate [69]. Monocytes are specialized antigen presenting cells (APCs) that present surface MHC molecule-bound antigens to activate T cells. The exhaustion of these APCs potentially facilitates the immunosuppression during sepsis. Sepsis induces altered monocyte-T cell interactions because of reduced expressions of co-stimulatory molecules on monocytes. Indeed, monocytes in sepsis patients are found to express much lower levels of CD40, CD80 and CD86 [70-72]. On the contrary, PD-L1 surface expression is up-regulated on monocytes from septic mice models as well as sepsis patients, correlated with T cell exhaustion and immunosuppression via PD-1/PD-L1 signaling pathway [60]. Monocyte PD-L1 expression can be used as an independent predictor of 28-day mortality in patients with septic shock [73].

Neutrophils are the most abundant leukocytes in the circulation and play a crucial role in sepsis as the first line of defense in protecting the body from microbial invasion. The interaction between neutrophils and other immune cells is necessary for the resolution of excessive inflammation as well as effective host defense [74]. Exhausted neutrophils with aberrant immune responses to infection have been observed in septic animal models and patients. Excessive bacterial products and pro-inflammation cytokines in sepsis induce the loss of CD62L expression but elevated integrin expression (e.g. CD11b) on the surface of neutrophils [75,76]. In addition, CXCR2 expression is reduced in the neutrophils of mice and patients with severe sepsis. Prolonged neutrophil stimulation results in up-regulation of iNOS and GRK2, which further promotes CXCR2 internalization [77]. Therefore, neutrophils exhibit reduced rolling and migratory capacity and fail to be recruited to the primary infection foci. Instead, these exhausted neutrophils tend to form pathogenic aggregations in the vital organs, which can be mimicked through in vitro examination [8]. The antimicrobial activities of neutrophils are also compromised during sepsis. Excessive bacterial load activates complement system, and high levels of C5a suppress the phagocytic function and ROS production of neutrophils. Various studies with septic mouse models and sepsis patients have revealed impaired phagocytosis, oxidant production as well as oxidative burst capacity of septic neutrophils [78,79]. Furthermore, neutrophils from septic mice and patients can induce immunosuppression and T cell apoptosis in a cell-contact dependent manner via the surface expression of PD-L1. Thus, the PD-L1 level on neutrophils, which is positively associated with sepsis severity, may serve as a biomarker for the prognosis of septic patients [80]. Despite its clinical significance, the mechanisms of neutrophil exhaustion during sepsis are still poorly understood. We have recently reported that neutrophils treated with LPS *in vitro* for a prolonged period develop a phenotype of elevated ICAM1, CD11b and PD-L1 expression as well as enhanced swarming and aggregation, which resembles the exhausted neutrophil phenotypes seen in sepsis. Importantly, the exhaustive profiles are significantly alleviated in TRAM deficient neutrophils after prolonged LPS challenge as compared with wild type neutrophils. TRAM mediated neutrophil exhaustion may be dependent upon Src family kinases (SFK) and STAT1 activation, since SFK inhibitor can effectively block neutrophil exhaustion cause by prolonged LPS treatment. Furthermore, TRAM deficiency is protective against the development of severe systemic inflammation and multi-organ damage in mice [8]. These data unveil a critical function of TRAM in promoting neutrophil exhaustion.

Innate immune memory during the pathogenesis of cancer

The phenotype and functionalities of myeloid cells (e.g., monocytes, macrophages, and neutrophils) are substantially changed by tumor-induced systemic environment and microenvironment, so that these cells usually acquire pro-tumor functions to promote cancer progression. On the other hand, the tumor associated innate immune cells may provide ideal targets for fighting against cancer.

The number of circulating monocytes significantly increases in both humans and mice bearing tumors. Among several cancer types, patients with high blood monocyte counts have a poorer disease prognosis, and the ratio of lymphocytes to monocytes has become a prognostic factor for lung cancer, colorectal cancer and ovarian cancer [81,82]. The increased monocyte levels may be caused by two reasons: enhanced migration from bone marrow to circulation, and increased myelopoiesis. Patients with pancreatic cancer exhibit elevated circulating monocyte levels associated with decreased monocyte abundance in the bone marrow. CCL2, a critical for monocyte recruitment, is commonly present at higher levels in serum of both mice and humans with cancer, facilitating the egress of monocytes from the bone marrow [83]. Cancer is usually accompanied by elevated serum levels of cytokines that are involved in the myeloid cell differentiation and survival, such as macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) [84-86]. Excessive production of these cytokines and tumor associated low-grade inflammation promote reprogramming of myelopoiesis. As a result, hematopoietic stem cell (HSC), common myeloid progenitor (CMP) and granulocyte-monocyte progenitor (GMP) populations are expanded, while common lymphoid progenitor (CLP) and megakaryocyte-erythrocyte progenitor (MEP) are not significantly altered. For example, increased frequency of HSC and GMP populations is observed in peripheral blood of patients with various types of solid tumors, indicating that tumor associated environment favors myeloid hematopoiesis and expansion of circulating monocytes [87-90]. In addition to increased numbers, the phenotype of monocytes is also profoundly influenced by tumors. One of the well documented features of cancer-educated monocytes is the acquisition of immunosuppressive properties. The monocytes from healthy individual express high level of HLA-DR (the protein of MHC II) on the surface, while HLA-DR level is significantly down-regulated in the monocytes of cancer patients [91,92]. The high level of CD14 HLA-DR^{low} monocytes is correlated with the lower levels of tumor-specific T-cells in the circulation of cancer patients. The patients with lower levels of CD14^{+} HLA-DR^{low} monocytes are more responsive to immune checkpoint blockade therapy [93,94]. The surface expression of CD86, a co-stimulatory molecule for T cell activation, is also reduced on cancer educated monocytes, inhibiting T cell function [91,92]. Furthermore, monocytes in cancer patients have enhanced expression and activity of arginase-1, which limits the availability of L-arginine to T cells [95,96]. Up-regulation of PD-L1 and GPNMB may also contribute to the immunosuppressive activity of monocytes in cancer [97]. Intriguingly, monocytes from cancer patients exhibited increased level of phosphorylated STAT3, and STAT3 is potentially activated in the healthy monocytes after co-culture with cancer cells. Inhibition of STAT3 attenuates the immunosuppressive activity of cancer educated monocytes [95,98]. Therefore, STAT3 may be a crucial transcription factor for the reprogramming of monocytes by tumor specific environment.

Based on the pro-tumor characteristics of monocytes, a series of regimens have been developed to reverse monocyte reprogramming. Specific antibody against CCR2 or small molecules that block CCR2 signaling remarkably restrain the growth and metastasis of tumor cells in mouse models of lung, breast, prostate, pancreatic and liver cancers [82]. A recent clinical study has revealed that oral administration of PF-04136309, a small molecule CCR2 antagonist, effectively reduces circulating monocytes and tumor-associated macrophages (TAMs) in pancreatic cancer [99]. Angiotensin II serves as a key regulator of cancer-induced myelopoiesis, and angiotensin-converting enzyme (ACE) inhibitors may suppress the excessive generation of pro-tumor monocytes. Enalapril, an ACE inhibitor, has been shown to reduce monopoiesis and TAMs as well as prolong the survival of mice with lung tumors [100]. Neutralization of tumor-derived GM-CSF reduces the emergence of $CD11b^+$ $Gr\text{-}1^+$ myeloid cells, leading to elevated anti-tumor activity of T cells and restrained tumor growth [101]. Arginase inhibition may also be an alternative approach to mitigate monocyte-mediated arginine depletion and consequent immunosuppression. Accordingly, a small molecule arginase inhibitor has been developed, which can increase plasma and tumor arginine levels, enhance anti-tumor T cell responses, prime immunity toward a pro-inflammatory state, and reduce tumor growth in mouse cancer models [102]. Application of STAT3 inhibitors have also yield promising anti-tumor outcomes by boosting anti-tumor immunity in mice. TAM receptor tyrosine kinases promote STAT3 phosphorylation, and administration of UNC4241, an inhibitor against TAM receptor tyrosine kinases, significantly alleviates the immunosuppressive activity of monocytes in a mouse model of melanoma [103].

Neutrophil is one of the major components of tumor-infiltrating innate immune cells. Cancer patients with poor prognosis often have an expanded pool of tumor-associated neutrophils (TANs), which exhibit complex and contradictory functions, promoting or limiting tumor growth [104]. The pro-tumor neutrophils produce high levels of VEGF, MMP9 and HGF, which facility tumor angiogenesis. MMP9 production and NET formation from neutrophils may provide ideal environment and niche for tumor cell intravasation and metastasis. Similar as cancer-educated monocytes, some TANs also possess immunosuppressive properties and suppresses T cell proliferation via deprivation of L-arginine [105]. Compared with circulating and splenic neutrophils, TANs secrete higher amount of CCL7 (a Tregs chemoattractant) that promotes the recruitment of Tregs to tumor, thereby forming an immunosuppressive microenvironment [106]. On the other hand, some neutrophils also exert anti-tumor activities by secreting cytotoxic mediators (ROS) to induce tumor cell apoptosis [105]. Intriguingly, accumulating data have suggested that the interaction between neutrophils and T cells is indispensable for the proper anti-tumor response of adaptive immunity [107,108]. A subset of TANs has been found to exhibit both neutrophil and APC characteristics in the patients with lung cancer, and these cells can boost anti-tumor T cell responses [109]. The molecular mechanisms underlying the differential function of neutrophils are not well understood. A recent study has indicated that Tollip, an innate immunity signaling adaptor molecule, may contribute to the neutrophil reprogramming in a mouse model of colorectal

cancer. Tollip deficient neutrophils have STAT5-dependent elevation of CD80 and reduction of PD-L1 as compared to wild type counterparts. Therefore, Tollip deficient neutrophils may potently activate T cells to exert antitumor activity. Adoptive transfer of Tollip-deficient neutrophils but not Tollip-deficient monocytes promotes tumor immune surveillance and reduces colorectal cancer burden *in vivo*. Thus, Tollip may serve as a target to modulate the decision-making process of neutrophils for future cancer therapy [110].

In recent years, the concept of trained immunity has drawn increasing attention due to its potential for the treatment of diseases such as cancer. Trained immunity represents an epigenetic and metabolic reprogramming process of innate immune cells to acquire long-term function and memory property [111]. Instillation of Bacillus Calmette-Guérin (BCG) can prime monocytes from the patients with bladder cancer into a pro-inflammatory and anti-cancer profile, mediated by the autophagy genes ATG2B and ATG5 [112]. The patients who exhibit low responsiveness to trained immunity have a higher chance of recurrence and tumor progression [15]. In LPS primed macrophages, 70% of the inducible genes are the targets of Jumonji domain containing 3 (JMJD3), also known as lysine demethylase 6B (KDM6B) [113]. KDM6B can stabilize tumor suppressor gene p53 [114], and higher KDM6B expression is a prognostic indicator for better survival in neuroblastoma patients [115]. Thus, LPS-trained macrophages via KDM6B may have clinical potential for cancer therapy. Fungal-derived polysaccharide β-glucans have been used to treat various cancers for a long time, and they are also potential inducers to promote trained immunity. A series of studies have revealed that β-glucan treatment leads to marked phenotypical and functional alterations of monocytes/macrophages, such as elevated cytokine production, enhanced phagocytic capacity, and increased ROS generation [111,116]. A recent study has shown that training with β-glucan leads to a transcriptomic and epigenetic rewiring of granulopoiesis in mice with melanoma, which subsequently induces the anti-tumor phenotype of TANs. Importantly, the mice receiving a single injection of β-glucan still exhibit a significant inhibition of tumor growth after 28 days, indicating long-term anti-tumor effects of neutrophil-mediated trained Immunity [117].

1.5. Concluding Remarks

Despite these exciting advances, the field of innate immune memory has only been revealed as the very tip of an iceberg closely intertwined with almost all aspects of human pathophysiology. Innate leukocyte may adopt highly diverse disease and context-dependent memory states, requiring single cell approaches to further clarify their unique contribution to distinct pathogenesis of inflammatory diseases. Memory leukocytes may further propagate their phenotypes to neighboring cells, establishing unique memory niche within local environments [118]. Future efforts are needed to finely map the establishment as well as propagation of innate memory through genetic and chemical approaches in tracking the ontogeny and propagation of memory innate leukocytes, in response to varying degrees of signal strengths and intensities

within complex host immune environments. Harnessing the potential of reprograming innate memory would hold enormous promise in the treatment of both acute and chronic human diseases.

1.6. Abbreviations

ABCA1	ATP Binding Cassette Subfamily A Member 1
ABCG1	ATP-binding cassette sub-family G member 1
ACE	Angiotensin-converting enzyme
APC	Antigen Presenting Cells
ApOE	Apolipoprotein E
ATG	Autophogy-related gene
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
CCL	C-C Motif Chemokine Ligand
CCR	C-C Chemokine receptor
CLP	Cecal Ligation and Puncture
CMP	Common myeloid progenitor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C motif chemokine receptors
DAMP	Damage-associated molecular pattern
ERK1/2	Extracellular signal-regulated kinase 1/2
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-monocyte progenitors
GPNMB	Glycoprotein-Nmb
GRK2	G protein-coupled receptor kinases
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
ICAM1	Intercellular Adhesion Molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK-M	Interleukin-1R-associated-kinase- M
JMJD3	Jumonji domain containing 3

KDM6B	Lysine demethylase 6B
LAG-3	Lymphocyte-activating gene
Ldlr	Low Density Lipoprotein Receptor
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
M-CSF	Macrophage colony-stimulating factor
MAL	MyD88-adapter-like
MCP	Monocyte Chemoattractant Protein
MEP	Megakaryocyte-erythrocyte progenitor
MMP	Matrix metalloproteinases
MYD88	Myeloid differentiation factor 88
NET	Neutrophil extracellular trap
NOX2	NADPH oxidase 2
oxLDL	Oxidized low density lipoprotein
PAMP	Pathogen associated molecular pattern
PD-1	Programmed cell death protein-1
PD-L1	Programmed death-ligand 1
PI3K/AKT	Phosphatidylinositol-3-Kinase and Protein Kinase B
SFK	Src family kinases
SR-A	Scavenger Receptor Class A
SR-B1	Scavenger Receptor Class B type 1
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T cell receptor
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	Toll/IL-1R domain-containing adaptor-inducing IFN-β-related adaptor molecule
TRIF	Toll/IL-1R domain-containing adaptor-inducing IFN-β
VEGF	Vascular endothelial growth factor

Table 1. Key features of innate and adaptive immune memories.

	Innate immune memory	**Adaptive immune memory**
Generation mechanism	Competitive signaling circuitries	Genetic recombination
Propagation mechanism	Inter-cellular communications	Clonal expansion
Duration	Relatively short-lived	Long-lasting
Stability	Prone to adaptation	Stable

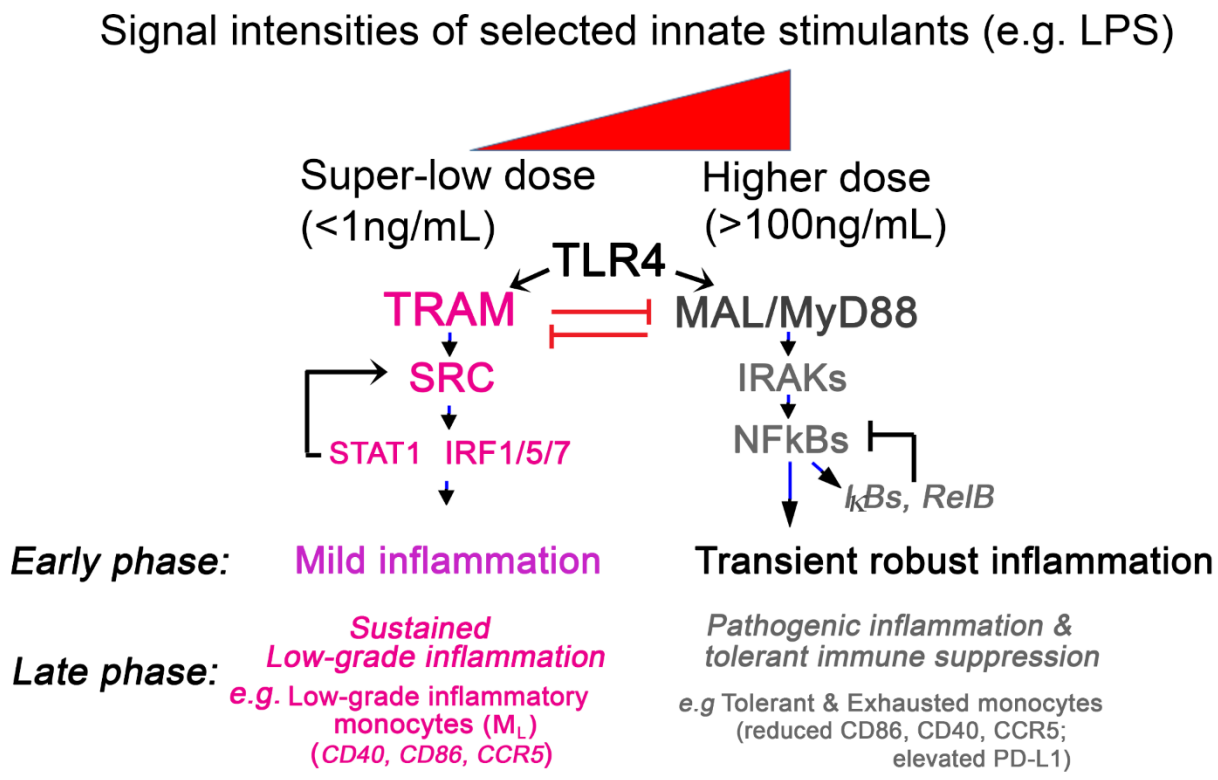

Figure 1. Illustration of innate memory dynamics based on signal strength and duration.

Innate immune leukocytes such as monocytes and macrophages can finely sense the strength and duration of external danger signals (e.g. lipopolysaccharide, LPS), and undergo distinct adaptations to generate dynamic memory states. In the case of LPS, a prolonged challenge with subclinical super-low dose LPS (<1ng/mL) will induce a sustained low-grade inflammatory states due to the positive-feedback signals involving mutually activating TRAM adaptor, SRC kinases, IRF1/5/7, and STAT1. In contrast, while higher dose LPS acutely induce a transient and robust inflammatory response through the activation of NFkB, prolonged stimulation with higher LPS signals will trigger the expression of inhibitory kBs (IkBs) and RelB, leading to a tolerant state with reduced expression of inflammatory mediators such as CD86, CD40 and CCR5. Tolerant leukocytes still maintain a skewed expression of profile of selected immune-suppression genes such as PD-L1, and eventually adopt an exhausted state characterized by pathogenic inflammation and immune suppression.

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Chapter 2: TRAM-related TLR4 pathway antagonized by IRAK-M mediates the expression of adhesion/co-activating molecules on low-grade inflammatory monocyte

2.1 Abstract:

Low-grade inflammatory monocytes critically contribute to the pathogenesis of chronic inflammatory diseases such as atherosclerosis. Elevated expression of co-activating molecule CD40 as well as key adhesion molecule CD11a are critical signatures of inflammatory monocytes from both human patients with coronary artery diseases as well as in animal models of atherosclerosis. In this study, we report that subclinical super-low dose LPS, a key risk factor for low-grade inflammation and atherosclerosis, can potently trigger the induction of CD40 and CD11a on low-grade inflammatory monocytes. Subclinical endotoxin-derived monocytes demonstrate an immune-enhancing effects and suppress the generation of regulatory $CD8^{+}CD122^{+}$ T cells, which further exacerbate the inflammatory environment conducive for chronic diseases. Mechanistically, subclinical endotoxemia activates TRAM-mediated signaling processes leading to the activation of MAPK and STAT5, responsible for the expression of CD40 and CD11a. We also demonstrate that TRAM-mediated monocyte polarization can be suppressed by IRAK-M. IRAK-M deficient monocytes have increased expression of TRAM, elevated induction of CD40 and CD11a by subclinical dose endotoxin, and are more potent in suppressing the CD8 regulatory T cells. Mice with IRAK-M deficiency generate an increased population of inflammatory monocytes and reduced population CD8 T regulatory cells. In contrast, mice with TRAM deficiency exhibit a significantly reduced inflammatory monocyte population and an elevated CD8 T regulatory cell population. Together, our data reveal a competing intra-cellular circuitry involving TRAM and IRAK-M that modulate the polarization of low-grade inflammatory monocytes with immune-enhancing function.

2.2 Introduction:

Persistent low-grade inflammation and associated chronic diseases such as atherosclerosis are leading health concerns with staggering economic tolls world-wide [1-4]. Clinical and basic studies indicate that the recruitment and retention of polarized low-grade inflammatory monocytes to the vasculature followed by their interaction with adaptive immune T cells collectively contribute to the initiation and progression of atherosclerosis [5, 6]. Elevated expression of co-stimulatory molecules such as CD40 and adhesion molecules such as CD11a on innate leukocytes are well-documented from human patients with coronary heart disease [7-9]. However, molecular mechanisms responsible for the generation of low-grade inflammatory monocytes with elevated CD40 and CD11a are not well understood.

One of the key risk factors for atherosclerosis is the subclinical endotoxemia in circulation due to compromised mucosal barrier and gut leakage, a common complication in patients with low-grade inflammation; obesity; infection; and aging [10-12]. Innate immune cells such as monocytes/macrophages can be potently polarized by subclinical low dose endotoxin/lipopolysaccharide (SLD-LPS) into a non-resolving low-grade inflammatory state with preferential expression of inflammatory mediators such as MCP-1, IL-12, CCR2, and CCR5[13-16]. We previously reported that polarized monocytes by subclinical dose LPS contribute to the progression of atherosclerosis and unstable plaques with enlarged necrotic areas [13]. However, the effects of subclinical low dose LPS on the expression of CD40 and CD11a remain to be determined.

Mechanistically, LPS is recognized by the Toll-like receptor 4 (TLR-4) on monocytes and can activate either MyD88-dependent (MyD88/Mal) or MyD88-independent (TICAM-1/TRIF and TICAM-2/TRAM) pathways [17, 18]. While higher dosages of LPS utilizes MyD88 to induce robust yet transition monocyte activation, we and others recently documented that subclinical dose LPS preferentially signals through TRAM instead of MyD88 to induce the expression of selected inflammatory mediators such as IL-12 and CCR5[15, 19]. Consistent with our mechanistic observation, TRAM deficient mice were shown to have reduced pathogenesis of atherosclerosis [20]. The interleukin-1 receptor associated kinases (IRAKs) function further downstream of the TLR4 adaptor molecules such as MyD88/Mal, with IRAK-M serving as a potent inhibitor of the TLR4 pathway [21-23]. We reported that IRAK-M deficient monocytes have elevated expression of MCP-1 and that IRAK-M deficient mice develop more severe atherosclerosis [13]. However, it is unclear whether IRAK-M may also modulate TRAM-mediated monocyte polarization and adhesion molecule expression.

Capitalizing on these studies, we aim to define whether subclinical low dose LPS may potently induce the expression of co-stimulating molecule CD40 and adhesion molecule CD11a, through TRAM or IRAK-M

mediated signaling processes in monocytes. Using primary bone marrow monocytes harvested from wild type, TRAM or IRAK-M knockout (KO) mice, we examined the expression of CD40 and CD11a expression through RT-PCR and flow cytometry analysis. We further characterized the regulation of downstream signaling molecules such as MAP kinase p38, tyrosine kinase c-Abl, as well as key transcription factor STAT5 involved in the expression of CD40 and CD11a [24-34]. The effects of polarized monocytes by subclinical dose LPS on the modulation of regulator CD8 T cells were examined both *in vitro* and *in vivo*.

2.3 Materials and Methods:

Experimental Mice and Cell Culture

Wild Type (WT) C57BL/6 and IRAK-M$^{-/-}$ were purchased from Jackson's Laboratory. TRAM$^{-/-}$ mice were from Dr. Holger Eltzschig (University of Texas Houston) as we described previously [15]. All experimental procedures were in compliance with guidance from the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the IACUC (Institutional Animal Care and Use Committee) of Virginia Tech. Bone marrow cells were harvested as we described previously from the tibias and femurs of WT, IRAK-M$^{-/-}$ or TRAM$^{-/-}$ mice, and cultured in complete RPMI (containing 10% FBS, 1% penicillin-streptomycin, and 1% L-Glutamine) supplemented with 10ng/mL M-CSF (PeproTech, Rocky Hill, NJ; #315-02). Cells were treated with either PBS (control) or SLD-LPS (100pg/mL) and cultured for 5 days at 37˚C in a humidified 5% CO2 atmosphere. Culture medium was replaced with fresh media, fresh PBS and LPS was added every 2 days as we described previously [13, 14].

Co-culture of monocytes with purified T cells:

Bone marrow cells from WT, IRAK-M$^{-/-}$ or TRAM$^{-/-}$ mice were treated with PBS or SLD-LPS (100pg/mL) and cultured for 5 days as described above. On day 5, cultured monocytes were harvested into a single cell suspension. T cells were purified from splenocytes via negative selection per manufacturer's instruction (Stem Cell #19851). A 1:1 ratio (10^6 cells/mL) of purified T cells to monocytes were co-cultured for 48 hours in the presence of 50ng/mL recombinant murine IL-15 (Peprotech #210-15) to preferentially maintain CD8 T cell population as described [35-38].

In Vivo Mouse Model

ApoE$^{-/-}$ mice purchased from the Jackson Laboratory were crossed with IRAK-M$^{-/-}$ mice (provided by Dr. Rischard A. Flavell at Yale University School of Medicine) to obtain ApoE$^{-/-}$ IRAK-M$^{-/-}$ mice. The ApoE$^{-/-}$ and ApoE$^{-/-}$IRAK-M$^{-/-}$ mice (6-10 weeks old) were fed with High Fat Western Diet (Harlan Teklad 94059) for two months with routine monitoring on a weekly basis. The spleen was then harvested for flow cytometry analysis.

Reagents

LPS (*Escherichia coli* 0111:B4) was purchased from Sigma Aldrich. Primary anti-STAT5 (#94205S, 1:1000 in 5% BSA), anti-p38 (#921281, 1:1000 in 5% BSA), anti-phospho-p38 (#4511S, 1:500 in 5% BSA.), anti-c-Abl (#28625, 1:500 in 5% BSA), anti-phospho-c-Abl (#2861S, 1:500 in 5% BSA) and anti-GAPDH (#2118, 1:1000) antibodies were purchased from Cell Signaling. Primary anti-oxCaMKII antibody (#36254, 1:1000, 5% BSA) was purchased from Genetex. Primary anti-TRAM/TICAM2 antibody (#AF4348, 1:200 in 5% BSA) was purchased from Novus Biological. Anti-β-actin (HRP) (# 47778, 1:1000 in 5% BSA) was purchased from Santa Cruz. Secondary anti-rabbit IgG horseradish peroxidase-conjugated antibody (#7074, 1:2000 in 5% BSA) was purchased from Cell Signaling. Primers for *musCD40, mus CD11a* and *musBactin* were synthesized by Integrated DNA Technologies are as follows:

mus *CD40* Forward: 5'GCT ATG GGG CTG CTT GTT GA 3'

mus *CD40* Reverse: 5'ATG GGT GGC ATT GGG TCT TC 3'

mus *CD11a* Forward: 5'GAA GCT GAG CAG CCT TGT C 3'

mus *CD11a* Reverse: 5' CCC GTC ACT TGG ATG AGG AT 3'

mus *Bactin* Forward: 5' ACT GTC GAG TCG CGT CCA 3'

mus *Bactin* Reverse: 5' ATC CAT GGC GAA CTG GTG G 3'

Flow Cytometry

The bone marrow cells at day 5 as well as cells obtained post- co-culture as described above were harvested and blocked with Fc block (BD Biosciences, #553141) followed by staining with anti-CD40 (PE Biolegend #124609), anti-Ly6C (PE-Cy7 Biolegend #128018), anti-CD11a (APC Biolegend #101119), anti-CD11b (APC-Cy7 Biolegend # 101226), anti-CD8 (APC-Cy7 Biolegend # 100713), anti-CD122 (APC Biolegend # 123214) antibodies. Samples were analyzed with a FACS Canto II (BD Biosciences), and data were analyzed with FlowJo (Ashland, OR).

Quantitative RT-PCR

Total RNA was isolated using TRizol, complementary DNA strand was synthesized using reverse transcription kit (Applied Biosystems #4368813), and real-time PCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA; #1725274) on CFX96 real-time PCR instrument (Bio-Rad). Relative expression levels of different transcripts were calculated using double delta CT method and

normalized to the expressions of a house-keeping gene *musBactin*. Graphs and statistical analysis were performed using GraphPad Prism v7.0.

Western Blot

Western Blot was performed as previously described [14]. Briefly, cells were lysed in 2% SDS lysis buffer containing protease inhibitor cocktail (Sigma #P8340), phosphatase inhibitor 2 (Sigma #P5726) and) and phosphatase inhibitor 3 (Sigma #P0044). Cell lysates were incubated on ice for 15 min and denatured at 95°C for 5 min. Protein concentration was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories; #5000112). Proteins were separated by size in 10% Acrylamide, followed by transfer to PVDF membranes. Post-transfer, membranes were blocked with 5% milk for an hour followed by probing with targeted protein antibodies overnight. Finally, HRP-linked Anti-rabbit IgG antibody and Advansta ECL detection kit (VWR #490005-020) were used to detect the plots. ImageJ software (NIH) was used for relative protein quantification.

Statistical analysis

Graphs were made and statistical analyses were performed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA). Student's *t*-test was used to determine the significance, where $P<0.05$ was considered statistically significant.

2.4 Results:

Low-grade inflammatory monocytes polarized by subclinical dose LPS exhibit elevated levels of co-stimulatory molecule CD40 and adhesion molecule CD11a

Previous studies reveal that monocytes/macrophages subjected to a prolonged challenge with pathologically relevant subclinical low dose SLD-LPS can potently express pro-inflammatory cytokines, chemokines, as well as chemokine receptors associated with chronic inflammatory diseases [11, 13, 14]. In addition to cytokines and chemokines, co-stimulatory molecules such as CD40 and adhesion molecules such as CD11a are highly expressed in human monocytes collected from patients with coronary artery diseases [7-9]**.** Given the significance of monocyte retention at inflamed tissues and their communication with adaptive T cells in disease pathogenesis, we herein examined the expression levels of CD40 and CD11a in monocytes stimulated with SLD-LPS. Murine bone marrow derived monocytes (BMDM) were cultured with or without 100 pg/mL LPS for 5 days to drive the polarization of low-grade inflammatory monocytes as reflected in the expansion of $Ly6C^{pos}$ monocyte populations [14]. The expression levels of *Cd11a* and *Cd40* mRNAs were measured by real-time PCR. As shown in Figure 1a and 1b, monocytes with prolonged LPS challenge exhibited significantly elevated levels of *Cd11a* and *Cd40* mRNAs as compared to control monocytes. We further corroborated our observation by examining the surface protein levels of CD11a and CD40 through flow cytometry analysis. As shown in Figure 1c and 1d, prolonged SLD-LPS challenge significantly induced the population of inflammatory $Ly6C^{++}$ $CD11a^{+}$ as well as $Ly6C^{++}$ $CD40^{+}$ monocytes respectively.

Frequent interactions between innate monocytes and T cells occur during disease pathogenesis and contribute to either T cell activation or regulation [39, 40]. With particular relevance, enhanced CD8 T cell activation and reduced $CD8^{+}$ $CD122^{+}$ T regulatory cells are closely related to enhanced pathogenesis of atherosclerosis, both in animal models and human patients with coronary artery diseases [41-43]. Although monocytes have been implicated in the modulation of $CD8^{+}$ $CD122^{+}$ T regulatory cells, molecular mechanisms were not well understood [41, 43]. Given our observation that low-grade inflammatory monocytes express elevated levels of CD11a and CD40, we then tested whether low-grade inflammatory monocytes may selectively reduce the $CD8^{+}CD122^{+}$ T regulatory cell population *in vitro*. Monocytes polarized with either PBS or 100pg/mL LPS were co-cultured with purified T cells in the presence of IL-15 for two additional days which preferentially sustains CD8 T cells [35-38]. As shown in Fig 1e, CD8T cells co-cultured with LPS-polarized monocytes exhibited significantly reduced levels of CD122 as compared to ones co-cultured with control monocytes.

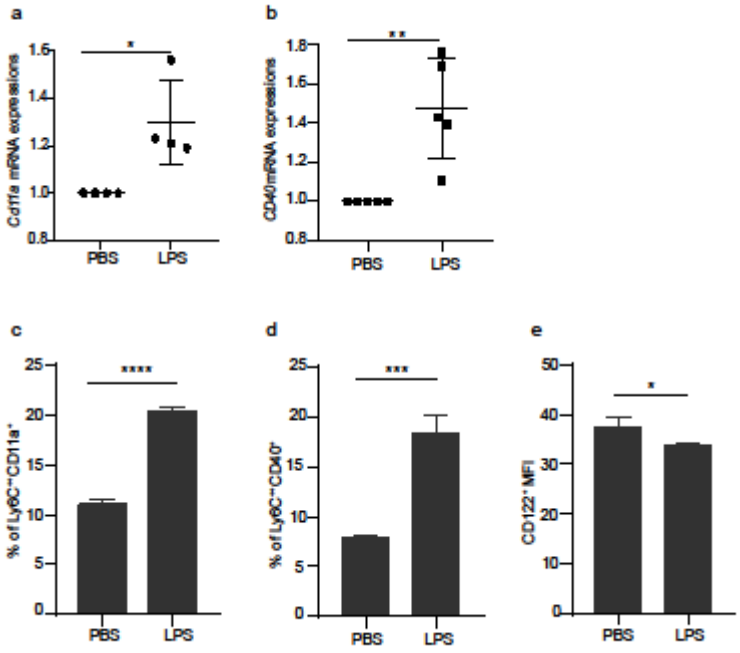

Figure 1. SLD-LPS induces expressions of CD40 and CD11a in BMDM. BMDMs were treated with SLD-LPS (100 pg/mL) or PBS on for 5 days. Relative mRNA expressions of *Cd1a* (a) and *Cd40* (b) normalized to *Bactin* were determined by real-time RT-PCR. Frequency of Ly6C^{++} CD11a^{+} (c) Ly6C^{++} CD40^{+} (d) populations were determined and quantified by flow cytometry. (e) Purified T cells were co-cultured for 48 hours with monocytes previously primed by SLD-LPS. The mean fluorescent intensities (MFI) of CD122 in CD8^{+} T cells were examined and quantified by flow cytometry. Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student *T* test.

SLD-LPS activates intra-cellular signaling processes involved in the expression of CD40 and CD11a

To further understand the underlying molecular mechanisms, we examined the involvement of key signaling molecules involved in the expression of CD11a and CD40 in polarized monocytes. As previous studies suggest that low-grade inflammatory monocytes accumulate reactive oxygen species and cellular stress [11, 44], we tested the activation status of stress kinase p38 as well as oxidized Ca2+/calmodulin dependent protein kinase II (CamKII). oxCAMKII is a downstream molecule oxidized and activated by ROS [45, 46]. Studies in other cellular systems suggest that LPS treatment leads to an increase in oxidative activation of CamKII, further enhancing inflammation signaling [45, 47]. Thus, we tested the levels of activated p38 (p-p38) and oxCAMKII in monocytes polarize by SLD-LPS as compared to PBS control. We observed that SLD-LPS polarized monocytes exhibited significantly elevated levels of p-p38 and oxCAMKII as compared to control monocytes as shown in Figure 2a and 2b respectively.

Previous studies in other cellular systems also suggest that ROS-mediated activation of c-Abl and STAT5 may be critically involved in the expression of CD40 and CD11a [25, 28, 31-34, 48]**.** Although higher doses

of LPS were shown to activate STAT5 [24-27] potentially through JAK2-independent and c-Abl dependent pathway [28-30, 49], it is not known whether pathologically relevant SLD-LPS may similarly activate c-Abl and STAT5. We observed that SLD-LPS can potently induce the activated form of c-Abl (phospho-cAbl) as well as STAT5 in monocytes stimulated with SLD-LPS (Figure 2 c and d, respectively). Collectively, our data suggest that SLD-LPS may induce cellular adhesion and co-stimulatory molecules through activating both the p38 and STAT5 signaling networks.

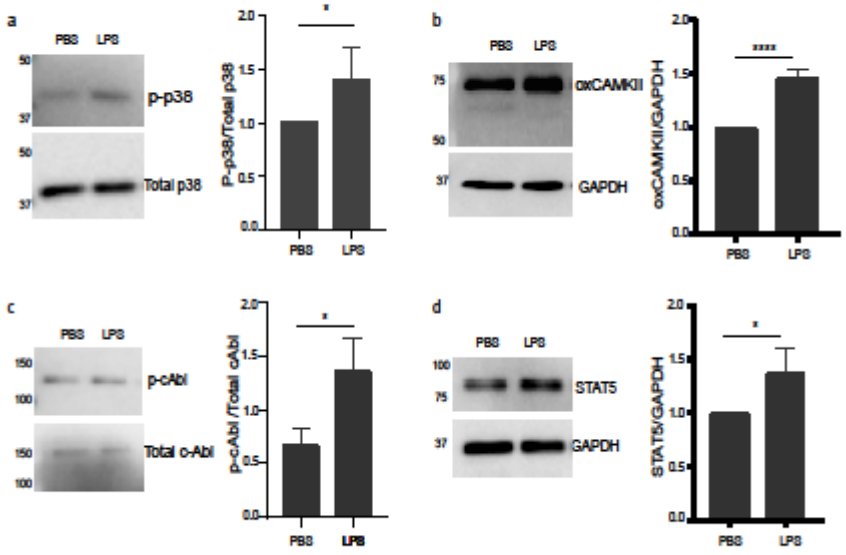

Figure 2. Activation of key signaling molecules in monocytes by SLD-LPS. BMDMs were treated with SLD-LPS (100 pg/mL) or PBS for 5 days. The levels of p-p38, oxCaMKII, p-cAbl, and STAT5 were determined by Western blot (a-d, respectively). Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; ****P < 0.0001; Student *T* test.

IRA-M deletion preferentially expand the population of inflammatory Ly6C^{++} monocytes

Interleukin-1 receptor associated kinase (IRAK)-M, also known as IRAK-3, is an immune suppressor of innate leukocytes [21-23]. Macrophages from IRAK-M KO mice exhibit reduced tolerance and enhanced pro-inflammatory responses as compared to WT controls when treated with LPS [21]. IRAK-M KO mice develop severe atherosclerosis with polarized monocytes expressing elevated levels of MCP-1 [13]. However, it is still unclear which sub-population of monocytes are predominantly modulated by IRAK-M. We therefore compared the activation profiles of WT and IRAK-M KO monocytes challenged with SLD-LPS. Consistent with our previous findings [13, 50], prolonged super-low dose LPS challenge led to the expansion of both inflammatory CD11b^{+}Ly6C^{+} as well as CD11b^{+}Ly6^{++} WT monocytes (Figure 3 a and

b). Interesting, IRAK-M deletion preferentially expanded the classical $CD11b^{+}Ly6C^{++}$ inflammatory monocytes as compared to WT cells treated with SLD-LPS (Fig 3).

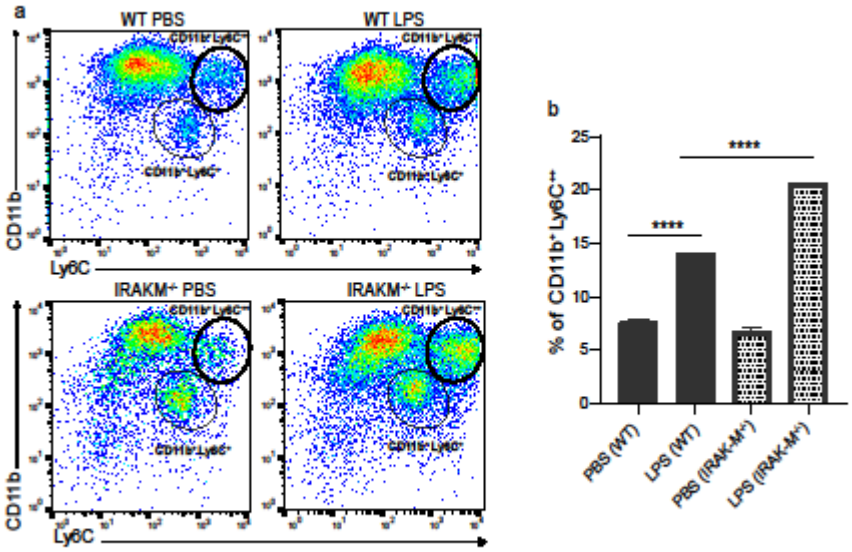

Figure 3. IRAK-M deletion leads to the expansion of inflammatory $CD11b^{+}Ly6C^{++}$ monocytes. WT and IRAK-$M^{-/-}$ monocytes were treated with SLD-LPS (100 pg/mL) or PBS for 5 days, and the $CD11b^{+}Ly6C^{++}$ population was determined and quantified by flow cytometry (**a** and **b**, respectively). Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). ****$P < 0.0001$; Student *T* test.

IRAK-M deficiency further enhances the expression of CD40 and CD11a on inflammatory monocytes

IRAK-M KO mice exposed to ovalbumin exhibit elevated infiltration of airway inflammatory cells, higher proinflammatory cytokine levels in lung homogenates and enhanced macrophages activation represented by elevated expression of co-stimulatory molecules [51, 52]. Based on our findings in Figure 3, we tested whether IRAK-M may also modulate the expression CD11a and CD40, specifically in $Ly6C^{++}$ monocytes polarized by SLD-LPS. Indeed, as measured by flow cytometry, we observed that the population of $Ly6C^{++}CD11a^{+}$ and $Ly6C^{++}CD40^{+}$ monocytes were significantly expanded in WT BMDMs cultured by SLD-LPS for 5 days. Strikingly, the population of $Ly6C^{++}CD11a^{+}$ and $Ly6C^{++}CD40^{+}$ monocytes were further expanded in IRAK-M KO BMDMs cultured with SLD-LPS as compared to WT BMDMs (Figure 4a and 4b). Given the expansion of $Ly6C^{Pos}$ monocytes due to IRAK-M deletion, we further tested whether IRAK-M KO monocytes may further reduce the $CD8^{+}CD122^{+}$ T regulatory cell population. Through the co-culture experiment, we observed that the MFI of CD122 were significantly reduced within $CD8^{+}$ T cells (representative of CD8T regulatory cells) following co-incubation with IRAK-M KO monocytes as

compared to T cells co-incubated with WT monocytes (Figure 4c). Our data extend previous observations regarding the inhibitory role of IRAK-M during the polarization of low-grade inflammatory monocytes mediated by pathologically relevant SLD-LPS.

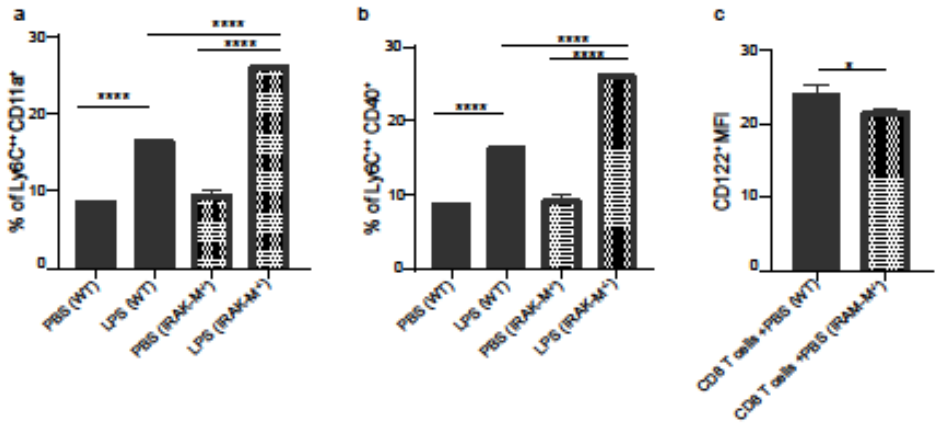

Figure 4. IRAK-M deficiency enhances the expression of CD40 and CD11a on inflammatory monocytes. WT and IRAK-M$^{-/-}$ BMDMs were treated with SLD-LPS (100 pg/mL) or PBS for 5 days. Frequencies of Ly6C^{++}CD11a^{+} (a) and Ly6C^{++} CD40^{+} monocytes (b) in WT and IRAK-M$^{-/-}$ BMDM were quantified via flow cytometry. (c) Purified T cells were co-cultured for 48 hours with either WT or IRAK-M$^{-/-}$ monocytes. The mean fluorescent intensities (MFI) of CD122 in CD8 T cells were examined and quantified by flow cytometry. Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; ****P < 0.0001; Student *T* test.

IRAK-M negatively modulates monocyte polarization by SLD-LPS through suppressing TLR4 adaptor molecule TRAM

Mechanistically, IRAK-M negatively regulates the MAPK and inflammatory signaling pathway following TLR4 activation [21, 53]. We herein confirmed the effects of IRAK-M in low-grade inflammatory monocytes polarized by SLD-LPS. We observed that IRAK-M KO monocytes challenged with SLD-LPS exhibited elevated levels of phosphorylated p38 (Fig. 5a). IRAK-M was also shown to suppress STAT5 in neutrophils [54]. We found that IRAK-M KO monocytes polarized with SLD-LPS exhibited elevated levels of phospho-c-Abl as well as STAT5 as compared to their WT counterparts (Fig 5b and 5c respectively). Since emerging studies suggest that TRAM instead of MyD88 is critically involved in mediating the effects

of SLD-LPS in monocytes [15, 50], we further tested whether IRAK-M may also modulate TRAM levels. Interestingly, we observed that TRAM protein levels were elevated in IRAK-M KO monocytes as compared to WT monocytes following the stimulation of SLD-LPS (Figure 5d). Our data suggest a novel mechanism that IRAK-M deletion may enhance monocyte polarization by SLD-LPS through increasing TRAM-mediated intra-cellular signaling processes.

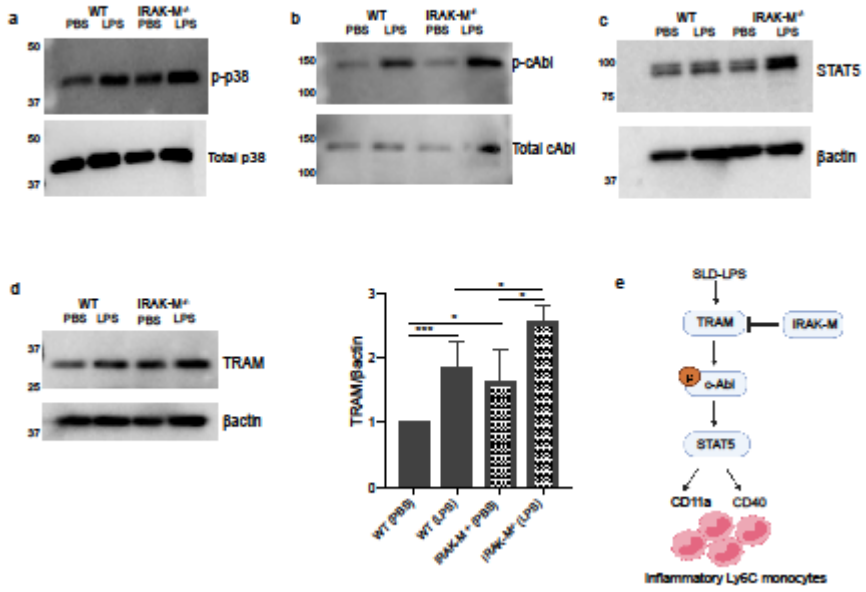

Figure 5. Enhanced molecular signaling and elevated TRAM levels in IRAK-M deficient monocytes. BMDMs from WT and IRAK-M$^{-/-}$ mice were treated with SLD-LPS (100 pg/mL) or PBS for 5 days, and the levels of p-cAbl, STAT5 and p-p38 were determined by Western Blot (a-c). Protein levels of TRAM were determined by Western Blot and quantified using imageJ (NIH) (d). The proposed mechanism was represented in the illustration (e). Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; **P < 0.01; Student *T* test.

TRAM deficiency reduces the population of CD11b^{+}Ly6C^{++} inflammatory monocytes

With emerging evidence suggesting TRAM as a key mediator for sustaining non-resolving inflammation in monocytes, we examined the impact of TRAM deficiency on the monocyte polarization by SLD-LPS. As shown in Figure 6, we observed that TRAM deficiency completely ablated the expansion of CD11b^{+}Ly6C^{++} population upon stimulation with SLD-LPS. At the mechanistic level, we observed that STAT5 induction by LPS was also completely ablated in TRAM KO monocytes (Fig 6 c). Our data further confirm that TRAM is critically responsible for mediating the effects of subclinical super-low dose LPS. Collectively, our data reveal a novel toggle switch involving IRAK-M and TRAM, involved in modulating the expansion of inflammatory CD11b^{+}Ly6C^{++} monocytes.

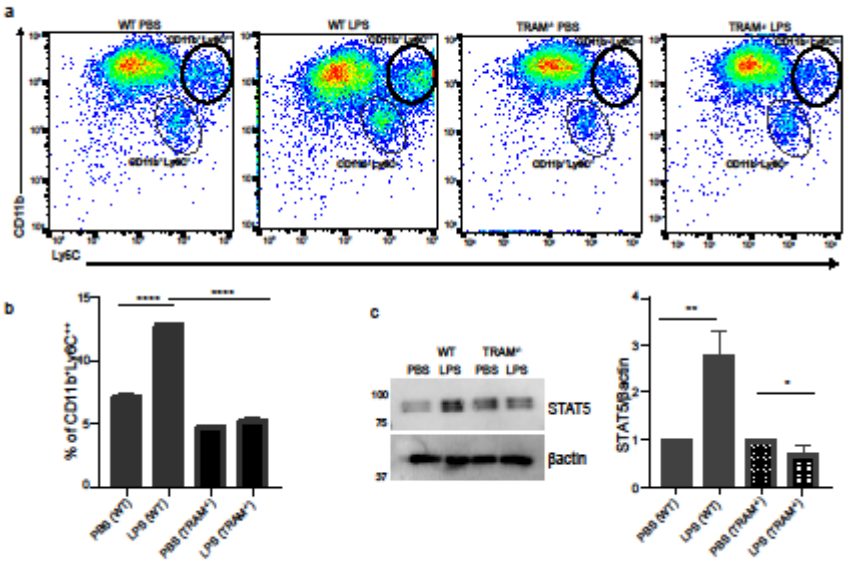

Figure 6. TRAM deficiency abolishes the expansion of Ly6C monocytes. WT and TRAM$^{-/-}$ BMDMs were treated with SLD-LPS (100 pg/mL) or PBS for 5 days and the CD11b^{+}Ly6^{++} population was determined and quantified by flow cytometry (a and b, respectively). Protein levels of STAT5 was determined by Western Blot and quantified using imageJ (NIH) (c). Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; **P < 0.01; ****P < 0.0001; Student *T* test.

TRAM deficiency dampens the expression of CD40 and CD11a

Since we observed that TRAM deficiency significantly reduces the population of CD11b^{+}Ly6C^{++} inflammatory monocytes, we further tested whether TRAM deficiency may also impact the levels of CD11a and CD40 post-SLD-LPS stimulation. We observed that Ly6C^{++}CD11a^{+} as well as Ly6C^{++}CD40^{+} populations were significantly reduced in TRAM KO BMDM as compared to the WT counterparts (Figure 7 a and b, respectively). We further tested whether monocytes with TRAM deletion may modulate CD122 levels on co-cultured CD8^{+} T regulatory cells. Purified T cells were co-cultured with TRAM KO monocytes as described in the methods section. We observed that the levels of CD122 were significantly elevated in CD8T cells co-cultured with TRAM deficient monocytes as compared to WT monocytes (Figure 7 c). Together, our data suggest that TRAM deletion could potentially dampen low-grade inflammation by reducing the expression of adhesion and co-activating molecules on monocytes while enhancing regulatory CD8T cells.

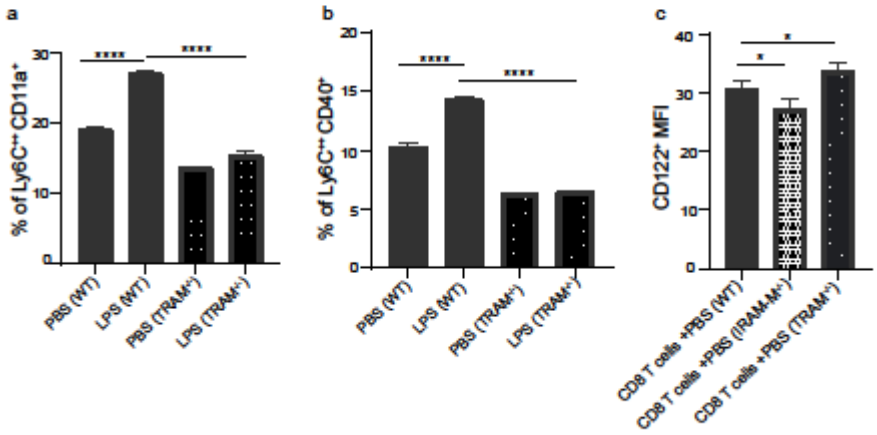

Figure 7. SLD-LPS fails to induce CD40 and CD11a in TRAM deficient monocytes. Frequency of $Ly6C^{++}CD11^{+}$ (a) and $Ly6C^{++}CD40^{+}$ (b) population in WT and $TRAM^{-/-}$ BMDM after 5-day SLD-LPS (100 pg/mL) treatment were analyzed and quantified using flow cytometry. Purified T cells were co-cultured for 48 hours with either WT or $TRAM^{-/-}$ monocytes. The mean fluorescent intensities (MFI) of CD122 in CD8 T cells were examined and quantified by flow cytometry (c). Data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). *P < 0.05; ****P < 0.0001; Student *T* test.

CD40 and CD11a levels are elevated in monocytes collected from IRAK-M KO mice, and reduced in monocytes from TRAM KO mice

We next examined the *in vivo* relevance of our *in vitro* observations. We utilized the ApoE deficient mouse model commonly used in the atherosclerosis study. $ApoE^{-/-}$, $ApoE^{-/-}$/IRAK-$M^{-/-}$ and $ApoE^{-/-}$/$TRAM^{-/-}$ mice were fed with high fat diet for two months as previously described to induce the pathogenesis of atherosclerosis [13, 55-57]**.** At the end of the feeding regimen, splenic monocytes were analyzed for the expression levels of CD11a through flow cytometry. As shown in Fig 8, we observed expansions of $CD11b^{+}Ly6C^{++}$ monocytes as well as $Ly6C^{++}CD11a^{+}$ monocytes from $ApoE^{-/-}$/IRAK-$M^{-/-}$ mice compared to $ApoE^{-/-}$ mice (Fig 8a and b). We further examined the levels of $CD8^{+}CD122^{+}$ T regulatory cells. As shown in Fig 8 c, the levels of CD122 within the CD8 T population (representative of CD8T regulatory cells) were significantly reduced in $ApoE^{-/-}$/IRAK-$M^{-/-}$ mice as compared to $ApoE^{-/-}$ mice.

In sharp contrast, the populations of $CD11b^{+}Ly6C^{++}$ monocytes as well as $Ly6C^{++}CD11a^{+}$ monocytes were significantly reduced in splenocytes harvested from $ApoE^{-/-}$/ $TRAM^{-/-}$ mice as compared to $ApoE^{-/-}$ mice (Fig 8 d and e). Correspondingly, the levels of CD122 within the CD8T cells were significantly elevated

in ApoE$^{-/-}$/TRAM$^{-/-}$ mice as compared to ApoE$^{-/-}$ mice (Figure 8f). Together, our *in vivo* data suggest that IRAK-M negatively modulates TRAM-mediated monocyte polarization and communication with adaptive T cells in vivo, potentially contributing to the pathogenesis of atherosclerosis.

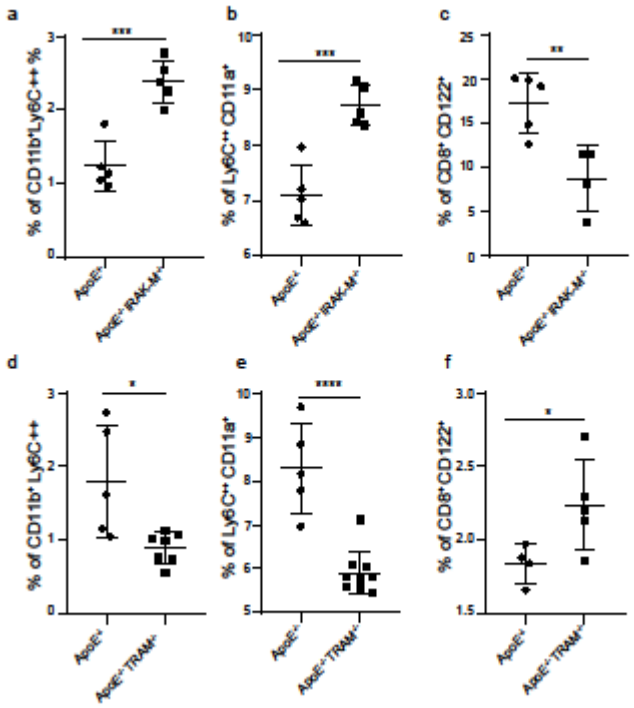

Figure 8. IRAK-M deficiency enhances while TRAM deficiency reduces inflammatory phenotypes in atherosclerotic mice. ApoE$^{-/-}$, ApoE$^{-/-}$ IRAK-M$^{-/-}$ and ApoE$^{-/-}$ TRAM$^{-/-}$ mice were fed with a high-fat diet for two months. The frequencies of CD11b^{+} Ly6C $^{++}$ cells (a and d); Ly6C^{++} CD11a^{+} cells (b and e) and CD8^{+} CD122^{+} T cells (c and f) in splenocytes were analyzed and quantified by flow cytometry. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student *T* test.

2.5 Discussion:

This study reveals the potent induction of adhesion molecular CD11a and co-activating molecule CD40 on low-grade inflammatory monocytes by subclinical super-low dose LPS, regulated by a competing intra-cellular circuitry involving TRAM and IRAK-M. IRAK-M deficient monocytes have elevated CD11a and CD40 induction by SLD-LPS, and exhibit an elevated immune-enhancing effect by suppressing CD8 T regulatory cells. Deficiency in IRAK-M, the TLR4 pathway suppressor, causes an increase in TLR4 signaling adaptor TRAM as well as downstream signaling processes, leading to the elevated expression of CD11a and CD40. In contrast, the induction of CD11a and CD40 is significantly attenuated in TRAM deficient monocytes.

Our data better clarify the phenotypic nature of low-grade inflammatory monocytes related to the pathogenesis of chronic inflammatory disease. The generation of low-grade inflammatory monocytes under

chronic inflammatory conditions is well recognized as a crucial contributor for the pathogenesis of diseases such as atherosclerosis, through secreting inflammatory mediators as well as accumulating lipids and forming foam cells [13, 58]. Monocytes further contribute to the pathogenesis of atherosclerosis through enhanced retention within atherosclerotic plaques and amplification of local inflammatory environment by interacting with adaptive immune cells [6, 13, 59, 60]. Elevated expression of key adhesion molecule CD11a as well as co-activating molecule CD40 are critical signatures of inflammatory monocytes from both human patients with coronary artery diseases as well as in animal models of atherosclerosis [7-9]. Subclinical endotoxemia is a key risk factor for low-grade inflammation and atherosclerosis [10, 12, 13, 61]. Extending previous reports that demonstrated an induction of soluble inflammatory mediators in monocytes challenged with subclinical super-low dose LPS, our current data reveal that monocytes polarized by subclinical super-low dose LPS adopt a low-grade inflammatory state with potent immune-enhancing effects, characterized by elevated expression of adhesion molecule CD11a as well as co-activating molecule CD40, capable of amplifying immune activation through suppressing the CD8T regulatory cells both *in vitro* and *in vivo*.

Mechanistically, our data identify a competing intra-cellular network involving IRAK-M and TRAM that modulates the expression of CD11a/CD40 in low-grade inflammatory monocytes challenged with subclinical super-low dose LPS. Previous studies suggest that IRAK-M is a potent suppressor of TLR4 signaling in monocytes and macrophages, primarily through suppressing MyD88-mediated assembly of IRAK1 activation complex [21]. IRAK-M deficient monocytes have elevated expression of pro-inflammatory cytokines when challenged with LPS [21, 52]. Extending these observations, our current study further reveal that IRAK-M specifically suppresses the $CD11b^{+}Ly6C^{++}$ inflammatory monocytes as well as the expression of CD40 and CD11a in low-grade inflammatory monocytes induced by SLD-LPS. Together with previous studies reporting that infiltrating monocytes within inflamed tissues were largely of $Ly6C^{pos}$ origin [62-64], our current study helps to explain increased monocytes/macrophage contents observed in atherosclerotic plaques of experimental animals with IRAK-M deletion [13]. The suppression of anti-inflammatory CD8T regulatory cells by the expanded population of $Ly6C^{++}$ monocytes due to IRAK-M deficiency may further exacerbate the inflammatory environment propagating the pathogenesis of atherosclerosis. Our current study provides an initial clue for intervening the complex interaction of innate and adaptive immune cells through altering either IRAK-M or TRAM in monocytes. Based on our limited phenotypic observation, future in-depth analyses are clearly needed in order to better define molecular and cellular dynamics involved in the cross-talk among monocytes and adaptive immunity.

Intriguingly, we observed that IRAK-M deficiency in the low-grade inflammatory monocytes exhibit elevated expression of TRAM, a unique adaptor molecule downstream of TLR4. This is consistent with

previous reports that TRAM, instead of MyD88, is the key adaptor that mediates the low-grade inflammatory responses to super-low dose LPS [15, 50]. We observed that TRAM deficient monocytes have significantly reduced population of $CD11b^{+}Ly6C^{++}$ inflammatory monocytes as well as reduced expression of CD11a and CD40 following stimulation with super-low dose LPS. Our current finding complements a previous *in vivo* study showing reduced atherosclerosis pathogenesis in mice with myeloid deletion of TRAM instead of MyD88 [20]. Together with previous findings that subclinical dose LPS can sustain the polarization of low-grade inflammatory monocytes through reducing the expression of IRAK-M [13], our data reveal a mutually inhibitory toggle switch composed of IRAK-M and TRAM that modulates the polarization of inflammatory monocytes. Subclinical endotoxemia may switch on the inflammatory polarization through reducing IRAK-M and enhancing TRAM.

Collectively, our study reveals an immune-enhancing phenotype of low-grade inflammatory monocytes with elevated expression of adhesion molecular CD11a and co-activating molecule CD40. Our data also clarifies the unique role of IRAK-M in modulating TRAM-mediated polarization of low-grade inflammatory monocytes capable of interacting with adaptive immune environment within the inflamed tissues. These findings provide important clues for future translational studies toward systems characterization of pathogenic monocytes involved in the pathogenesis of atherosclerosis.

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Chapter 3: Development of exhausted memory monocytes and underlying mechanisms

3.1 Abstract:

Pathogenic inflammation and immuno-suppression are cardinal features of exhausted monocytes increasingly recognized in septic patients and murine models of sepsis. However, underlying mechanisms responsible for the generation of exhausted monocytes have not been addressed. In this report, we examined the generation of exhausted primary murine monocytes through prolonged and repetitive challenges with high dose bacterial endotoxin lipopolysaccharide (LPS). We demonstrated that repetitive LPS challenges skew monocytes into the classically exhausted Ly6C^{hi} population, and deplete the homeostatic non-classical Ly6C^{lo} population, reminiscent of monocyte exhaustion in septic patients. scRNAseq analyses confirmed the expansion of Ly6C^{hi} monocyte cluster, with elevation of pathogenic inflammatory genes previously observed in human septic patients. Furthermore, we identified CD38 as an inflammatory mediator of exhausted monocytes, associated with a drastic depletion of cellular NAD^+; elevation of ROS; and compromise of mitochondria respiration, representative of septic monocytes. Mechanistically, we revealed that STAT1 is robustly elevated and sustained in LPS-exhausted monocytes, dependent upon the TRAM adaptor of the TLR4 pathway. TRAM deficient monocytes are largely resistant to LPS-mediated exhaustion, and retain the non-classical homeostatic features. Together, our current study addresses an important yet less-examined area of monocyte exhaustion, by providing phenotypic and mechanistic insights regarding the generation of exhausted monocytes.

3.2 Introduction:

Sepsis is a life threatening disease caused by dysregulated host inflammatory response leading to organ dysfunction (1, 2). Traditionally, a biphasic immune response with initial hyper-inflammation followed by immunosuppression depicted septic conditions, with the latter immuno-paralysis primarily contributing to long-term sepsis-related illness and infection (2-4). It is now accepted that both inflammatory and anti-inflammatory responses are simultaneously activated during sepsis, and dominance of one response over the other leads to dysfunctional immune response followed by exacerbated septic conditions (5). Leukocyte transcriptome data also reveal a simultaneous increase in expression of genes involved in systemic inflammatory and compensatory immunosuppressive response post-severe injury (6). Over 20% of patients that recover from sepsis either die within the first year after recovery from secondary infections or the survivors suffer from imbalances in immune responses due to prolonged inflammation and immune suppression (7). Chronic immune dysfunction in sepsis survivors is irreversible (8) emphasizing the urgency to thoroughly investigate sepsis-related immune dysfunction. However, cellular and molecular mechanisms that underlie the generation of pathogenic inflammation and immuno-suppression are still poorly understood.

Immune dysfunctions in monocytes of sepsis patients have been well-noticed through basic and translational studies. Despite a reduction of inflammatory cytokines, monocytes from septic patients exhibit elevated production of reactive oxygen species (ROS) as well as nitric oxide (NO), potentially contributing to pathogenic inflammation (9). Septic monocytes also have compromised CD86 and HLA-DR (MHC-II in mice) expressions while maintain elevated PD-L1 expressions, collectively correlated with immunosuppressive phenotypes (2, 10, 11). Flow cytometry based studies further identify that the classical monocytes (murine $Ly6C^{hi}$, and human $CD14^{hi}CD16^{lo}$) may exhibit inflammatory natures with elevated ROS production (12, 13). In contrast, the non-classical monocytes (murine $Ly6C^{-}$, and human $CD14^{lo}CD16^{hi}$) play important beneficial roles in maintain vasculature homeostasis and reducing pathogenic inflammation (12, 13). Infiltration of $Ly6C^{pos}$ monocytes in early as well as late phases of sepsis has been reported in experimental murine models (5, 7). Recent studies also reveal a sharp decrease of the human non-classical monocyte populations in septic patients as well as patients with severe COVID-19 (7, 14, 15). Despite these important phenotypic observations, mechanisms responsible for the development of pathogenic inflammatory and immuno-suppressive classical monocytes are still poorly defined.

Previous *in vitro* studies with cultured monocytes recapitulating septic conditions almost solely focused on the aspect of suppressed expression of inflammatory cytokines commonly known as endotoxin tolerance (3, 16). Monocytes/macrophages with repetitive challenges of bacteria endotoxin Lipopolysaccharide (LPS) have blunted expression of inflammatory cytokines, partly due to MyD88-mediated induction of

NFkB transcriptional suppressors including RelB (17, 18). However, the traditional concept of endotoxin tolerance fails to capture the cardinal features of pathogenic inflammation and immuno-suppression manifested in both human septic patients and experimental murine sepsis models. Indeed, some limited studies suggest that "tolerant" monocytes are not transcriptionally inactive. Rather, they can still robustly express "non-tolerizable" genes involved in pathogenic inflammation and immuno-suppression (6, 19, 20). Given emerging data revealing a complex "exhausted" nature of septic monocytes capable of expressing pathogenic inflammatory genes and immuno-suppressive genes, it is necessary to conduct mechanistic studies to address the generation of exhausted pathogenic monocytes, which is currently lacking in the field.

To fill this critical gap, we performed studies with integrative approaches of scRNAseq examining gene expression profiles and flow cytometry analyses of key protein markers reflecting exhausted monocytes cultured in vitro with repetitive challenges of higher dose LPS. We validated the expansion of exhausted $Ly6^{hi}$ monocytes, and confirmed representative marker genes up or down-regulated in monocytes collected from septic patients in our in vitro culture system. Furthermore, we identified CD38 as a key marker that is robustly and persistently elevated in exhausted monocytes. CD38 is an ecto-enzyme involved in degrading and depleting both inter-cellular and intra-cellular NAD^+, and potentially propagate inter-cellular inflammation (21-24). We also observe a drastic depletion of cellular NAD^+, compromised mitochondria respiration and elevated cellular ROS in exhausted monocytes cultured with repetitive LPS challenges. Mechanistically, we observed that exhausted monocytes have drastically elevated STAT1 and KLF4 activation, dependent upon TRAM, the other arm of the TLR4 adaptor pathway. Together, our data reveal that sustained activation of STAT1 via TRAM pathway may be responsible for the generation of exhausted monocytes, with polarized induction of pathogenic inflammatory mediators such as CD38; and suppression of co-stimulatory molecule CD86.

3.3 Materials and Methods:

In vitro cell culture

Primary murine cells from either C57BL/6 (WT) or TRAM KO mice were used for in vitro cell culture. The WT mice were purchased from Jackson's Laboratory and TRAM KO mice with C57BL/6 background were kindly provided by Dr. Holger Eltzschig (University of Texas Houston) and regularly maintained in our laboratory. All animal procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee (IACUC) of Virginia Tech. Bone marrow cells were harvested from WT and TRAM KO mice as described previously (19, 25, 26). Primary cells were cultured in complete RPMI 1640 media with supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-Glutamine and 10ng/mL M-CSF (PeproTech, Rocky Hill, NJ; no. 315-02). Cells were treated with either PBS (control) or high-dose LPS (100 ng/mL) for 5 days at 37˚C in a humidified 5% CO2 atmosphere. Fresh complete media supplemented with M-CSF and treatments were added every 2 days as reported previously(19, 25, 26). As we previously characterized, bone marrow cells cultured under such condition were monocyte-like, loosely adherent, and did not express mature macrophage marker CD71 (27).

Reagents

LPS (Escherichia coli 0111:B4) was purchased from Sigma-Aldrich. Primary anti-STAT1 (#9172S, 1:1000 in 5% BSA) and anti-phospho-STAT1 (#9177S, 1:1000 in 5% BSA) antibodies were purchased from Cell Signaling. Anti-β-actin (HRP) (# 47778, 1:1000 in 5% BSA) antibody was purchased from Santa Cruz. Primary anti-KLF antibody (#ab129473, 1:1000 in 5% BSA) was purchased from Abcam. N-Acetyl-L-cysteine or NAC (#A9165) was purchased from Sigma-Aldrich. Fludarabine, a STAT1 activation inhibitor (S1491) was purchased from Selleck Chemicals.

Western blot analyses

Western blot was performed as previously described (25). Cells were lysed in 2% SDS lysis buffer containing protease inhibitor (Sigma, #P8340), phosphatase inhibitor 2 and 3 (Sigma, #P5726 and # P0044, respectively). Cell lysates were denatured at 95°C for 5 minutes and protein concentrations were determined using the Bio-Rad DC Protein Assay Kit (#5000112). Proteins were separated by electrophoresis using 10% Acrylamide gel and transferred to PVDF membranes. Membranes were then blocked with 5% milk for 1 h, followed by incubation with targeted primary antibodies at 4°C overnight and secondary HRP-conjugated anti-rabbit IgG antibody (#7074, 1:2000 in 5% milk) at room temperature for 1 h. Images were developed

with ECL detection kit (VWR, # 490005-020), and the relative expressions of target protein were quantified with ImageJ software (NIH).

NAD^+ assay

Amplite™ Fluorimetric cADPR-Ribose Assay Kit was used to determine the NAD^+ levels following manufacturer's instructions. Cytation^3 Image Reader (BioTek) was used to quantify NAD+ levels.

Flow cytometry

On Day 5, murine monocytes were washed with PBS, harvested, and blocked in with 1:100 Fc block (BD Biosciences, #553141). Cells were then stained with fluorochrome-conjugated antibodies against Ly6C (PE-Cy7; Biolegend #128018), CD11b (allophycocyanin-Cy7; Biolegend #101226), CD86 (FITC; Biolegend # 105006), MHCII (FITC; Biolegend # 107606), PD-L1 (APC; Biolegend # 124312) and CD38 (PE; Biolegend # 102708), for 30 minutes followed by washing with FACS buffer. Finally, cells were resuspended in (Life Technologies # P3566) in FACS buffer containing Propidium Iodide (PI, 1:200). To determine intracellular ROS levels, CellROX Deep Red Reagent (Life Technologies #C1049) was added to cell cultures 45min before harvesting and the cells were also co-stained with and Live/Dead (Life Technologies # L34970). Samples were analyzed with a FACS Canto II (BD Biosciences), and data were analyzed with FlowJo (Ashland, OR)

Seahorse Assay

Monocytes (10^6 cells/mL) were seeded in the Seahorse Bioscience XF24 (Agilent) cell culture plates and cultured for 5 days. Fresh media and treatments were added every 2 days. Cell Mito Stress Test was performed by sequentially treating the cells with 0.5 μM oligomycin, 0.6 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and 0.1 μM rotenone/antimycin A according to the manufacturer's instructions.

scRNA-seq and data analysis

Cell preparation and scRNAseq were performed as we previously described (27). Briefly, FACS-purified cells were resuspended in cold PBS supplemented with 0.04% BSA (VWR). About 1,000 cells were targeted for the experiment. Gel-Bead in Emulsions (GEMs) were generated using the 3′ V3 chemistry (Chromium Single Cell 3' Reagent Kit, 10X Genomics) and RT reaction was conducted in the C1000 touch PCR instrument (BioRad). Barcoded cDNA was obtained from the GEMs by Post-GEM RT-cleanup and amplified for 12 cycles. Amplified cDNA was subjected to enzymatic fragmentation, end-repair, A tailing, adaptor ligation and 10X specific sample indexing as per manufacturer's protocol. Libraries quality and quantity were determined using Bioanalyzer analysis. Libraries were then pooled and sequenced on

Illumina HiSeq platform (Novogene). Analyses of data were performed as we previously published (28) and briefly described below. Cell Ranger (version 3.1.0) from the 10X Genomics website (https://support.10xgenomics.com/single-cell-gene-expression/software) were used to annotate mouse reference genome, perform the alignment and mapping of sequenced reads, as well as the quantification of relative levels of gene expression. The default pipeline of Seurat (version 3.1.4) in R were used to perform quality control and data normalization. Doublets as well as cells with fewer than 200 unique genes were excluded. Genes that existed in fewer than 3 cells were also removed. A small cluster of neutrophils expressing *Ly6g* was excluded. Dimensionality reduction was performed by principal component analysis (PCA), and cells were clustered through UMAP analysis as we reported (28). Differentially expressed genes with significant differences among distinct clusters were identified. Selected genes from top 200 differentially expressed genes as well as patho-physiologically relevant players involved in pathogenic inflammation and immunosuppression related to sepsis were presented on the heat-map and the dot plot. scRNAseq data sets were deposited in the Genebank with the accession number GSE182355.

Statistical analysis

GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA) was used to generate graphs and to perform statistical analyses. Student's *t*-test (for two groups) or one-way analysis of variance (ANOVA) (For multiple groups) was used to determine the significance, where $P<0.05$ was considered statistically significant.

3.4 Results:

Generation of exhausted monocytes with prolonged and repetitive treatments of high dose LPS

To monitor the exhaustion of primary monocytes to persistent challenges of bacterial endotoxin LPS, we cultured murine monocytes derived from bone marrow for 5 days in the presence of M-CSF to maintain cell survival as we described (25, 26), with the repetitive challenges with either PBS or high dose LPS (100ng/mL) on Day 0, 2 and 4. We then harvested the cultured cells at day 1, 3, and 5 for flow cytometry analyses of CD86, MHCII and PD-L1. CD86 and MHCII are key immune-enhancing mediators, while PD-L1 is a well-recognized immune suppressor. A reduction of CD86 and MHCII coupled with an elevation of PD-L1 were shown as key signatures of monocyte exhaustion closely associated with the pathogenesis of sepsis (2). As shown in Fig 1 A and B, the initial LPS challenge led to an induction of CD86 and MHCII expression during the initial days of LPS challenge compared to PBS controls, as measured by flow cytometry analyses, consistent with previous findings (2). Expectedly, as the treatment regimen continued, the expression levels of MHCII and co-stimulatory molecule CD86 were reduced at day 5 of the treatment regimen compared to PBS controls, reminiscent of the development of LPS tolerance (Fig 1A and 1B, Supplementary Fig S1A and S1B). In sharp contrast, the expression of PD-L1 was not tolerized, and continually induced in monocytes following repetitive LPS challenge at day 3 and day 5 time points compared to PBS controls (Fig 1C, Supplementary Fig S1C). Collectively, our *in vitro* system of repetitive LPS challenges for a 5-day time period generated a representative monocyte exhaustion phenotype with reduced expression of MHCII and co-stimulatory molecule CD86, as well as continued induction of immuno-suppressor PD-L1, consistent with the exhausted monocyte phenotype observed in experimental animals and human sepsis patients (29-31). We therefore used the 5-day culture system to further characterize the exhausted monocytes in this study.

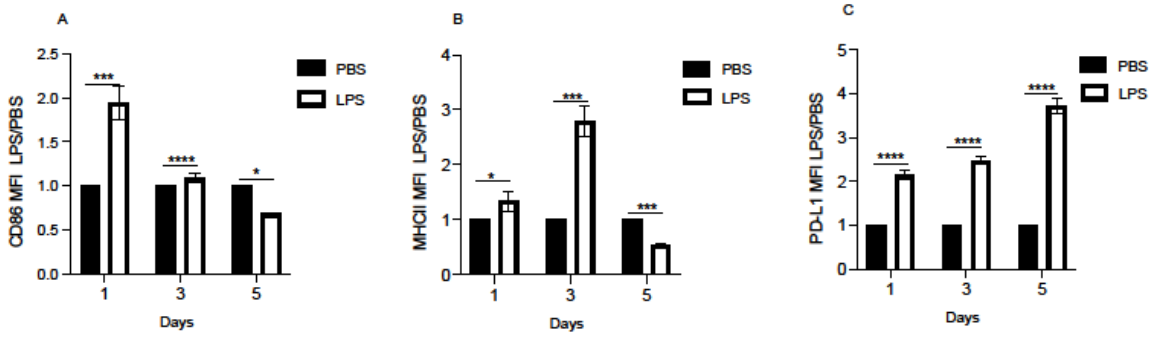
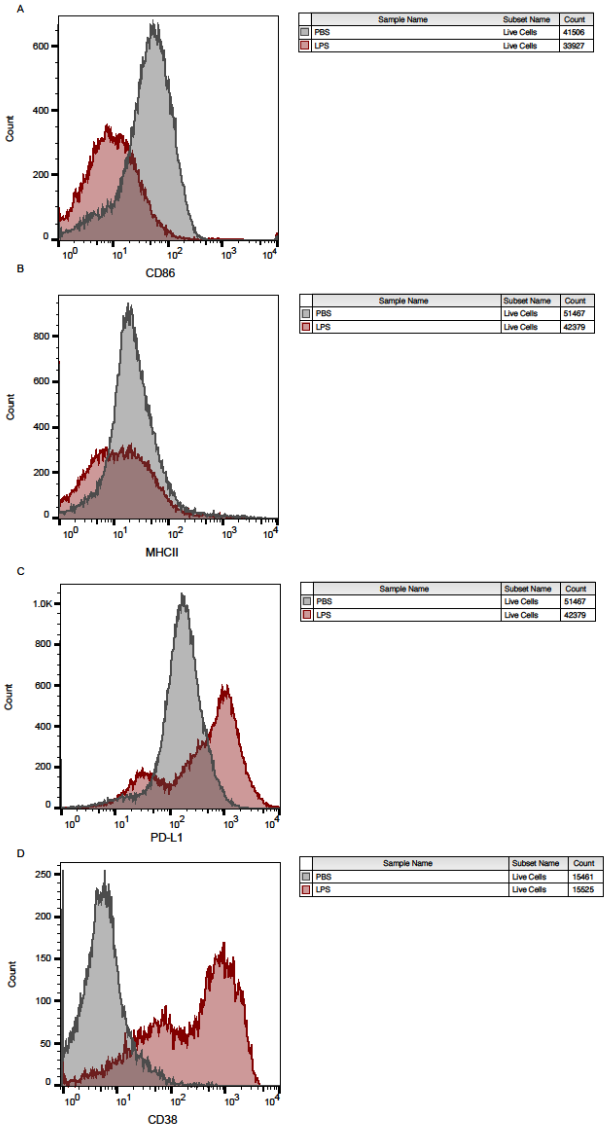


Figure 1: Generation of exhausted monocytes via repetitive LPS challenges. BMDMs were treated with PBS or high dose LPS (100ng/mL) for 5 days. (A-C) The Mean Fluorescent Intensity (MFI) of CD86, MHCII and PD-L1 on day 1, 3 and 5 were monitored using flow cytometry. The relative fold changes in MFIs of CD86, MHCII and PD-L1 comparing LPS-treated vs PBS-treated monocytes were determined and plotted in the graphs. Data were representative of three independent experiments, and error bars represent means ± SEM (n=3 for each group). *p <0.05, ***p < 0.001, ****p < 0.0001, Student *t* test.



Supplementary Figure S1. Representative histograms of monocyte tolerant and exhaustion markers.

BMDMs from WT mice were treated with PBS or high dose LPS (100ng/mL) for 5 days. (A-D) Representative histograms illustrating shifts in surface MFIs of CD86 (A), MHCII (B), PD-L1 (C) and CD38 (D) in live cells obtained via flow cytometry were presented.

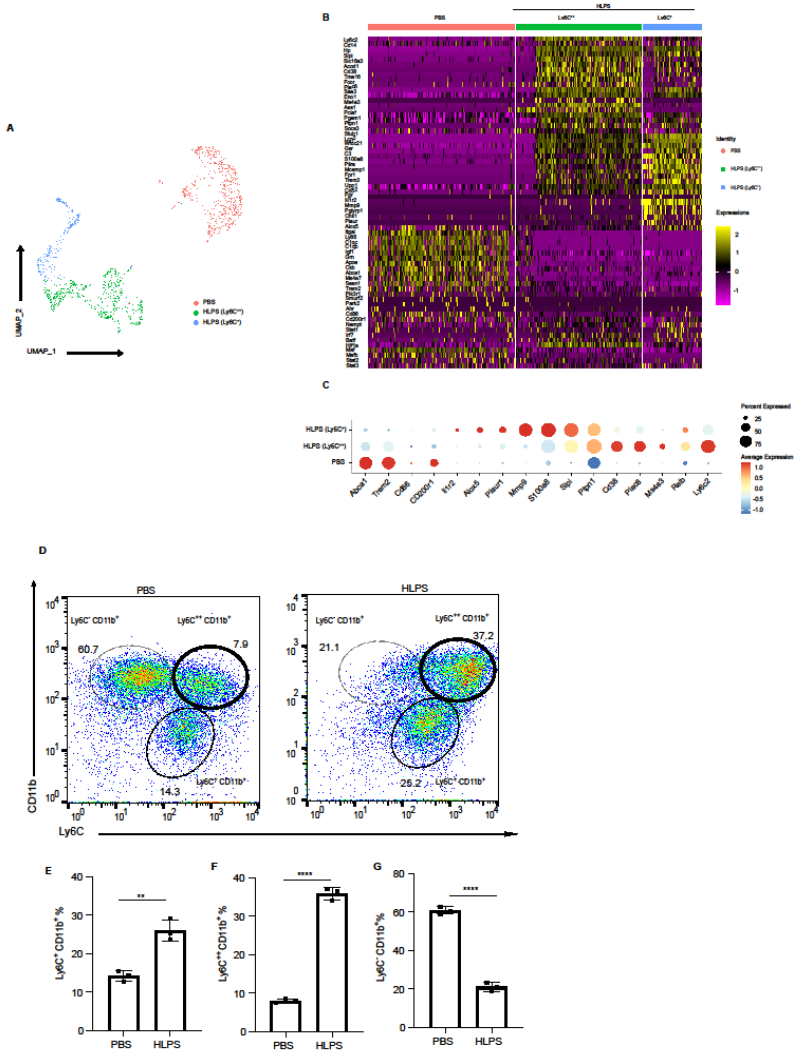
Systems analyses of exhausted monocytes reveal significantly elevated CD38

To further systematically define the nature of exhausted monocytes generated *in vitro*, we performed scRNAseq analyses of monocytes cultured for 5 days with PBS or high dose LPS. UMAP analyses indicated that cultured control monocytes largely clustered into a homogenous population, represented with negative expression of Ly6C (Fig 2 A-C), consistent with previous independent flow cytometry characterization of resting murine $Ly6C^{Neg}$ monocytes reported by us and others (32, 33). In contrast, monocytes cultured for 5 days with high dose LPS were polarized into a majority $Ly6C^{++}$ and a minority $Ly6C^{+}$ population (Fig 2 A-C). Figure 2 A-C were generated with the help of Ziyue Yi, a graduate student in the GBCB program. We independently confirmed a near complete shift of monocytes into the exhausted $Ly6C^{++}$ and $Ly6C^{+}$ populations and the disappearance of the resting $Ly6C^{Neg}$ monocytes via flow cytometry analyses (Fig 2 D-G). We also observed a decrease in the MFI of CD86, MHCII as well as an increase in the MFI of PD-L1 and CD38 in both $Ly6C^{+}$ and $Ly6C^{++}$ populations (Supplementary Fig S2 A-H). Our data is consistent with emerging studies in both experimental animals and humans with sepsis and/or polymicrobial infections including COVID-19 patients, demonstrating the reduction of human non-classical monocyte population (murine $Ly6C^{Neg}$ monocyte equivalent) and an increase of the exhausted monocyte population (human $CD14^{hi}$, murine $Ly6C^{hi}$ equivalent) (7, 14, 16).

Upon additional examination of selectively induced genes within LPS-exhausted monocytes, we identified additional representative genes independently reported to be highly elevated in septic monocytes from experimental animals and human septic patients, including *Cd38*, *Plac8, S100a8, Ptpn1, Lcn2, Ms4a3, Il1r2*, *Plaur,* and *Alox5* (34-39). Exhausted monocytes collected from septic patients not only expressed pathogenic inflammatory genes listed above, but also exhibit reduced expression of immune-enhancing genes such as CD86 (30). We identified the reduction of multiple genes involved in homeostasis and/or immune-enhancing processes including *Cd86, Cd200r, Apoe, Abca1, Trem2, Pik3r1, Smurf2*, and *Park2* (Fig 2B). Our data further validated our in vitro system in generating exhausted monocytes relevant to sepsis pathogenesis via repetitive high dose LPS challenges.

Persistent CD38 expression on monocytes has been shown to be involved in the establishment of sustained pathogenic inflammation, through depleting cellular source of NAD^{+} and compromising mitochondria function (40-42). Elevated CD38 expression was also implicated in propagating inflammation via inter-cellular communication (43). Upregulation of CD38 in septic patients has been reported previously (34), however detailed understanding on the role and regulation of CD38 in sepsis and monocyte exhaustion is still missing. We therefore validated the expression of CD38 protein on the surface of exhausted monocytes via flow cytometry analyses. Indeed, as shown in Figure 3, the levels of CD38 on monocytes kept on increasing upon repetitive LPS challenges (Fig 3A, Fig S1D). We further tested cellular levels of

NAD^+ and observed a drastic reduction in LPS-exhausted monocytes as compared to control monocytes (Fig 3 B). This is also consistent with a previous finding about reduced NAD^+ in liver tissues from a Cecal Ligation and Puncture (CLP)-induced sepsis model (44). Seahorse assays revealed a significant compromise of mitochondria respiration in LPS-exhausted monocytes as compared to control monocytes as shown in the graph (Fig 3C). The basal and maximum OCR/ug in PBS vs high dose LPS are also shown in Fig 3 D and E. Consequently, we documented a drastic increase of cellular ROS levels via staining with flow cytometry (Fig 3F). Collectively, our data revealed a sustained elevation of CD38 on in vitro exhausted monocytes, correlated with dysfunctional mitochondria and increased ROS, reminiscent of septic conditions from septic patients.



Figures 2 A-C were generated with the help of Ziyue Yi, graduate student in the GBCM program

Figure 2: scRNAseq analyses of exhausted monocytes.

(A-C) BMDMs were treated with PBS or high dose LPS (100 ng/mL) for 5 days and subjected to scRNA-seq analysis using Chromium Single Cell 3' Reagent Kit V3. PBS control treated ($Ly6C^-$) cluster and HLPS treated clusters ($Ly6C^+$ and $Ly6C^{++}$) are shown using UMAP plot (A), Heatmap (B), and Selected gene dot plot (C). (D) $Ly6C^+$ $CD11b^+$, $Ly6C^{++}$ $CD11b^+$ and $Ly6C^-$ $CD11b^+$ populations in the cell cultures challenged with PBS or high dose LPS for 5 days were analyzed with flow cytometry. (E-G) The frequencies of indicated populations were quantified. Around 1000 cells were subjected to scRNA-seq analysis. The flow cytometry data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). **p <0.01, ****p < 0.0001, Student's *t* test

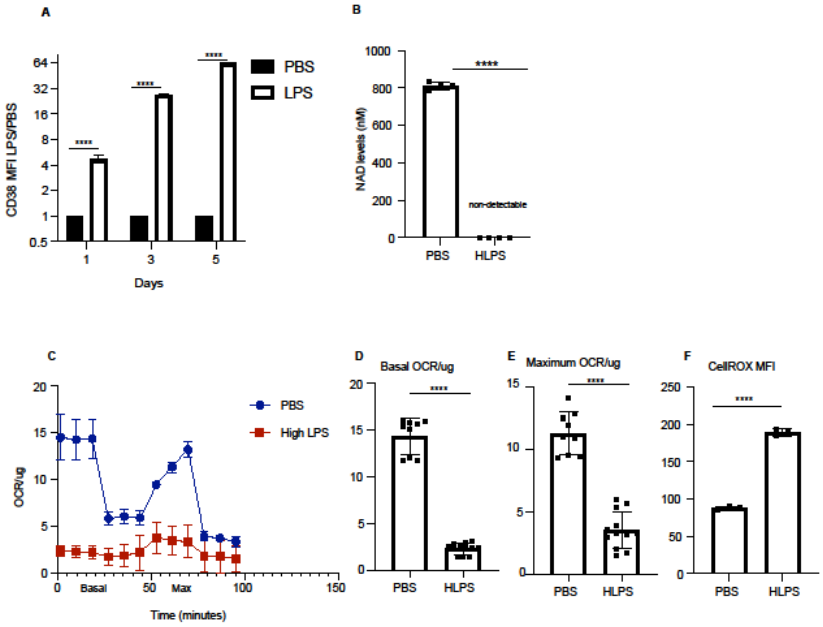
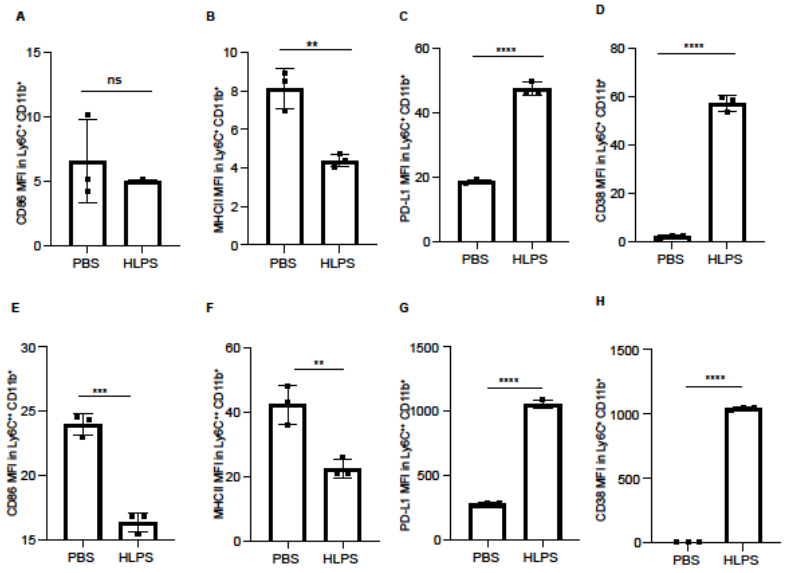


Figure 3. Exhausted monocytes express elevated levels of CD38 and exhibit compromised mitochondria respiration. BMDMs were stimulated with PBS or high dose LPS (100 ng/mL) for 5 days. (A) CD38 MFIs on Day 1,3 and 5 were analyzed with flow cytometry. The relative fold changes in MFIs of CD38 comparing LPS-treated vs PBS-treated monocytes were determined and plotted in the graphs. n=3. (B) NAD^+ levels in monocytes treated with either PBS or high dose LPS were measured on Day 5 using a fluorescence-based assay. n=4. (C) OCR/ug was determined by Seahorse assay. n=3. (D-E) The basal and maximum OCR/ug levels were quantified. $n \geq 9$. (F) Intracellular ROS levels (n=3) were determined using flow cytometry. The data are representative of at least three independent experiments, and error bars represent means ± SEM. ****$p < 0.0001$, Student's *t* test.



Supplementary Figure S2. Ly6C^{+} and Ly6C^{++} monocyte populations show exhausted phenotypes

BMDMs from WT mice were treated with PBS or high dose LPS (100ng/mL) for 5 days. (A-D) Surface expressions of CD86 (A), MHCII (B), PD-L1 (C) and CD38 (D) within the Ly6C^{+} CD11b^{+} population were determined with flow cytometry. (E-H) Similarly, expressions of CD86 (E), MHCII (F), PD-L1 (G) andCD38 (H) within the Ly6C^{++} CD11b^{+} population were determined with flow cytometry. The data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group). ****p < 0.0001, Student's *t* test.

Exacerbated STAT1 and KLF4 activation contribute to monocyte exhaustion

Next, we explored the molecular mechanisms that may lead to monocyte exhaustion. Our work and others through computational analyses suggest that STAT1 may serve as a key node for driving the polarization of inflammatory classical monocytes in murine monocytes and human monocytes with polymicrobial infections including COVID-19 (14, 28). This is also consistent with additional experimental studies, reporting that STAT1 levels are upregulated in sepsis models (45); and that STAT1 deficient mice are protected from cecal-ligation and puncture-induced experimental sepsis (46). However, these previous phenotypic observations did not address the role of STAT1 in monocyte exhaustion. We then performed Western blot analyses comparing control and LPS-exhausted monocytes, and indeed confirmed a drastic elevation of both the phosphorylated STAT1 as well as total levels of STAT1 in LPS-exhausted monocytes as compared to control monocytes (Fig 4 A-C).

In addition to STAT1, Kruppel-like factor 4 (KLF4) also plays a critical role in macrophage activation via LPS and IFNs (47). Role of KLF4 in inflammation is reported to be STAT-1 mediated(47, 48). Elevated KLF may not only work together with STAT1 in exacerbating pathogenic inflammation, but also serve as

a suppressor of CD86 expression (47, 48). Hence, we investigated whether there is a coupled induction of KLF4 in exhausted monocytes. Indeed, we observe a sharp upregulation of KLF4 proteins in exhausted monocytes as compared to the control counterparts (Figure 4 D and E).

To further test the causal role of STAT1 in driving monocyte exhaustion, we applied a selective STAT1 inhibitor to the monocyte culture. We pre-treated monocytes with 10uM fludarabine (STAT1 inhibitor) for 2 hours followed by 100ng/mL LPS treatment to attenuate STAT1 activation as shown in Fig 4 F and G. We further observed that fludarabine pre-incubation partially decreased the expression of CD38 and PD-L1 induced by LPS, while also partially restored the expression of CD86 as compared to LPS exhausted monocytes (Fig 4 H-J). Together, our data validated the role of STAT1 in driving monocyte exhaustion.

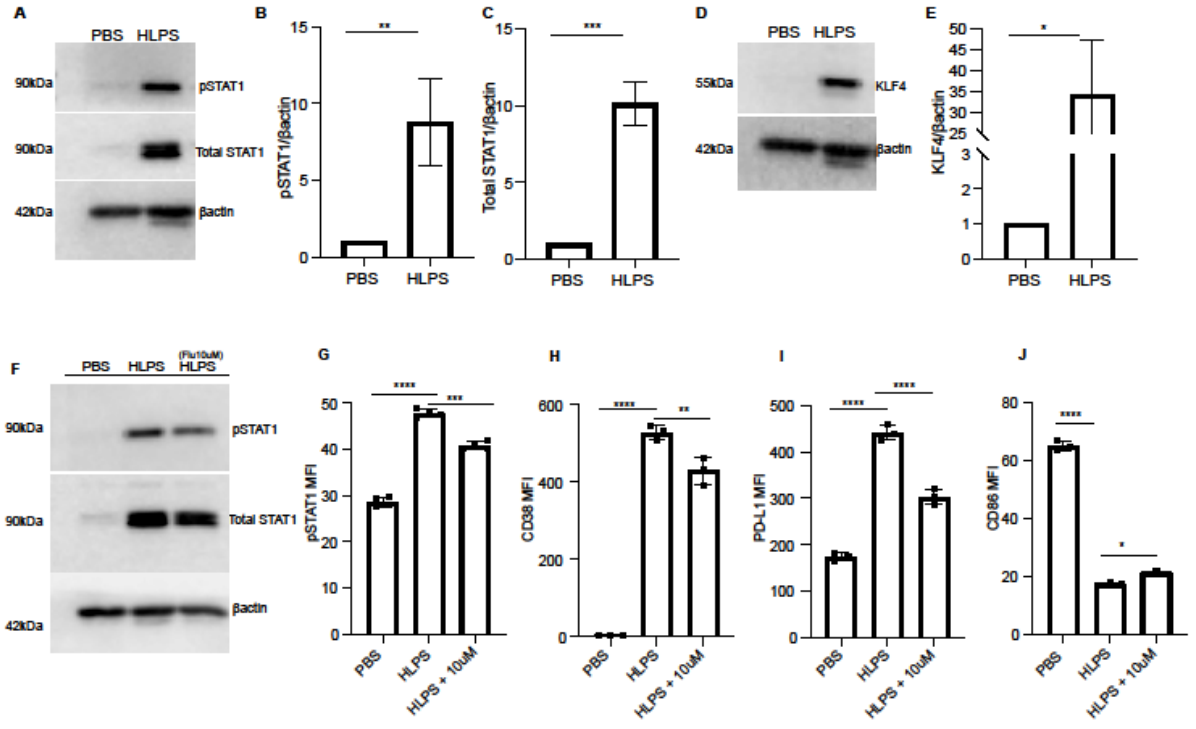


Figure 4. Exhausted monocytes exhibit sustained activation of STAT1. (A-E) BMDMs were stimulated with PBS or high dose LPS (100 ng/mL) for 5 days. The levels of phosphorylated STAT1 and total STAT1 were determined by Western blot, n=3. (A). The relative levels of pSTAT1 (B) and STAT1 (C) were normalized to β-actin. The level of KLF-4 was determined by Western blot, n=3 (D), and the relative level was normalized to β-actin (E). (F-J) BMDMs were pre-treated with STAT1 inhibitor (Fludarabine 10 uM) on Day 0 for 2 hours, followed by stimulation with PBS or high dose LPS (100 ng/mL). The levels of pSTAT1 and STAT1 were determined using Western blot, n=3 (F) and flow cytometry, n=3 (G). The expressions of CD38 (H), PD-L1 (I) and CD86 (J) were analyzed with flow cytometry, n=3. The data are representative of at least three independent experiments, and error bars represent means ± SEM. *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001, (B, C and E) Student's *t* test and (G-J) one-way ANOVA.

Scavenging cellular ROS reduces STAT1 activation and monocyte exhaustion

Our data reveal that LPS-exhausted monocytes exhibit elevated CD38; compromised mitochondria function and elevated ROS. Previous reports showing that elevated ROS can further lead to STAT1 activation, thus potentially forming a sustained positive feedback loop (49, 50). Thus, we further tested whether scavenging cellular ROS may disrupt such positive feedback loop and reduce monocyte exhaustion. By applying the ROS scavenger N-acetyl-l-cysteine (NAC), we observed a significant drop in cellular ROS from LPS-exhausted monocytes (Fig 5A). Consequently, we observed that NAC application led to a reduction of LPS-mediated STAT1 activation (Fig 5B and C). NAC application also partially reduced CD38 and PD-L1 levels in LPS-exhausted monocytes (Fig 5 D and E). On the other hand, NAC application partially restored the expression of CD86 in LPS-exhausted monocytes (Fig 5F).

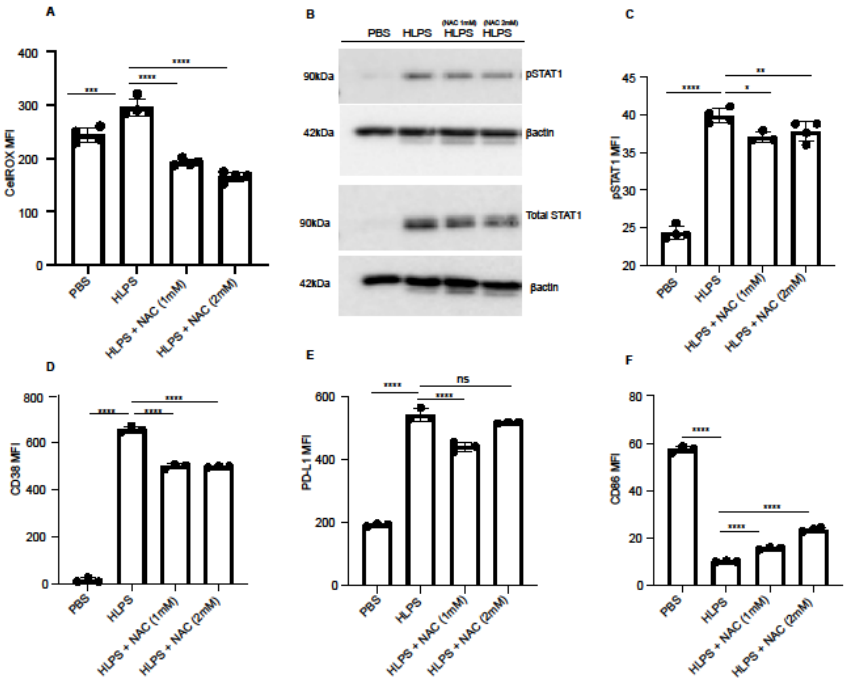


Figure 5. Scavenging ROS reduces STAT1 activation and monocyte exhaustion

BMDMs were stimulated with PBS or high dose LPS (100 ng/mL) for 5 days, and NAC was added to indicated cultures. (A) Intracellular ROS level was measured with flow cytometry, n=4. (B-C) The protein expression and phosphorylation of STAT1 was determined using Western blot, n=3 (B) and flow cytometry, n=4 (C). (D-F) The MFIs of CD38 (D), PD-L1 (E) and CD86 (F) were determined using flow cytometry, n=3. The data are representative of at least three independent experiments, and error bars represent means ± SEM. *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA.

TRAM is required for the induction of monocyte exhaustion

Given our previous report that TLR4 adaptor TRAM is responsible for STAT1 activation in exhausted neutrophils (51), we then further examined whether TRAM is responsible for monocyte exhaustion. To test this, we compared the exhaustion phenotype of WT and TRAM deficient monocytes challenged with repetitive LPS challenges. We observed that WT monocytes are readily exhausted into a majority $Ly6C^{++}$ population and a minority $Ly6C^{+}$ population (Fig 6 A-C). In sharp contrast, TRAM deficient monocyte are resistant to LPS exhaustion, and largely remain as the $Ly6C^{-}$ non-classical homeostatic population (Fig 6 A-D). More strikingly, the induction magnitudes of CD38 and PD-L1 by LPS on TRAM deficient monocytes were drastically attenuated as compared to WT monocytes challenged by LPS (Fig 6 E and F). On the other hand, the reduction of CD86 and MHCII by LPS on TRAM deficient monocytes was also dampened as compared to WT monocytes exhausted by LPS (Fig 6 G and H).

We further measured the ROS levels and mitochondrial oxygen consumption rate (OCR/ug) in TRAM deficient monocytes. We observed that LPS challenge failed to induce cellular ROS levels in TRAM deficient monocytes (Fig 6I). Likewise, the OCR/ug were comparable in PBS vs LPS-treated TRAM deficient monocytes (Fig 6 J-O). Collectively, our data reveal that TRAM is critically involved in the generation of exhausted monocytes by repetitive LPS challenges.

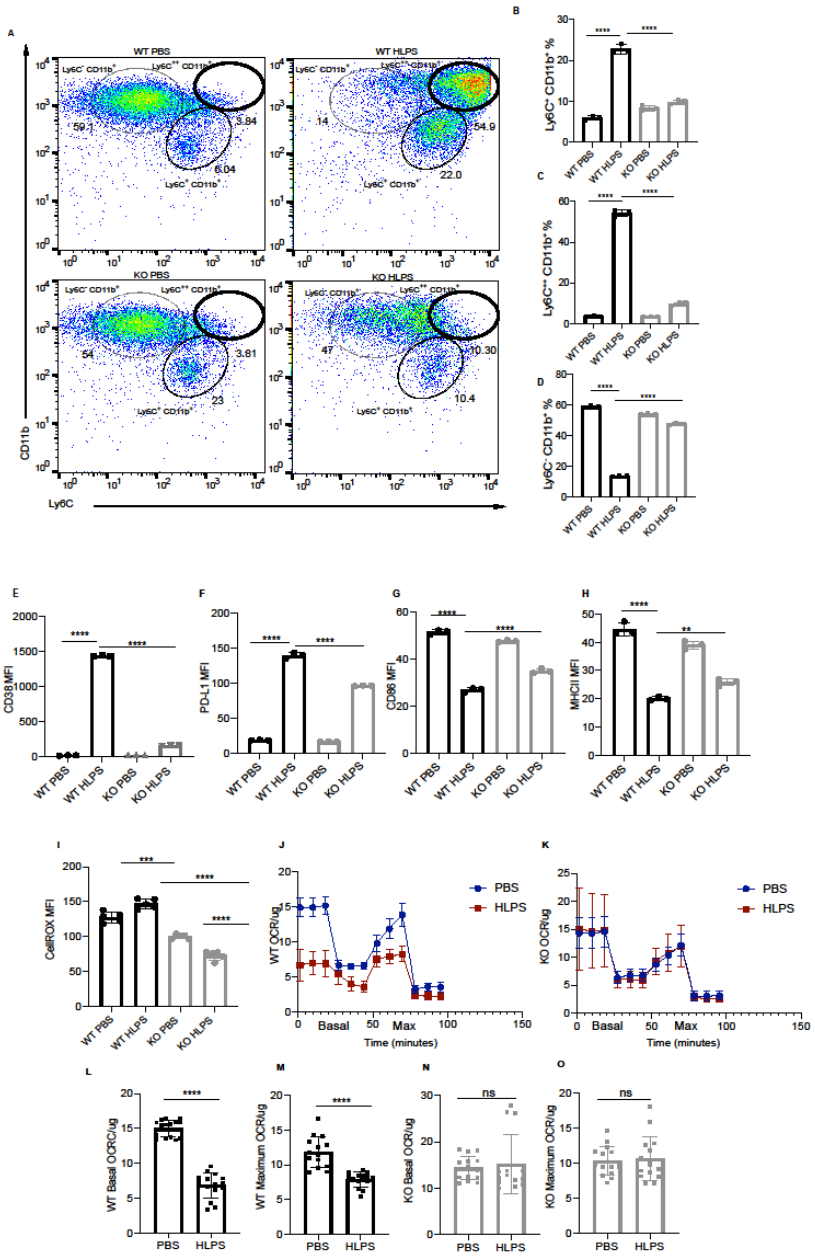


Figure 6. TRAM is required for the generation exhausted monocytes.

BMDMs from WT and TRAM KO mice were treated with PBS or high dose LPS (100 ng/mL) for 5 days. (A) Ly6C^{+} CD11b^{+}, Ly6C^{++} CD11b^{+} and Ly6C^{-} CD11b^{+} populations in the cell cultures were analyzed with flow cytometry. (B-D) The frequencies of indicated populations were quantified, n=3. (E-H) The MFIs of CD38 (E), PD-L1 (F), CD86 (G) and MHC-II (H) in WT and TRAM KO monocytes were determined with flow cytometry. (I) Intracellular ROS levels in WT and TRAM KO monocytes were determined with flow cytometry, n=3. (J-K) Mitochondrial OCR/ug levels in WT (J) and TRAM KO (K) monocytes were detected with Seahorse assay, n=5. (L-O) The basal and maximum OCR/ug levels in WT and TRAM KO were compared ($n \geq 14$). The data are representative of at least three independent experiments, and error bars represent means ± SEM. **p <0.01, ***p < 0.001, ****p < 0.0001, (B-I) one-way ANOVA and (L-O) Student's *t* test.

TRAM is required for the activation of STAT1 and KLF4 in exhausted monocytes

Since we observed a drastic upregulation of STAT1 and KLF4 in the exhausted monocytes, we further tested whether TRAM is required for their induction. We observed that the magnitudes of STAT1 and KLF4 activation were drastically attenuated in TRAM KO monocytes challenged with LPS, as compared to WT monocytes exhausted by LPS treatments (Figure 7 A-D). Together, our data show that the monocyte exhaustion generated via repetitive LPS challenges was dependent upon TRAM-mediated activation of STAT1. Finally, a schematic illustration of the potential molecular mechanisms partaking in monocyte exhaustion is shown in Fig 6E.

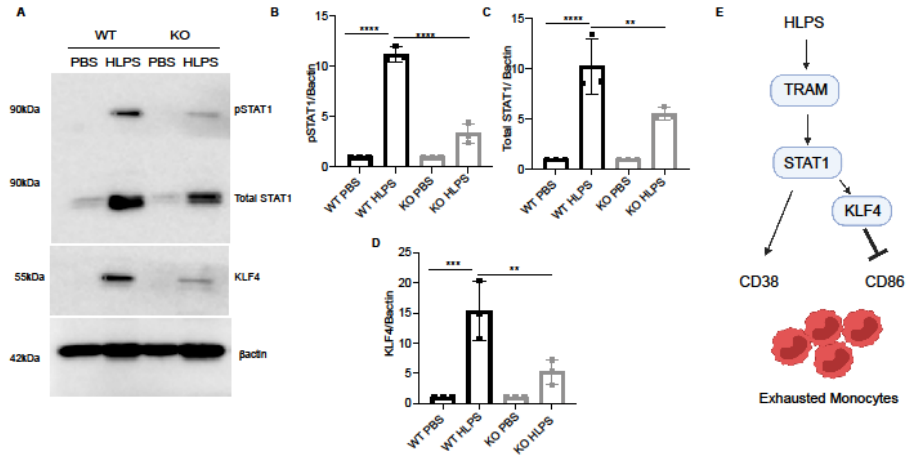


Figure 7. TRAM is required for the activation of STAT1/KLF4 involved in monocyte exhaustion. BMDMs from WT and TRAM KO mice were stimulated with PBS or high dose LPS (100 ng/mL) for 5 days. (A) The levels of pSTAT1, total STAT1 and KLF4 were determined by Western blot. (B-D) The relative levels of pSTAT1 (B), STAT1 (C) and KLF4 (D) were normalized to β-actin. (E) Schematic illustration summarizing potential mechanism involved in monocyte exhaustion. The data are representative of at least three independent experiments, and error bars represent means ± SEM (n=3 for each group) **p <0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA

3.5 Discussions:

In this report, we collected data that support the generation of exhausted Ly6C^{hi} monocytes exhibiting gene expression profiles of pathogenic inflammation and immuno-suppression characteristic of septic monocytes via repetitive challenges with high dose LPS. We identified CD38 as a novel marker for exhausted monocytes associated with depleted cellular NAD^+, compromised mitochondria function and elevated cellular ROS. Our mechanistic study further revealed that TRAM-mediated exacerbation of STAT1 activation is responsible for sustained CD38 expression and monocyte exhaustion.

Our findings expand and complement previous experimental systems in examining the fundamental dynamics of innate immune training and memory. Although earlier works predominately focused on endotoxin tolerance as reflected in reduced cytokine expressions from monocytes repetitively challenged with LPS (3, 16), some limited studies reported that "tolerant" cells can still robustly respond to LPS and express "non-tolerizable" genes (20, 52, 53). Earlier time-course studies with monocytes reported that upon LPS challenge, human monocytes initially express CD86 at an earlier time point and followed by a reduction of CD86 expression at later time points(54, 55). Our current study provided a systems analyses of monocytes following repetitive and prolonged LPS challenges. We confirmed previously findings that although LPS challenge initially induced the expression of CD86, repetitive and prolonged LPS challenges led to a reduction of CD86, consistent with the "tolerant" phenotype. Our previous study employing the same culture system also reported the development of tolerance in terms of suppressed expression of immune-enhancing inflammatory genes such as IL-12 and CCR5 when monocytes were persistently challenged with high dose LPS (56). It was well-recognized that not all genes are suppressed in "tolerant" monocytes, and many pathogenic inflammatory genes can still be robustly induced under "tolerant" settings (20, 56). Thus, the terminology of "tolerance" coined several decades of ago cannot fully capture the dichotomy of pathogenic inflammation and immuno-suppression phenotype of septic monocytes with persistent challenges of endotoxin, which may be better captured by the terminology of "exhaustion". Indeed, monocytes with repetitive LPS challenges still maintain robust induction of PD-L1. Our scRNAseq analyses also revealed the induction of SLPI, an anti-inflammatory molecule; IL-1R2, an antagonist of IL-1 signaling. Both of SLPI and IL-1R2 were shown to be elevated in septic monocytes collected from human patients (57, 58). Collectively, the elevation of immuno-suppressors such as PD-L1, SLPI and IL-1R2 and reduction of co-stimulatory molecules such as CD86 contribute to the immuno-suppressive phenotype characteristics of exhausted monocytes observed in clinical sepsis patients (11, 31, 57, 58).

In addition to well-defined immuno-suppressive molecules mentioned above, the exhausted monocytes generated in vitro also expressed representative pathogenic inflammatory genes identified in human septic patients including *S100a8*, *Lcn2*, and *Alox5* (35, 38). Furthermore, we identified elevated expression of

Plac8, *Plaur* and *Ms4a3* in the exhausted monocytes, consistent with recent single cell sequencing analyses and machine learning characterization studies of monocytes from human septic patients (14, 36-38). The inductions of pathogenic inflammatory genes are consistent with both murine and human studies. On the other hand, the independent scRNAseq analyses of human septic monocytes also revealed a subset of monocytes with reduced expression of class II MHC (37), consistent with the immuno-suppressive phenotype representative of monocyte exhaustion. Despite these important phenotypic findings, future detailed comparative studies are needed to better define evolutionarily conserved monocyte exhaustion phenotypes from mice to humans subjected to sepsis. Further, independent validation at protein levels should be performed to confirm the targets obtained from the scRNAseq-based gene expression studies. Together, our systems analyses reveal the generation of exhausted monocytes with cardinal features of pathogenic inflammation coupled with immuno-suppression representative of monocytes from septic patients.

Complementing our gene expression analyses, our flow cytometry-based measurements of key cell-surface protein molecules further validated key features of pathogenic inflammation and immuno-suppression. Upon exhaustion, the homeostatic non-classical Ly6C^{-} monocyte population almost completely disappeared, consistent with clinical studies of septic patients reporting the reduction of human counter-part of CD14^{lo}CD16^{hi} non-classical monocytes (37). Intriguingly, COVID-19 patients may experience similar monocyte exhaustion with a drastic reduction of non-classical monocyte population(14, 15), suggesting monocyte exhaustion as a universal mechanism underlying the pathogenesis of polymicrobial sepsis. It is also worth noting that there are two subsets of exhausted monocytes (the classical Ly6C^{++} and the intermediate Ly6C^{+}), although both share similar features of pathogenic inflammation/immuno-suppression markers. Our findings are consistent with recent human patients-based studies revealing the expansion of two subsets of human monocytes (classical and intermediate) in sepsis as well as severe COVID-19 patients (14, 15). The differential contributions of these two exhausted monocyte subsets to the severity of sepsis, however, will require future studies with animal models.

Among cell surface pathogenic inflammatory molecules, we identified a robust and persistent induction of CD38, an important enzyme in depleting both intra-cellular and inter-cellular NAD^{+} (21, 23). Although moderate and transient expression of CD38 may be beneficial, exacerbated and prolonged CD38 expression was implicated in depleting cellular NAD^{+} and causing run-away pathogenic inflammation (42). The previous study regarding CD38 regulation used the single LPS challenge system, and showed a transient induction of CD38 (42). In sharp contrast, under the prolonged septic stimulatory condition, we observed a sustained induction of CD38. Such sustained CD38 expression may be responsible for the depletion of cellular NAD^{+}, leading to the exhaustion phenotype we observed in this current study. Indeed, we observed

a drastic reduction of mitochondria respiration and elevated cellular ROS in monocytes with prolonged LPS challenges, another key feature of exhausted monocytes observed independently from septic models (40, 41, 59). Mitochondrial dysfunction has been directly associated with organ dysfunction in animal sepsis as well as septic patients (40). Excessive oxidative stress and inflammatory mediators inhibit mitochondrial respiration, destruct mitochondrial protein and lipid membrane and hence, are key pathogenesis for mitochondrial dysfunction in sepsis (40, 60). In age-related diseases, CD38 was shown as an important NAD-degrading enzyme further leading to mitochondrial dysfunction and CD38 deficiency maintains NAD^+/NADH ratio in mitochondria (23). Our data revealed that CD38 may serve as a novel cell-surface indicator of exhausted monocytes that may sustain and propagate pathogenic inflammation associated with sepsis.

Our current study not only systematically revealed the pathogenic inflammatory and immuno-suppressive features of exhausted monocytes, but also identified potential underlying mechanisms that involve sustained exacerbation of STAT1 activation. Our finding is consistent with a previous phenotypic report that demonstrated higher survival rates in septic mice with STAT1 deficiency as compared to WT controls (80% vs 10%) (46). Another independent study suggests that STAT1 may synergize with KLF4 in further skewing pathogenic inflammation (47). STAT1 was shown to be important in the upregulation of PD-L1 (61, 62) while KLF4 was shown to suppress CD86 expressions (63). Consistent with these earlier reports, we observed robust elevation of both STAT1 and KLF4 in exhausted monocytes. KLF4 may perform both pro- as well as anti-inflammatory functions, likely modulated by its context-dependent binding partners. It remained to be tested whether STAT1-coupled KLF4 may over-ride the beneficial effects of stand-alone anti-inflammatory effects of KLF4. The detailed co-operation among STAT1 and KLF4 in the context of monocyte exhaustion requires extensive future studies. Our current approach of applying STAT1 inhibitor partially attenuated monocyte exhaustion. We realize the limitation of the inhibitor approach due to its potential off-target effects and such approach may not be sufficient in finely characterize the involvement of STAT1 as well as intertwined ROS generation during the complex development of various monocyte exhaustion phenotypes. To better define complex mechanisms involved in the dichotic modulation of genes involved in pathogenic inflammation as well as immuno-suppression, future independent approaches with genetic STAT1 deletion would be warranted to further precisely determine the contribution of STAT1 during the generation of exhausted monocytes.

Our study also revealed the important contribution of TRAM adaptor molecule in the generation of exhausted monocytes. TRAM is a less studied TLR4 adaptor molecule (64), that may coordinate TLR4 signaling dynamics, in competition and/or cooperation with other known adaptor molecules such as MyD88 and TRIF. Both MyD88 and TRIF have been shown to facilitate the generation of immune-enhancing

monocytes (65-67). Innate stimulants such as beta-glucan can generate immune-enhancing trained immunity through MyD88 and/or TRIF dependent activation of PI3K pathway, and exert protective effects in experimental sepsis (68, 69). Some limited mechanistic studies suggest that these adaptor molecules may competitively function downstream of TLR4 and finely modulate monocyte activation dynamics ranging from priming, tolerance to exhaustion (56, 70-72). While TRAM may coordinate with TRIF in monocytes under low-grade inflammatory conditions, and facilitate the priming/immune-enhancing effects (56), our current data suggest that TRAM may also be uniquely involved in facilitating the generation of exhausted monocytes, perhaps independent of TRIF, which requires further clarification with detailed studies. Our data suggest a distinct role of TRAM in selectively propagating monocyte exhaustion via facilitating the sustained activation of STAT1 and KLF4. We demonstrated that TRAM deficiency drastically attenuated the induction of both STAT1 and KLF4 by LPS, and kept monocytes predominantly within the $Ly6C^-$ non-classical resting state. TRAM deficient monocyte also maintained proper mitochondria respiration following repetitive LPS challenges and balanced expression of CD86/PD-L1. Our findings not only reveal a unique mechanism for monocyte exhaustion, but also suggest that TRAM may serve as a promising target for mitigating monocyte exhaustion and preventing sepsis pathogenesis.

Data Availability Statement

scRNAseq data sets were deposited in the Genebank with the accession number GSE182355. All other relevant data are presented in this manuscript.

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Chapter 4 Conclusions and Future Directions:

In summary, our work highlights the dose-dependent effects of endotoxin on monocytes and the underlying mechanisms. In the first chapter we report the sustained expressions of costimulatory molecule (CD40) and adhesion molecule (CD11a) on monocytes persistently exposed to super low dose (SLD-LPS) lead to non-resolving inflammation. In addition, we also report that the low-grade inflammatory monocytes influence neighboring T cells by suppressing regulatory T cell functions as shown my reduced CD122 expressions in CD8 T cells co-cultured with low-grade inflammatory monocytes. Our novel findings emphasize that priming of monocytes by SLD-LPS can lead to a long-term exacerbation of chronic inflammation. Mechanistically we report that the prolonged inflammatory phenotypes are dependent upon TRAM (TLR4 adaptor molecule).

Our findings further raise insightful questions regarding the effects of these dynamic monocytes in different microenvironments and context of the disease. We have previously reported that low grade inflammatory monocytes can exacerbate atherosclerosis and impair wound healing (1, 2). In contrary, it is fair to question whether the low grade inflammatory monocytes may have a deleterious or beneficial effects in tumor environment. It is shown that monocytes can have anti-tumoral functions as they can exhibit cytokine-induced cell death of malignant cells as well as phagocytose tumor-derived materials (3). On another note, priming monocytes with SLD-LPS and persistent exposure to SLD-LPS assists in development of innate immune memory, where monocytes are initially "trained" for a more robust second response. Other stimuli such as oxLDL, Beta glucan and mycolic acid also play a role in training innate immune cells for enhanced second response(4).

In the second chapter we portray novel characteristics of exhausted monocytes repeatedly treated with high dose LPS. We report that exhausted monocytes share immunosuppressive as well as pathogenic inflammatory phenotypes as supported by consistent elevation of CD38 in the exhausted monocytes. In addition, exhaustion is associated with elevated ROS and compromised mitochondrial respiration. These novel findings suggest to reevaluate the term "tolerance" as not all monocyte subpopulations are immunosuppressive and pathogenic inflammatory genes are still upregulated. We also identified that monocyte exhaustion is also TRAM-pathway dependent. Understanding the characteristics of exhausted monocytes is important as it can provide insights on early treatments as well as investigate long term effects of sepsis.

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