

MODULATION OF NEUTROPHIL FUNCTIONS AND ANTI-TUMOR IMMUNE
RESPONSES BY INNATE SUPPRESSORS

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ABSTRACT

Neutrophils are known to be key innate defenders through performing vital and diverse functions such as degranulation, oxidative burst, and generation of extracellular trap (NET). Recent data suggest that neutrophils may also play key roles in modulating tissue inflammatory/immune environment by secreting soluble mediators as well as surface-attached co-activators. Furthermore, neutrophils may adopt distinct functional states either conducive or detrimental for tumor-growth through cellular contact with cancer cells and/or other immune cells such as T helper cells. However, molecular mechanisms that modulate functional adaptations of neutrophils are not well understood. The objective of my thesis is to identify the role of Tollip, a novel TLR signaling adaptor molecule, in modulating neutrophil functions by suppressing the inflammatory signaling pathway. Preliminary data from our lab suggest Tollip deficient neutrophils may be programmed to exhibit enhanced anti-tumor activities. Based on these novel findings, I tested the hypothesis that neutrophils also have subsets with different functions similar to monocyte/macrophages, and Tollip deficient neutrophils may be programmed into an enhanced anti-tumor state through upregulating inflammatory signaling processes and mediators.

GENERAL AUDIENCE ABSTRACT

Cancer immunotherapy gained instant popularity overnight after former president of the United States, Jimmy Carter, announced successful treatment of his metastatic melanoma. Since then, FDA has approved the first immunotherapy in the summer of 2017. Current cancer immunotherapy focuses heavily on the potential of T cells to target unregulated cells in the host. However, time and specificity have proven a difficult challenge to overcome. Innate immune cells may circumvent these challenges and present equal potential as therapy in the fight against cancer. Neutrophils are one of the innate immune cells that provide first line of defense against pathogens. Neutrophils can clear danger or maintain the situation until more cells arrive to help clear the danger. They were originally thought to confer simple and transient effects, but emerging data suggest they may have more diverse role in host defense. We propose programming neutrophils will make the cells less susceptible to cancer manipulation and provide enhanced protection against cancer establishment and maintenance.

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INTRODUCTION

In January 2000, Douglas Hanahan and Robert Weinburg published a review article in *Cell* titled “The Hallmarks of Cancer,” which provided a framework about how cancer has adapted to flourish in the host. While the framework provides an easy-to-understand picture for general public and some preliminary insights into multiple ways cancer therapies can be directed for researchers, it ignored a key component regarding the tumor interaction with the host immune environment. Since then, the authors revised the review paper to include a number of additional “hallmarks” and “enabling characteristics” that specifically relate to cancer-host immune responses. Emerging literature supports that tumors, both solid and non-solid, may evade host immune responses by suppressing 1) phagocytosis, 2) antigen presentation, 3) dendritic cell activation, and 4) cytotoxic T cell effector function. Moreover, cancer cells have shown to modulate immune cells to actively assist in their cell growth and metastasis. While literature focuses on the interactions of already established cancer cells and host immune cells, there is a gap in knowledge in how the host homeostasis becomes disrupted and the dysregulation of host immune system contributes to non-resolving inflammation and chronic diseases.

Currently, personalized medicine and individual therapy have garnered momentum as T cells have delivered unprecedented results in the war on cancer. While it has shown promising results, the therapy preparation may take weeks as it would require specific engineering of a unique subset of T cells expressing a tumor-specific T cell receptor (TCR). Unfortunately, not many have the luxury to wait weeks on end until the treatment becomes available. In addition, T cell therapy is highly specific while cancer cells are known to mutate like no other cells in the body. As the book *Emperor of All Maladies: A Biography of Cancer* by Dr. Siddhartha Mukherjee puts it, cancer

cells are the “super-version” of host cells: they can hijack metabolism and signaling pathways to grow faster[1-4], modulate neighboring cells to provide additional support for their growth[5, 6], evade death signaling[7, 8], and migrate to different regions of the body to flourish when one road closes on them[9, 10]. Not surprisingly, more eyes are turning to the other weapon found in the host, innate immune cells, as we hit a wall with adaptive immune cells. Though innate immune cells were previously known to confer transient defense against invading pathogens and damaged cells, new data are emerging to contradict the old findings. For my study, the interaction between cancer cells and innate immune cells, more specifically, the polarization that occurs in neutrophils and possible pathways involved in immune-related tumorigenesis, are systematically examined.

Neutrophils are the most abundant innate immune cells that first establish contact with pathogens, clear known danger and debris, send out signaling to recruit other immune cells, train other immune cells for future action, and inaugurate homeostasis once danger has been cleared. They are known to employ nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathways to confer their actions. Not much has been known about these cells until recent years due to their fragility outside of the host and short life span. However, new findings suggest the cells can prolong their lifespan to more than 5 days, instead of 8 hours as previously known, when certain conditions are met in the microenvironment.

Contradictory data has been published thus far where neutrophils seem to play dual roles of inflammation in cancer: tumor-promotion and tumor-inhibition [11-13]. This may stem from lack of current understanding in the complex nature of neutrophils. Similar to M1 and M2 in macrophages, the concept of N1 and N2 surfaced not too long ago. However, this schematic simplifies the actions of neutrophils and limits the heterogeneity I found in our lab. Neutrophils may exhibit a spectrum of activation and actions dependent on external signals. This information

is especially relevant in the tumor microenvironment where a number of leukocytes have been shown to promote tumor growth instead of boosting surveillance, elimination, and clearance. Cancer cells have found ways to bypass the host defense mechanism and modulate the cells to assist in their maintenance, uncontrolled growth, and migration. For my thesis, I aim to identify the communication that occurs between neutrophils and cancer cells and find ways to impede neutrophil polarization that assist in cancer cell growth.

The focus of my project is to identify the molecular, cellular, and physiological nature of neutrophils in homeostatic and tumor microenvironments. I used chemically-induced murine colon tumor model to mimic colitis symptoms in mice. Then I used different knockout mice to confirm the role of enhanced NF- κ B signaling in tumorigenesis. Tollip has been previously recognized as inhibitory protein in NF- κ B signaling pathway (Figure 1). A number of roles has been identified for Tollip, such as its role in endosome acidification, initiation and completion of autophagy, lysosome fusion[14-16]. Not surprisingly, it has been associated with diseases such as atherosclerosis, infection, wound repair, and neurodegenerative diseases. However, its role in neutrophil polarization and its effect on tumorigenesis in the colon has yet been explored. Tollip may enhance neutrophil clearance and signal macrophages to switch from M1 to M2, marking pro-inflammation to pro-resolving phase in inflammation. By deleting the inhibitory gene, the host may experience enhanced acute inflammation versus non-resolving chronic inflammation, which is often found in cancer patients. Lastly, I used *in vitro* assays with tumor cell lines to examine the cellular and molecular mechanism behind the neutrophil function in tumorigenesis, and whether neutrophils can be modulated to confer improved anti-tumor activities. The long term goal is to manipulate host/patient neutrophils to serve as a potential therapy to prevent and/or treat cancer.

To achieve this objective, I posed following questions to examine the role of neutrophils and neutrophil polarization in cancer:

Question # 1: *Do Tollip deficient mice exhibit an improved anti-tumor activity due to enhanced neutrophil functions?*

Our data shows that Tollip deficient mice develop fewer colon polyps when challenged with AOM and DSS. Based on results showing greater number of neutrophils, as well as reduced PDL-1 expression on the neutrophils, we hypothesize that the improved anti-tumor activity may arise from enhanced immune function of neutrophils in Tollip deficient mice. To test this hypothesis, I examined the efficacy of adoptively transferred neutrophils from WT and Tollip deficient mice in modulating tumorigenesis in recipient WT mice challenged with AOM and DSS. Clinical observation, immunohistochemistry, and flow cytometry approaches were used for the proposed studies.

Question # 2: *Do Tollip deficient neutrophils directly suppress tumor cell growth?*

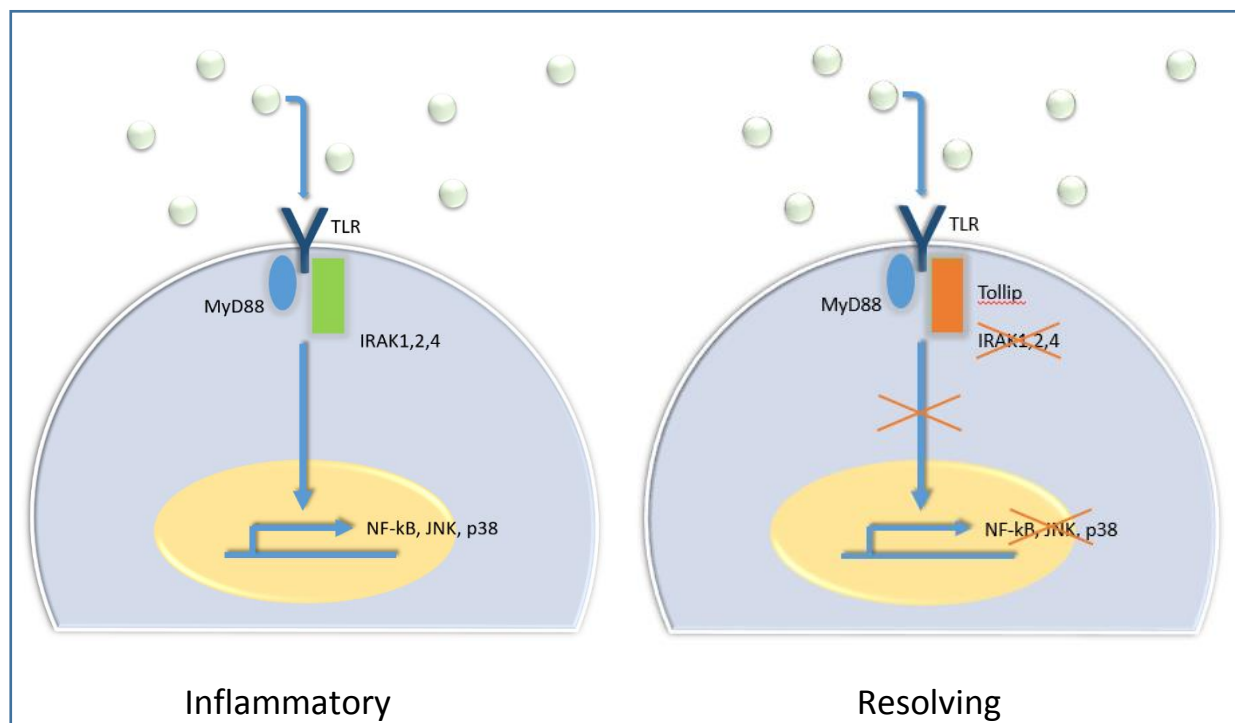
The cellular mechanism behind cell-to-cell communication (i.e., whether neutrophil confers its function in cancer via direct cell-to-cell contact or solely by secreted cytokines and chemokines) is not well known. Despite neutrophils being excellent secretors of chemokines and granule enzymes, current studies have focused heavily on the neutrophils' ability to clear foreign particles through phagocytosis and NETosis, or their interaction with T cells to clear danger. However, direct communication between neutrophils and tumor cells may influence tumor cell survivability to a greater extent than previously acknowledged. To further examine this novel aspect, I tested whether direct cell-to-cell contact versus soluble mediators from neutrophils may be responsible for enhanced anti-tumor defense of Tollip deficient neutrophils. I observed that Tollip deficient neutrophils are much more efficient in suppressing tumor cell proliferation/growth as compared to wild type neutrophils, potentially due to elevated levels of miR-34. Findings suggest that direct cell-to-cell contact is not necessary for the anti-tumor activities in neutrophils and that Tollip deficient neutrophils secrete higher levels of miR-34.

Question # 3: What are the molecular mechanisms responsible for enhanced neutrophil functions related to anti-tumor immune defense?

Although Tollip has been well studied in monocytes by suppressing NFkB pathways, less is known about its function in neutrophils. I tested the molecular mechanisms for neutrophil reprogramming due to Tollip deficiency through examining some of the key signaling molecules involved. In addition, I tested other NF-kB signaling molecules, such as IRAK-M and PHLPP, in neutrophil re-programming. IRAK-M is another related innate immune suppressor, and PHLPP is a key phosphatase that may associate with Tollip. Through analyzing neutrophils deficient in these molecules both *in vivo* and *in vitro*, I aimed to gain a systematic understanding of neutrophil programming dynamics as it relates to the modulation of anti-tumor immune defense.

These questions have been examined in an integrative manner, woven into several manuscripts, and organized into the thesis chapters below.

Figure 1. Graphical abstract of Tollip-dependent inhibition of NF-kB signaling.



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CHAPTER 1. LITERATURE REVIEW

Programming and memory dynamics of innate leukocytes during tissue homeostasis and inflammation

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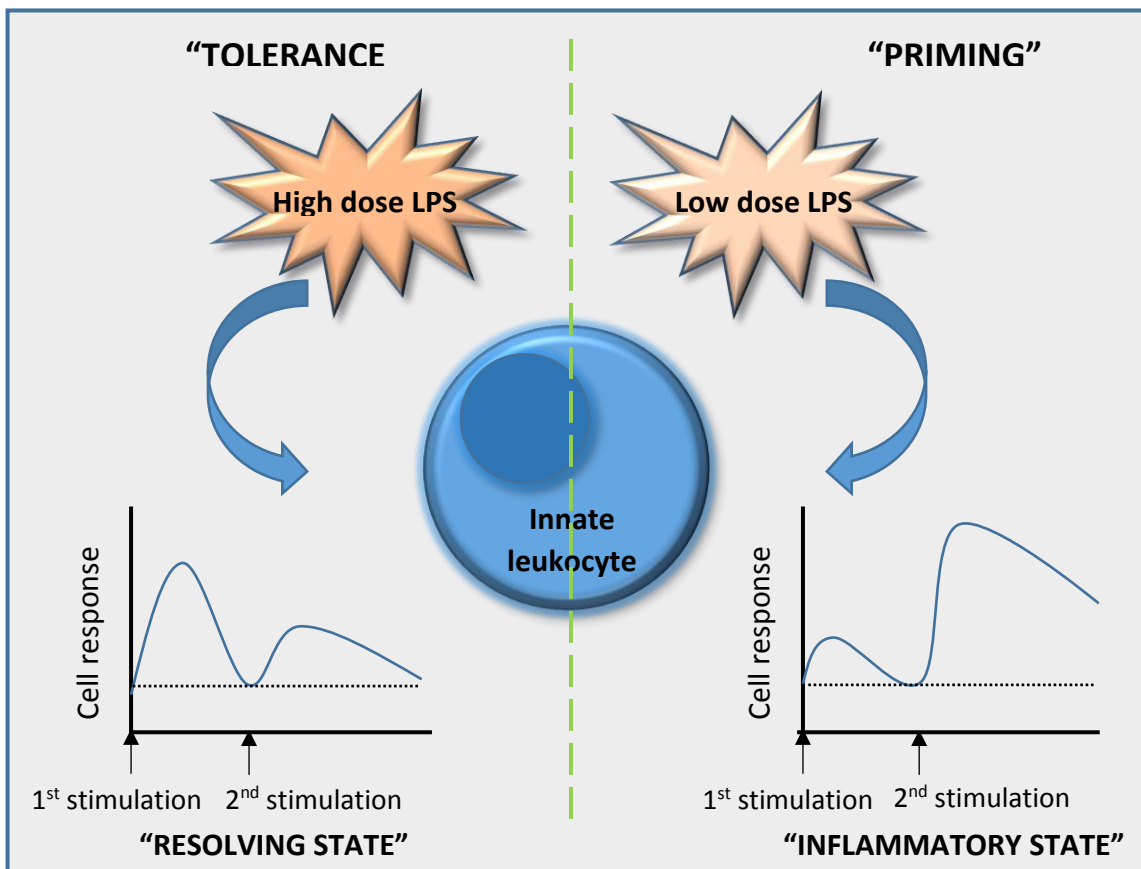
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ABSTRACT

The field of innate immunity is witnessing a paradigm shift regarding “memory” and “programming” dynamics. Past studies of innate leukocytes characterized them as first responders to danger signals with no memory. However, recent findings suggest that innate leukocytes, such as monocytes and neutrophils, are capable of “memorizing” not only the chemical nature but also the history and dosages of external stimulants. As a consequence, innate leukocytes can be dynamically programmed or reprogrammed into complex inflammatory memory states. Key examples of innate leukocyte memory dynamics include the development of primed and tolerant monocytes when “programmed” with a variety of inflammatory stimulants at varying signal strengths. The development of innate leukocyte memory may have far-reaching translational implications, as programmed innate leukocytes may affect the pathogenesis of both acute and chronic inflammatory diseases. This review intends to critically discuss some of the recent studies that address this emerging concept and its implication in the pathogenesis of inflammatory diseases.

GRAPHICAL ABSTRACT



INTRODUCTION

Previous studies have provided extensive information with regard to the effector functions of innate leukocytes, as well as critical signaling pathways that selectively control these functions. Key pathways initiated by well-characterized receptors, such as TLRs, receptors for cytokines and chemokines, growth receptors, and scavenger receptors, have been widely examined and reviewed [1–3]. Despite these important advances, significant gaps in knowledge still remain concerning the complex programming dynamics that modulate potential innate leukocyte memory responses during tissue homeostasis and inflammation. The rich repertoire of dynamically programmed monocytes and neutrophils can be seen in both acute and chronic inflammatory conditions, including septic shock and cancer. Better understanding of the integrated cellular and molecular circuitries that program distinct memory states of innate leukocytes may lead to effective strategies for restoring leukocyte homeostasis and treating complex inflammatory diseases.

MOLECULAR MECHANISMS FOR PROGRAMMING DYNAMICS OF INNATE LEUKOCYTES

One of the classic examples of leukocyte memory dynamics is the differential priming and tolerance of monocytes/M ϕ s in response to distinct stimulants with varying signal strengths [4, 5]. Primed monocytes/M ϕ s may adopt a nonresolving inflammatory state conducive to the propagation of chronic inflammatory diseases, such as atherosclerosis and impaired wound healing [6, 7]. In contrast, tolerant monocytes/M ϕ s may facilitate tissue homeostasis and resolution of inflammation [8–10]. The establishment of monocyte/M ϕ tolerance has been best studied in the context of endotoxin tolerance [11]. At the molecular level, the accumulation of negative regulators of endotoxin signaling networks, such as IRAK-M, Tollip, suppressor of cytokine signaling, and methyltransferase, has been strongly associated with the development of endotoxin tolerance [12, 13]. These negative regulators may attenuate cellular signaling, as well as epigenetically remodel and suppress selected chromatin associated with the expression of proinflammatory mediators [13, 14]. In addition to endotoxin, TLR7 and TLR9 agonists may similarly induce monocyte tolerance with similar molecular mechanisms [15, 16].

In contrast to tolerance, monocytes/M ϕ s may adopt a nonresolving, “primed” inflammatory state when challenged with IFN- γ , β -glucan, or super-low dose endotoxin [17–20]. Epigenetic remodeling of chromatin induced by IFN- γ or β -glucan may allow “poised” induction of proinflammatory gene expression [17, 21]. Metabolic reprogramming may also occur by favoring glycolysis [22]. Altered metabolism may further promote epigenetic remodeling and chromatin loosening of inflammatory gene promoters. In addition to epigenetic programming, clearance/disruption of negative signaling regulators, such as AKT, ERK, Tollip, and IRAK-M,

may play critical roles during the establishment of nonresolving inflammatory monocytes [6, 16, 23]. These negative regulators are critical for the establishment of homeostatic tolerance [12, 24], and suppression of these regulators may allow for the opposite response, thereby priming monocytes/M ϕ s to a nonresolving inflammatory state/phenotype.

To achieve selective priming and tolerance of monocytes, the signaling adaptor molecules MyD88 and TRIF may be differentially used [15]. MyD88-dependent signaling was shown to be responsible for the induction of negative-feedback regulators required for tolerance adaptation [25]. In contrast, the adaptors TRAM/TRIF may be selectively involved in the establishment of a nonresolving inflammatory state through the disruption of negative modulators [15]. At the functional level, TRAM/TRIF deficiency has been associated with reduced chronic inflammation in an animal model of atherosclerosis [26].

Monocyte and tolerance may reflect the tip of an iceberg concerning highly complex dynamics of monocyte memory, adaptation, and programming. Recent proteomics and genomics studies reveal complex and diverse profiles of monocyte differentiation and activation [27]. The duration and amplitude of monocyte responses may vary as a result of diverse differentiation/adaptation states, based on the nature of stimulating signals, the signaling strength and history, as well as the spatial constraint within tissues and organs [19, 28, 29]. This is in contrast to the simplistic M1/M2 view of monocyte/M ϕ activation. Although initially the M1/M2 concept may provide a reference point to understand monocyte regulation, accumulating evidence suggests that monocyte differentiation/adaptation is much more complex and may include a spectrum of phenotypes as opposed to a discrete M1 or M2 phenotype [28, 30–32].

Beyond monocytes/Mφs, past studies revealing complex behaviors of neutrophils also hint at complex programming and memory dynamics. Neutrophils serve as the first responders to invading pathogens and make up a majority of the innate immune cells in circulation. It is well known that neutrophils can perform diverse functions, such as phagocytosis, chemotaxis, toxin secretion via degranulation, and generation of NET [33]. With respect to degranulation, neutrophils have at least 3 distinct types of granules that have unique functions and can be differentially secreted in a context-dependent fashion [34]. Primary granules may be secreted when neutrophils encounter bacteria, virus, or fungi, whereas secondary granules are predominantly secreted for bactericidal purposes. Tertiary granules are secreted during neutrophil migration and may digest collagen, allowing cells to pass through the basement membrane of blood vessels. The order of secretion typically is as follows: tertiary > secondary > primary. However, the mechanisms behind how neutrophils decide when to secrete certain types of granules remain poorly understood. Emerging studies suggest that neutrophils may also be dynamically programmed into unique functional states. For example, different dosages of signaling molecules, such as IL-8, have been shown to affect differential granule secretion through the activation of either Ca^{2+} -dependent or independent respiratory burst [34].

The generation of neutrophil NET has also drawn significant attention as a result of its important roles in both antimicrobial defense and inflammation [35]. The breakdown and release of nuclear chromatin with either unmodified or citrullinated histones may serve as microbial traps to contain infectious agents with potential inflammatory complications to the host tissues. Although the exact nature of NETs and their pathophysiological functions are still under debate, it is clear that

complex dynamics of NET regulation exist. Our recent study suggests that analogous to monocyte programming dynamics, neutrophil NET formation may be similarly sensitive to dynamic modulation in response to varying dosages of endotoxin [36]. We reported that a higher signal strength of LPS challenge may program/enable the subsequent generation of neutrophil NET through the induction of ERK [36]. In sharp contrast, subclinical super-low dose endotoxin reduces ERK activity, thereby reducing subsequent NET-forming potential [36]. Our study serves as a prelude for future dynamic analyses of neutrophil function, both at basic and translational levels, as we will discuss in a later section of this review.

Neutrophil programming dynamics are also manifested in other cellular functions, such as oxidative burst. Robust production of ROS by activated neutrophils, known as respiratory burst, is crucial for pathogen clearance. Respiratory burst is mediated by NADPH oxidase, which is composed of 4 cytosol subunits and 2 transmembrane subunits. Neutrophil priming initiates the translocation and phosphorylation of the NADPH oxidase cytosol subunits, potentiating the assembly of the complete NADPH oxidase enzyme complex and ROS production following secondary challenges [37]. Dynamic modulations of neutrophil oxidative burst can be achieved through exposure to diverse agonists. For example, AGEs, commonly seen in chronic inflammatory conditions such as diabetes, can prime neutrophil respiratory burst. In a study testing the effects of various doses of AGE (50–800 mg/ml) on neutrophil ROS production, it was observed that neutrophils primed with 200 mg/ml AGE elicited maximum production of ROS with subsequent stimulation of fMLP, whereas neutrophils trained with higher dosages of AGE (400 or 800 mg/ml) exhibited a dampened ROS production [38]. Similar phenomenon have also been observed in neutrophils trained with MAFP, a phospholipases A2 inhibitor. Pretreatment

with low dosages of MAFP ($<1\ \mu\text{M}$) enhanced superoxide production in neutrophils stimulated with fMLP. In sharp contrast, pretreatment with high-dose MAFP ($>1\ \mu\text{M}$) caused a dramatic reduction of superoxide production [39]. In addition to chemical or biologic compounds, live microbe interactions may dynamically program neutrophil oxidative burst. For example, infection with a fungal pathogen *Candida albicans* could dose dependently induce ROS production in human neutrophils. The ROS production reached a maximum plateau in human neutrophils infected with *C. albicans* at $\text{MOI} = 2$ and subsequently declined in infected neutrophils at higher MOIs [40].

CELLULAR MECHANISMS FOR INNATE LEUKOCYTE PROGRAMMING DYNAMICS

Emerging data suggest that proper assembly of signaling platforms within subcellular organelles is critical for the dynamic modulation of innate leukocyte activation. Spatial coordination of signaling molecules occurs not only at the cellular membrane but also near intracellular membrane compartments, such as autophagosomes, mitochondria, lysosomes, and peroxisomes. Recent studies suggest that dynamic coordination among these subcellular organelles may play key roles during the programming and memory of innate leukocytes [41, 42].

For example, effective autophagy was shown to be required for not only the differentiation but also for the homeostatic maintenance of monocytes/M ϕ s [43, 44]. Autophagy is a process that clears away cellular debris through the formation of the autophagosome and subsequent fusion with lysosome [45]. Multiple highly coordinated autophagic processes, including the induction, nucleation, elongation, and fusion of the autophagosome (with the lysosome), may modulate cellular homeostasis [46]. Impaired autophagy at either of these steps may lead to M ϕ inflammatory activation through the induction of inflammatory cytokines [47]. We recently reported that defective completion of the autophagy process via disruption of autolysosome fusion may skew innate monocytes into a nonresolving inflammatory state with elevated expression of selective inflammatory mediators [7]. Disrupted autolysosome fusion may cause an accumulation of inflamed autophagosomes triggering activation of the transcription factor IRF5 [7, 15]. We also identified Tollip as a key mediator in the proper formation of the autolysosome [48]. Tollip deficiency in M ϕ s leads to a disruption of lysosome fusion with autophagosome and contributes to elevated expression of inflammatory mediators, such as CCR5 [48]. On the other hand,

compounds, such as tauroursodeoxycholic acid (TUDCA), can facilitate effective autolysosome fusion and may have a beneficial effect in restoring innate M ϕ homeostasis [7].

Besides autophagosomes, mitochondria may also serve as key platforms for coordinating the inflammatory activation and homeostasis of innate leukocytes [49]. Upon challenges with inflammatory signals, inflammatory signalosomes and molecules, such as mitochondrial antiviral-signaling proteins and TNFR-associated factors, have been shown to translocate to the proximity of the mitochondrial membrane. These signaling molecules not only initiate the expression programs of inflammatory genes but also coordinate the dynamics of mitochondria biogenesis and metabolic function [44]. Metabolic switches within mitochondria, from lipid fuel to glycolysis, may, in turn, contribute to the inflammatory activation of monocytes/M ϕ s [50]. Although the connection between metabolism and inflammation has drawn immense interest in the field, the intertwined dynamics still require systems studies for thorough comprehension.

Another intriguing yet often ignored organelle is the peroxisome, which serves as a key detoxification site for microbes, as well as toxic organic molecules [42]. Signaling molecules, such as mammalian target of rapamycin complex 1 (mTORC1) and Janus kinase/signal transducers and activators of transcription (JAK/STAT), have been shown to assemble near the peroxisome membrane and participate in key inflammatory activation, as well as antimicrobial responses in innate M ϕ s [42, 51]. Given the relative abundance of peroxisomes and relative lack of mitochondria in neutrophils when compared with other innate immune cells, peroxisomes may have a particularly significant role in neutrophils. Peroxisome biogenesis contributes to the formation of neutrophil granules, as well as inflammatory activation, by supplying membrane ether

lipid and sustaining neutrophil viability [52]. Although neutrophils are relatively short lived (with a circulating half-life of ;8 h in humans), their lifespan can be drastically prolonged following inflammatory stimulations for an average of 5.4 d, both *in vitro* and *in vivo* [53–56]. However, the regulatory mechanisms responsible for the complex modulation of neutrophil survival and inflammatory activation are not well understood. Further studies are needed to elucidate better these emerging roles of the peroxisome in neutrophil activation.

CROSS-TALKS WITH ADAPTIVE IMMUNE CELLS

Given the close interaction of innate and adaptive leukocytes, mutual cross-talks undoubtedly occur that may impact the dynamic programming of both innate and adaptive immune cells. For example, cellular contacts between NK cells and neutrophils were shown to promote caspase-dependent neutrophil apoptosis via NKp46- and Fas-dependent mechanisms [57]. On the other hand, cytokines released by activated NK cells, such as IFN- γ , GM-CSF, and TNF- α , may exert feedback effects on neutrophils by expanding neutrophil lifespan, increasing ROS production, boosting neutrophil phagocytic potential, and upregulating the expression of neutrophil activation markers [58, 59]. Activated CD4 and CD8 T cells can promote neutrophil activation and survival in coculture systems, and human neutrophils cocultured with $\gamma\delta$ T cells exhibit elevated expressions of CD64 and HLA-DR [55, 60]. Furthermore, IL-17 produced by Th17 cells stimulates epithelial cells to secrete G-CSF, as well as chemokines, including CXCL1, CXCL2, and CXCL8, which not only enhance neutrophil recruitment but also induce neutrophil activation [61]. Given the focus of this review on innate programming, we will keep the discussion of this fascinating topic brief. Future studies with systems approaches are highly warranted and will undoubtedly reveal highly complex, dynamic, and pivotal cross-talks among multitudes of innate and adaptive leukocytes in both healthy and diseased tissue environments.

INNATE LEUKOCYTE PROGRAMMING DYNAMICS IN HEALTH AND DISEASE

Given the increasing recognition of innate leukocyte memory and programming dynamics, its contribution to the pathogenesis of inflammatory diseases warrants systematic re-evaluation. Instead of simply serving as rudimentary effector cells for antimicrobial defense and inflammation, reprogrammed memory innate leukocytes may serve as key players in the initiation, propagation, and sustainment of diverse inflammatory processes conducive for distinct inflammatory diseases. The following sections will highlight some of the recent advances that consider innate memory dynamics in disease.

Innate memory dynamics in sepsis

Sepsis poses a serious health concern in critical care units worldwide [62]. Despite extensive efforts at basic and translational levels, there has not been any U.S. Food and Drug Administration-approved drug or effective cure to treat sepsis. The extremely complex dynamic that underlies the pathogenesis of sepsis is largely to blame for the lack of cure [11, 63]. With particular relevance, the complex dynamics are manifested in the drastically disrupted homeostasis of innate leukocytes [11, 64]. Disappointment from previous studies calls for novel efforts to define the innate leukocyte dynamics and underlying mechanisms. Altered monocyte homeostasis in sepsis is reflected in a drastic, early upswing of inflammatory processes, followed by a late-phase tolerance. Existing attempts that primarily focus on addressing one-sided (tolerance) modulation of monocyte activation may explain the lack of therapeutic efficacy.

Likewise, the restoration of neutrophil homeostatic balance should also be considered. Neutrophils in healthy individuals are capable of functions, such as chemotaxis, oxidative burst, degranulation, and the generation of an extracellular trap (NET) [33, 65–67]. In sharp contrast, neutrophils collected from septic patients and animal models exhibit compromised chemotaxis toward bacterial sources and reduced NET generation [64]. Septic neutrophils exhibit selective “migratory paralysis” and fail to migrate properly toward microbial chemoattractants, such as fMLP [66]. Knockout mice with FPR2 (fMLPR) deficiency have significantly worse sepsis outcomes compared with wild-type mice [68]. On the other hand, septic neutrophils tend to express higher levels of CCR5, capable of trafficking neutrophils to vital host organs and causing multiorgan inflammation and failure [69, 70]. Furthermore, septic neutrophils have reduced NET forming potential, a vital antimicrobial defense mechanism to clear microorganisms [36, 65]. We recently reported that the “paralytic” neutrophil phenotype could be recapitulated *in vitro* through programming with super-low dose LPS. We demonstrated that super-low dose LPS selectively reduced the levels of FPR2, increased CCR5 levels, reduced NET-forming potential on cultured neutrophils, and increased sepsis severity [36]. At the mechanistic level, we reported that Tollip deficiency is critically involved in the establishment of paralytic neutrophils, as a result of reduced ERK/AKT activity [71]. Tollip-deficient mice have exacerbated sepsis severity [71]. Consistent with our mechanistic studies, a human genetic study revealed that defective human Tollip expressions are associated with increased human sepsis severity, whereas higher Tollip expressions are correlated with better sepsis outcomes [72].

Innate memory dynamics in atherosclerosis

Atherosclerosis and related cardiovascular complications are among the leading causes of death in industrialized countries [73]. The programming of low-grade inflammatory monocytes may play a key role during the pathogenesis of atherosclerosis. Sustained low-grade inflammatory monocytes and Mφs are key drivers for the formation of atherosclerotic plaques [74]. Despite growing interest in low-grade inflammation and its contribution to atherosclerosis, the mechanisms responsible for the sustained low-grade inflammation are not well understood [75].

Previous studies have primarily focused on the concept of “excessive” inflammatory signals causing exacerbated atherosclerosis [76]. However, recent data show that excessive inflammatory signals tend to trigger compensatory anti-inflammatory tolerance [5, 77]. Thus, simple emphasis on the excessive positive signals represents an overly simplistic view with regard to the complex dynamics of atherosclerosis pathogenesis. In collaboration with computational systems biologists, we have integrated both experimental and computational approaches, which revealed that the dysfunction of homeostatic negative regulators may also be critical for the sustaining nonresolving inflammatory monocytes [6, 7, 18, 23, 78]. Experimentally, we reported that chronic injection of super-low dose LPS can establish nonresolving, low-grade inflammatory monocytes *in vivo* and contribute to exacerbated atherosclerosis [6].

In addition to monocytes/Mφs, programming of inflammatory neutrophils may occur during the pathogenesis of atherosclerosis and may contribute to reduced plaque stability [79, 80]. Future systems studies are needed to understand better the programming dynamics of innate leukocytes as they relate to the temporal and spatial modulation of atherosclerosis.

Innate memory dynamics in wound repair

In the context of wound repair, an initial influx of inflammatory monocytes into the cutaneous wound bed is critical for the early phase of vascular sprouting [81]. Subsequent transition to anti-inflammatory monocytes would enable proper wound closure and tissue regeneration during the resolving phase [81, 82]. This temporal switching process from an inflammatory state to a subsequent anti-inflammatory state is vital for proper wound healing. Persistent existence of inflammatory monocytes in the wound bed may be one of the primary culprits for hard-to-heal wounds, such as those seen in diabetic patients [83]. We recently reported that the programming of nonresolving, low-grade inflammatory monocytes by subclinical endotoxemia can contribute to delayed wound healing in a murine skin-excision wound model [7]. The establishment of nonresolving inflammatory monocytes is facilitated by the disruption of lysosome fusion, causing the accumulation of IRF5, a signature transcription factor associated with inflammatory monocytes [7].

Innate memory dynamics in cancer

Dynamic programming of innate leukocytes has also been increasingly recognized in the tumor environment [84]. TAMs often exhibit a tumor-promoting phenotype with increased expression of growth factors and reduced expression of immune-stimulating cytokines, such as IL-12 [85]. Likewise, tumor tissues may secrete chemokines and other factors that actively recruit neutrophils to and reprogram neutrophils in the vicinity of tumor cells [86]. Much like TAM, tumor-associated neutrophils are shown to secrete molecules to assist in tumorigenesis [87–91].

The modulation of the programming dynamics of innate monocytes/Mφs and neutrophils may be a novel approach in the future treatment of cancer. We reported that disruption of IRAK-M, a key negative regulator of innate immunity signaling, can benefit host immune defense toward both xenografted tumors, as well as chemically induced tumorigenesis [92, 93]. IRAK-M deficiency skews innate monocytes away from the TAM phenotype. Furthermore, IRAK-M deficiency programs neutrophils into an immune-modulatory state with increased bacterial-killing activity, increased migratory potential toward inflammatory signals, as well as heightened expression of immune-stimulating molecules, such as MHC class II and L-selectin [93].

Innate memory dynamics in autoimmune diseases

Innate immune cell programming dynamics may play a key role in the pathogenesis of autoimmune diseases as well. Circulating monocytes from patients with SLE were shown to exhibit features resembling DCs. SLE serum skews the differentiation of healthy monocytes into DCs that can potently activate both T cells and B cells [94]. At the mechanistic level, a recent study unveiled modified gene-expression profiles in SLE monocytes, with elevated expression of the chemokine receptor CCR7, as well as molecules related to IFN signaling. These events may potentiate the acquisition of APC-like functions in SLE monocytes and facilitate SLE pathogenesis [95].

Besides monocytes and DCs, neutrophils from SLE patients also display dramatic abnormalities. SLE neutrophils are inclined to form aggregation as a result of their intravascular activation by autoantibodies and nucleosomes [96]. A subset of specialized neutrophils has been identified in

peripheral blood mononuclear cell (PBMC) fractions of lupus patients, termed LDGs, which exhibit a surface phenotype similar to healthy neutrophils but differ in their nuclear morphology. LDGs are programmed into a proinflammatory state, with elevated expression of IFNs and TNF, as well as immune-stimulatory bactericidal proteins [97–99]. In addition, SLE neutrophils constitutively generate NETs to release proinflammatory cytokines and dsDNA in the absence of infection or additional stimulus [99, 100].

Differentially programmed innate immune cells have been observed in the pathogenesis of RA as well. The frequency of CD14^{high}CD16⁺ intermediate monocytes is increased in peripheral blood and synovial fluid of patients with chronic RA. These monocytes express high levels of CCR1, CCR5, ICAM-1, and TLR2 [101]. The lifespans of monocytes/Mφs are prolonged in RA patients, with persistently elevated levels of proinflammatory cytokines, chemokines, and matrix metalloproteinases [102, 103]. Monocytes/Mφs from RA patients express higher levels of HLA-DR, adhesion molecules, and costimulatory molecules, such as CD40, CD80, and CD86, which are conducive for the polarization of CD4 T cells toward Th1 and Th17 subsets [103]. In addition, the cytokines, immune complexes, and rheumatoid factors in synovial fluid may collectively induce neutrophil activation and granular exocytosis, which contribute to cartilage destruction [104]. Enhanced NETosis can be observed in circulating neutrophils, as well as those found in the synovial fluid of RA patients. Moreover, NET-forming neutrophils infiltrate into rheumatoid nodules and skin. Neutrophil NETs serve as a key source of citrullinated autoantigens and immunostimulatory molecules that robustly boost the inflammatory responses in RA [105].

CONCLUSIONS AND FUTURE DIRECTIONS

Innate leukocytes use complex and integrated signaling circuitries at both the molecular and subcellular levels to adapt and respond dynamically to diverse inflammatory environments. With the help of systems analyses [106, 107], combined with genomic and proteomic approaches [108], these integrated molecular signaling dynamics are beginning to be elucidated. Future efforts that combine single-cell analyses with systems and omics approaches are necessary to gain full insight to the spectrum of the memory dynamics of innate leukocytes.

At the pathophysiological level, programmed memory innate leukocytes may exert profound effects that control the magnitude and duration of diverse inflammatory processes. The *in vivo* relevance of innate leukocyte memory should be vigorously studied with animal models that exhibit relevant pathophysiological states. Future mechanistic studies with model organisms should be corroborated with translational studies using human blood samples from healthy human donors and patients. Collectively, better understanding of innate leukocyte memory dynamics will provide clues for restoring leukocyte homeostasis and treating inflammatory diseases.

ABBREVIATIONS

AGE = advanced glycation end products,

AKT = protein kinase B

CD = cluster of differentiation

DC = myeloid dendritic cell

FPR2 = N-formyl peptide receptor 2

IRAK-M = IL-1R-associated kinase M

IRF = IFN regulatory factor

LDG = low-density granulocyte

M1 = classically activated macrophage

M2 = alternatively activated macrophage

MAFP = methyl arachidonyl fluorophosphonate

MOI = multiplicity of infection

NET = neutrophil extracellular trap

RA = rheumatoid arthritis

ROS = reactive oxygen species

SLE = systemic lupus erythematosus

TAM = tumor associated macrophage

Tollip = Toll-interacting protein

TRAM = Toll-IL-1R domain-containing adapter-inducing IFN- β -related adaptor molecule

TRIF = Toll-IL-1R domain-containing adapter-inducing IFN- β

AUTHORSHIP

C.L., S.G., and L.L. wrote the manuscript. Y.Z. and A.R. provided thoughtful discussion and comments.

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DISCLOSURES

The authors declare no conflicts of interest.

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CHAPTER 2

Enhanced tumor immune surveillance through neutrophil re-programming due to Tollip deficiency

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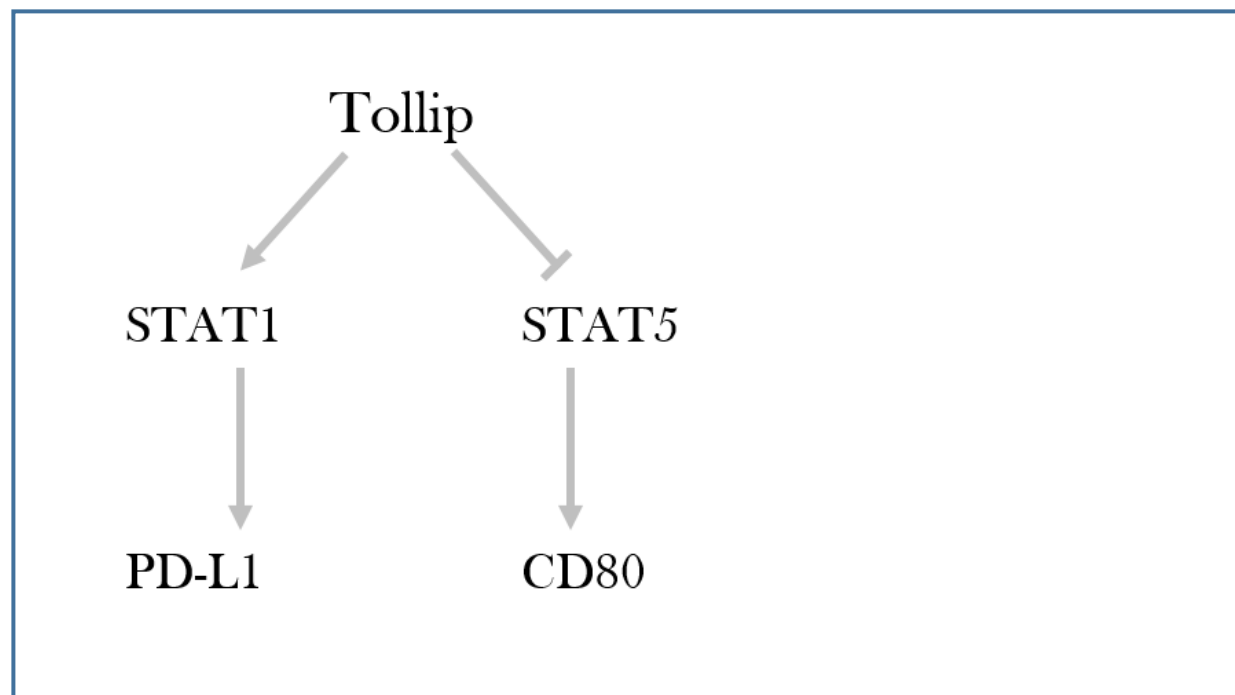
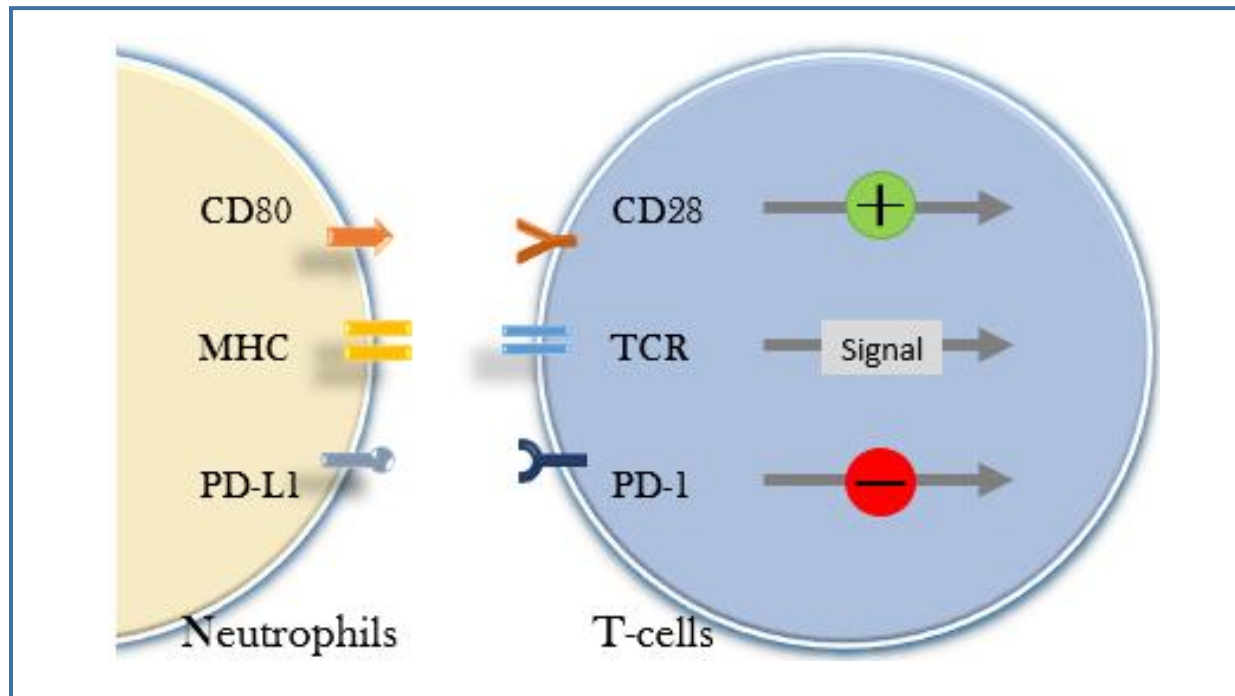
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ABSTRACT

Although tumor immune environment is increasingly recognized to play a key role in the modulation of tumorigenesis and tumor regression, most of the attention related to tumor-immune therapies in the past has been paid to adaptive immune cells. The role and regulation of innate leukocytes such as neutrophils during the modulation of tumor immune environment remain controversial and less defined. Here we observed that the selective deletion of Tollip, a key innate immune cell modulator, renders enhanced tumor immune surveillance in a chemically induced colon tumor model. Tollip deficient neutrophils significantly elevate T cell activation through enhanced expression of co-stimulatory molecule CD80, and reduced expression of inhibitory molecule PD-L1. Mechanistically, Tollip deficiency increases STAT5 levels and reduces STAT1, responsible for the expression of CD80 and PD-L1 respectively in neutrophils. Through the adoptive transfer study, we demonstrate that Tollip deficient neutrophils are sufficient to transfer enhanced tumor immune surveillance and reduce tumor burden *in vivo*. Our data reveal a novel re-programming strategy of neutrophil function conducive for enhancing anti-tumor immune environment.

GRAPHICAL ABSTRACT



INTRODUCTION

Decades of extensive studies have provided compelling evidence supporting a role for the immune system during the complex dynamics of tumor initiation, progression and regression [1]. Most notably, the roles of adaptive immune cells such as T cells are well appreciated through recognizing tumor-specific antigens and coordinating anti-tumor functions [2]. Recent advances suggest that innate immune cells including dendritic cells, monocytes and neutrophils play vital roles in facilitating the anti-tumor functions of T cells, through affecting the expression and activities of immune check point genes such as PD-L1 [3]. Despite these exciting advancements, it still remains less understood with regard to the roles and mechanisms of innate immune cells during the modulation of tumor-immune environment.

Among tumor infiltrating innate immune cells, neutrophil is one of the major constituents [4]. Solid tumor patients with poor prognosis tend to have expanded pools of tumor-associated neutrophils [4-6]. Although the mechanisms are not well understood, neutrophils are known to exhibit complex and often opposing functions that either facilitate or prevent tumor initiation and growth [7]. Differential expressions of neutrophil cell surface molecules (e.g. PD-L1) as well as secretory mediators may contribute to the opposing functions of neutrophils in either augmenting or suppressing adaptive T cell activation. However, molecular mechanisms underlying the differential activation of neutrophils are not known.

Tollip is an innate immunity signaling adaptor molecule expressed in myeloid cells [8]. Initially recognized as an inhibitor for the TLR signaling pathway, recent studies suggest that Tollip may

modulate cellular autophagy and other pathways in monocytes [9, 10]. Its role in modulating neutrophil function and tumor immune environment has not been studied.

We aim to test the hypothesis that Tollip may contribute to the differential activation of neutrophils and that Tollip deficient neutrophils may alter tumor immune environment. We tested the tumor burden, immune environment, and neutrophil function of WT and Tollip deficient mice bearing colon tumors chemically induced by AOM-DSS. We also performed adoptive transfer studies to specifically examine the role of Tollip deficient neutrophils in modulating immune environment and tumor growth. We observed that Tollip deficient mice have reduced tumor burden and enhanced neutrophil function in promoting T cell proliferation. Tollip deficient neutrophils have elevated co-stimulatory molecule CD80 and reduced co-inhibitory molecular PD-L1, through the induction of STAT5 and reduction of STAT1. Our study reveals Tollip as a novel molecular check-point that governs the decision-making processes of neutrophils in modulating tumor immune environment.

RESULTS

Tollip deficiency reduces colitis-associated tumorigenesis

In the previous study of acute DSS-induced colitis, we observed that Tollip^{-/-} mice exhibit more severe acute colitis as compared to WT mice, due to elevated leukocyte infiltration and inflammation in the gut tissue [11]. In the current study, we tested the severity of colorectal tumorigenesis in Tollip^{-/-} mice with the Azoxymethane (AOM)-dextran sulfate sodium salt (DSS) model which is a well-defined colorectal cancer model. Age and gender compatible mice (WT and Tollip^{-/-}) were injected *i.p.* with a single dose of AOM (10 mg/kg) followed by three cycles of 2% DSS in drinking water. While all mice survived the experimental periods (Fig. S1), WT mice developed significant amount of colon tumors throughout the distal as well as other segments of the colons (Fig. 1B and 1C). In contrast, Tollip^{-/-} mice exhibited 50% reduction in both microscopic and macroscopic polyps as compared to WT mice (Fig. 1B and 1C). We also monitored the whole body health conditions including weight loss, stool consistency, colorectal bleeding throughout the experimental course. Consistent with the acute colitis observations, we observed slightly severe disease scores from Tollip^{-/-} mice following the initial cycle of DSS as compared to WT mice. In contrast, toward the end of the final DSS cycle, WT mice had much worse clinical scores including stool bleeding, consistent with more severe tumor burdens (Fig. S2, S3, and S4).

In addition to the general body outlook, we further performed histological assessment of the colon tissues. H&E staining showed more severe colon inflammation and alterations of epithelial structure in WT mice as compared to Tollip^{-/-} mice (Fig. 1D). In addition, WT mice with the AOM-

DSS treatment exhibited pervasive Ki67 staining throughout the colon tissues which served as a molecular marker for hyper-proliferative cells and tumorigenesis (Fig. 1E). In contrast, Tollip^{-/-} mice similarly challenged with AOM-DSS demonstrated significantly reduced Ki67 positive cells in the colon (Fig. 1E and 1F). Furthermore, the cellular levels of β -catenin were also decreased in the colon sections from Tollip^{-/-} mice as compared to WT mice (Fig. 1G and 1H). Collectively, our data reveal that Tollip deficient mice have reduced colon tumor formation when challenged with AOM-DSS. Lastly, the expression of p53, a marker for distressed cell and most often mutated gene found in tumor cells, were increased in the colon sections in WT mice compared to Tollip^{-/-} mice (Fig. 1I and 1J).

Tollip deficiency enhances anti-tumor innate immune checkpoints and facilitates inflammation homeostasis

Since Tollip is recognized as a key modulator of innate immune cells, we asked whether enhanced anti-tumor defense in Tollip deficient mice may be due to more effective anti-cancer checkpoints from innate immune cells. To test this, we first measured the key innate checkpoint molecules such as PD-L1 and CD80 expressed on neutrophils. As shown in Fig. 2A, splenic neutrophils from naïve Tollip^{-/-} mice expressed significantly less PD-L1 and higher CD80 as compared to naïve WT mice. This trend remained at the end of AOM-DSS cycle (Fig. 2A). The percentages of neutrophils within blood and colon tissues were similar among WT and Tollip mice before and after AOM-DSS challenge (Fig. S5).

We next examined CD4 and CD8 T cells in WT and Tollip^{-/-} mice. At the end of the final DSS cycle, Tollip^{-/-} mice had significantly higher amount of both CD4 and CD8 T cells as compared to WT mice within the lamina propria where colonic leukocytes home into (Fig. 2B). The numbers of CD8T cells in the spleen were also significantly higher from Tollip^{-/-} mice as compared to WT mice (Fig. S5). Correlated with elevated T cell populations, we observed elevated levels of IFN γ and IL-12 within colon tissues of Tollip^{-/-} mice as compared to WT mice following AOM-DSS challenge (Fig. 2C).

Despite elevated T cells and enhanced T cell promoting neutrophils in Tollip^{-/-} mice, we observed reduced circulating inflammatory cytokine IL-1 β in Tollip^{-/-} mice challenged with AOM-DSS as compared to WT mice (Fig. 2D). In contrast, circulating plasma levels of TGF- β were significantly higher in Tollip^{-/-} mice challenged with AOM-DSS as compared to WT mice (Fig. 2D). Other inflammatory surface markers of circulating neutrophils such as CD14 and CCR5 were also significantly lower in Tollip^{-/-} mice as compared to WT mice (Fig. 2E). Together, our data reveal that Tollip deficiency facilitates the resolution of chronic inflammation during AOM-DSS induced colon tumorigenesis. Our findings also support the hypothesis that Tollip^{-/-} neutrophils may enhance tumor-immune defense through facilitating T cell proliferation and activation.

Tollip mediates the immunosuppression of neutrophils in suppressing T cell proliferation

It is well-noticed that tumor-associated neutrophils have immune-suppressive effects by suppressing T cell proliferation [4]. To test the hypothesis that Tollip may facilitate the suppressive

effects of neutrophils, we performed *in vitro* co-culture studies. Increased production of granulocyte macrophage colony-stimulating factor (GM-CSF) has been observed in mucosa of patients with inflammatory bowel disease and rodents subjected to experimental colitis [12]. GM-CSF was also shown to promote the generation of myeloid-derived suppressor cells [13]. We cultured bone marrow neutrophils from WT or Tollip^{-/-} mice in GM-CSF overnight, and subsequently co-cultured with CFSE-labeled allogeneic splenocytes in anti-CD3 coated plates. GM-CSF primed neutrophils showed typical immunosuppressive phenotype, as evident from reduced T cell proliferation with the addition of neutrophils (Fig. 3A). However, compared with WT neutrophils, Tollip^{-/-} neutrophils had significantly less immunosuppressive effects on the proliferation of both CD4 and CD8 T cells (Fig. 3A). Consistent with *in vivo* results, Tollip^{-/-} neutrophils had increased expression of CD80 and decreased PD-L1 expression (Fig. 3B). To confirm the involvement of PD-L1 or CD80 on Tollip neutrophils during the modulation of T cell proliferation, we applied the blocking antibodies in the co-culture assays. In the presence of anti-CD80 antibody during the co-culture, T cell proliferation were further blocked (Fig 3C). In contrast, in the presence of anti-PD-L1 antibody, the suppression of WT neutrophils on T cell proliferation was partially removed (Fig. 3D). These data suggest that Tollip^{-/-} neutrophils have reduced suppressive effects on T cell proliferation through an increase of CD80 and a decrease of PD-L1 expression.

Tollip deficient neutrophils enhance T cell activation and survival

Neutrophils may not only affect the proliferation, but also the survival and activation of T cells. We further tested the effects of Tollip^{-/-} neutrophils on T cell activation and survival *in vitro* through the co-culture assay. For the T cell activation measurement, we tested the surface expression of CD62L and CD107α through flow cytometry, as well as secretants such as INFγ and granzyme B by ELISA. Following 1-day co-culture of GM-CSF-primed neutrophils, both CD4 and CD8 T cells cultured with Tollip^{-/-} neutrophils exhibited significant down-regulation of CD62L as compared to cells co-cultured with WT neutrophils, an indication of enhanced T cell activation (Fig. 4A). The populations of CD107α expressing CD8 T cells were also significantly elevated during the co-culture with Tollip^{-/-} neutrophils as compared with WT neutrophils (Fig. 4B). As measured by ELISA, the secreted levels of INFγ and Granzyme B from the co-cultures with Tollip^{-/-} neutrophils were significantly higher as compared to the co-cultures with WT neutrophils (Fig. 4B). We further measured the viability of T cells following 3 day co-culture. Both CD4 and CD8 T cells co-cultured with Tollip^{-/-} neutrophils exhibited significantly higher survival rate as compared to T cells co-cultured with WT neutrophils (Fig. 4C). Collectively, our data reveal that Tollip^{-/-} neutrophils enhance T cell activation as well as survival as compared with WT neutrophils.

Tollip neutrophils have elevated STAT5 activation and reduced STAT1 activation

We next examined the underlying molecular mechanisms responsible for the neutrophil reprogramming due to Tollip deficiency. Previous studies reported that STAT5 and STAT1 are differentially involved in the expression of CD80 and PD-L1, with STAT5 promoting the

expression of CD80 [14] and STAT1/STAT3/IRF1 promoting the expression of PD-L1 [15, 16]. Thus, we tested the activation of STAT1, STAT5 as well as other key signaling molecules comparing WT and Tollip^{-/-} neutrophils. We observed that the phosphorylation levels of STAT1, STAT3 and IRF1 were all reduced in Tollip^{-/-} neutrophils as compared to WT neutrophils (Fig. 5A). This is consistent with reduced PD-L1 expression in Tollip^{-/-} neutrophils. In contrast, we detected an increased phosphorylation of STAT5 and p65 in Tollip^{-/-} neutrophils as compared to WT neutrophils, consistent with elevated expression of CD80 in Tollip^{-/-} neutrophils (Fig. 5B). We also noticed elevated phosphorylation of CAMKII in Tollip^{-/-} neutrophils (Fig. 5C). These mechanistic observations are consistent with elevated CD80 expression in Tollip^{-/-} neutrophils. Correspondingly, we performed quantitative measurement of STAT1/STAT5 phosphorylation through flow cytometry. As shown in Fig. 5B, Tollip neutrophils had significantly elevated levels of p-STAT5 and reduced levels of p-STAT1. Our data reveal that the dichotomy of elevated STAT5 activation and reduced STAT1 activation due to Tollip deficiency may underlie the polarized CD80/PD-L1 expression in Tollip deficient neutrophils conducive for an effective T cell response toward tumor immune surveillance.

Adoptive transfer of Tollip^{-/-} neutrophils is sufficient to dampen colitis-associated tumor progression

To directly test whether neutrophil deficiency in Tollip is responsible for an effective anti-tumor effect *in vivo*, we next performed the adoptive transfer experiment. Purified neutrophils from either WT or Tollip^{-/-} mice were injected weekly via *i.v.* to WT mice subjected to AOM-DSS challenge

as described in the Method Section. We observed that WT mice receiving Tollip^{-/-} neutrophils exhibited a marked reduction in the tumor load as compared to the WT mice receiving WT neutrophils (Fig. 6A and 6B). The overall body weight as well as colon length were similar among these groups at the end of the study (Fig. S6). However, H&E staining revealed reduced inflammation in mice transfused with Tollip^{-/-} neutrophils (Fig. 6C). Ki67 and β -catenin staining revealed that the colons from WT mice transfused with Tollip^{-/-} neutrophils had reduced β -catenin and Ki67 positive cells (Fig. 6D). Our data indicate that transfusion of Tollip^{-/-} neutrophils is sufficient to render protection against AOM-DSS induced colon tumorigenesis.

We further examined the levels and activation status of CD4 and CD8 T cells in mice transfused with WT or Tollip neutrophils and subjected to the AOM-DSS challenge. As shown in Fig. 6E, mice transfused with Tollip^{-/-} neutrophils had more splenic cell counts of CD4 and CD8 T cells. Furthermore, CD8 T cells in mice transfused with Tollip^{-/-} neutrophils demonstrated significantly elevated activation status as reflected in the higher percentage of CD62L^{low} as well as Granzyme B^{positive} CD8 T cells (Fig. 6F). Our data reveal an enhanced *in vivo* anti-tumor immunity due to transfusion of Tollip^{-/-} neutrophils.

DISCUSSION

Our current study reveals that, through Tollip deletion, neutrophils can be uniquely programmed to serve as a highly effective anti-tumor immune modulator. Several lines of data support this novel conclusion. First, we observed that Tollip deficient mice have reduced colon tumor development when subjected to the AOM-DSS challenge. Second, we found that Tollip deficient neutrophils are re-programmed to be conducive for T cell proliferation, survival and activation. Third, we demonstrated that the transfusion of Tollip deficient neutrophils into WT mice is sufficient to alleviate AOM-DSS induced colon tumor formation.

Our data provide a fresh perspective for the emerging and potentially important roles of neutrophils during the modulation of tumor immune environment. Emerging basic and translational studies with experimental models and human cancer patients suggest complex repertoires of tumor-associated myeloid cells that may either promote or inhibit tumor progression [4]. Although tumor tissues are known to have expanded pools of neutrophils that often correlate with aggravated tumor growth [4-6], it is not well understood how tumor-associated neutrophils are programmed at molecular level to either facilitate or suppress tumorigenesis. Earlier studies led to the hypothesis that neutrophils may be differentially activated into either an N1 tumor-promoting state or an N2 tumor-promoting state [17]. However, such categorization of neutrophils still lacks phenotypic and mechanistic clarity. Our data collected in this study extend these emerging studies and provide mechanistic insights regarding the role of neutrophil reprogramming in tumor growth. Consistent with previous reports, we observed that wild type neutrophils exhibit suppressive function toward T cell proliferation, survival and activation *in vitro* partly through immune check point PD-L1,

resembling the tumor-promoting and T cell-suppressing effects of tumor-associated neutrophils *in vivo*. In contrast, Tollip^{-/-} neutrophils promote the survival and activation of CD4 and CD8 T cells. Recent studies reveal the significance of innate immune checkpoint molecules such as PD-L1 in suppressing T cell function and promoting tumor immune-evasion [3]. Our current study not only confirms the critical involvement of PD-L1 and CD80 on neutrophils in modulating T cell function, but also reveals that Tollip deficiency reprograms neutrophils into a T cell-promoting state with significantly reduced PD-L1 and elevated CD80. Our data suggest an innovative approach for reprogramming neutrophils through Tollip deletion for enhanced tumor immune-surveillance.

Our current study also provides molecular mechanisms responsible for the reprogramming of tumor-suppressing neutrophils. The immune check-point T cell inhibitory molecule PD-L1 expression on neutrophils was shown to be under the control of STAT1, STAT3 and IRF1 [15, 16]. We observed that Tollip deficiency leads to reduced activation of STAT1, STAT3 and IRF1 in Tollip^{-/-} neutrophils that is consistent with reduced PD-L1 expression. On the other hand, STAT5 is responsible for the expression of co-stimulatory molecules such as CD80 on neutrophils [14]. Our finding that Tollip deficient neutrophils have elevated STAT5 activation provides a mechanistic explanation for elevated CD80 expression. Future detailed biochemical analyses are clearly needed to further determine the molecular mechanisms responsible for the differential activation of STAT1 and STAT5 by Tollip.

From a translational perspective, our data reveal a novel potential of using “reprogrammed” innate leukocytes such as neutrophils in tumor treatment. There has been a resurgence in cancer immune-

therapy, given the intriguing yet limited success through engineered T cells combined with checkpoint inhibitors [2]. Recent studies also suggest that subsets of neutrophils may hold potential in suppressing cancer cell growth, although underlying mechanisms for the differentiation/activation of tumor-suppressing neutrophils are not clear [4]. Our adoptive transfer data with Tollip deficient neutrophils not only provide a proof-of-principle for the translational potential of reprogrammed neutrophils in cancer treatment, but also reveal critical mechanisms for effective reprogramming of tumor-fighting neutrophils through Tollip deletion.

Collectively, our current study reveals that neutrophils engineered with Tollip deletion can be effectively reprogrammed into a novel state to exhibit an effective anti-tumor immune defense. Mechanistically, we demonstrate that Tollip deficient neutrophils can potently activate the functions of both CD4 and CD8 T cells. However, we cannot rule out the possibility that reprogrammed neutrophils due to Tollip deletion may also affect other tumor-fighting immune cells such as NK cells or innate lymphocytes, which should be systematically examined in future studies. Mechanistic studies regarding molecular regulatory networks related to Tollip regulation are also needed in order to better characterize the therapeutic potential of Tollip-targeting drugs in designing precision cancer medicine.

MATERIALS AND METHODS

Mice

Wild type (WT) C57BL/6 mice and Tollip^{-/-} mice were bred and maintained in the animal facility at Virginia Tech in accordance to approved Animal Care and Use Committee protocol. All littermate mice were 8-10 weeks of age and 25–30 g weight when experiments were initiated.

Experimental design

WT and Tollip^{-/-} mice received a single intraperitoneal injection of azoxymethane (AOM, Sigma-Aldrich) at a dose of 10mg/kg body weight. A week after AOM injection, the mice were given three cycles of 2% dextran sulfate sodium salt (DSS, MP Biomedicals) for 5 days followed by 14 days of normal drinking water. After the last water cycle mice were sacrificed and tissues were harvested for further analysis. A schematic protocol is illustrated in Figure 1A. Body weight, stool consistency, bleeding was measured as part of clinical score (score 0-4, with higher score corresponding to worse condition). Polyp formation was labeled macro- and micro-polyp depending on the size equal to or greater than 2mm versus less than 2mm, respectively. Independent experiments of AOM-DSS induced colorectal tumorigenesis were conducted more than 3 times, and for every experiment there were at least 5 mice in each group.

Histology

Histological analyses of colon tissues were performed on freshly frozen OCT (Optimal-Cutting-Temperature compound)-embedded, and sectioned slides (5µm). Slides were fixed in 4% neutral buffered formalin for 5 min. Hematoxylin and eosin (H&E) staining were performed.

Immunofluorescence

Immunofluorescence analyses were performed on freshly frozen OCT-embedded, and sectioned slides (5µm). At least 6 mice from WT and Tollip^{-/-} mice were used for the study. For the

measurement of Ki67 (Abcam), β -catenin (Cell Signaling), and p53 (Cell Signaling), sections were fixed in 4% neutral buffered formalin for 5 min, and stained with anti-mouse primary antibodies (1:100) followed by a biotinylated anti-Ig secondary Ab (BD eBiosciences) and streptavidin-PE or FITC. DAPI was used to stain nucleus. Multiple viewing fields from each slide were captured under fluorescent microscope. Pixel values reflecting the fluorescent intensities of each viewing field were quantitated with the NIH ImageJ software.

Immunohistochemistry

Sections from WT and Tollip^{-/-} were co-stained with anti-myeloperoxidase (MPO) (Abcam; 1:100 dilution) and anti-CD3 (Abcam; 1:100 dilution). Secondary staining was performed using VECTASTAIN Elite ABC-HRP Kit followed by DAB Peroxidase Substrate kit, or ImmPRESSTM-AP Anti-Rat IgG Alkaline Phosphatase (AP) Polymer Detection Kit, followed by ImmPACT Vector Red AP Substrate. All kits were purchased from Vector laboratory. Staining was performed according to the manufacturer's instructions.

Immunoblotting

Bone marrow neutrophils were purified by 65% percoll gradient and the purity was >90% confirmed by Ly6G+CD11b⁺ staining. For neutrophil culture, purified neutrophils were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin and with GM-CSF (1 ng/ml) overnight. Naive or cultured neutrophils were harvested in SDS lysis buffer containing protease and phosphatase inhibitors as previously described [18]. Briefly, equal amount of protein was applied to SDS-PAGE and transferred to PVDF membranes (BioRad). The membranes were blocked with 5% non-fat dry milk, and then incubated with primary anti-phospho STAT1 (cell signaling), anti-STAT1 (cell signaling), anti-phospho-p38 (Cell Signaling), anti-p38 (Cell Signaling), anti-phospho-p65 (Cell Signaling), anti-p65 (Cell Signaling), IRF5 (Cell Signaling), oxCaMKII (Millipore) or β -actin antibody (Santa Cruz), and anti-rabbit or mouse IgG secondary antibody (Cell Signaling) according to the manufacturer's instructions. The immunoblots were developed by a chemiluminescence ECL detection kit (Thermo Scientific).

Adoptive transfer of neutrophils

Bone marrow neutrophils from donor mice (WT or Tollip^{-/-}) were purified (>90% confirmed by flow cytometry) using EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell), according to the manufacturer's instruction. Recipient WT mice were transfused twice (post DSS day 5 and day 12) per DSS-resting cycle through intravenous injection with 2.4×10^6 WT or Tollip^{-/-} neutrophils suspended in 200 µl sterile PBS. Detailed timeline was illustrated in Fig. S6A.

ELISA

The levels of TNF-α and LTB4 in plasma were measured using ELISA kits purchased from R&D system, according to the manufacturer's instructions.

Lamina propria cell isolation

Colons were opened longitudinally, and cleaned by flushing with ice-cold PBS. Single-cell suspension was prepared using Lamina Propria dissociation Kit (MACS). Briefly, the colons were cut into fine pieces, and incubated with HBSS containing 5mM EDTA, 5% FBS, and 1 mM DTT to remove epithelial cells. The remaining pieces were then incubated with HBSS containing 5% FBS and enzyme mix using the gentleMACS dissociator. The cells were washed, passed through 70µm strainer, and resuspended in FACS buffer for further flow cytometry analyses.

Flow cytometry

Fluorescent-conjugated anti-mouse antibodies specific for PD-L1, CD80, CD14, CD11b, CD4, CD8, Ly6G were purchased from BioLegend. Propidium iodide (PI) was also added to determine the cell viability. Peripheral blood cells and splenocytes were harvested from WT and Tollip^{-/-} mice as previously described [19]. The cells were washed in FACS buffer (HBSS supplemented with 2% FBS and 0.02% sodium azide) and stained with fluorescently-labelled antibodies for 20 min on ice. Stained cells were analyzed with a FACSCanto II (BD Biosciences). FACS plots shown were analyzed with FlowJo (Ashland, OR).

T cell proliferation assay

Splenocytes were labelled with 5,6-carboxyfluorescein diacetate succinimidyl (CFSE) (Invitrogen, Molecular Probe), according to the manufacturer's instructions. CFSE-labeled splenocytes were stimulated with plate-bound anti-mouse CD3 antibody (eBioscience, clone: xxx). Neutrophils purified from bone marrow were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin and with GM-CSF (1 ng/ml) for 24 hours. CFSE-labeled splenocytes were mixed with cultured neutrophils at a 1:1 ratio and co-cultured in CD3 coated plates for 72 hours. CFSE signals were analyzed by flow cytometry on gated CD4⁺ and CD8⁺ cells. In blocking experiments, antibodies against CD80 or PD-L1 (BioLegend) were added to the co-culture at the concentration of 1 µg/ml. To test the viability of T cells after co-culture, neutrophils and T cells were treated as above but plated in 96-well U bottom plate without CD3 coating.

Statistical analysis

Statistical analysis was performed with Prism software (GraphPad Software 6.0). Values were expressed as means $\pm$ SD. The significance of the differences was assessed by Student's t-test (two sided). Sample sizes are in line with similar reported studies and can yield sufficient power for observing changes with proper statistical analyses. Data used fall into typical Gaussian distribution, with similar variances between groups. $P < 0.05$ was considered statistically significant.

Data availability

Data presented in this study are available upon reasonable request from the corresponding author.

ACKNOWLEDGMENTS

We appreciate the help of Li Lab members for assistance.

FUNDING

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AUTHOR CONTRIBUTIONS

L.L. and Y.Z. conceived the project, designed the studies, proposed the experiments, and wrote the manuscript. Y.Z. and C.L. performed the experiments.

COMPETING INTERESTS

The authors declare that there is no competing interest.

DATA AND MATERIALS AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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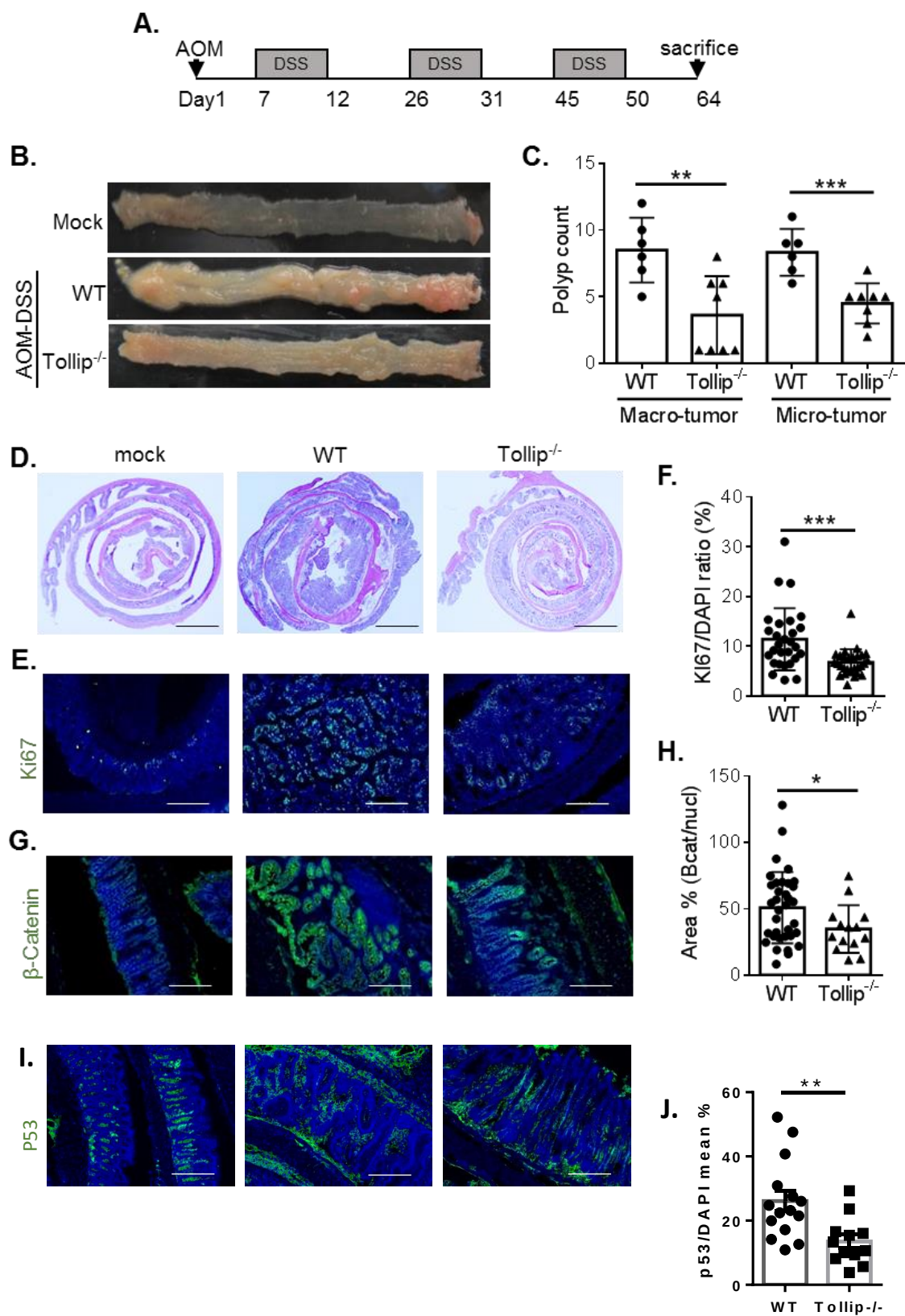


Fig. 1. Tollip deficiency decreased tumorigenesis in the AOM-DSS mouse model of colon cancer. (A) Schematic protocol of AOM-DSS treatment. (B) Representative images of colons from WT and Tollip^{-/-} mice treated with AOM-DSS or naïve mice. (C) Graphical representation of tumor burden in WT (n=6) and Tollip^{-/-} (n=8) mice. Diameter of tumors greater than or equal to 2mm defined as “macro” tumor; diameter of tumors less than 2mm defined as “micro” tumor. (D) H&E stained sections of colon from WT or Tollip^{-/-} mice treated with AOM-DSS treated or naïve mice. Colons were collected in swiss rolls at the end of AOM-DSS regimen. Scale bar represents 2.0mm. (E) Immunofluorescent analysis of Ki67 in colons of from WT or Tollip^{-/-} mice treated with AOM-DSS treated or naïve mice. Scale bar represents 200µm. (F) Quantitative analysis of Ki67 staining. (G) Immunofluorescent analysis of active β-catenin in colons from WT or Tollip^{-/-} mice treated with AOM-DSS treated or naïve mice. Scale bar represents 200µm. (H) Quantitative analysis of active β-catenin staining. (I) Immunofluorescent analysis of p53 in colons from WT or Tollip^{-/-} mice treated with AOM-DSS treated or naïve mice. Scale bar represents 200µm. (H) Quantitative analysis of p53 staining.* p<0.05, ** p<0.01, *** p<0.001.

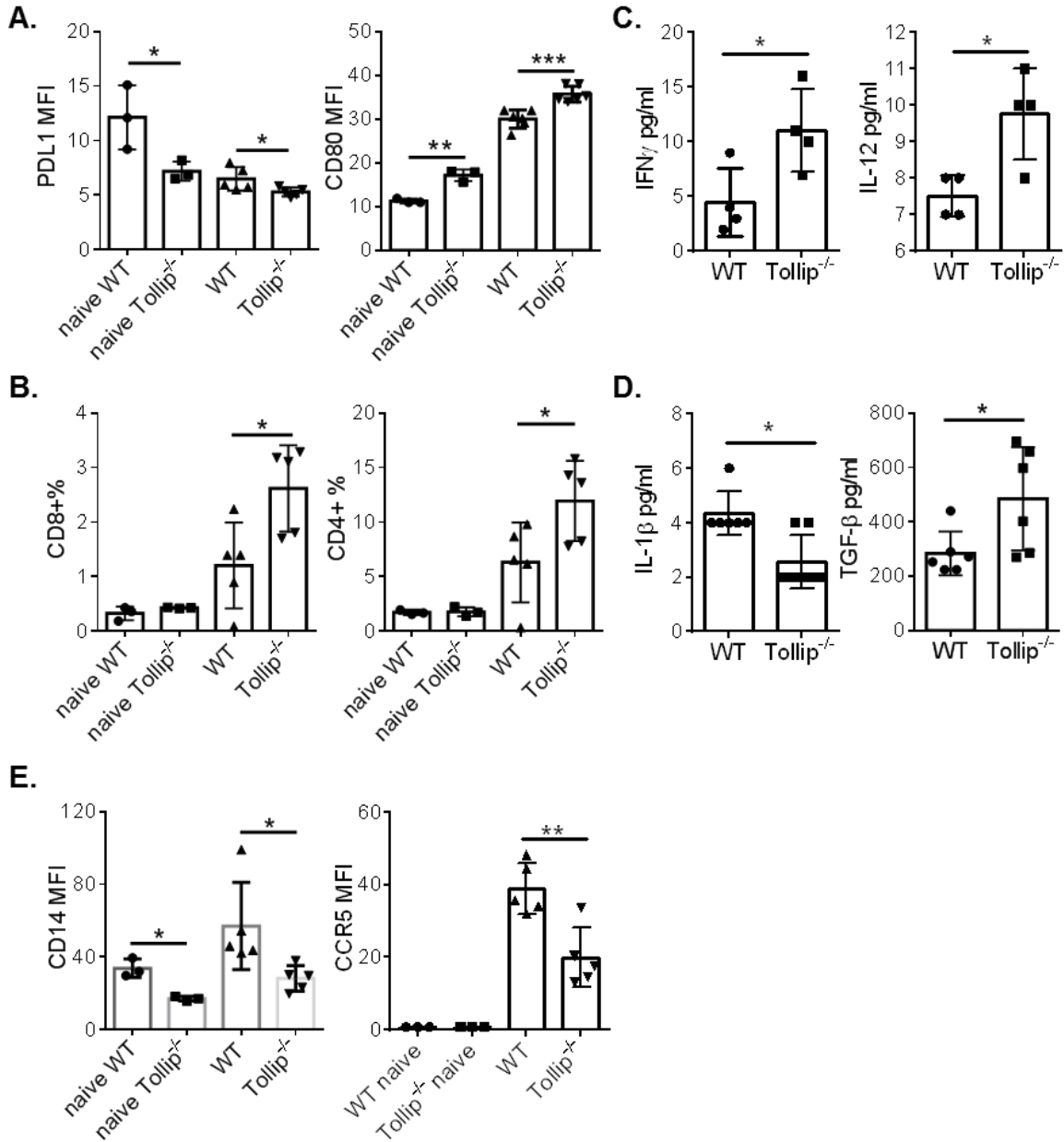


Fig. 2. Tollip deficiency enhanced anti-tumor innate immune checkpoints. (A) PD-L1 and CD80 expression on the neutrophils in the spleens from WT or Tollip^{-/-} mice with AOM-DSS treatment or naïve mice. (B) Percentages of CD4⁺ and CD8⁺ cells in the colon lamina propria from WT or Tollip^{-/-} mice with AOM-DSS treatment or naïve mice. (C) Cytokine profiles of colons collected from WT or Tollip^{-/-} mice treated with AOM-DSS. (D) Cytokine profiles of plasma collected from WT or Tollip^{-/-} mice treated with AOM-DSS. (E) CD14 and CCR5 expression on the surface of neutrophils in the blood. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

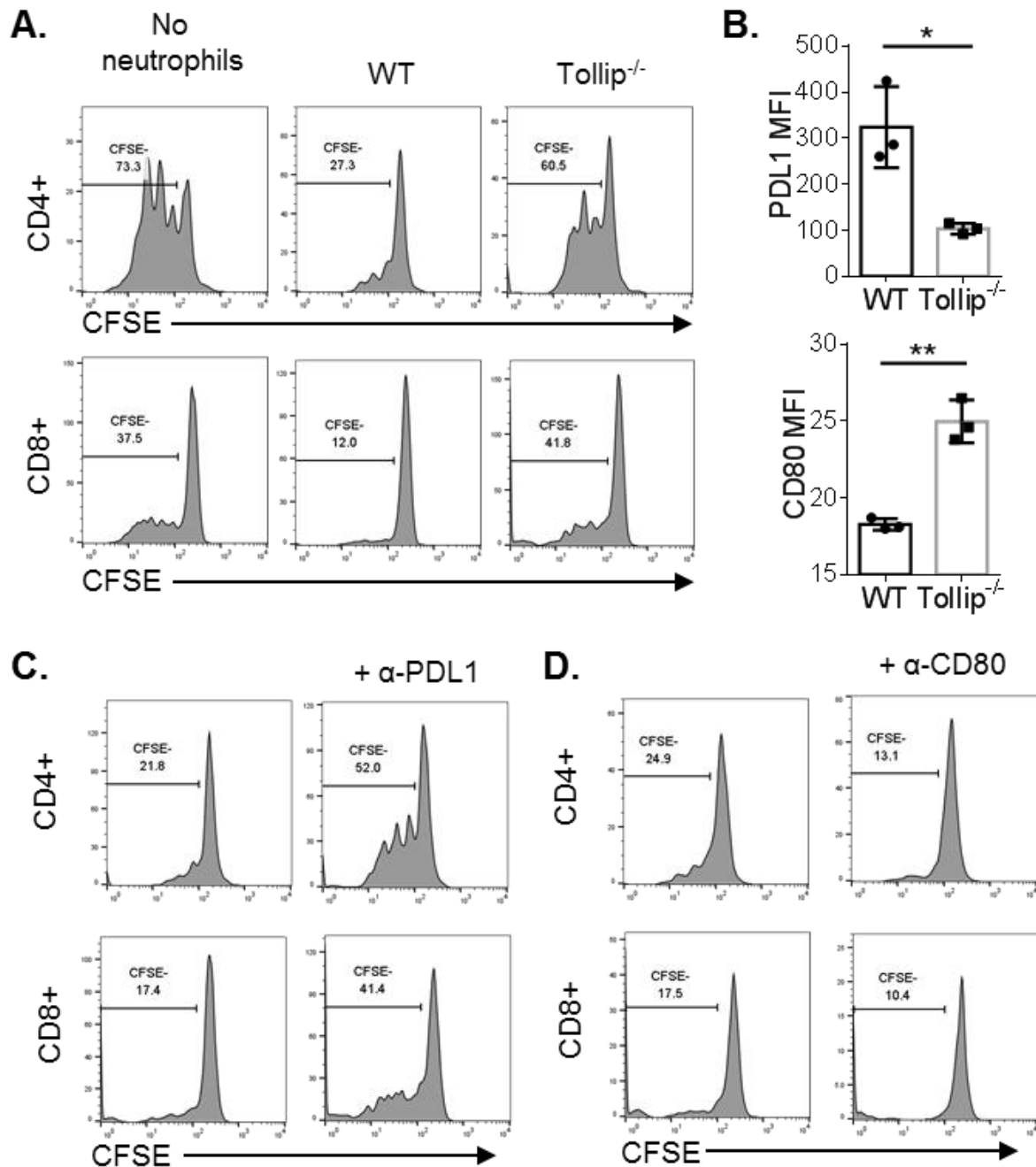


Fig. 3. Tollip deficiency released the neutrophil suppression on T cell proliferation via PD-L1/CD80. (A) CFSE-labeled splenocytes were cocultured with GM-CSF primed neutrophils in the anti-CD3 antibody coated plates for 72 hours. Representative results are shown. (B) PD-L1 and CD80 expression on GM-CSF primed neutrophils. * $p < 0.05$, ** $p < 0.01$. (C) In the presence of anti-PD-L1 antibody, CFSE-labeled splenocytes were cocultured with GM-CSF primed WT neutrophils in the anti-CD3 antibody coated plates for 72 hours. (D) In the presence of anti-CD80 antibody, CFSE-labeled splenocytes were cocultured with GM-CSF primed WT neutrophils in the anti-CD3 antibody coated plates for 72 hours.

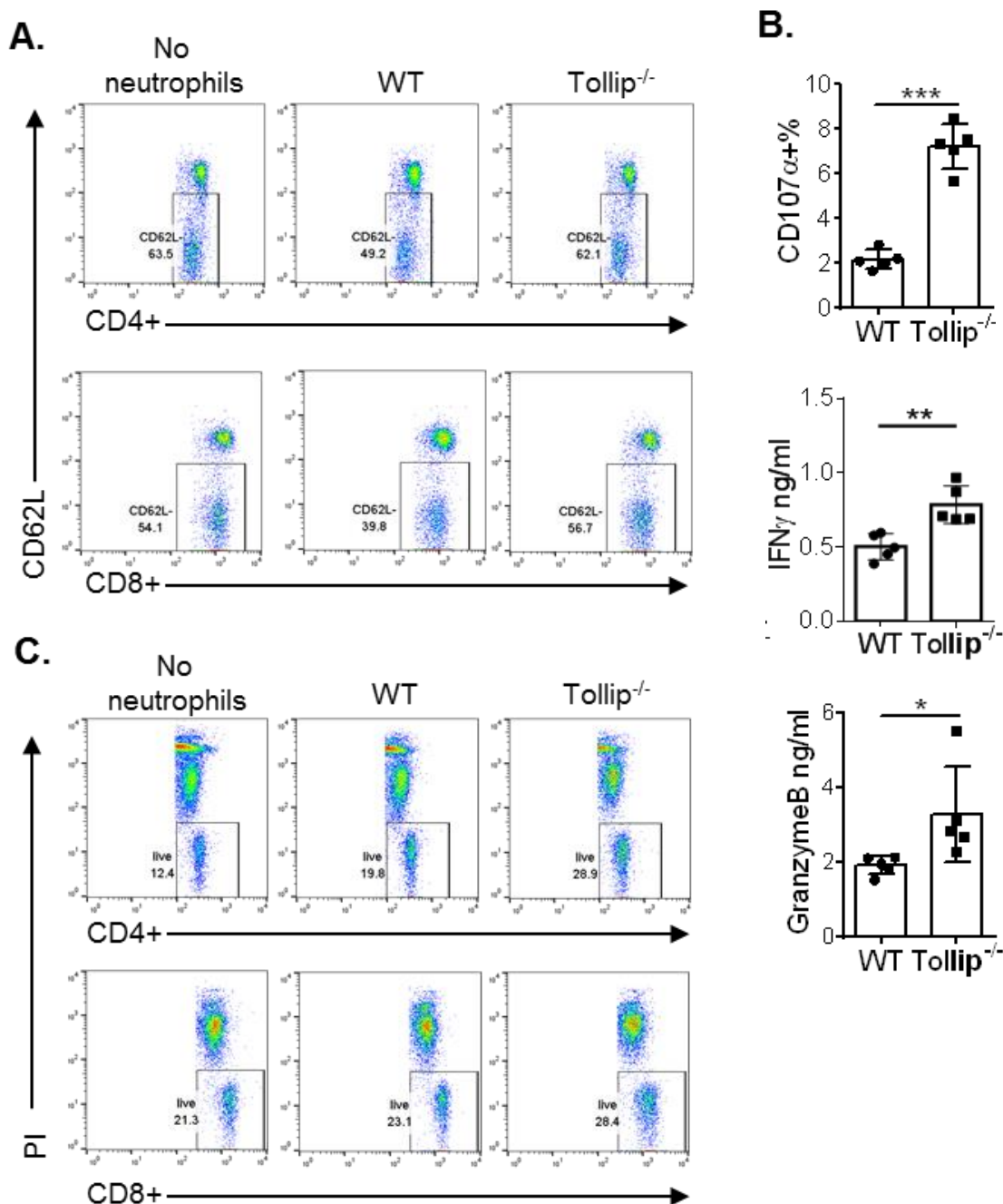


Fig. 4. Tollip^{-/-} neutrophils facilitated T cell activation and survival. (A) Splenocytes were cocultured with GM-CSF primed neutrophils (WT or Tollip^{-/-} in the anti-CD3 antibody coated plates for 24 hours, then CD62L levels on CD4+ or CD8+ T cells were measured by flow cytometry. Representative results are shown. (B) After co-culture, CD107a positive cells were analyzed in CD8+ cells and conditional medium was subjected to ELISA analysis. * p<0.05. (C) Splenocytes were cocultured with GM-CSF primed neutrophils (WT or Tollip^{-/-} for 72 hours, before cell viabilities were tested.

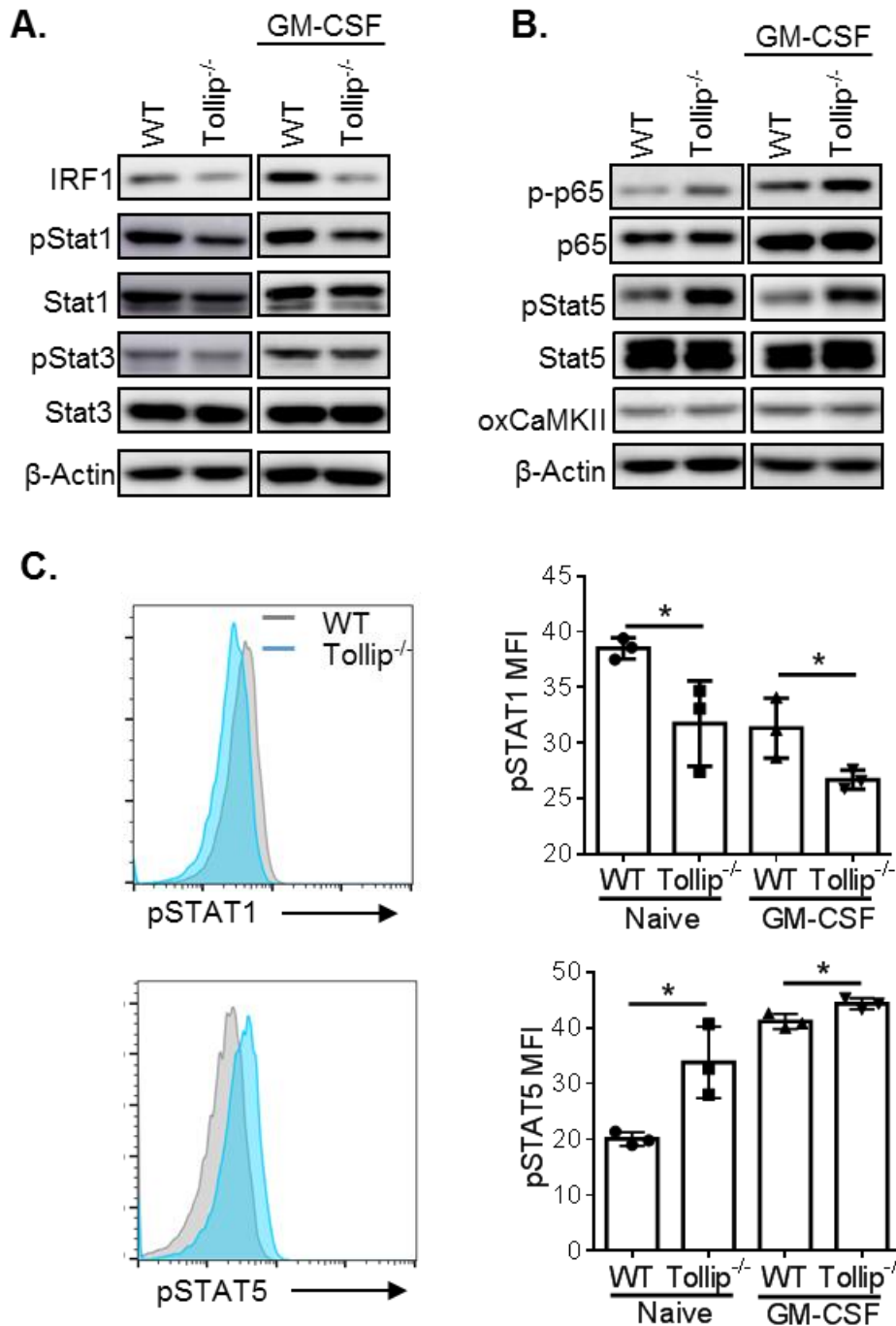


Fig. 5. Tollip deficiency released the neutrophil suppression on T cell proliferation via PD-L1/CD80 signaling pathway. (A) Immunoblotting analysis of STAT1, STAT3 and IRF1 in lysates from fresh bone marrow neutrophils or neutrophils primed with GM-CSF overnight. (B) Immunoblotting analysis of STAT5, p65, and CaMKII in lysates from fresh bone marrow neutrophils or neutrophils primed with GM-CSF overnight. (C) Flow cytometry analysis of phospho-proteins in fresh bone marrow neutrophils or neutrophils primed with GM-CSF overnight, pre-gated on Ly6G⁺ cells. * $p < 0.05$.

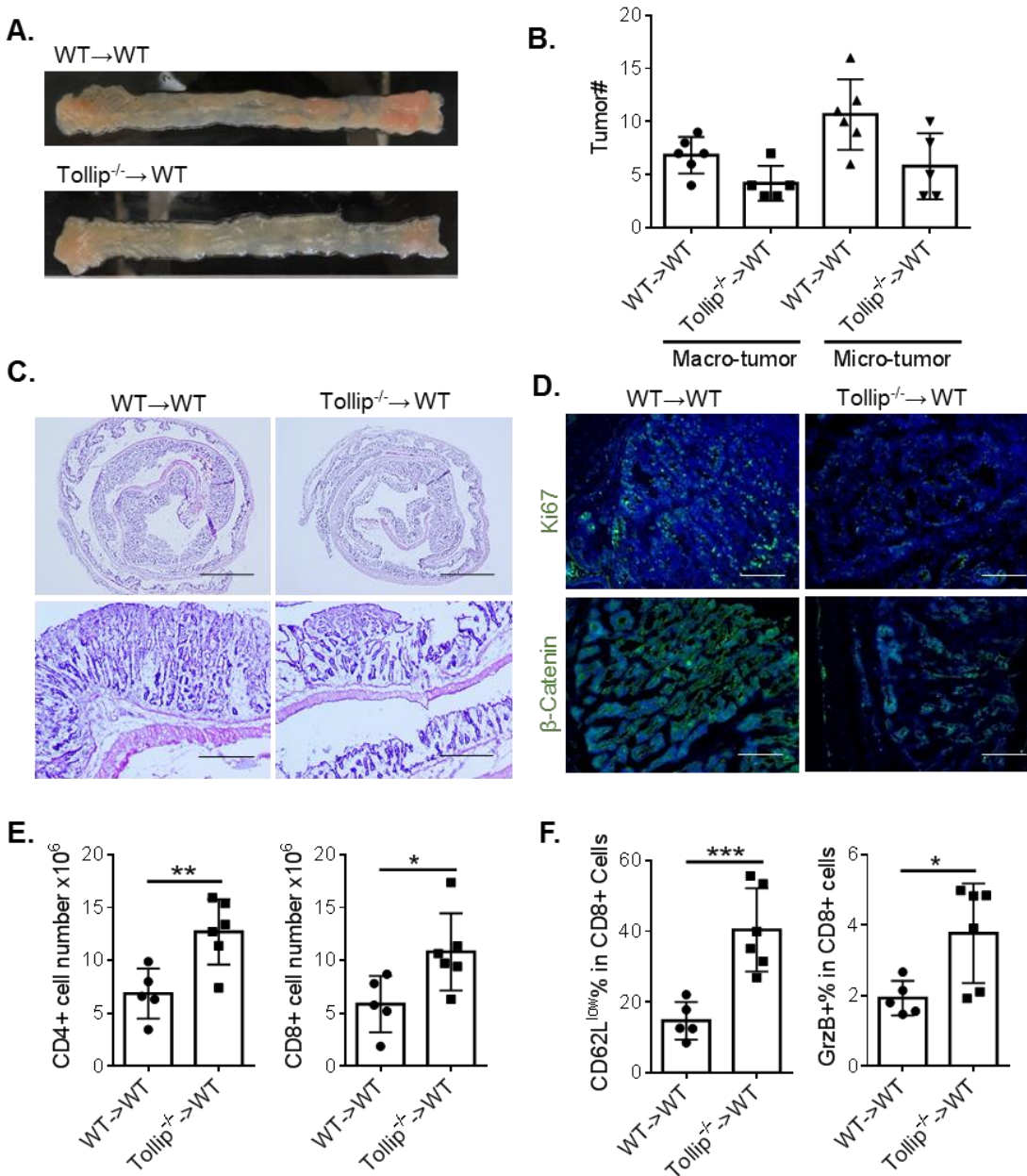


Fig. 6. Adoptive transfer of Tollip^{-/-} neutrophils to WT mice slows down colitis-associated cancer progression. (A) Representative images of colons from WT mice received WT or Tollip^{-/-} neutrophils on Day 64. (B) Graphical representation of tumor burden in WT mice received WT or Tollip^{-/-} neutrophils. N=6 each group. (C) H&E stained sections of colon from the mice received WT or Tollip^{-/-} neutrophils. Colons were collected in swiss rolls at the end of AOM-DSS regimen. Scale bar represent 2.5mm (top) and 0.5mm (bottom). (D) Immunofluorescent analysis of Ki67 and β-catenin. Blue color is DAPI staining. Scale bar represents 200μm. (E) CD4+ and CD8+ cell counts in the spleens from the mice received WT or Tollip^{-/-} neutrophils. (F) Percentages of CD62L^{low} in CD8+ T cells. Percentage of Granzyme B positive cells in CD8+ T cells. * p<0.05, ** p<0.01, *** p<0.001.

SUPPLEMENTARY MATERIALS

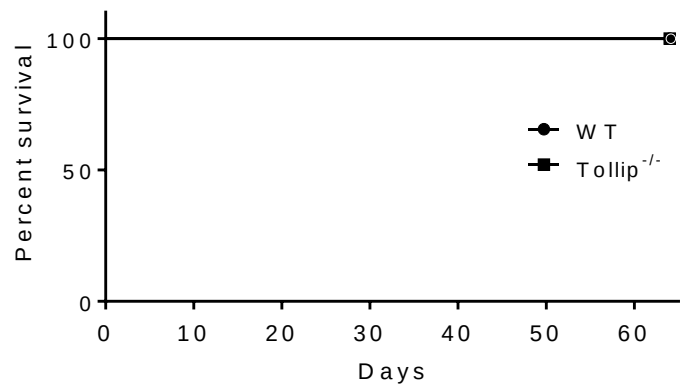


Fig. S1. Survival curve. Representative survival curve of WT and Tollip^{-/-} mice treated with AOM-DSS treatment. N=5 each group.

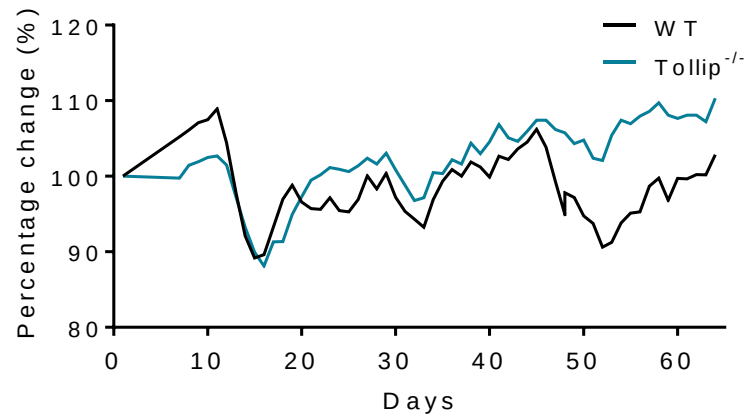


Fig. S2. Body weight change. Body weight change curves of WT and Tollip^{-/-} mice during AOM-DSS treatment. N=5 each group, and values were expressed as means.

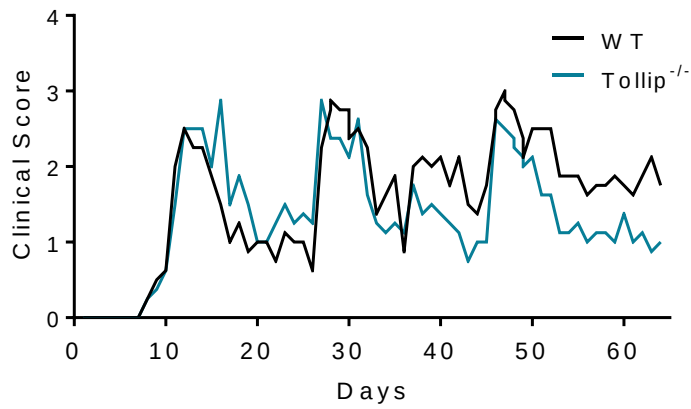


Fig. S3. Stool clinical score evaluations. Stool clinical scores including stool consistency and bleeding of WT and Tollip^{-/-} mice. N=8, values were expressed as means.

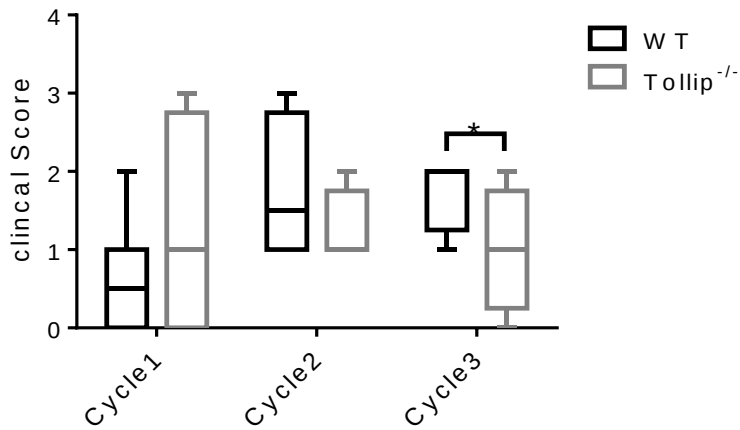


Fig. S4. Comparison of stool clinical scores. Stool clinical scores including stool consistency and bleeding of WT and Tollip^{-/-} mice were collected and compared at the end of each cycle (rest day 14). N=8, values were expressed as means± SD. * p<0.05.

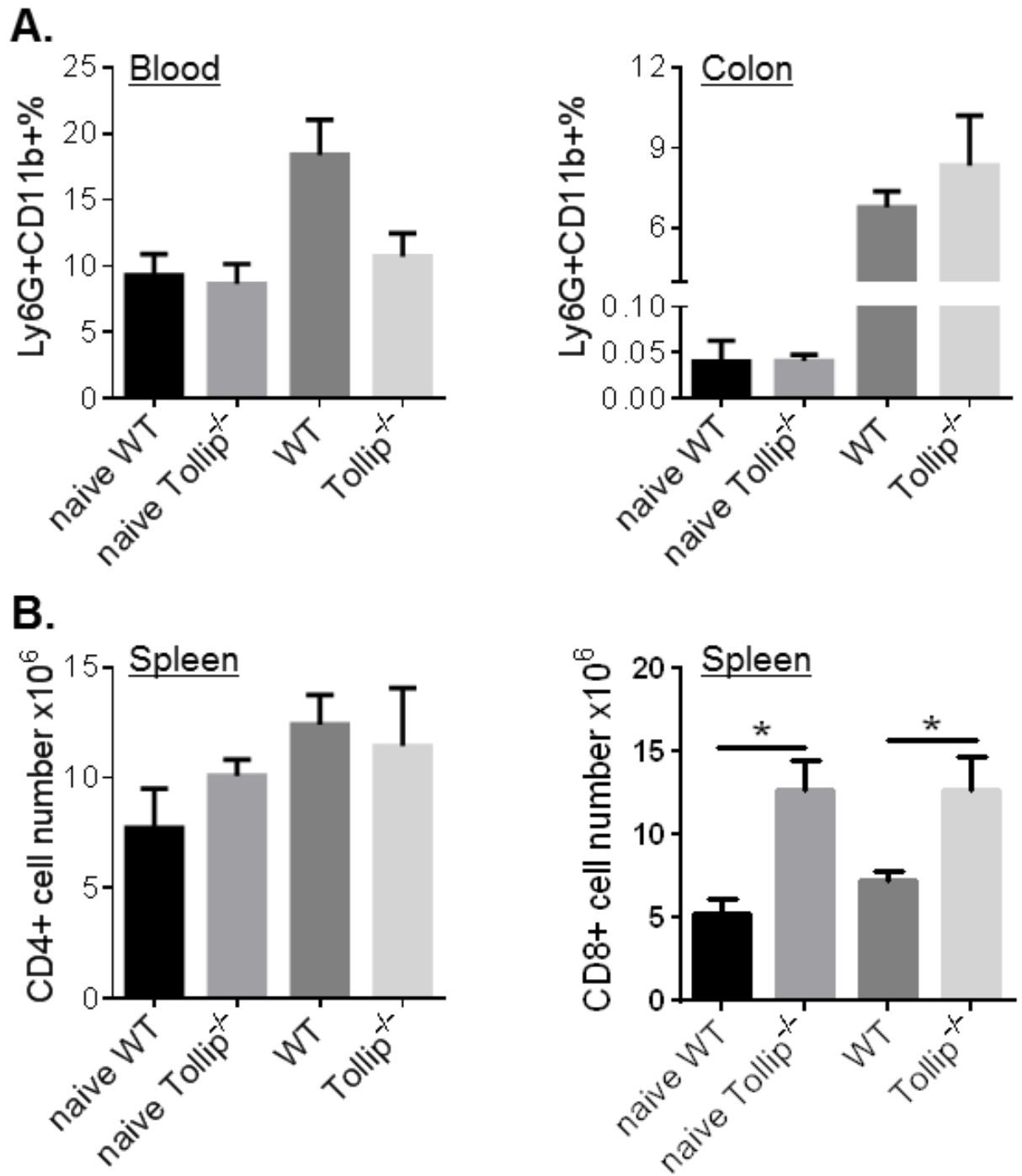


Fig. S5. Elevated T cell population was observed in Tollip deficiency mice. (A) Neutrophil (Ly6G+CD11b+) percentages in the blood and colon from naïve WT and Tollip^{-/-} mice, or AOM-DSS treated WT and Tollip^{-/-} mice. (B) CD4+ and CD8+ cell counts in the spleens from WT or Tollip^{-/-} mice with AOM-DSS treatment or naïve mice.

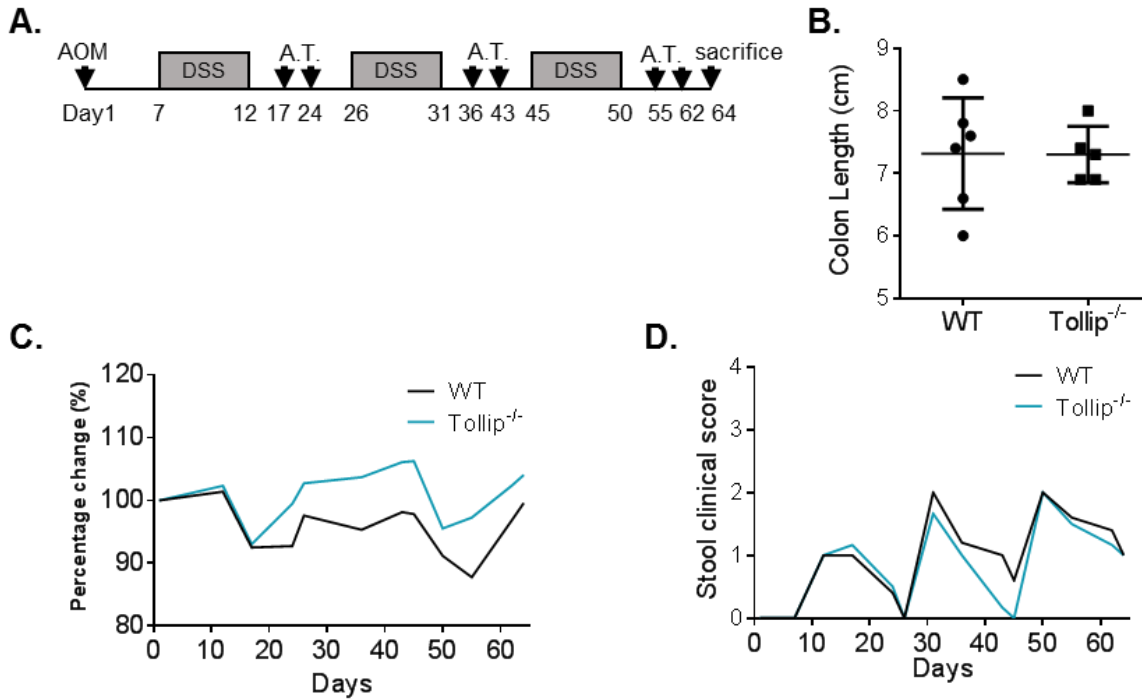


Fig. S6. (A) Schematic protocol of AOM-DSS treatment with adoptive transfer (A.T.) of WT or Tollip^{-/-} neutrophils to WT mice. **(B)** Colon length from mice received WT or Tollip^{-/-} neutrophils at the end of AOM-DSS regimen. **(C)** Body weight change curves of the mice received WT or Tollip^{-/-} neutrophils during AOM-DSS treatment. **(D)** Stool clinical scores including stool consistency and bleeding of the mice received WT or Tollip^{-/-} neutrophils.

CHAPTER 3

Genetically deficient neutrophils or pre-conditioned neutrophils are sufficient to transfer enhanced immune anti-tumor effects

Christina Lee, Liwu Li

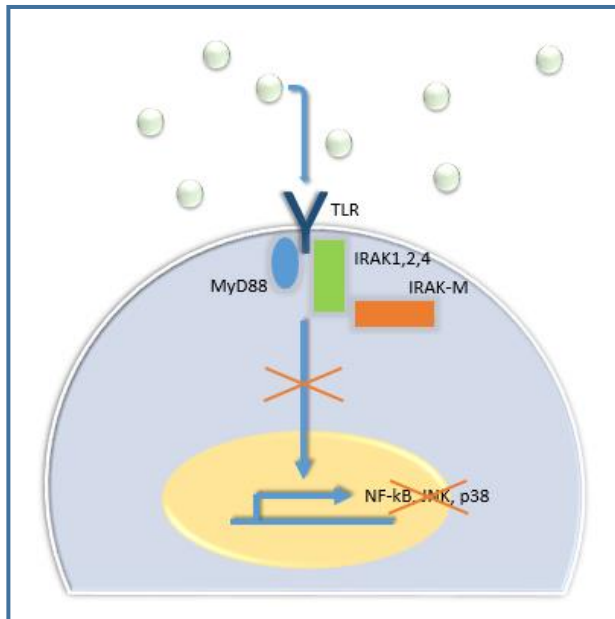
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ABSTRACT

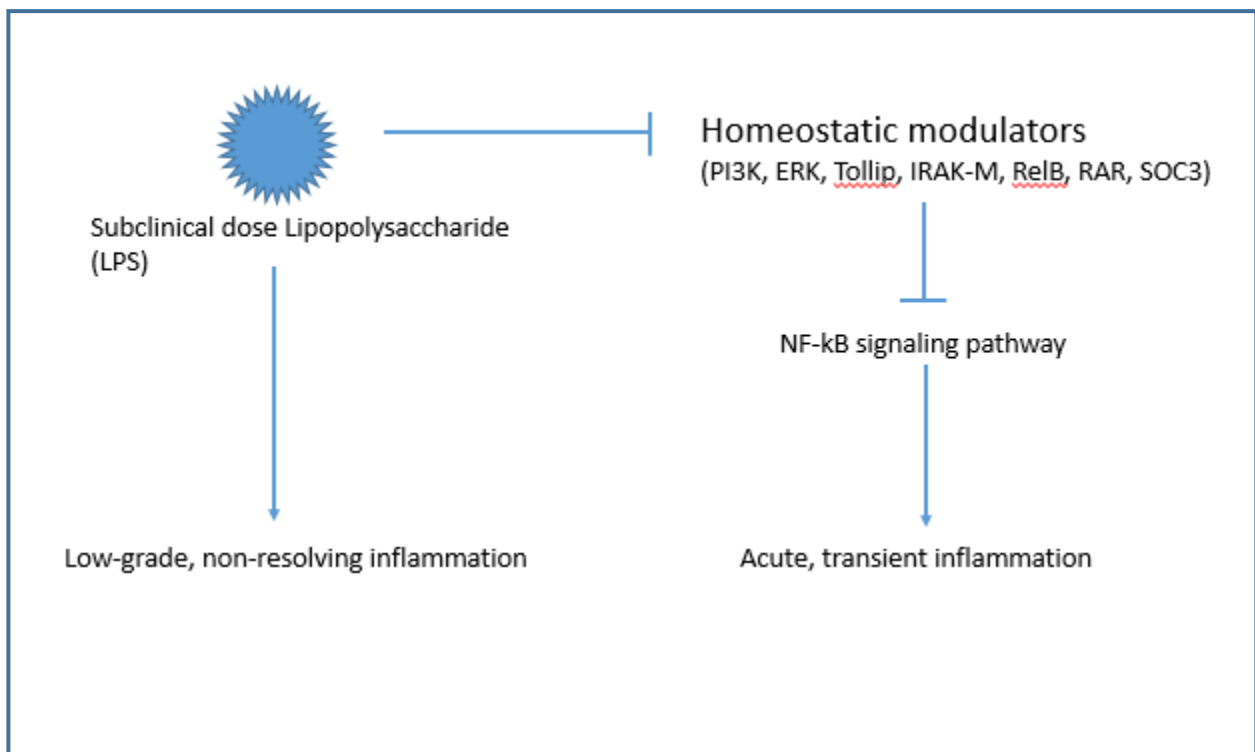
In previous chapter, we have shown Tollip knockout in mice provide protection against chronic DSS caused degradation and polyp formation in mice gut. In this chapter, we identify the precise role of neutrophil in the phenomena found in the previous chapter via adoptive transfer method. In addition to Azoxymethane (AOM, a carcinogen) and dextran sodium sulfate (DSS, an inflammatory reagent) challenge, which recapitulated colorectal cancer symptoms in mice, we injected wild type mice (WT) with WT neutrophils, gene knockout neutrophils, or monocytes to observe any differences that may arise from different immune cells introduced to mice under chronic AOM-DSS challenge. Improved clinical scores and lower polyp formation were observed in mice that received super low dose LPS stimulated neutrophils and IRAK-M^{-/-} neutrophils, while slightly worse colitis symptoms and polyp formation were observed in mice that received Tollip^{-/-} monocytes. The benefits from the adoptive transfer were not as dramatic as when the mice had the universal knockout of the genes. However, this provides a wider possibility in innate immune cell modulation via inhibition of suppressor genes for IBD and colorectal cancer therapy purpose.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Adoptive transfer with **Tollip^{-/-} monocytes** does not confer significant protection in colon against AOM-DSS challenge
- Adoptive transfer with **IRAK-M^{-/-} neutrophils** confers protective effect in colon against AOM-DSS challenge, similar to the adoptive transfer with **Tollip^{-/-} neutrophils**.
- Adoptive transfer with **super-low dose LPS stimulated neutrophils** confers significant protection in colon against AOM-DSS challenge.



INTRODUCTION

Cancer cells have shown to prematurely switch immune cells from pro-inflammatory to pro-resolving state in order to exploit immune cells to cater their unregulated growth and migration [1-6]. Neutrophils may not escape this phenomena. However, neutrophil modulation as seen in previous chapter with Tollip deficiency may allow us to polarize the cells into a phenotype that is more beneficial to the host under inflammatory stress. In continuation to our findings of possible cell polarization to host benefit against polyp development in chronic AOM-DSS challenged mice via adoptive transfer with Tollip deficiency neutrophils (Chapter 2), we further identified other possible modulated immune cells that may confer similar anti-tumor protection in mice gut challenged with chronic AOM-DSS stress. In this study, we observe the effect of super low dose stimulated LPS neutrophils, IRAK-M KO neutrophils, and Tollip KO monocytes in recipient WT mice under AOM-DSS.

Literature suggest super low dose LPS may prime the immune cells to confer exaggerated inflammatory phenotype. While lasting low grade inflammation in host is detrimental to host health and cause numerous chronic diseases, acute inflammation may enhance neutrophil function to better target and/or surveillance for dangerous cells like cancer cells. Not surprisingly, we found that super low dose LPS inhibit homeostatic modulators such as Tollip and IRAK-M (see graphical abstract). Based on this reasoning, adoptive transfer with IRAK-M^{-/-} and subclinical dose LPS stimulated neutrophils may confer silimiar protection as Tollip^{-/-} neutrophils in mice gut challenged with chronic AOM-DSS stress. We found super low dose LPS injection to mice under acute DSS challenge protected the host from DSS responsible gut degradation (further explored in Chapter 5). However, whether the effect arises specifically from neutrophil needs to be elucidated.

Universal IRAK-M KO mice also showed benefits to host under acute and chronic inflammation but it is unclear whether the effect arise from neutrophil (see Chapter 5).

In order to further confirm the protective effect found in the adoptive transfer study with Tollip deficient neutrophil (Chapter 2), we proceeded adoptive transfer with Tollip KO monocytes. Monocytes are one of the better characterized innate immune cells in current literature [7, 8]. However, their role under inflammatory state have been divisive. Adoptive transfer with polarized cells may prevent premature functional switch in host under AOM-DSS stress and may better arrest the polyp development in the host. We hypothesize neutrophil modulation may enhance host surveillance of abnormal cells and target cancer cells by being resistant to tumor microenvironment. Findings from this experiment will further confirm that neutrophils may be artificially modulated to a subset more beneficial to the host under inflammatory stress and may be used in IBD and colorectal cancer therapy in the future.

METHODS

Mice

Wild type (WT) C57BL/6, Tollip^{-/-}, and IRAK-M^{-/-} mice were bred and maintained in the animal facility at Virginia Tech in accordance to approved Animal Care and Use Committee protocol. All littermate mice were 10-11 weeks of age and 25–30 g weight when experiments were initiated.

Adoptive transfer of neutrophils

WT mice received a single intraperitoneal injection of azoxymethane (AOM, Sigma-Aldrich) at a dose of 10mg/kg body weight. A week after AOM injection, the mice were given three cycles of 2% dextran sulfate sodium salt (DSS, MP Biomedicals) for 5 days followed by 14 days of normal drinking water. Bone marrow neutrophils from donor mice (WT and IRAK-M^{-/-}) were purified (>90% confirmed by flow cytometry) using EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell) according to the manufacturer's instruction. Monocytes from Tollip^{-/-} mice were harvested from bone marrow and cultured 5 days with G-CSF before injection to mice. Recipient WT mice were transfused twice (post DSS day 5 and day 12) per DSS-resting cycle through intravenous injection with 2.4×10^6 WT neutrophils, overnight super low dose (100 pg/ml) LPS (Sigma) stimulated WT neutrophils, IRAK-M^{-/-} neutrophils, or Tollip^{-/-} monocytes suspended in 200 μ l sterile PBS. After the last water cycle mice were sacrificed and tissues were harvested for further analysis. Body weight, stool consistency, bleeding was measured as part of clinical score (score 0-4, with higher score corresponding to worse condition. See below for detail.). Polyp formation was labeled macro- and micro-polyp depending on the size equal to or greater than 2mm versus less than 2mm, respectively.

IBD Clinical Score Determination (0 Normal - 4 Poor condition)

Weight Grading <0-4>

- 0 No weight loss
- 1 Weight loss of 1—5%
- 2 Weight loss of 5.1—10%
- 3 Weight loss of 10.1—20%
- 4 Weight loss of more than 20%

Stool Grading <0-4>

- 0 Normal: dry and hard
- 1 Soft/Well formed: does not change round shape when pressed together
- 2 Soft/NOT well formed: round shape but mushy when pressed together
- 3 Pasty: came out long and gooey
- 4 Diarrhea: has no shape and does not come out clean (sticks to anus)

Bleeding Grading <0-4>

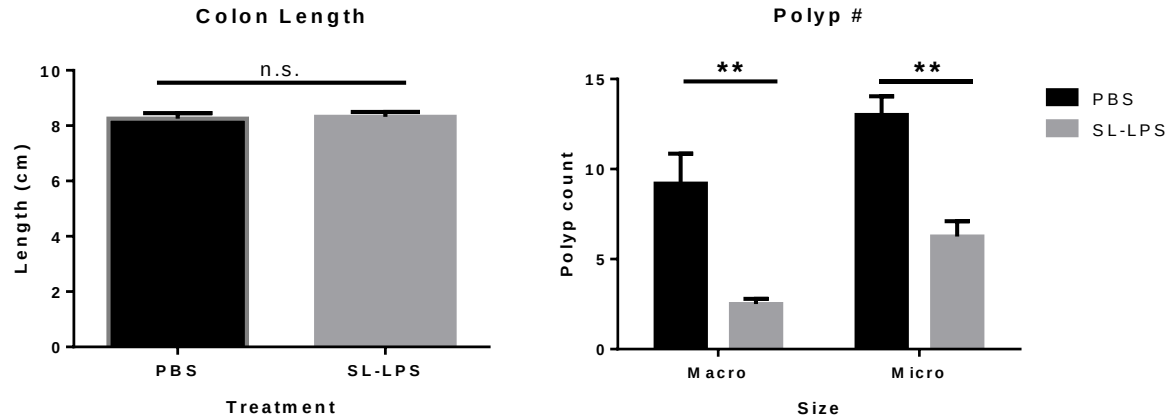
- 0 None
- 1 Occult bleeding: hint of blood
- 2 Visible bleeding: bright red (no question about the presence of blood)
- 3 Dark red: stool is soaked in blood
- 4 Gross bleeding: excess blood from anus

RESULTS

Super low dose LPS stimulated neutrophils confer protection in mice under AOM DSS challenge.

We found adoptive transfer with neutrophils over the course of AOM-DSS model significantly reduce polyp formation in mice that received Tollip deficient neutrophils but not in mice that received wild type neutrophils (Chapter 2). In order to confirm whether neutrophils that have chemically induced NF- κ B signaling can also confer anti-tumor effect, neutrophil stimulated with super low dose LPS overnight were injected to WT mice under AOM-DSS. Super low dose LPS stimulation has been shown to confer priming effect in immune cells by inhibiting homeostatic modulators. Since super low dose LPS also modify neutrophils via inhibition of homeostatic modulators, LPS stimulated neutrophils may present similar results to the adoptive transfer with Tollip KO neutrophil in mice under AOM-DSS. While colon lengths were comparable between the mice that received unstimulated neutrophils and LPS stimulated neutrophils, polyp formation was significantly lower in mice that received LPS stimulated neutrophils (Figure 1A). It is understandable that the injection with LPS stimulated neutrophils present more pronounced protection than the adoptive transfer with Tollip^{-/-} neutrophils since LPS stimulation may polarize neutrophils to a higher degree of pro-inflammatory phenotype by influencing signaling molecules higher up in the NF κ B signaling pathway. Lower polyp formation is supported by clinical scores as well (Figure 1B). Mice that received super low dose LPS stimulated neutrophils fared better than mice that received unstimulated neutrophils in overall weight change, stool consistency, and bleeding condition by the end of AOM-DSS cycle. This study further confirms the possibility of neutrophil modulation in cancer therapy.

A.



B.

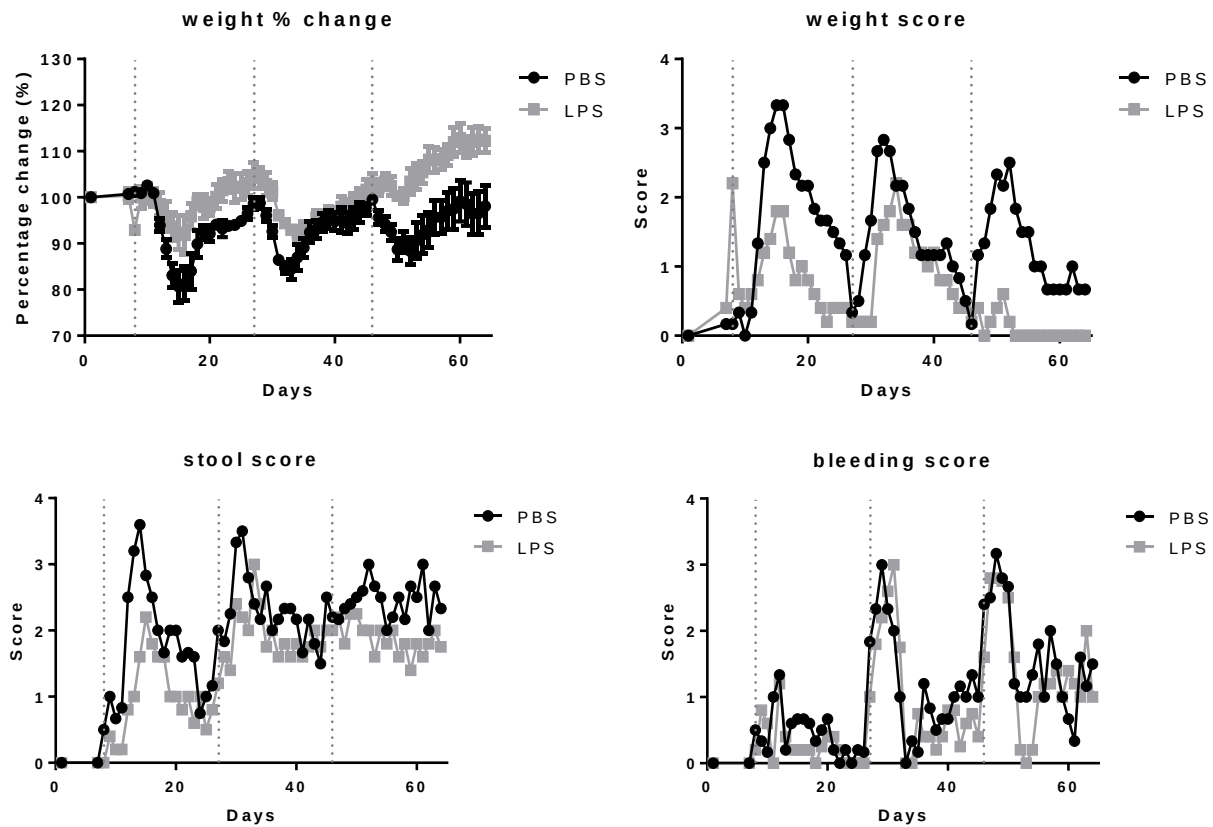
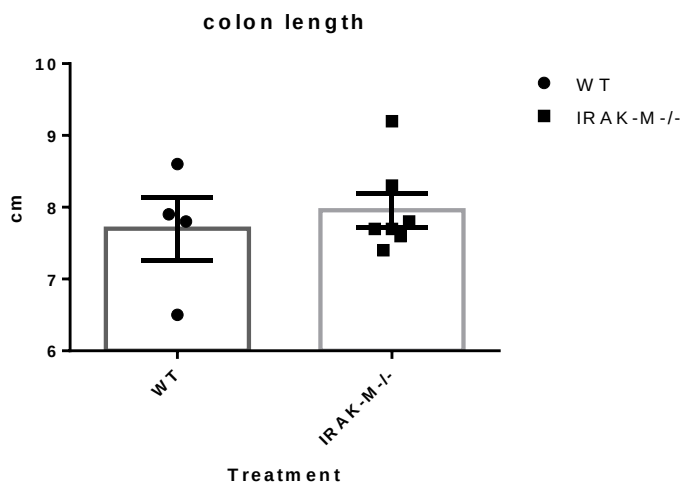


Figure 1: Tollip deficient neutrophils reduces chemically induced polyp formation in mice under AOM-DSS. A) Significantly higher number of polyp is found in the gut of WT mice that received unstimulated neutrophils compared to WT mice that received LPS stimulated neutrophils, albeit nonsignificant difference in colon length. Macropolyp, size ≥ 2 mm, micropolyp, size < 2 mm. (Male, 11 weeks old at the start of experiment; unstimulated, n=5; LPS stimulated, n=6). B) Weight change from day 0, stool consistency, and bleeding score recorded over 64 day time course.

IRAK-M KO neutrophils confer protection in mice gut under AOM DSS challenge.

IRAK-M is another negative regulator of NF κ B signaling pathway that has been found to exacerbate IBD symptoms in mice under acute and chronic DSS challenge (further explored in Chapter 4 and 5). It provides inhibition in MyD88-TIR signaling cascade that is similar to the Tollip-dependent regulation. The signaling molecule may influence the neutrophil polarization similar to Tollip (Chapter 2). In this study, we introduce IRAK-M KO neutrophils to recipient WT mice under AOM-DSS challenge to identify the role of IRAK-M in neutrophil in regards to tumorigenesis in mice gut. While the beneficial trend was present in mice that received the IRAK-M^{-/-} neutrophils, differences in colon length, polyp formation, and clinical score were not significant as seen in adoptive transfer with Tollip^{-/-} neutrophils or in universal IRAK-M KO mice (Figure 2) [9]. This may be explained by the fact that IRAK-M in other cells may compensate for the IRAK-M deficient neutrophils that are introduced to the host system. Eradication of all neutrophils in the host mice before the gene KO neutrophil injection may not be a plausible option since a complete neutrophil depletion may be fatal and/or cause other complications in mice.



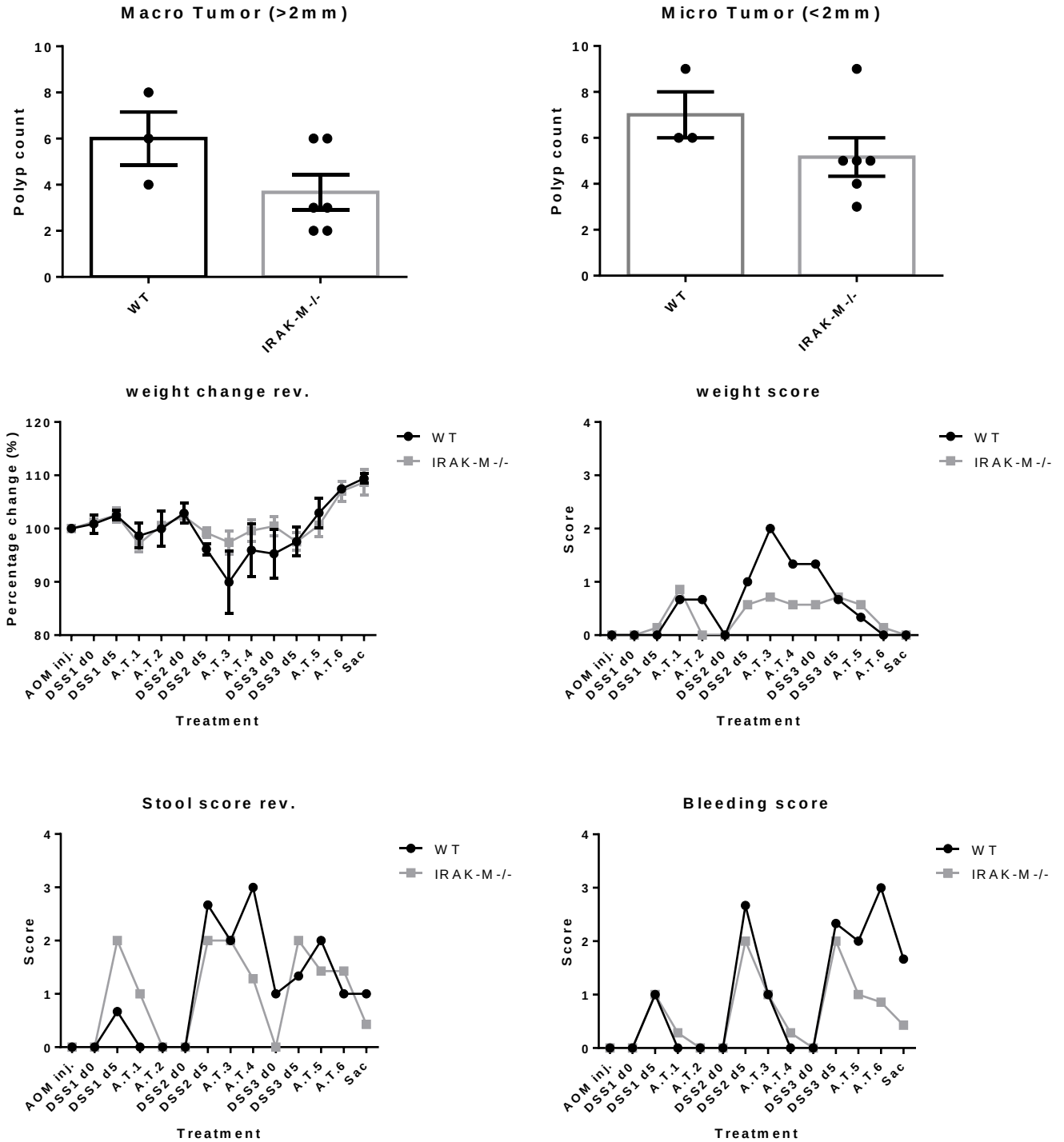
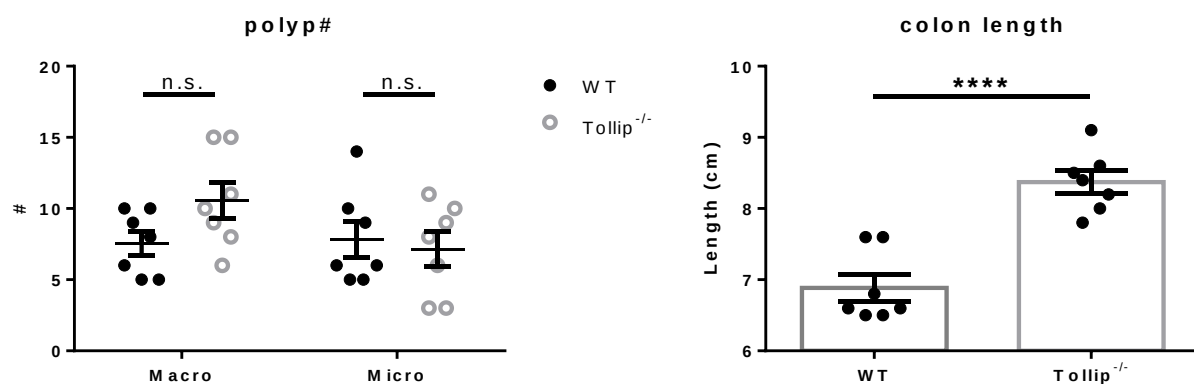


Figure 2. Adoptive Transfer with IRAK-M^{-/-} neutrophil provides protection against AOM-DSS. Colon length and polyp formation at the end of day 64. Weight change from day 0, stool consistency, and bleeding score recorded over 64 day time course (Male; 10 weeks old at the start of experiment; WT, n=4; IRAK-M, n=7).

Tollip KO monocytes confer no protection in mice gut under AOM DSS challenge.

In order to isolate the benefits of Tollip KO polarization to neutrophils, we proceeded to introduce monocytes with Tollip deficiency to recipient WT mice under AOM-DSS. Monocytes are better characterized innate leukocytes and a number of polarized phenotypes that arise from different stimulations are better defined in literature. However, Tollip deficient-dependent polarizations in monocyte are less understood, especially in context of tumorigenesis in gut. Surprisingly, there were no benefits observed in the WT mice that received Tollip^{-/-} monocytes (Figure 3). A number of polyp formations observed suggests Tollip^{-/-} in monocytes may be harmful to the host under inflammatory stress. WT mice that received Tollip^{-/-} monocytes fared slightly worse in all categories of clinical scores albeit having significantly longer colon lengths compared to WT mice that received WT neutrophils. The findings highlight the importance of neutrophils in tumorigenesis suppression and the possibility of neutrophil modulation in future IBD and colorectal cancer therapy.



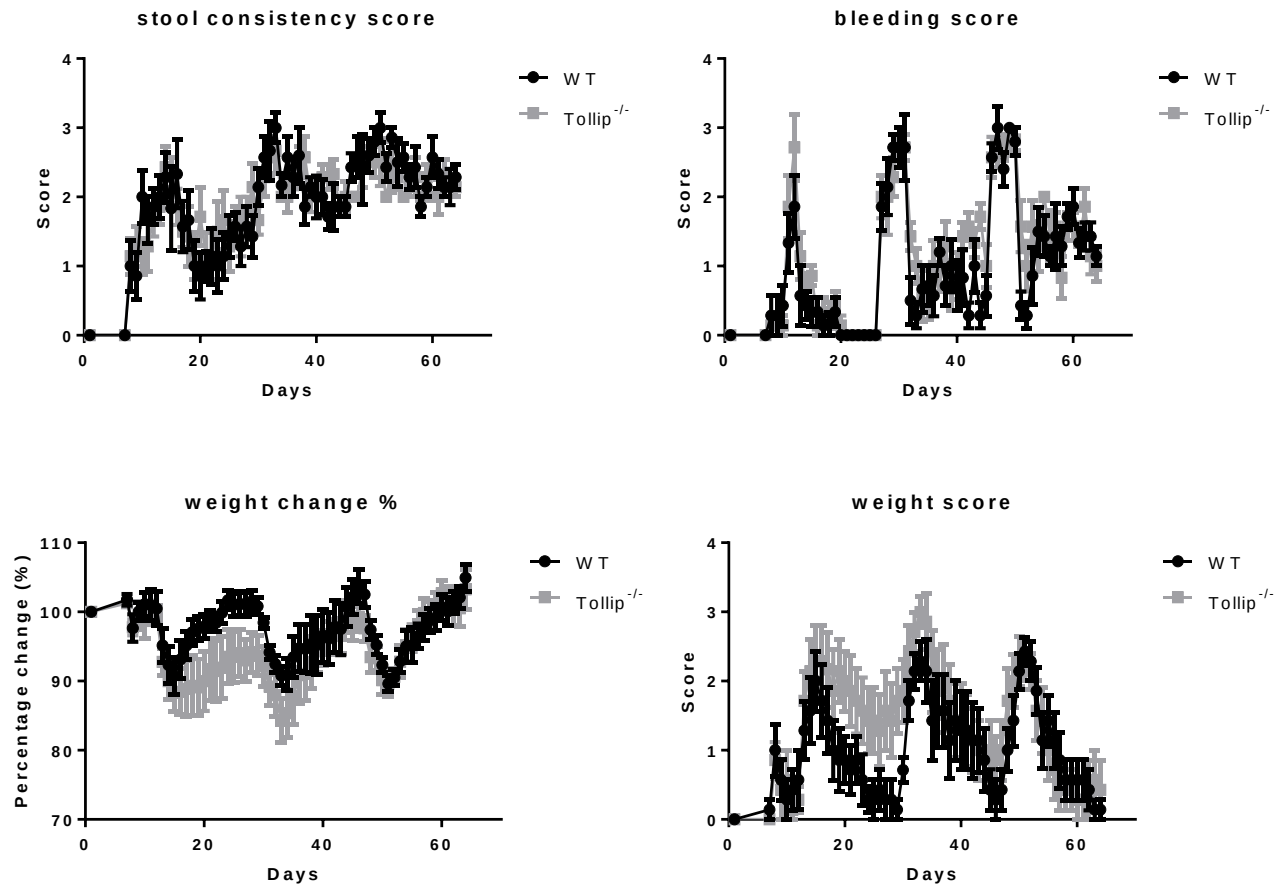


Figure 3. *Adoptive Transfer with Tollip^{-/-} monocytes provides no protection to mice under AOM-DSS. Colon length and polyp formation at the end of day 64. Weight change from day 0, stool consistency, and bleeding score recorded over 64 day time course (Male; 10 weeks old at the start of experiment; mice that received WT monocytes, n=8; mice that received Tollip^{-/-} monocytes, n=8).*

CONCLUSION

Neutrophils make up more than half of leukocytes in a healthy individual and are one of the first responders to invading pathogens. Due to their low mitochondria and DNA concentration, it was originally thought that neutrophils have transient and limited functions exclusively allocated to antibacterial activities. However, current research find that neutrophils may comprise more dynamic function than previously known. Apart from phagocytosis, neutrophils have been reported to clear or hold microorganisms via degranulation, oxidative burst, NET formation, as well as recruit and activate other immune cells and initiate autophagy that signal M1—M2 switch, conferring both pro- and anti-inflammation in an intricate balance. Similar to monocyte/macrophages, neutrophils may exhibit N1 and N2 phenotypes (and possibly more) when encountered with different stimuli in their microenvironment [10].

Higher neutrophil count has been noted in cancer patients, albeit contradicting outcomes, which further reinforce the possibility of different neutrophil subsets conferring different functions in host [11-13]. Despite recurring evidence pointing to neutrophils' susceptibility to polarization based on microenvironment signaling, surprisingly little is known about the cellular and molecular mechanism behind the cell polarization.

Modulation of neutrophils via the inhibition of suppressors in NF- κ B signaling pathway may allow the cell to polarize to a pro-inflammatory state that is resistant to further modification under inflammatory stress in host. Findings in this study also suggest there may be more than one way to manipulate neutrophils to the host benefit. Super low dose LPS stimulation of neutrophils have shown to prime the cells to better suppress tumorigenesis. IRAK-M^{-/-} neutrophils also have shown potential to protect the host under chronic AOM-DSS stress. On the other hand, monocytes, which

have been the focus of many innate immune cell studies may not provide as much benefit to host under chronic inflammation-related diseases. It is necessary for this study to be further optimized (i.e., injection timing and concentration of neutrophils in adoptive transfer) to confer the maximum protection to the host and translated to human study. Neutrophil manipulation in the host may prove to be a great therapeutic tool against chronic colitis and colorectal cancer.

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CHAPTER 4

Tollip deficient neutrophils reduce cancer cell growth through secreted soluble mediators

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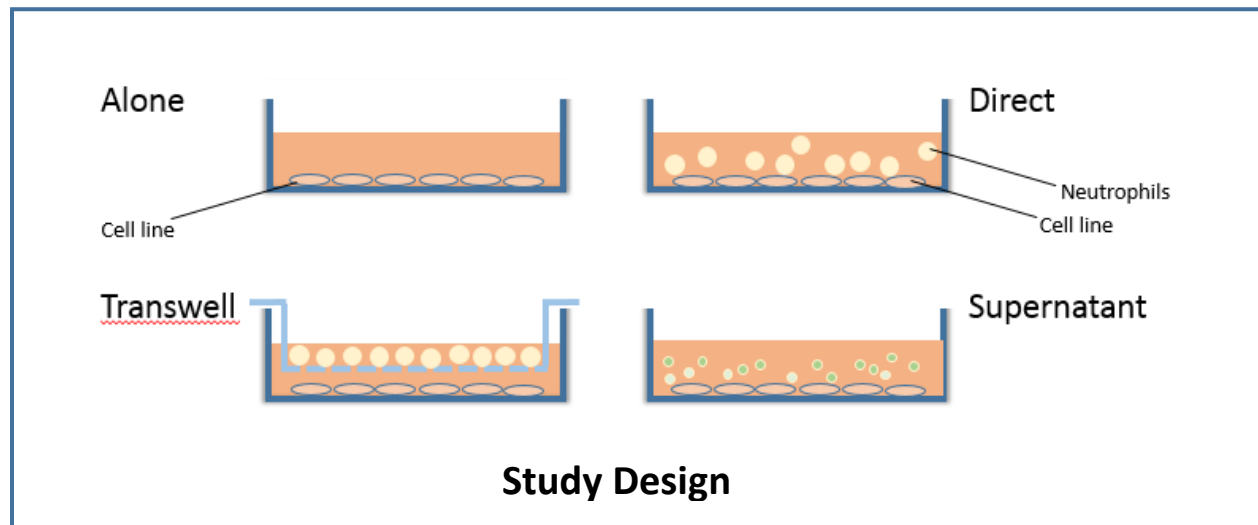
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ABSTRACT

In addition to their interaction with T cells to modulate colon tumorigenesis (see Chapter 2), neutrophils may also have a direct effect on tumor cell growth. However, such effect and underlying mechanisms are poorly studied nor understood. In this study, we tested whether LPS-conditioned neutrophils and Tollip deficient neutrophils may also exhibit enhanced anti-tumor effect *in vitro* in the absence of other immune modulatory cells such as T cells. We observed that tumor cell lines grow faster in the presence of the wild type neutrophils but less in the presence of Tollip deficient neutrophils and LPS-conditioned neutrophils. Mechanistically, we observed that Tollip deficient neutrophils and LPS-conditioned neutrophils attenuated tumor cell growth through inducing cell death as measured by Annexin V staining. Using *in vitro* assay with cancer cells and intact neutrophils or secreted media by cultured neutrophils, we observed that secretant from neutrophil is sufficient to account for the anti-tumor growth effect. We further observed that Tollip deficient neutrophils express higher levels of miR-34, which is linked with anti-tumor effect. Together, our study provides a novel concept of direct anti-tumor effect of neutrophils through potentially secreting tumor-modulating miRNAs.

GRAPHICAL ABSTRACT



INTRODUCTION

Previously, neutrophils were known to confer simple and transient defense for the host. However, current literature supports the concept of neutrophils with more complex functions. They can not only secrete cytokines and chemokines to recruit other immune cells and express surface receptors to communicate and modulate environment, but also miRNAs that can potentially modulate neighboring cell growth. By doing so, neutrophils may change the phenotype of neighboring cells to match the needs of the host. There may be a mutual adaptation between neutrophils and cancer cells that has not been discovered.

In this chapter, we focus on *in vitro* assays to test the hypothesis that neutrophil deficient in Tollip may modulate tumor cell growth *in vitro* through such mechanism. We test whether neutrophils can directly suppress cancer cells, either via direct contact or through secreted mediators. MiR-34a is one of the possible mediators that may be involved in the communication between neutrophils and cancer cells. It is well characterized miRNA that has been shown to be downregulated in several different tumor types[1-3]. It is a direct transcriptional target of the onco-suppressor gene p53, which is one of the most commonly mutated genes in variety of cancer cells. Genetic deletion of Tollip in neutrophils may be able to compensate downregulation of the miRNA bestowed by the cancer cells. Based on our previous finding that low dose LPS can inhibit the function of Tollip, we also tested the efficacy of LPS-conditioned neutrophils on tumor cell growth *in vitro*. The findings may provide better understanding of the nature behind the cancer-host immune interaction as well as possible therapeutic measures that can be targeted in the future.

METHODS

Mice

Wild type (WT) C57BL/6, Tollip^{-/-}, IRAK-M^{-/-} mice were bred and maintained in the animal facility at Virginia Tech in accordance to approved Animal Care and Use Committee protocol. Mice were age (7-16 weeks of age at the time of use) and gender matched in each experiment.

Neutrophil Isolation

Bone marrow neutrophils were isolated and purified using (1) 3 layer (82%, 65%, 55%) percoll (Sigma Aldrich) and NaCl (Sigma Aldrich) gradient; (2) 1 layer (65%) percoll NaCl gradient; (3) EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell). Purity was checked using FACSCanto II (BD Biosciences). Cells were labeled with Ly6G and CD11b (BioLegend) for neutrophils, and purity ranged from mid 80% - low 90%. Stained cells were analyzed with a FACS plots shown were analyzed with FlowJo (Ashland, OR).

Co-culture Assay

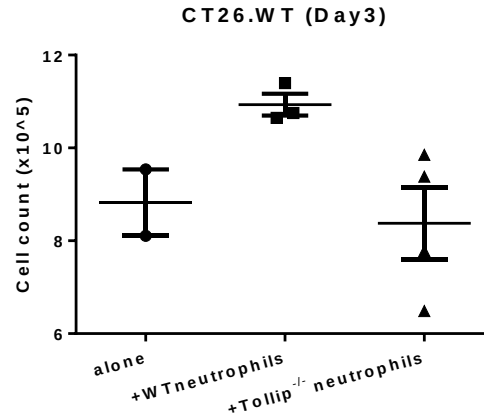
CT26.WT (murine colon carcinoma), CMT93 (murine rectum polyploidy carcinoma), and HCT116 (human colon carcinoma) were purchased from ATCC. MCF10A (human breast epithelial) were given to us by Dr. Eva Schmelz at Virginia Tech. Cell lines were cultured alone or with primary murine neutrophils at 1:5 and 1:10 ratio. Neutrophils were replenished every day, every other day, every three days, or once at the beginning of the experiment based on the length of experiment. Neutrophils were co-cultured with cell lines as fresh +/- super low dose LPS (100pg/ml) or 1ng/ml GM-CSF, or after 24 hour stimulation. Neutrophils were co-cultured with cell lines directly, on transwell (0.4um), or indirectly via supernatant. Supernatant from neutrophils were collected after 30 minute or 24 hour stimulation with neutrophils.

RESULTS

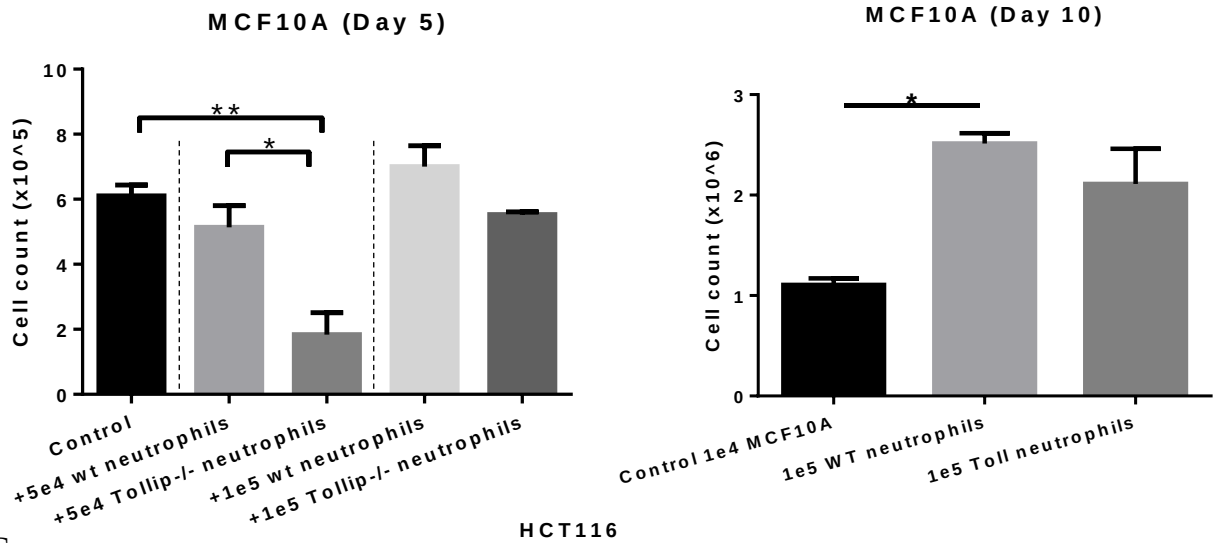
Tollip^{-/-} neutrophils suppress cell line proliferation in vitro.

In order to confirm the communication between cancer cell and neutrophil, I carried out co-culture assays. Starting with mouse colon carcinoma, CT26.WT, I found that WT neutrophils from mouse bone marrow assist in cancer cell growth (Figure 1A). Interestingly, when Tollip^{-/-} neutrophils are added to cancer cell line, the rate of cell growth decreased or remained the same as when the cancer cell line was grown without neutrophils. Previous literature varied greatly in target-to-effector ratio. However, I found that a minimum of 1:5 cancer cell-to-neutrophil ratio was necessary for this effect. However, the greater the target to effector ratio, the better the inhibitory effect was observed. Once the trend has been confirmed in multiple trials, I moved to human cell lines, MCF10A (human breast cell line) and HCT116 (human colorectal carcinoma), to confirm that the cancer cell-neutrophil communication is universal in mouse and human. Again, the cancer cell numbers were *significantly* increased when the cells were co-cultured with the WT neutrophils but not with the Tollip KO neutrophils (Figure 1B,C; Supplementary Figure 1). The trend persisted even at day 10. Neutrophils from both gender mice were tested with similar results (Supplementary Figure 2). In order to make sure live neutrophils are present in co-culture, neutrophils from bone marrow was replaced every other day or every three days in five day culture and ten day culture, respectively. For the following experiments, we focused on MCF10A cells since HCT116 grew at much faster rate than other cell lines that we use. It is important to note that, albeit non-significant, the cell lines proliferated at a greater rate when the cells were co-cultured with neutrophils than when the cells were cultured alone in extended time period.

A.



B.



C.

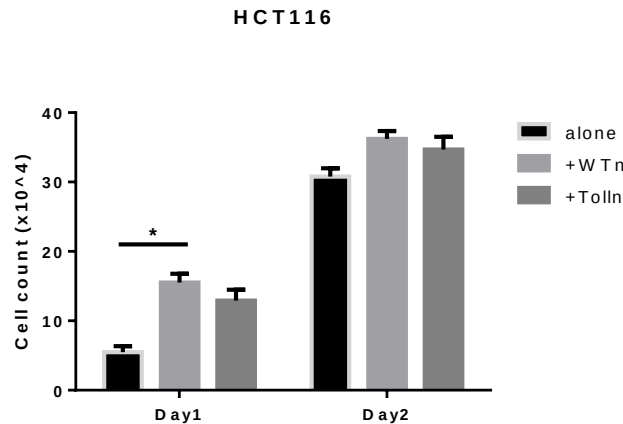


Figure 1. Minimum 1:5 target to effector ratio is necessary for cell line growth suppression in *Tollip*^{-/-} neutrophil. (A) CT26.WT—neutrophil co-culture, 1:5 target-to-effector ratio. (Male; 13 weeks old; WT, n=3; *Tollip*^{-/-}, n=4) (B) MCF10A—neutrophil co-culture, 1:5 and 1:10 target-to-effector ratio. MCF10A cell number at day 5 and day10 (Female; 9 weeks old; WT, n=3; *Tollip*^{-/-}, n=3). (C) HCT116—neutrophil co-culture, 1:5 target-to-effector ratio (Female; 16wks old; WT, n=3; *Tollip*^{-/-}, n=3)

Neutrophil stimulated with GM-CSF confer greater suppression on cell line growth.

In order to examine whether polarized neutrophils assist cell lines in their growth in the same manner as previously seen, artificially polarized neutrophils are introduced to co-culture. GM-CSF, a possible pro-inflammatory stimulant for neutrophils, was added to the co-culture at the time of the neutrophil addition. CT26.WT cells (murine colon carcinoma) cultured with 30 minute GM-CSF stimulated WT neutrophils showed slight inhibition in cell proliferation. However, the inhibition is more pronounced with the unstimulated Tollip KO neutrophil co-culture (Figure 2A). We proceeded the experiment with over-night GM-CSF stimulated neutrophils to make certain that neutrophils are well polarized prior to coming in contact with the cancer cells (Figure 2B). We found greater suppression in cell growth inhibition, especially in the cell line and Tollip^{-/-} neutrophil co-culture. The same experiment was carried out with different cell lines, CMT-93 (murine polyploid rectum carcinoma) and MCF10A, to see whether this is a universal phenomenon. Both mouse cell line and human cell line show the similar trend when they are co-cultured with over-night GM-CSF stimulated neutrophils (Figure 2C,D). Again, overnight GM-CSF stimulated Tollip^{-/-} neutrophils exhibit the strongest inhibition in cell line growth.

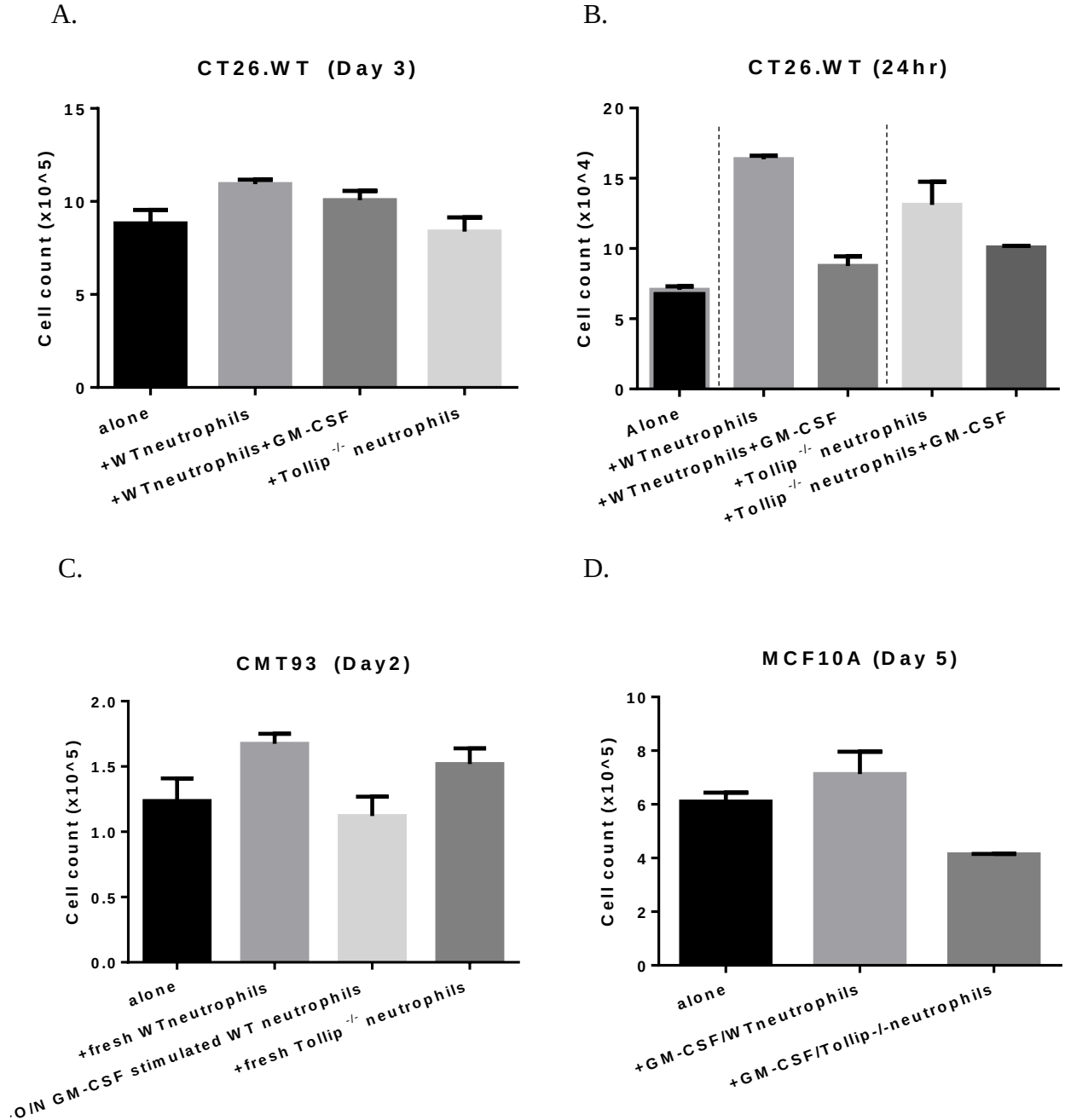


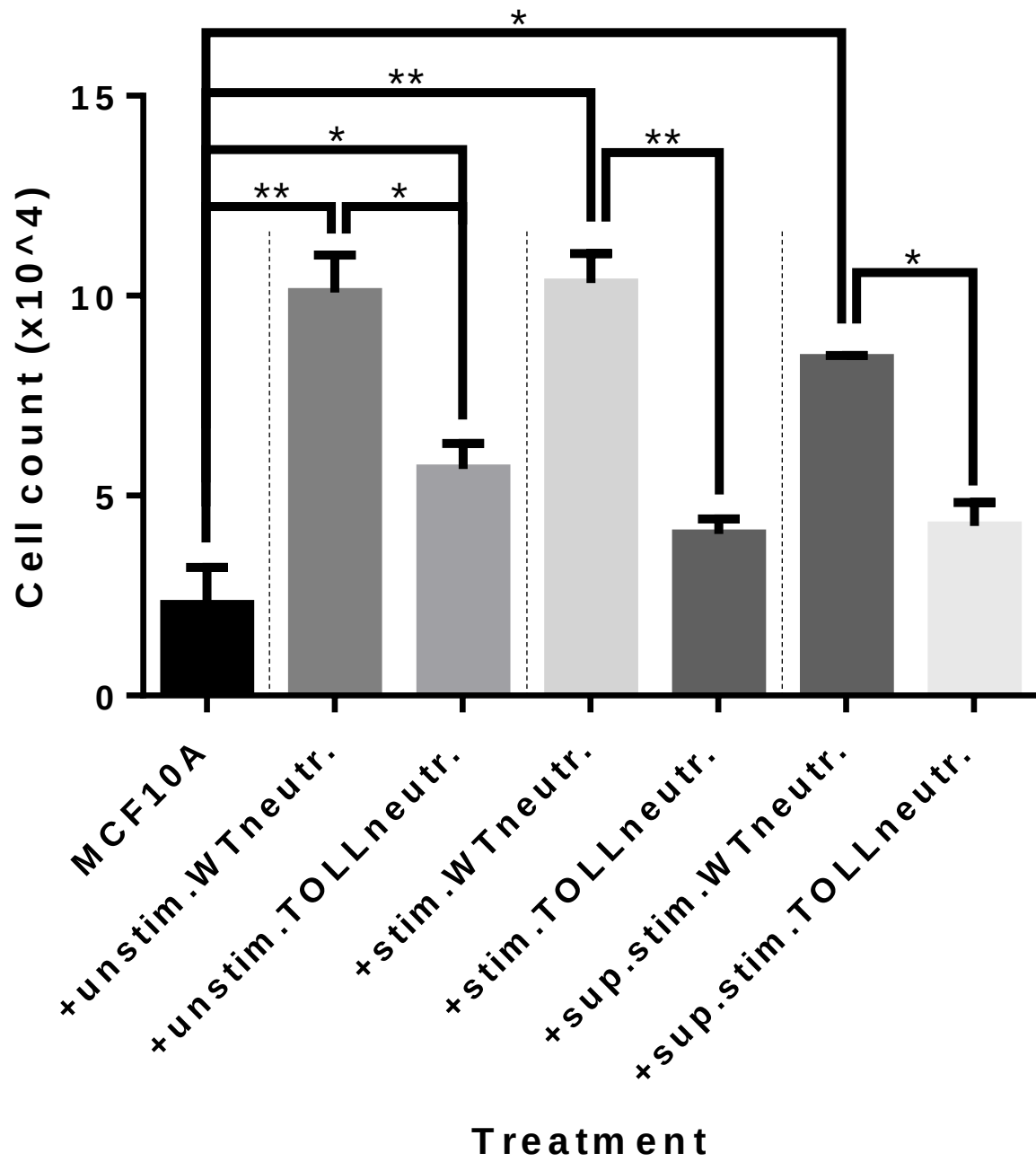
Figure 2. GM-CSF stimulation enhance neutrophil suppressive effect on cell line growth. (A) CT26.WT—neutrophil co-culture (Male; 13 weeks old; WT, n=3; Tollip^{-/-}, n=4) (B) CT26.WT—O/N GM-CSF stimulated neutrophils (Female; 12 weeks old; WT, n=4; Tollip^{-/-}, n=4). (C) CMT93—O/N GM-CSF stimulated neutrophils (Male; 11 weeks old; WT, n=4; Tollip^{-/-}, n=4) (D) MCF10A—O/N GM-CSF stimulated neutrophils. 1:10=T:E. Fresh neutrophils every 3days. (Female; 9 weeks old; WT, n=3; Tollip^{-/-}, n=3)

Supernatant from neutrophil is sufficient to confer inhibition in cell line proliferation.

Neutrophils can clear pathogens via phagocytosis and NETosis but it is unclear whether neutrophils suppress cancer cell line proliferation via direct contact. In this study, we investigate the mechanism behind neutrophil cell line suppression. Surprisingly, supernatant from neutrophils conferred similar, or better, suppression in cell line proliferation compared to direct co-culture (Figure 3A). Again, similar trend is found between the WT and the Tollip^{-/-} neutrophils as in earlier studies: Tollip deficiency neutrophils are superior in cell line proliferation suppression compared to WT neutrophils. Unstimulated, overnight GM-CSF stimulated, and corresponding supernatant were all significantly different between WT and Tollip^{-/-} neutrophil. Overnight GM-CSF stimulated Tollip^{-/-} neutrophils conferred the greatest suppression, closely followed by supernatant from overnight GM-CSF stimulated Tollip^{-/-} neutrophils and unstimulated Tollip^{-/-} neutrophils. WT neutrophils significantly assisted cell line growth in unstimulated, overnight GM-CSF stimulated, and corresponding supernatant co-culture. On the other hand, Tollip^{-/-} neutrophils significantly supported cell line growth in unstimulated co-culture only. The trend persist to day 4 and day 5 (Supplementary figure 3A,B). Experiment with the same setting is carried out with CT26.WT cell lines to ascertain the phenomena is universal. Tollip^{-/-} neutrophils better inhibited CT26.WT cell proliferation in transwell and supernatant (Figure 3B). Fresh neutrophils (+/- GM-CSF) may not be adequate time for neutrophils to polarize to effectively confer inhibitory effect on MCF10A cells. However, overnight stimulation may (terminally) polarize neutrophils to a phenotype that can better suppress MCF10A cell growth. Results suggest molecules and enzymes secreted by neutrophils in the media may be responsible for significant inhibitory effects seen in MCF10A cell growth over extended period of time.

A.

MCF10A (Day 3)



B.

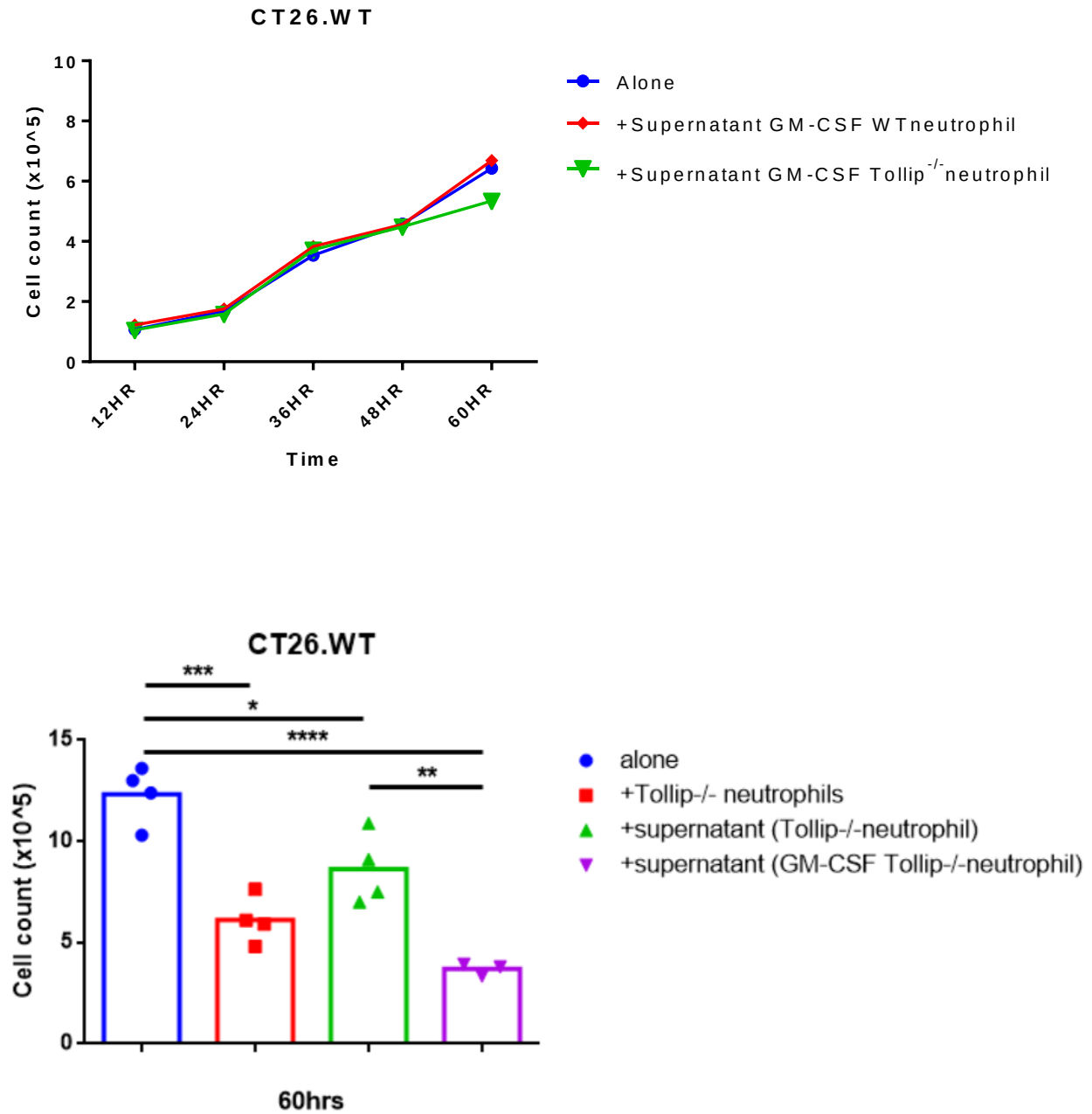


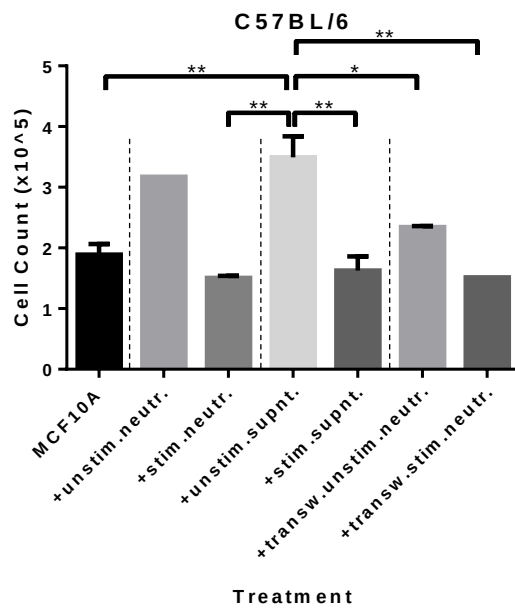
Figure 3. Supernatant from WT and Tollip^{-/-} neutrophils are enough to confer growth suppression in MCF10A and CT26.WT cells. (A) MCF10A—O/N GM-CSF stimulated neutrophil co-culture, 1:5=T:E (Male; 12 week old; WT, n=3; Tollip^{-/-}, n=3). (B) CT26.WT— O/N GM-CSF stimulated neutrophil co-culture (Female; 8weeks & 12weeks old; WT, n=4; Tollip^{-/-}, n=4).

Acute LPS stimulation confers similar suppression in cell line proliferation.

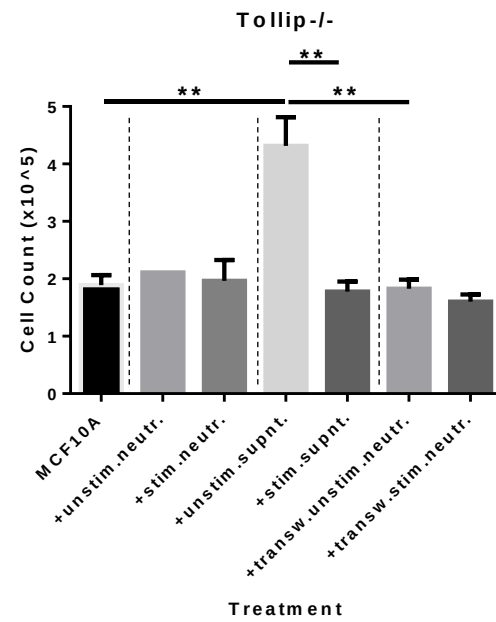
In continuation of the acute and chronic *in vivo* studies with LPS injection and LPS stimulated neutrophil adoptive transfer, respectively, neutrophils were stimulated 30 minutes with super low dose LPS (100pg/ml) for extended co-culture study. In addition to supernatant, transwell is added to the system for the following study. Transwell allow neutrophils and cell lines to secrete signaling molecules in the presence of the other cells without direct contact. Surprisingly, we did not see a difference in suppression capability between the direct contact, supernatant, and transwell (Figure 4A). Stimulated neutrophils once again showed enhanced suppression in all categories (direct co-culture, supernatant, and transwell) with 30 minute LPS stimulation. Similar experiment was carried out using the Tollip KO neutrophils and the IRAK-M KO neutrophils. Interestingly, Tollip KO neutrophils suppressed cell line proliferation in all categories save for unstimulated supernatant, which showed similar growth rate as the supernatant from unstimulated WT neutrophils (Figure 4B). MCF10A cells cultured with the unstimulated neutrophils, supernatant, and transwell neutrophils were slightly higher than its counter stimulated conditions with Tollip KO neutrophils. IRAK-M KO neutrophil experiment exhibited similar result to WT neutrophil experiment with even greater inhibition (Figure 4C). MCF10A cell count in transwell with stimulated IRAK-M KO neutrophils were too low for accurate measure. Supernatant from the unstimulated neutrophils conferred the least suppression in cell line proliferation than in any other conditions, which indicate inactive/naïve neutrophils do not secrete much, if any, pro-inflammatory cytokines. This also supports the notion that the neutrophils secrete different cytokines and mediators dependent on the signals they receive from their environment. With neutrophil modulations, MCF10A cells may not be able to polarize neutrophils to a phenotype that can cater their growth. WT, Tollip^{-/-}, and IRAK-M^{-/-} neutrophils confer different degree of

inhibition, which indicate possible different pathways of signaling and/or time frame of activation in each neutrophils. Based on the results, cell contact may not be necessary for the inhibition of MCF10A cell growth. However, extended stimulation may be required for more drastic inhibition.

A.



B.



C.

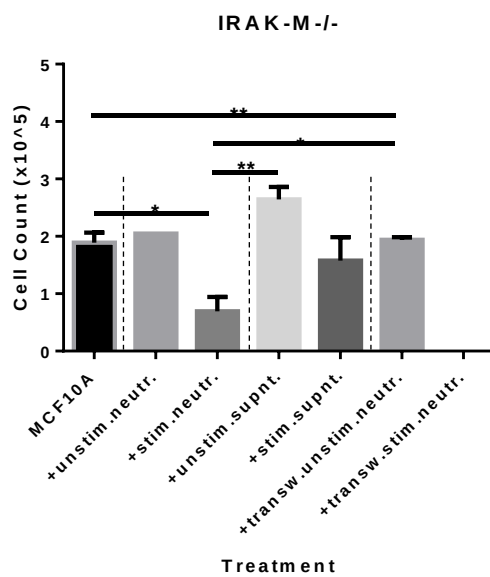


Figure 4. Direct contact is not necessary to suppress MCF10A cells with acute LPS stimulated neutrophils. Neutrophils were added every other day for 5 days. (Male; 7 weeks old; WT, n=3; Tollp^{-/-}, n=3)

Higher necrosis and apoptosis detected in MCF10A cells when co-cultured with Tollip^{-/-} neutrophils.

In order to determine the cause of lower cell numbers when the cells are co-cultured with Tollip^{-/-} neutrophils, we stained the MCF10A cells with Annexin V and PI. By running the samples through flow cytometry, we were able to measure the percentage of cells that are in live (PI^{low}AnnexinV^{low}), necrotic(PI^{high}AnnexinV^{low}), and apoptotic stage. Neutrophils may modulate MCF10A cell state and trigger the cells to die at a faster rate via necrosis or apoptosis. Surprisingly, we found both lower numbers of live cells and higher number of necrotic and early apoptotic (PI^{low}AnnexinV^{high}) MCF10A cells when the cells are co-cultured with the supernatant from overnight stimulated GM-CSF neutrophils (Figure 5). Interestingly, there were higher number of late apoptotic (PI^{high}AnnexinV^{high}) cells when MCF10A cells are co-cultured with overnight GM-CSF stimulated neutrophils directly. This indicate direct contact between cancer cell and neutrophil may confer separate pathway of suppression than when the cells communicate indirectly. It is also important to note that MCF10A cells cultured alone have the lowest live cells and the highest early apoptotic cells. This indicate neutrophils support MCF10A cell proliferation via deterring the cells from going into apoptotic stage. Supernatant from overnight GM-CSF stimulated Tollip^{-/-} neutrophils is able to bring the apoptotic effect similar to that of MCF10A cells cultured alone. Specific stimulation time or GM-CSF dosage may confer more pronounced effect, which needs to be identified in future study.

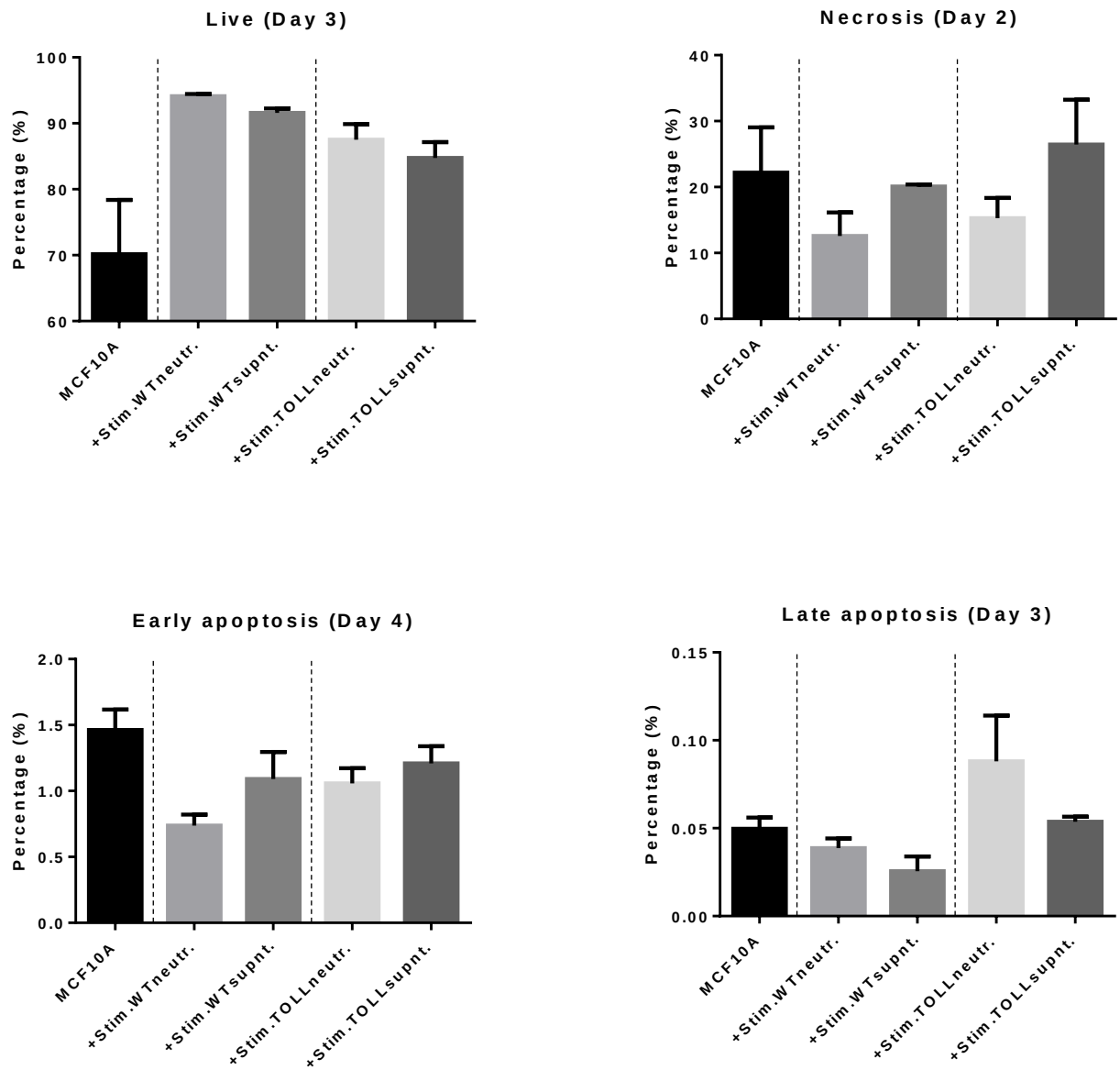


Figure 5. Tollip in neutrophil modulate necrosis and apoptosis state in MCF10A cells. (1 layer gradient; male; 12weeks old; WT, n=3; Tollp^{-/-}, n=3)

Tollip^{-/-} neutrophils have lower PD-L1 expression.

The effect of lower PD-L1 expression in Tollip^{-/-} mouse neutrophil compared to WT mouse neutrophil has been previously discussed in Chapter 2. It is understood that the lower expression of PD-L1 is necessary for adaptive immune cell function. However, it may also have more direct effect in cancer and neutrophil communication. We proceeded with qRT-PCR to confirm the flow cytometry results in Chapter 2. Compared to the PD-L1 expression in the WT bone marrow neutrophils, the PD-L1 expression in the Tollip^{-/-} neutrophils in blood and spleen were much lower than WT neutrophils under AOM-DSS challenge (Figure 6A). We observed similar trend in neutrophils from *in vitro* assay when using overnight super low dose LPS stimulation (Figure 6B) and GM-CSF stimulation (Supplementary Figure 4). Overexpression of PD-L1 carries cell death signal that leads to apoptosis in cell. Lower expression in Tollip^{-/-} neutrophils indicate neutrophils with Tollip^{-/-} may live and activate longer than WT neutrophils.

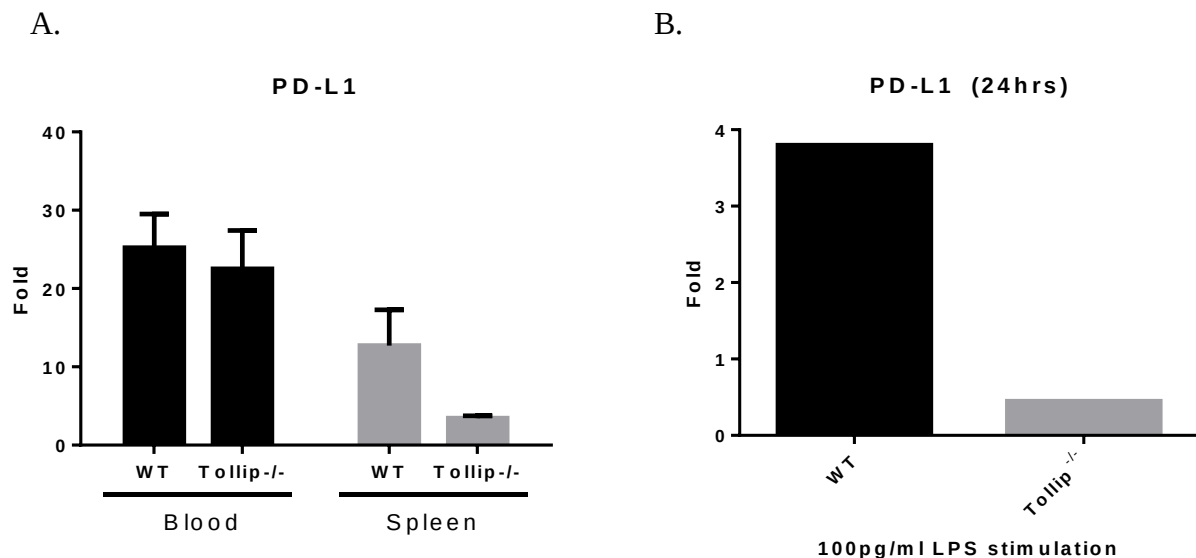
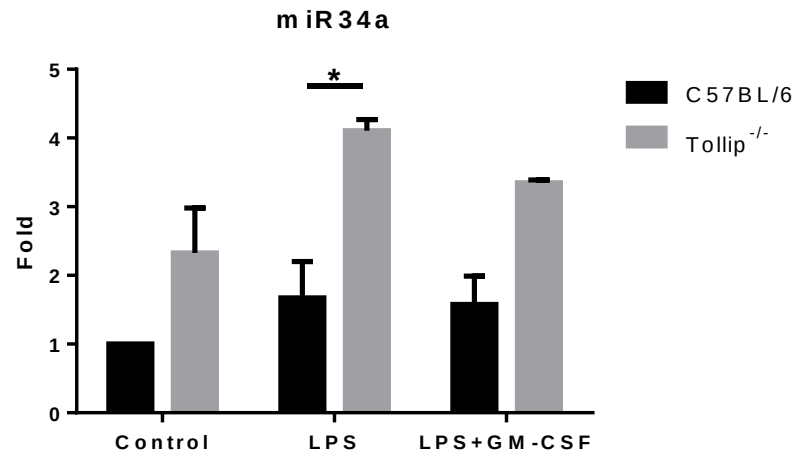


Figure 6. PDL-1 level is enhanced in WT neutrophil compared to Tollip^{-/-} neutrophil. (A) Neutrophils isolated from mice under chronic AOM-DSS challenge (Male; 11 weeks old; WT, n=2; Tollip^{-/-}, n=2). (B) Neutrophils collected after 24 hrs incubation with 100pg/ml LPS (Male; 8 weeks old; WT, n=1; Tollip^{-/-}, n=1).

Tollip suppresses miR-34a expression.

MiRNAs are found to regulate as many as 60% of mRNA and may also be highly involved in immune cell functions than previously known. MiR-34 is one of the well characterized miRNAs that has been shown to be downregulated in several different tumor types. The gene is involved in many cellular processes such as apoptosis, inhibition of proliferation, inhibition of migration, inhibition of invasion, and inhibition of differentiation. We hypothesize that Tollip may be involved in the regulation of miR-34 secretion, which invariably influence neutrophil function and tumor growth in host. It is a direct transcriptional target of the onco-suppressor p53, which is one of the most commonly mutated genes in variety of cancer cells. qRT-PCR shows higher expression of miR-34a in naïve Tollip^{-/-} neutrophils and overnight super low dose LPS +/- GM-CSF stimulated Tollip^{-/-} neutrophils compared to the counter WT neutrophils (Figure 7A). In order to ascertain miR-34a effect on CT26.WT cell proliferation, anti-miR-34a was added to the co-culture assay. Tollip^{-/-} neutrophils were superior in CT26.WT cell growth suppression compared to WT neutrophils (Figure 7B). Overall, supernatant is better at inhibiting CT26.WT cell growth. Co-Culture with the overnight GM-CSF stimulated neutrophils and supernatant from the stimulated neutrophils suppressed CT26.WT cell growth better than their counter unstimulated conditions. However, the effect was much reduced with the addition of miR-34a inhibitor. Except the CT26.WT co-culture with GM-CSF stimulated neutrophils, Tollip^{-/-} neutrophils suppression were non-significant compared to WT neutrophils in all categories. This indicates miR-34a may be responsible for the neutrophil suppression in cancer cell proliferation and may be targeted in the future therapy.

A.



B.

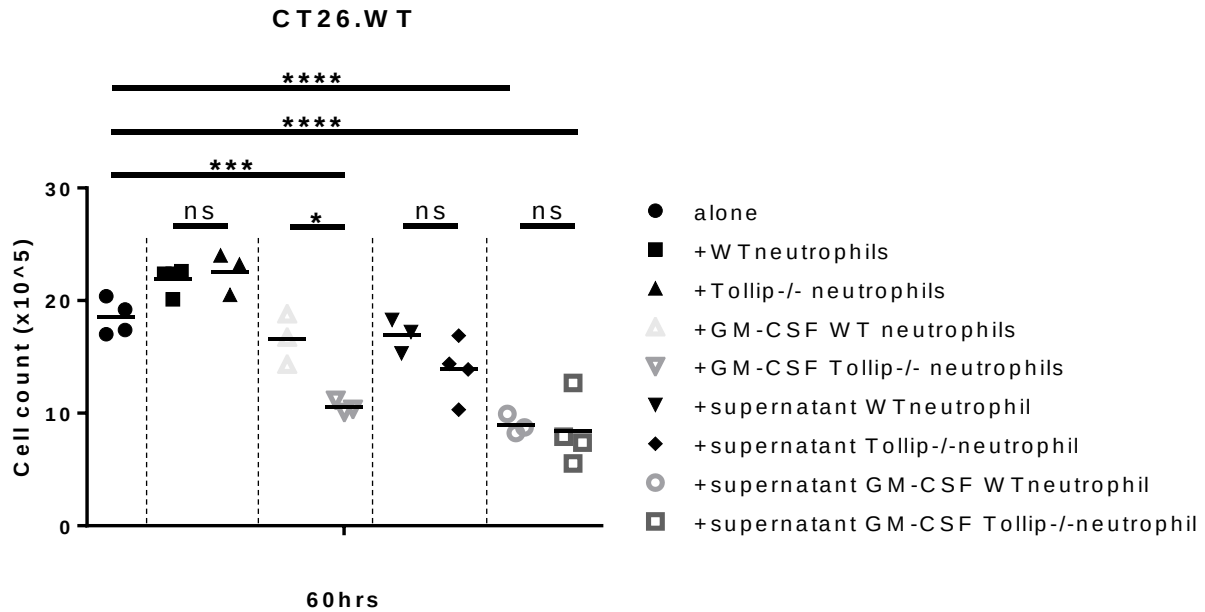


Figure 7. miR-34 level is suppressed in WT neutrophil compared to Tollip^{-/-} neutrophil. (A) Overnight super low dose LPS +/- GM-CSF stimulated and unstimulated neutrophils (Female; 8 weeks old; WT, n=3; Tollip^{-/-}, n=3) (B) CT26.WT—neutrophil co-culture with miR-34a inhibitor. CT26.cell count after 60hrs (Male; 8 weeks old; WT, n=4; Tollip^{-/-}, n=4).

CONCLUSION

Cancer cells have shown to utilize the host immune cells to support their needs. Tollip in neutrophils may be one of the ways cancer cells modulate the immune cells to establish, maintain, and grow. In this chapter, we found that neutrophils can be programmed with external signaling molecules, such as GM-CSF and LPS, to confer different suppression capability in four different cell line growth. Pre-conditioning the neutrophils with either super low dose LPS or genetically deleting Tollip shifted neutrophils into an enhanced anti-tumor phenotype as seen by their elevated suppressive effect on the various cell line proliferation. We also found that direct contact between neutrophils and cell line is not necessary for neutrophils to suppress cell line proliferation. Neutrophils are excellent secretors of cytokines and soluble mediators. Both the supernatant from overnight neutrophil culture and the transwell proved effective in neutrophil suppression in cell line proliferation. Similar to GM-CSF, super low dose LPS stimulation also enhanced neutrophil suppression in cell line proliferation. Interestingly, both higher necrosis and apoptosis were detected in MCF10A cells when the cells were co-cultured with Tollip^{-/-} neutrophils, but not with WT neutrophils. This is consistent with the literature report that WT neutrophils may adopt a pro-tumor growth effect *in vivo*. Our findings that Tollip deficient neutrophils exhibiting an opposite anti-tumor effects are novel and hold potential translational significance. Mechanistically, we found that Tollip^{-/-} neutrophils have lower PD-L1 (confirmed *in vitro* and *in vivo*) and higher miR-34a expression as compared to WT neutrophils. The elevated levels of miR-34 might potentially explain the anti-tumor effects of the Tollip deficient neutrophils, which can be tested further in future studies.

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SUPPLEMENTARY FIGURES

1.

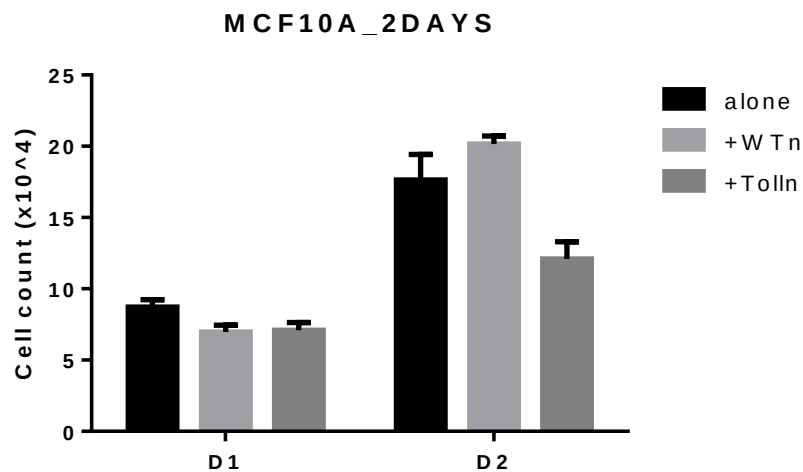
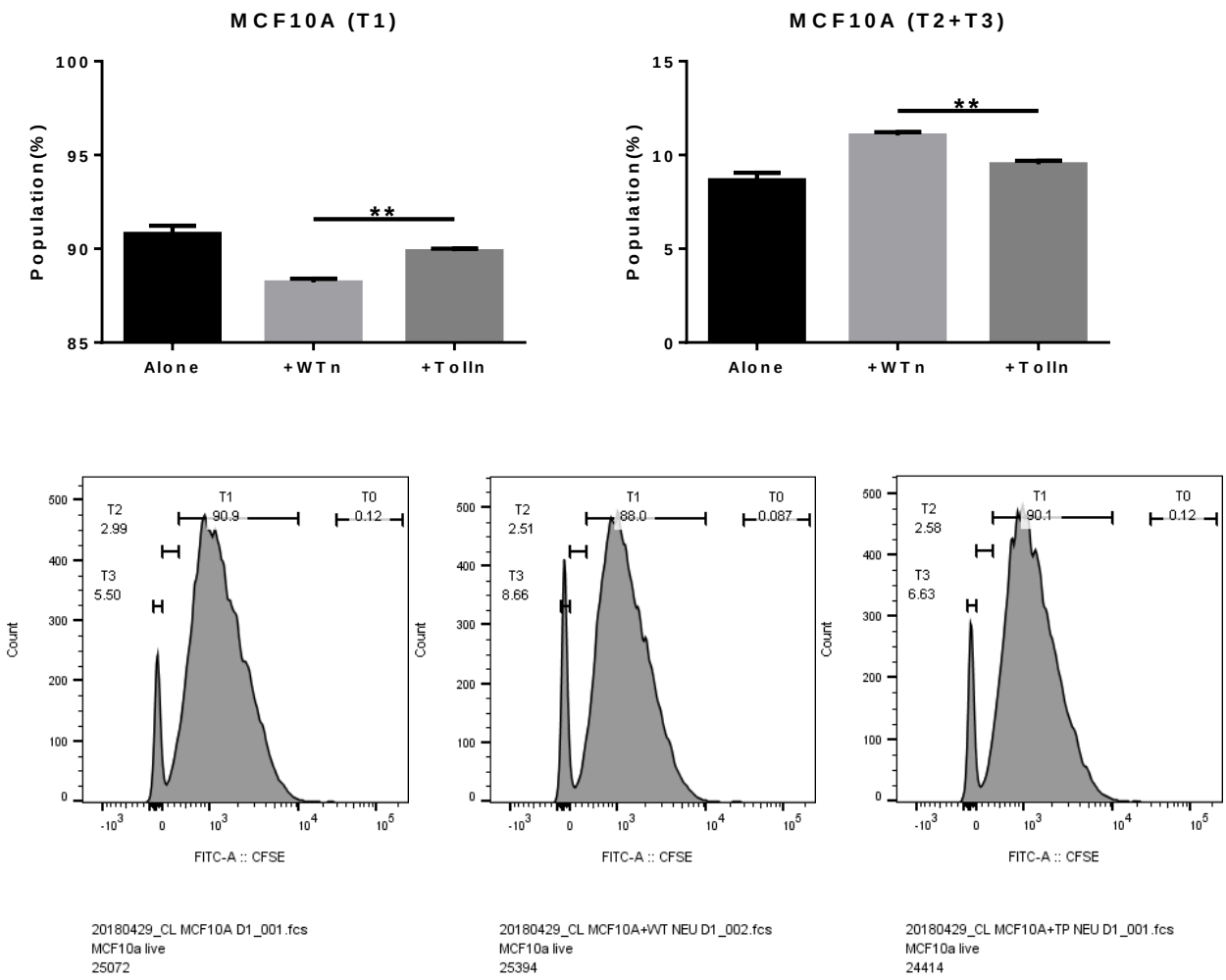


Figure 1S. Similar neutrophil suppression on cell line growth is found when using male and female mice neutrophils. Although Tollip^{-/-} neutrophil suppressed cancer growth in cancer cell line, when using male mice neutrophil, there were greater suppression than when using female mice neutrophil. So we looked at cancer cell number at earlier time point to assess the difference seen in Figure 1. Female mice neutrophil show similar trend as male mice neutrophil in day 1 but cancer cell with neutrophil catches up to cancer cells without neutrophil by day 2. The Tollip^{-/-} neutrophil still conferred better suppression than WT neutrophil in all trials.

2.

CFSE DAY1



CFSE DAY2

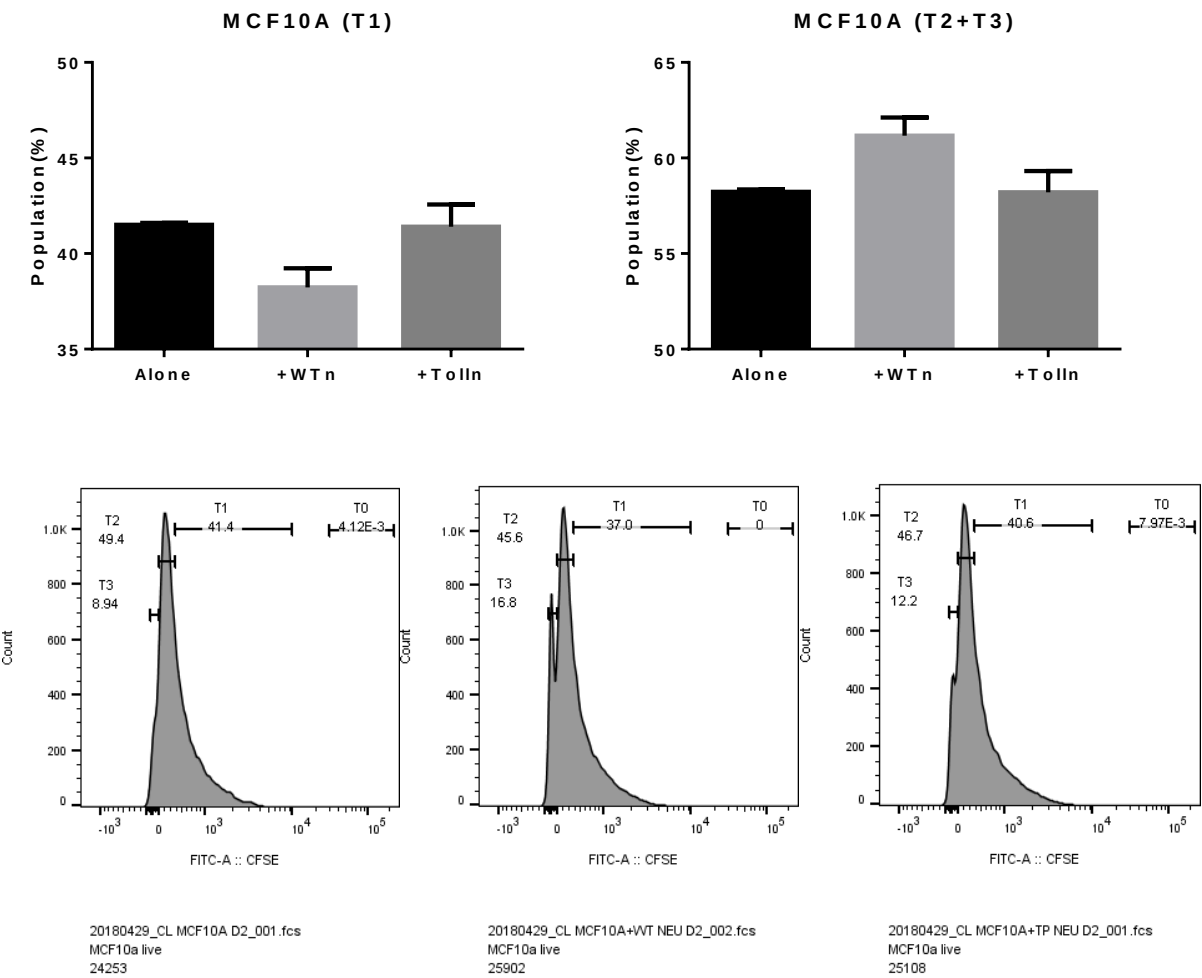


Figure 2S. MCF10A cell proliferation staining at Day 1 and Day 2. Lower % in T1 indicate cells have divided at higher rate and have shifted to T2 and T3 emission.

3.

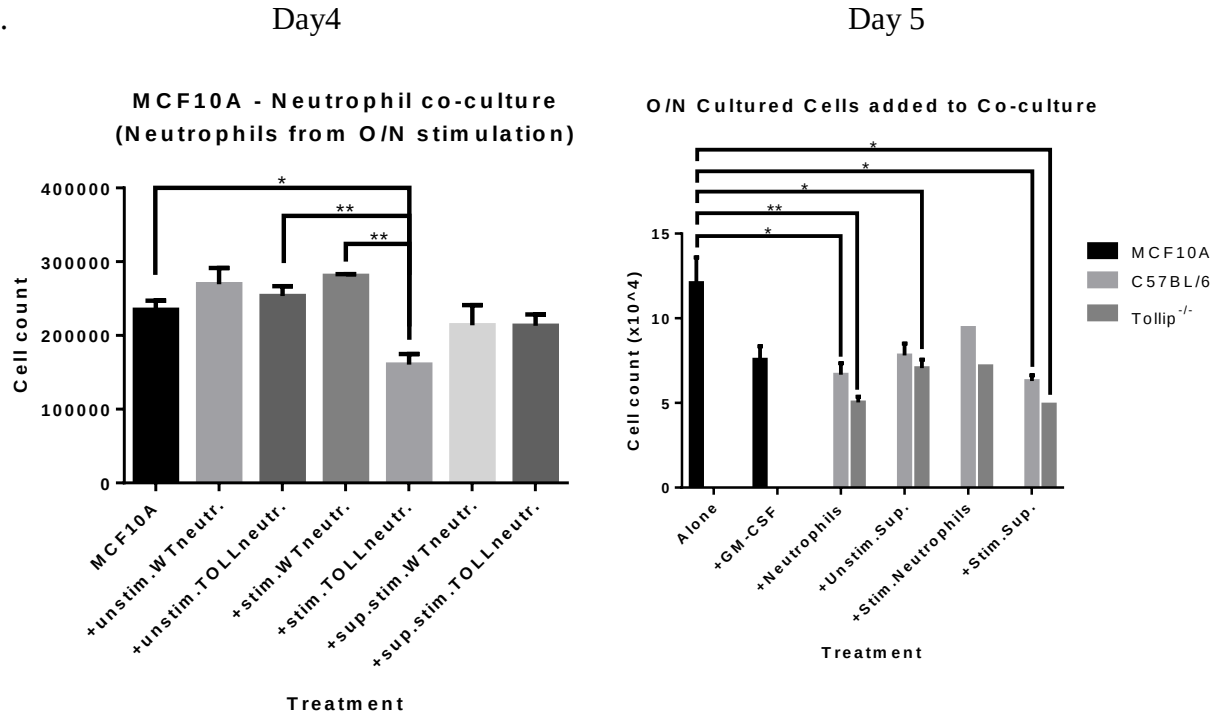


Figure 3S. Overnight 1ng/ml GM-CSF stimulated neutrophils confers significant suppression in MCF10A cell proliferation in Day 4 and Day 5 compared to MCF10A cell alone, unstimulated Tollip^{-/-} neutrophils, and stimulated WT neutrophils. Neutrophils were added to MCF10A cells every other day at 1:5 target-to-Effector ratio. Although the data yield non-significance between WT and Tollip^{-/-} neutrophils, MCF10A cells with Tollip^{-/-} neutrophil (or supernatant from Tollip^{-/-} neutrophils) are consistently lower in number compared to WT neutrophils (or supernatant from WT neutrophils), which may indicate enhanced programming/polarization when Tollip protein is absent. The results are comparable to earlier experiments.

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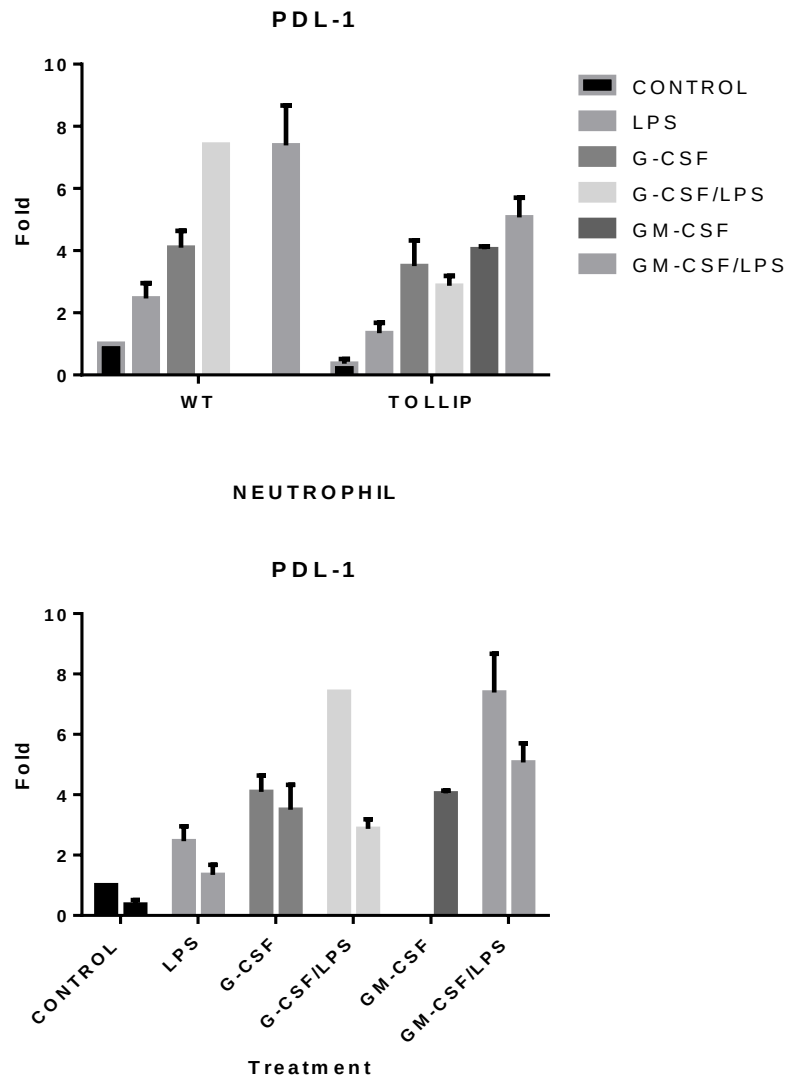


Figure 4S. qRT-PCR. O/N stimulation with 100pg/ml LPS, 100ng/ml G-CSF, +/-1ng/ml GM-CSF. 1:20 target to effector.

CHAPTER 5

Suppressor molecules modulate acute colitis outcome

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ABSTRACT

Immune cells are excellent defenders of host health against foreign pathogens and maintaining host homeostasis. They can confer pro-inflammatory or anti-inflammatory responses depending on the external signalings they receive from their microenvironment. Consequently, immune cells are found to be polarized in a state that is more harmful to the host in many inflammatory diseases. In this paper, we explore whether priming immune cells provide protection in the mice gut against chemically induced inflammation. Super low dose LPS is injected to mice prior to the DSS treatment in order to determine whether primed immune cells are able to provide protection for mice under colonic distress. We also explore different negative regulators in NFkB signaling pathways, IRAK-M and Tollip, using the knockout mice models to identify possible targets in the NFkB signaling pathway for therapeutic purposes.

INTRODUCTION

Current literature suggest high plasticity in immune cells which allows them to switch their phenotype that is appropriate to host need. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway has been well defined over the years. The signaling pathways encompass numerous negative regulators in order to control inflammation and maintain homeostasis. Pathogen associated molecular pattern (PAMP) will instigate pro-inflammatory response in host to attack, hold, and clear the danger. Once foreign particles are cleared, anti-inflammatory response sets in to rebuild the damaged tissue and maintain homeostasis. There is an intricate balance between the pro-inflammatory and the anti-inflammatory response so that the host is protected without further damaging tissue or exhausting its defense.

In a number of inflammatory diseases, such as atherosclerosis and inflammatory bowel disease, a high number of immune cells are found in immature polarization. One of the ways to impede immature polarization in immune cells to anti-inflammatory state is to downregulate negative regulators of NF κ B signaling pathways. Previous data from our lab suggest immune cell polarization may be dependent on external signaling from environment [1-6]. We found that with high dose polyliposaccharide (LPS), cells may encompass “tolerant” phenotype and exhibit limited inflammatory response when second challenge is presented. On the other hand, super low dose LPS pushed immune cells to a “primed” state that allowed for enhanced inflammatory function when second challenge is presented. Based on the preliminary data, we assume the introduction of low grade endotoxin will not instigate a full inflammatory response but “prime” the immune cells for following challenge. Classical pathway of NF κ B involves MyD88-TIR activation. Two of the most prominent negative regulators are Interleukin-1 receptor-associated kinase 3 (IRAK-M) and

Toll-interacting-protein (Tollip)[7, 8]. Once PAMP signal activates toll-like-receptor (TLR), MyD88 binds to the Toll/interleukin 1 receptor (TIR) and initiates a series of activation process, the signal can be depressed when IRAK-M or Tollip binds to IRAK and TIR respectively. However, the exact role of its signaling molecules in regards to environmental challenge is not well understood. Thus, it is pertinent to understand what causes the inappropriate polarization in immune cells and whether this process can be reversed.

In order to investigate whether “primed” immune cells can provide protection in mice gut under acute inflammation, we injected mice with well-known endotoxin, LPS. We used suboptimal dose of LPS that has been shown to confer “priming” effect on immune cells. Then we proceeded to test whether IRAK-M or Tollip knockout mice can provide enhance immune response and provide protection against acute inflammation in the mice gut. Furthermore, in order to isolate the immune cell activity to neutrophils, which comprises the majority of the immune cells in a healthy person, we implemented neutrophil cre-IRAK-M floxed mice for further observation. Dextrane sodium sulfate is a well known chemical that induces inflammation in the gut, mimicking symptoms similar to inflammatory bowel disease (IBD) in human. We used the same scoring guide from previous chapters to measure IBD progression in mice.

METHODS

Mice

Wild type (WT) C57BL/6, Tollip^{-/-}, IRAK-M^{-/-} mice were bred and maintained in the animal facility at Virginia Tech in accordance to approved Animal Care and Use Committee protocol. Neutrophil Cre-IRAK-M flox (CRE+) mice were provided by Dr. HongWei Chu at National Jewish Health, CO. Mice were age (between 7-26 weeks of age at the time of use) and gender matched for each experiment.

Experimental Design

Mice were given 3% dextrane sodium sulfate (DSS, MP Biomedicals) in drinking water for five days and normal water for 7 days and sacrificed at day 3, 5, 6, 8, or 12. Super low dose LPS at 100pg/mice were injected i.p. daily from day 0 of DSS cycle. Mice conditions, such as body weight, stool consistency, and bleeding, were graded according to scale created in lab (see below for detail). Mice colons were extracted and measured for length at the termination of experiment.

Histology

Histological analyses of colon tissues were performed on freshly frozen OCT (Optimal-Cutting-Temperature compound)-embedded, and sectioned slides (5µm). Slides were fixed in 4% neutral buffered formalin for 5 min. Hematoxylin and eosin (H&E) staining were performed.

IBD Clinical Score Determination (0 Normal - 4 Poor condition)

Weight Grading <0-4>

- 0 No weight loss
- 1 Weight loss of 1—5%
- 2 Weight loss of 5.1—10%
- 3 Weight loss of 10.1—20%
- 4 Weight loss of more than 20%

Stool Grading <0-4>

- 0 Normal: dry and hard
- 1 Soft/Well formed: does not change round shape when pressed together
- 2 Soft/NOT well formed: round shape but mushy when pressed together
- 3 Pasty: came out long and gooey
- 4 Diarrhea: has no shape and does not come out clean (sticks to anus)

Bleeding Grading <0-4>

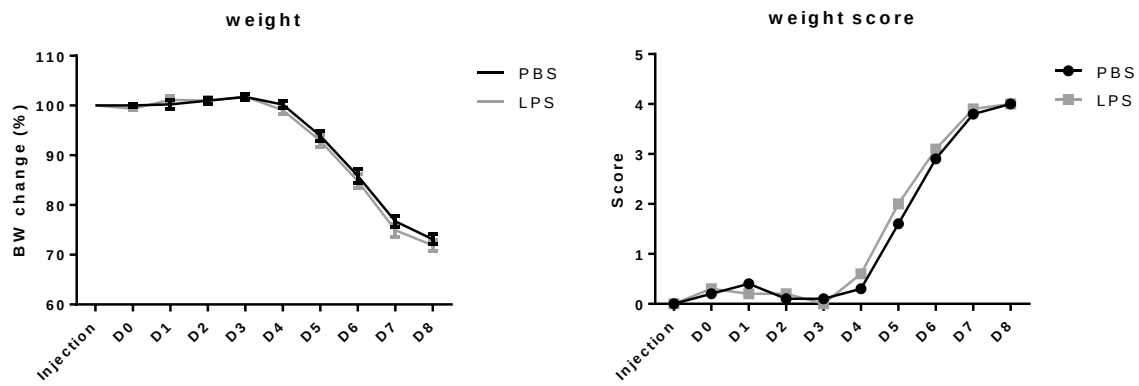
- 0 None
- 1 Occult bleeding: hint of blood
- 2 Visible bleeding: bright red (no question about the presence of blood)
- 3 Dark red: stool is soaked in blood
- 4 Gross bleeding: excess blood from anus

RESULTS

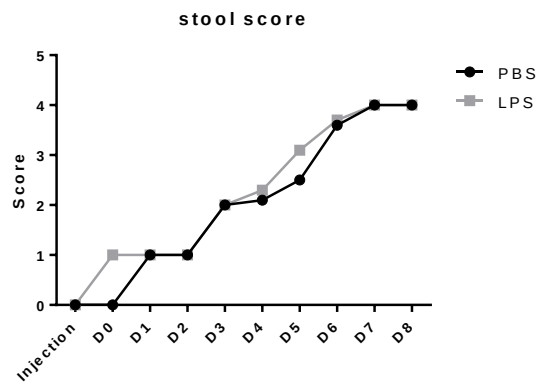
Super-low dose LPS injection exacerbates IBD symptoms.

Immune cells are capable of polarizing to “priming” or “tolerance” state depending on the dosage of their first endotoxin encounter. When cells are stimulated with super-low dose LPS (100pg/ml), a mild inflammatory response was observed. However, upon the second stimulation, the cells displayed much greater pro-inflammatory phenotype. On the other hand, stimulation with high dose LPS (1ug/ml) instigated a typical inflammatory response initially followed by much depressed pro-inflammatory response upon second stimulation. Based on these *in vitro* findings, we were able to modulate immune cells *in vitro* to a phenotype that may be more beneficial *in vivo* in chronically distressed mice. In order to investigate whether primed/hyper immune cells are capable of providing protection in the colon against acute DSS challenge, mice were injected intraperitoneally with suboptimal dose of LPS daily at the start of DSS treatment. Surprisingly, weight and stool condition were similar between the groups that received PBS and LPS injection (Figure 1. A,B). However, the group that received LPS injection exhibited more blood in the stool, which is supported by greater scarring tissue and shortened colon length found at day 5 and day 12 of the experiment (Figure 1. C,D,E). Base on the *in vivo* data, we conclude that injecting mice with immune cells primed with super-low dose LPS contributes to worsened IBD symptoms.

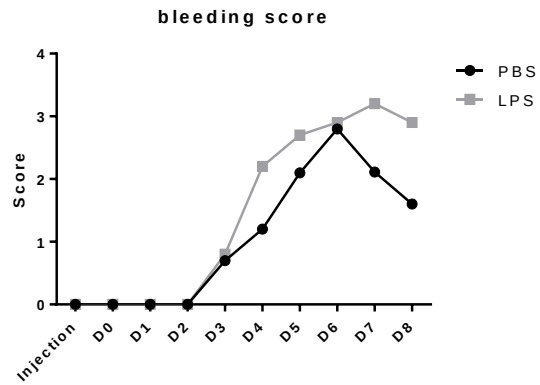
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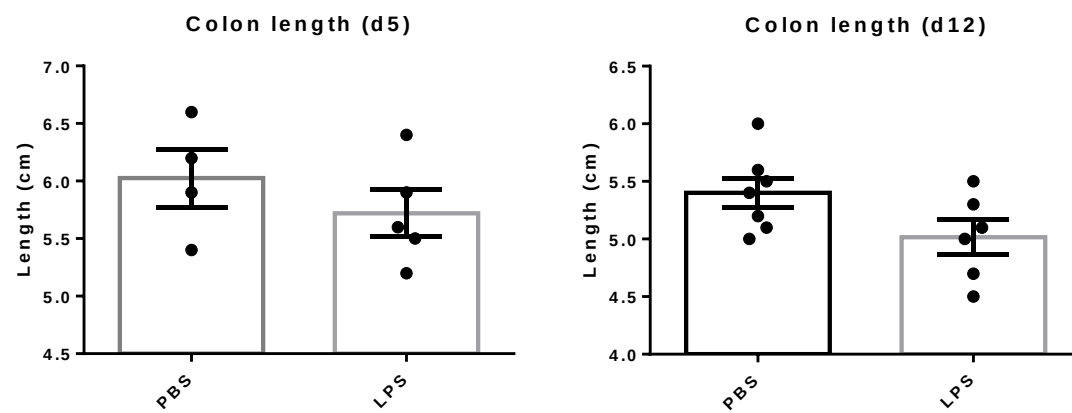
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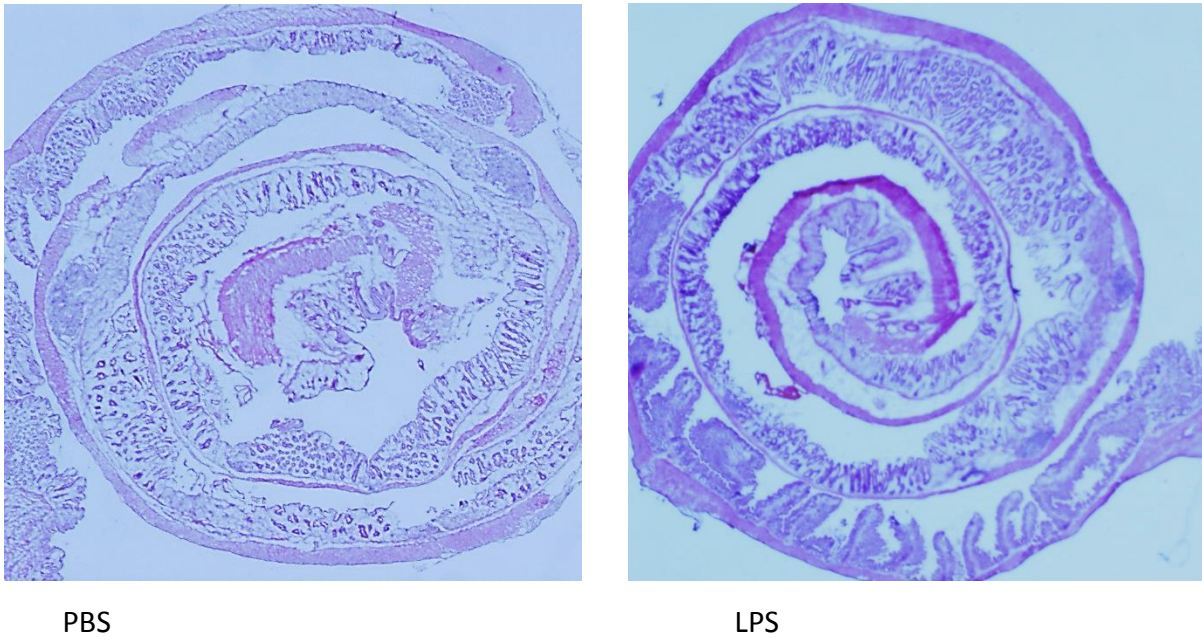


Figure 1. Mice injected with super-low dose LPS exhibit worse clinical score under acute DSS challenge. (A) Body weight change curve from day 0 of WT mice receiving PBS or LPS during the course of five days DSS and 3 days normal water. (Male; 8wks old; PBS, n=10; LPS, n=11). (B) Stool consistency score of WT mice receiving PBS or LPS during the course of five days DSS and 3 days normal water. (Male; 8wks old; PBS, n=10; LPS, n=11). (C) Bleeding from rectum score for WT mice receiving PBS or LPS during the course of five days DSS and 3 days normal water. (Male; 8wks old; PBS, n=10; LPS, n=11). (D) Colon length of WT mice receiving PBS or LPS after five days of DSS (Data are mean $\pm$ SEM; male; 9wks old; PBS, n=4; LPS, n=5), and five days of DSS plus 7days of drinking normal water. (Data are mean $\pm$ SEM; male; 7wks old; PBS, n=10; LPS, n=10). (E) H&E stained sections of colon from WT mice receiving PBS or LPS during the course of five days DSS and 3 days normal water. (Male; 7wks old; PBS, n=10; LPS, n=10).

IRAK-M knockout mice fares better than WT and Tollip knockout mice under acute DSS challenge.

Tollip and IRAK-M are both negative regulators in MyD88-TLR dependent NFkB signaling pathway. Tollip competes with IRAK in TIR binding site once MyD88-TIR are activated. On the other hand, IRAK-M binds with TIR bounded IRAK to inhibit further phosphorylation. Interestingly, Tollip knockout mice and IRAK-M knockout mice show varying degree of protection under acute DSS challenge compared to WT mice. Colon lengths were measure at day 3 and day 6 during the DSS treatment to measure the extent of scarring in colon: the more extensive the scarring in the colon, the shorter the colon lengths. The baseline for WT, Tollip^{-/-}, and IRAK-M^{-/-} mice colon lengths were comparable prior to DSS treatment (not shown). However, by day three, Tollip^{-/-} mice colon were shown to be the least affected by the DSS challenge (Figure 2A). By day six, IRAK-M^{-/-} mice colon show the least amount of change in length from the start of the DSS challenge (Figure 2B). Clinical data supports the protective effect found in colon length during and after the DSS treatment. IRAK-M^{-/-} mice fared better than WT mice in all categories of clinical scores—body weight change, stool consistency, and bleeding (Figure 3).

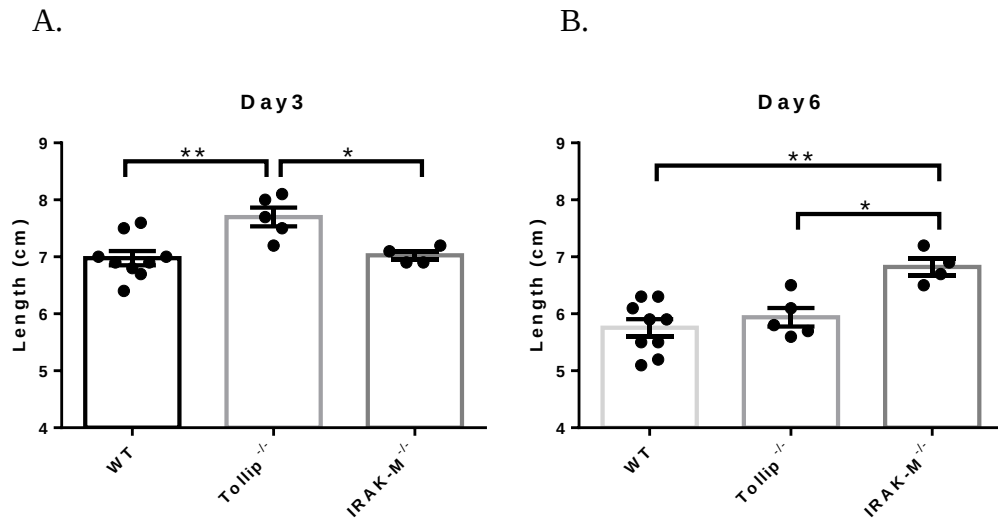


Figure 2. Different knockout mice display distinctive protective phenotype under acute DSS challenge. Colon length of WT, Tollip^{-/-}, and IRAK-M^{-/-} mice at day three and day six of DSS challenge (Data are mean $\pm$ SEM; WT, n=5; Tollip^{-/-}, n=5; IRAK-M^{-/-}, n=4).

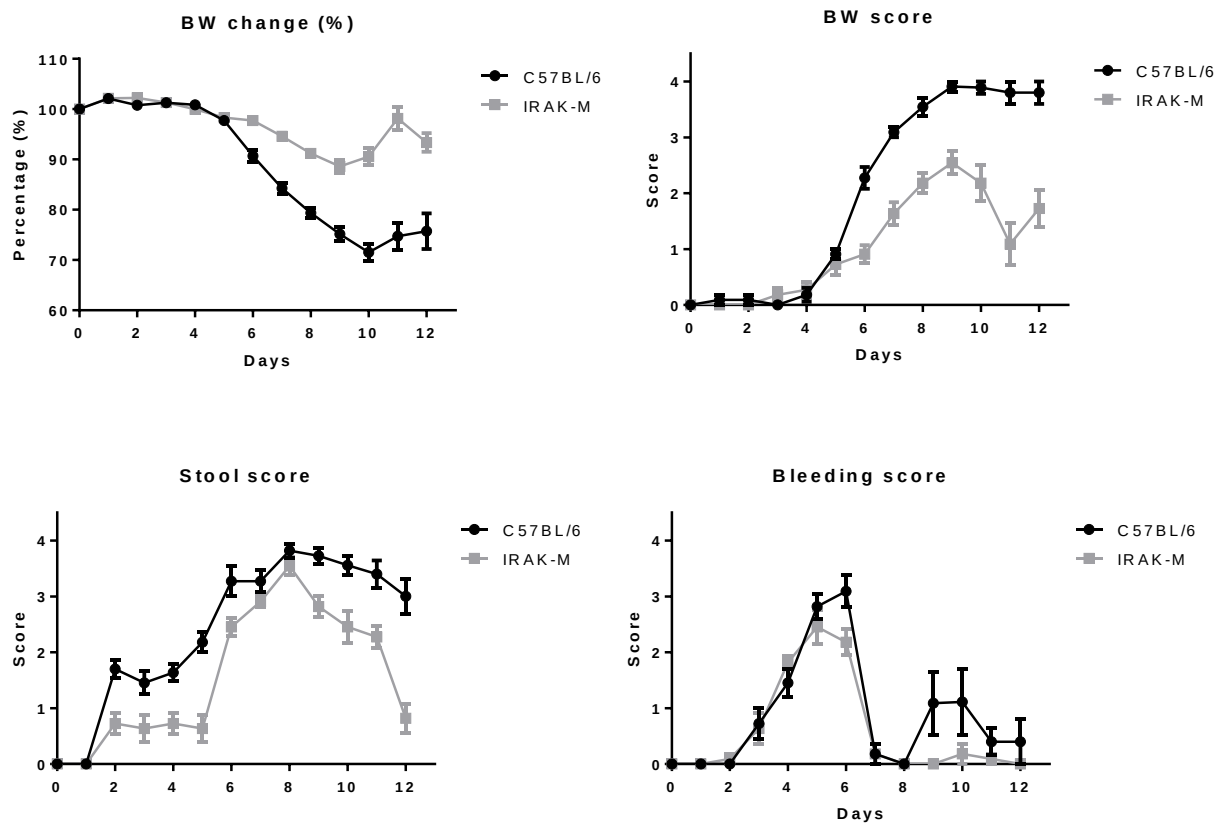
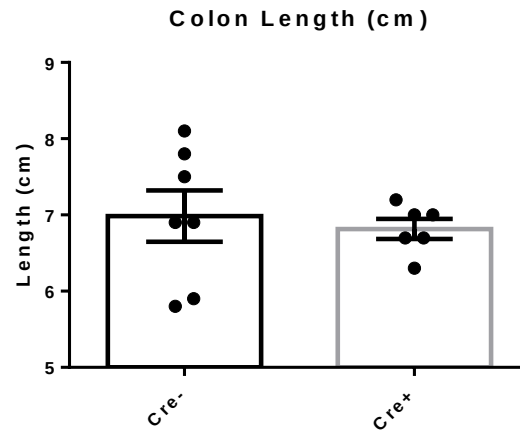


Figure 3. *IRAK-M*^{-/-} mice under acute DSS challenge yield better clinical score compared to WT mice under acute DSS challenge. Weight change from day 0, stool consistency, and bleeding from rectum are graded daily based on scoring guide 0-4 (Data are mean $\pm$ SEM; male; 7wks old; WT, n=11; *IRAK-M*^{-/-}, n=11).

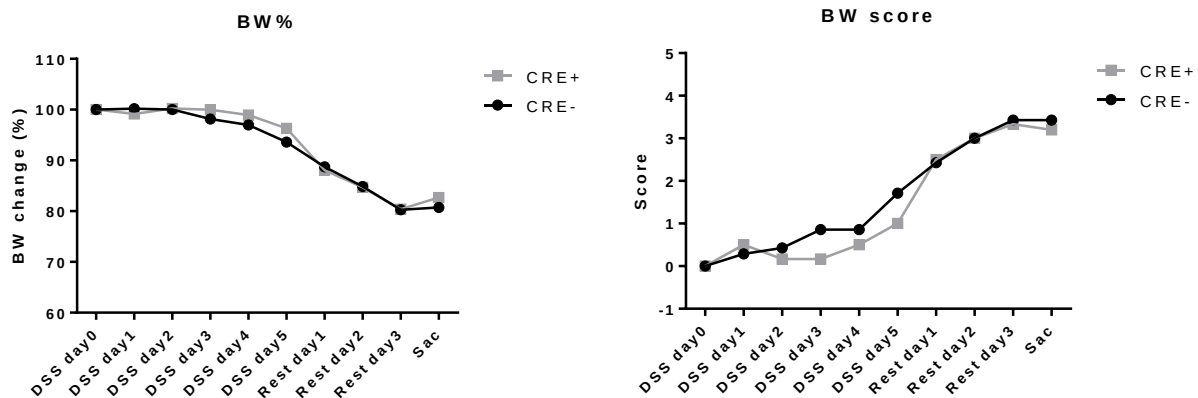
IRAK-M in neutrophil is not the vital factor in conferring protection in mice colon during acute DSS challenge.

To further investigate the effect of IRAK-M in neutrophils while mice are under acute DSS challenge, neutrophil Cre-IRAK-M flox mice were utilized. CRE-positive mice have the IRAK-M gene floxed out from neutrophils while IRAK-M were present in all the other cells. This will allow us to see the direct effect of IRAK-M in neutrophil on colon under acute DSS challenge. We focused on neutrophils because neutrophils comprise majority of innate immune cells in a healthy host and are known to be the first immune cells to arrive at the site of distress. Since degradation of colon inevitably may also allow gut microbiome to infiltrate mucosa and peritoneum, we assumed that neutrophils may play an important role in gut protection against the DSS challenge. Surprisingly, we did not find significant difference between the Cre-positive and Cre-negative mice (Figure 4). Based on this findings, we conclude that IRAK-M in neutrophils bestow less effect in terms of protection in gut when mice are under acute DSS challenge. We have not measured the expression of IRAK-M in other immune cells and surrounding tissues, which may be higher to compensate for the lost expression in neutrophils, and may be masking the true effect of IRAK-M in neutrophils in Cre-positive mice. IRAK-M in neutrophils may still play an important role in colon protection. Further study is warranted.

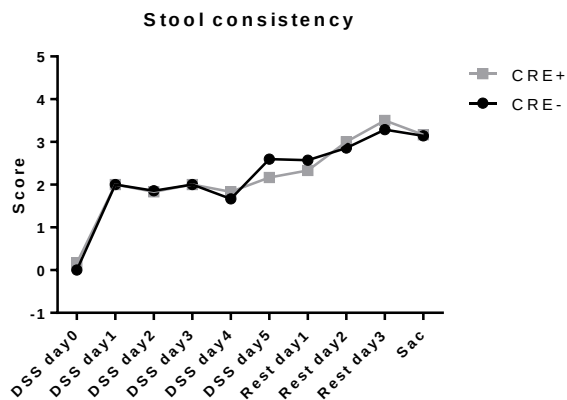
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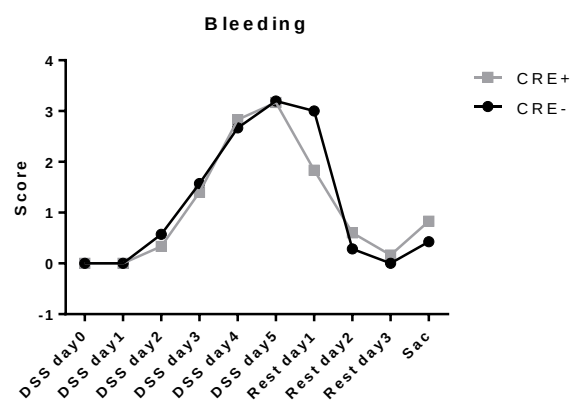


Figure 4. *IRAK-M*-negative neutrophil mice under acute DSS challenge does not show any beneficial effect compared to *IRAK-M*-positive neutrophil mice. Weight change from day 0, stool consistency, and bleeding from rectum are graded daily based on scoring guide 0-4 (Data are mean $\pm$ SEM; mixed gender; 18-26wks old; Cre-neg, n=7; Cre-pos, n=6).

DISCUSSION

It is well known that the immune system protects the host from invading pathogens, clear damaged cells, and maintain homeostasis. However, immune cells are also known to polarize to an immature phenotype before danger signal has yet been cleared or persist when danger signal no longer exists. Disrupted homeostasis is often found in inflammatory diseases. In this study, we use chemically induced colitis in mice and introduce artificial pro-inflammatory environment into system. We polarize the host immune cells with super-low dose LPS to “prime” the cells in parallel to the DSS treatment. The purpose of the study is to see whether external modification of the host immune cells can confer better protection in mice colon against DSS challenge. Surprisingly, we found that heightened immune response is harmful to mice under the acute DSS challenge. However, certain knockout of negative regulators in NFkB signaling show a different degree of protection in mice under the acute DSS challenge. We also found that IRAK-M in neutrophil is not the major impactor in the protective effect found in mice under the acute DSS challenge, albeit neutrophils being the major immune cells in the host body. In regards to the acute colitis and the DSS challenge, “tolerant” immune cells may confer better protection in colon since host immune cells may already be in a hyper-active state due to their contact with high dose LPS in the host system. Three methods to confirm this is through flow cytometry reading of the immune cell markers and single cell analysis of mice under DSS challenge to profile immune cell state. Lastly, measurement of colon length and clinical score in high-dose LPS injection in mice under acute DSS challenge may further confirm or refute the findings of this study. Nevertheless, the findings in this study have identified possible signaling molecules in NFkB signaling pathway, Tollip and IRAK-M, that may be further studied to modulate inflammation in the acute colitis and the DSS challenge.

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CHAPTER 6

The effects of PHLPP-1 deficiency on chronic colitis

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ABSTRACT

PHLPP is a novel signaling molecule involved in reducing cell proliferation and survival. However, its role in modulating inflammation is unclear. We are interested in PHLPP due to a previous unpublished screening of Tollip-interacting molecules through co-immunoprecipitation assays. Given our findings about the involvement of Tollip in colitis, we examined the effects of PHLPP deficiency in colitis model as well. In this study, we look at the physiological differences between WT and PHLPP-1^{-/-} mice challenged with AOM-DSS. One injection of AOM followed by three cycles of DSS is an established protocol to induce polyp formation in mice gut. It produces symptoms similar to human patients with colitis. Colon length, polyp formation, and clinical scores are measured. We found differences in the production of several pro-inflammatory genes between WT and PHLPP-1^{-/-} neutrophils challenged with LPS *in vitro*. We observed that PHLPP-1^{-/-} neutrophils have enhanced pro-inflammatory gene upregulations only under the low grade inflammation condition as compared to WT neutrophils. This may potentially explain the reduced polyp formation found in the PHLPP-1^{-/-} mice after the chronic AOM-DSS challenge. The findings also imply another promising gene to target in the the future therapy for colon cancer.

INTRODUCTION

According to the CDC report 2015, approximately 1.3% of the U.S. population (3 million people) has been diagnosed with the inflammatory bowel disease (IBD). While a number of factors have been found to contribute to its onset, ongoing dysregulated inflammation/homeostasis in the gut are commonly found in the patient population. Chronic inflammation in the gut further gives rise to tissue damage and gene toxicity, which may proceed to an increased chance of mutation in the host cell and cancer development. It has been reported that low levels of tumor suppressor genes, such as PH Domain and Leucine Rich Repeat Protein Phosphatase 1 (PHLPP-1), are present in colitis and colon cancer patients[1]. However, it is unclear whether the low PHLPP-1 expression found in host under chronic inflammatory state is beneficial to patients with colitis or colon cancer. Interestingly, CO-IP screening of Tollip-interacting molecules show dichotomy expression of PHLPP.

PHLPP-1 inhibits a number of cell signaling pathways, one of which is MEK/ERK pathway that promotes cell proliferation[2]. Another pathway PHLPP-1 is involved in is Akt signaling, which is involved in cell survival[3]. Non-specific suppression of all cell survival and proliferation and promotion of apoptosis, via inhibition of PKC signaling, may not be the best manner to protect host from chronically inflamed colon. Also, immune cells of IBD and colon cancer patients are found to be less competent in terms of their function compared to immune cells from healthy donors. In this paper, we utilize AOM, a known carcinogen, and DSS, a detergent, combination to recapitulate colitis and colon cancer in mice to study the effects of PHLPP-1 in colitis and tumorigenesis in colon. Low dose endotoxin is commonly found circulating in IBD, colitis, and colon cancer patients, while high dose endotoxin is found in septic patients. We also identify upregulated genes in neutrophils in presence of low dose and high dose LPS. Data from qRT-PCR

will allow us to compare the neutrophil phenotype in WT and PHLPP-1^{-/-} mice under different inflammatory state via levels of pro-inflammatory markers present.

METHODS

Mice

Wild type (WT) C57BL/6 and PHLPP-1^{-/-} mice were bred and maintained in the animal facility at Virginia Tech in accordance to approved Animal Care and Use Committee protocol. Mice were age (10-14 weeks of age at the time of use) and gender matched in each experiment.

Experimental design

WT and Tollip^{-/-} mice received a single intraperitoneal injection of azoxymethane (AOM, Sigma-Aldrich) at a dose of 10mg/kg body weight. A week after AOM injection, the mice were given three cycles of 2% dextran sulfate sodium salt (DSS, MP Biomedicals) for 5 days followed by 14 days of normal drinking water. After the last water cycle, mice were sacrificed and tissues were harvested for further analysis. Body weight, stool consistency, bleeding condition were measured as part of clinical score (score 0-4, with higher score corresponding to worse condition, see next page for detail).

Neutrophil Isolation

Bone marrow neutrophils were isolated and purified using 3 layer (82%, 65%, 55%) percoll (Sigma Aldrich) and NaCl (Sigma Aldrich) gradient.

qRT-PCR

In order to measure levels of surface markers on neutrophils, total RNA was extracted by TRIzol (FisherScientific) method and converted into cDNA using SSO Advanced SYBR green mix (BioRad). Primers are listed below:

β-actin:	F	5'-TGTGATGGTGGGAATGGGTCAG-3'
β-actin:	R	5-TTTGATGTCACGCACGATTTCC-3'
CD14:	F	5'-TGCAACTTCTCAGATCCGAAGCCA-3'
CD14:	R	5'-ACCGTAAGCCGCTTTAAGGACAGA-3'
FPR2:	F	5'-TCTACCATCTCCAGAGTTCTGTGG-3'
FPR2:	R	5'-TTACATCTACCACAATGTGAACTA-3'
CCR5:	F	5'-GCTGTAGGAATGAGAAGAAGAG-3'
CCR5:	R	5'-GGCATAGATGACAGGGTTTAG-3'

IBD Clinical Score Determination (0 Normal - 4 Poor condition)

Weight Grading <0-4>

- 0 No weight loss
- 1 Weight loss of 1—5%
- 2 Weight loss of 5.1—10%
- 3 Weight loss of 10.1—20%
- 4 Weight loss of more than 20%

Stool Grading <0-4>

- 0 Normal: dry and hard
- 1 Soft/Well formed: does not change round shape when pressed together
- 2 Soft/NOT well formed: round shape but mushy when pressed together
- 3 Pasty: came out long and gooey
- 4 Diarrhea: has no shape and does not come out clean (sticks to anus)

Bleeding Grading <0-4>

- 0 None
- 1 Occult bleeding: hint of blood
- 2 Visible bleeding: bright red (no question about the presence of blood)
- 3 Dark red: stool is soaked in blood
- 4 Gross bleeding: excess blood from anus

RESULTS

PHLPP-1 deficient mice present more severe clinical symptoms than WT mice when under AOM-DSS challenge.

PHLPP-1 has been identified as a tumor suppressor gene in a healthy individual. In order to determine whether PHLPP-1 can confer protection in colon in mice under chronic AOM-DSS challenge, we ran a parallel study to compare the clinical symptoms between WT and PHLPP-1^{-/-} mice. Not surprisingly, PHLPP-1^{-/-} mice fared slightly worse than WT mice in terms of stool consistency and bleeding from rectum (Figure 1). Interestingly, or perhaps due to their initial heavier weight compared to WT, PHLPP-1^{-/-} mice did not lose as much weight as WT mice over the course of the chronic AOM-DSS challenge.

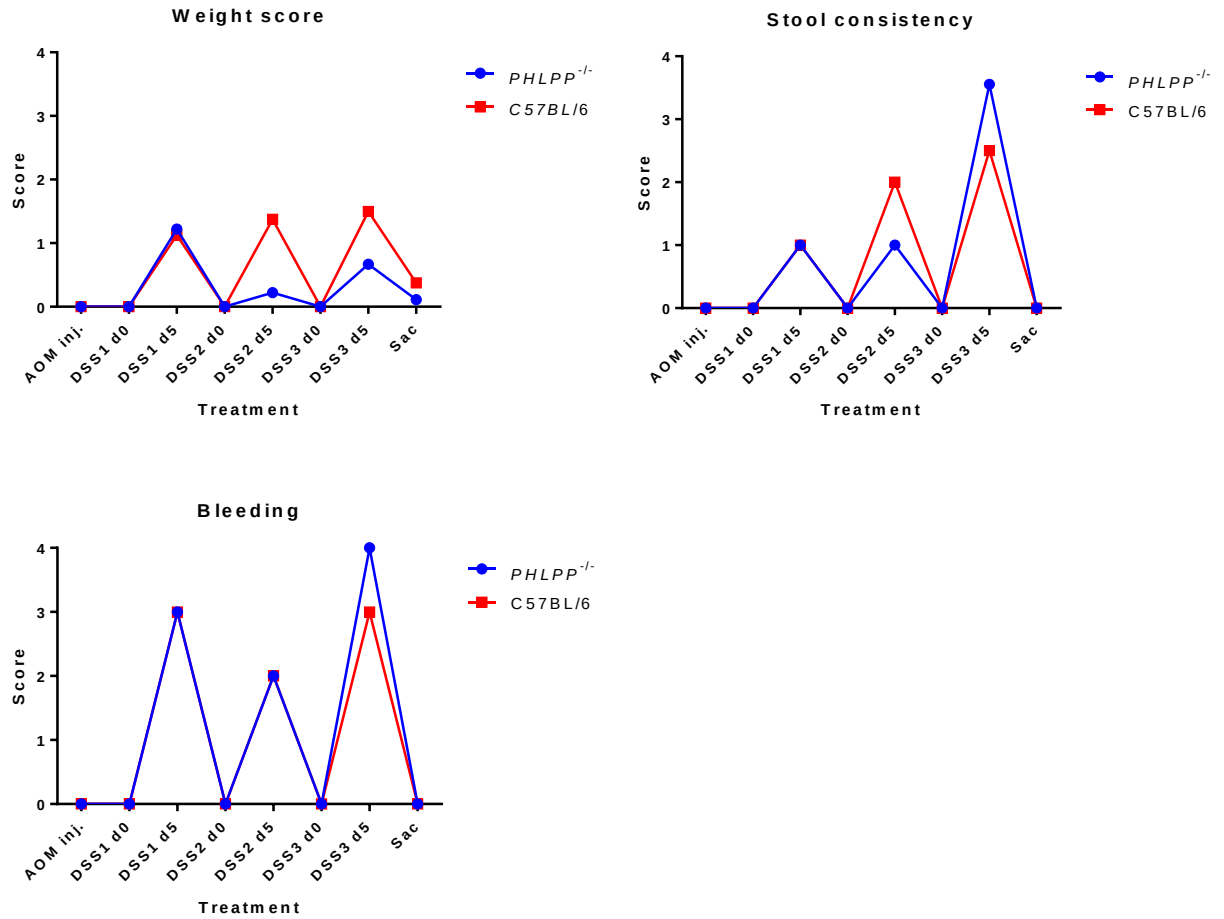


Figure 1. $PHLPP^{-/-}$ mice fare worse under chronic AOM-DSS challenge compared to WT. Weight change from day 0, stool consistency, and bleeding condition were recorded daily (Female; 10 weeks old at the start of experiment; WT, n=8; $PHLPP^{-/-}$, n=9).

PHLPP-1^{-/-} surpass in tumor growth suppression in mice colon under AOM-DSS.

Based on previous findings, it was believed that PHLPP conferred protection to host under chronic inflammatory state. Interestingly, we found no difference in colon length (Figure 2A) albeit greater bleeding and worse stool consistency found in the PHLPP-1^{-/-} mice (Figure 1). Upon examining the mice gut, we found significantly less polyp formation in PHLPP-1^{-/-} mice (Figure 2B). Although PHLPP-1 is a known tumor suppressor gene, it's role in immune cells is not well understood and may be detrimental to host in chronically inflamed condition.

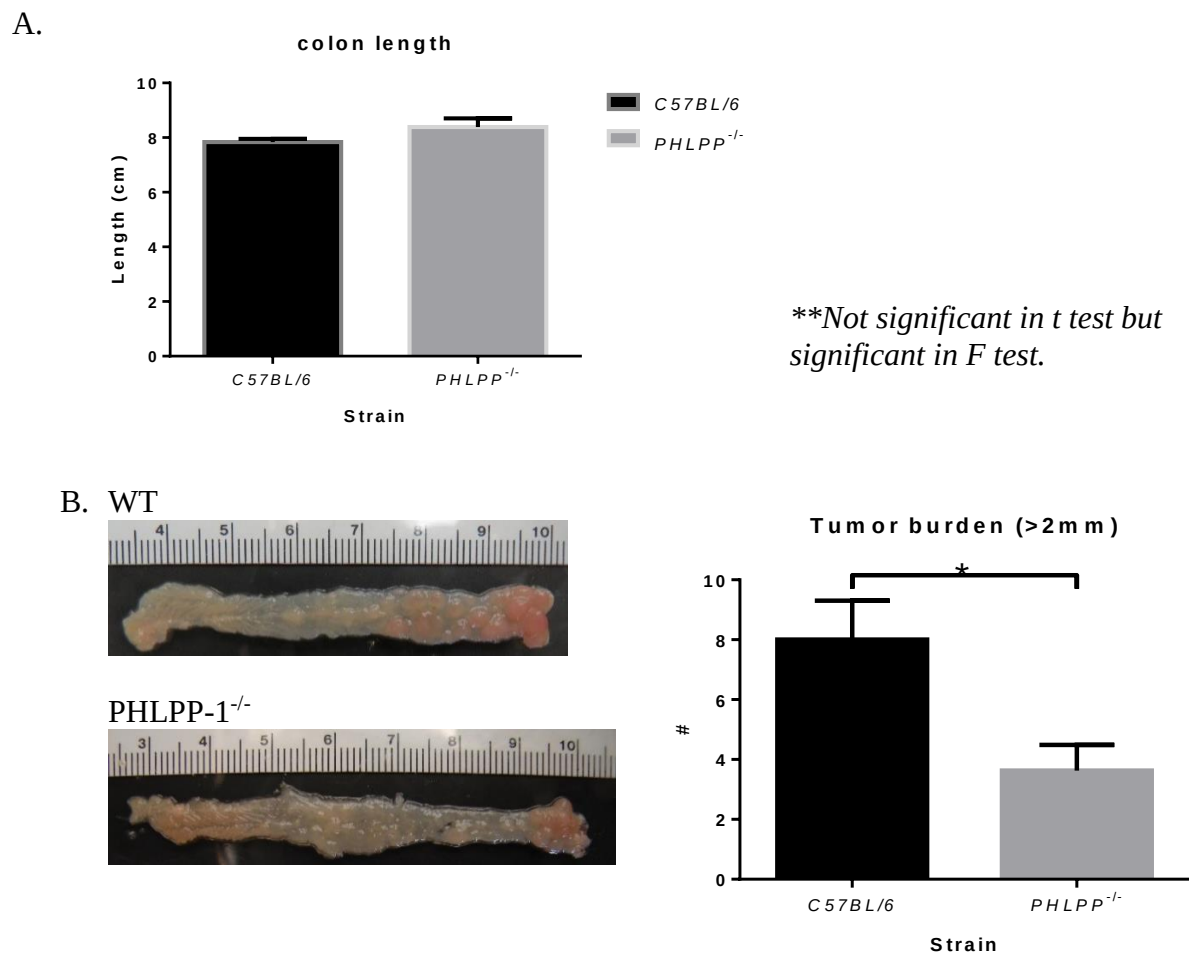


Figure 2. Physiological differences are found in gut after 64 days of AOM-DSS challenge between WT and PHLPP-1^{-/-} mice. Colon length and polyp (>2mm) number were measured (female; 10 weeks old at the start of experiment; WT, n=8; PHLPP-1^{-/-}, n=9).

Pro-inflammatory surface marker levels are enhanced in PHLPP-1^{-/-} neutrophils.

In chronic inflammation such as IBD and colon cancer, immune cells are thought to be primed with low grade endotoxin. In order to determine whether neutrophil surface markers support this finding, we proceeded with qRT-PCR of neutrophils, which are the major innate leukocytes in body. In order to mimic acute and chronic low grade inflammation, we stimulated neutrophils with 100pg/ml LPS and 10ug/ml LPS, respectively, in 0 hour and 72 hour culture and collected the cells for RT-PCR. Interesting, pro-inflammatory markers CD14, CCR5, and FPR2 were all upregulated in PHLPP-1^{-/-} neutrophils for super-low dose LPS but not in fresh or high-dose LPS (Figure 3). CD14 is a co- receptor for detection of bacterial LPS; CCR5 is a G-protein-coupled receptor on the surface of leukocytes, involved in receptor for chemokines in inflammation; and FPR2 is involved in chemotaxis mediating immune cell response to infection. Data indicate WT neutrophils have superior pro-inflammatory function than PHLPP-1^{-/-} neutrophils in healthy and highly inflamed settings such as sepsis, but perform less idealistically under chronic low grade inflammation. Immune cells from patients have shown to deliver less competent pro-inflammatory functions compared to immune cells collected from healthy donors.

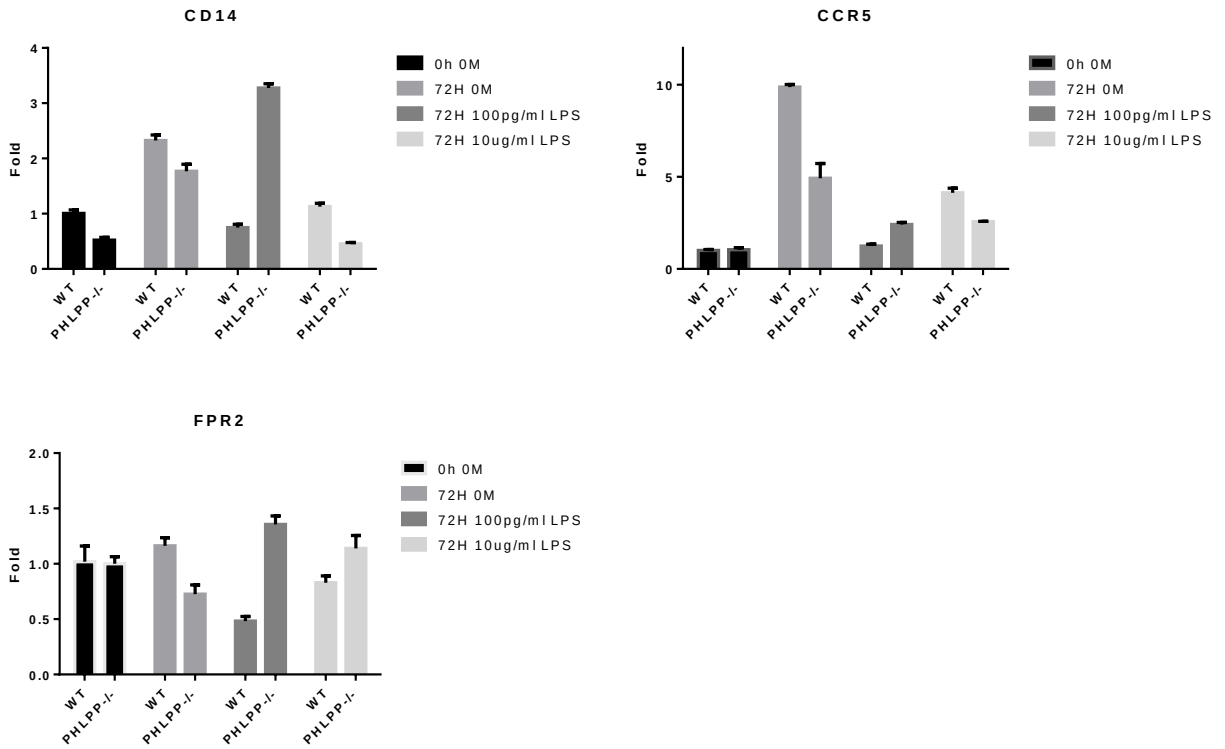


Figure 3. Enhanced neutrophil function found in *PHLPP*^{-/-} mice. qRT-PCR of neutrophils with or without LPS stimulation show differential expression of inflammatory profile in 72 hour culture.

DISCUSSION

Continued disruption in homeostasis in host gives rise to more complications in host health. However, the exact mechanism behind how unresolved IBD may lead to colon cancer is unclear. Toxicity and damage to host cells from a continuous pro-inflammatory state may give rise to increased mutation in host cell cycle. Immune cell may also be programmed to a state that is more harmful to host than beneficial under chronic inflammation. In addition, immune cells may become less competent from exhaustion in chronic inflammation in host. While PHLPP-1 is an essential tumor suppressor in a healthy individual, its role in different inflammatory diseases needs reassessment. Findings in this study suggest universal knockout of PHLPP in mice develop less polyp in gut under the chronic AOM-DSS challenge albeit worse clinical score. qRT-PCR results highlight the fact that the neutrophils from PHLPP deficient mice have higher levels of CD14, CCR5, and FPR2 compared to WT mice in chronic AOM-DSS study. This translates to a better pro-inflammatory function in PHLPP-1^{-/-} neutrophils than WT neutrophil when super low dose endotoxin are present in the system as often found in IBD and colon cancer patients. Further study is warranted to identify the cause of differences seen in PHLPP-1 function in different inflammatory states and ways to manipulate immune cells to better cater to host needs.

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CONCLUSION AND FUTURE DIRECTION

Colorectal cancer (CRC) is the third most prominent cancer in both men and women with more than 200,000 cases in the US each year. It leads to weight loss, diarrhea, and bleeding from the gut in advanced stage patients. Common therapies include radiation therapy and surgery. However, the recurrence and metastasis rate remains high. There is no cure as of today but ways to manage symptoms life-long. Numerous factors have been associated to cause CRC, including non-resolving inflammation. When inflammation persists, it can lead to tissue damage and gene toxicity that gives rise to mutated cells, leading to cancer. Low-grade inflammation may “prime” immune cells to confer exaggerated response in following attack, further exacerbating the tissue. On the other hand, premature switching of pro-inflammatory immune cells to pro-resolving immune cells provide cancer cells with means to evade immune surveillance, maintain uncontrolled growth, and proliferate beyond original establishment. Cancer cells may be utilizing some or all of these tactics to their benefit. Thus it is paramount to first identify the root cause of non-resolving inflammation and find ways to ascertain cancer cells do not “pinch”/grab-hold-of any opportunity to proliferate in host. (“pinch” referring to “crab-like” character of cancer where the name originates.)

Neutrophils are one of the first responders to invading pathogens and make up more than half of all leukocytes in a healthy individual. However, due to their transient nature, not much has been established about the cells. Other than their phagocytic and antimicrobial enzyme releasing activities, research has only recently uncovered more dynamic nature of the cells. Adoptive Transfer with specific knockout or modified neutrophils in chronic AOM DSS model revealed a dramatic difference in final outcome among mice that received the placebo, the WT neutrophil control group, and the experimental group. The number of macropolyp and micropolyp were

significantly reduced in the experimental groups: Tollip^{-/-}, IRAK-M^{-/-}, PHLPP-1^{-/-} mice, and WT mice that received super low dose LPS stimulated neutrophils. Clinical score and H&E staining of mice gut supports protective influence of modified neutrophils under mimicked colitis model. Future studies may involve determining the optimal concentration of neutrophils and timing for the adoptive transfer to be therapeutic and/or preventative in human.

Based on our findings, we have reasons to believe that signaling proteins, such as Toll-interacting-protein (Tollip), IL-1R-associated kinase M (IRAK-M), and PH Domain and Leucine Rich Repeat Protein Phosphatase 1 (PHLPP-1) may be major contributors in the cell polarization and heterogeneity. Tollip binds to either TLR or IL-1r and co-activate with MyD88 to suppress NF-kB and TGF-B signaling pathways during inflammation. IRAK-M binds to IRAK and suppresses NF-kB signaling pathway. As a result, Tollip and IRAK-M deficient mice have shown to have dysregulated inflammatory cytokine production in response to IL-1b and lipopolysaccharide (LPS). Our *in vivo* data suggests, with impaired Tollip or IRAK-M production, neutrophils may stay in a phenotype that is more beneficial to the host in regulating colorectal cancer development. Cancer cells may lose their advantage in polarizing neutrophils to clear additional neutrophils and switch immune cells from pro-inflammatory to pro-resolving state with Tollip and IRAK-M deficiency. PHLPP-1 is an important tumor suppressor gene that regulates cell proliferation and survival, and promotes apoptosis. Our *in vitro* study shows that PHLPP-1^{-/-} neutrophils have enhanced upregulation in pro-inflammatory genes under a low grade inflammation setting compared to WT neutrophils. These molecules have never been associated with the cell polarization and may greatly influence future research in neutrophils.

High neutrophil levels have been noted and associated with *both* better and worse outcomes in cancer patients. This may be explained by the plasticity we found in neutrophils in *in vitro* assay.

When naïve neutrophils were co-cultured with cell lines, there were significant increase in cell line proliferation rate. However, when neutrophils were stimulated with GM-CSF and/or super low dose LPS for an extended time, co-culture study shows that the cell line proliferation was further restrained. Moreover, Tollip deficiency further enhanced suppression in cell line growth. Our data indicates that neutrophils are capable of alternative differentiations based on signaling they receive from their microenvironment and have both pro- and anti- inflammatory phenotype, and possibly more subsets. The type of signaling molecules and duration of exposure may be further refined in future research for optimal outcomes.

In addition, there is limited information with regard to neutrophil direct communication with cancer cells in current literature. We found that with supernatant from neutrophils and transwell that physically separated neutrophils from cell lines were enough to confer inhibition in cell line growth in *in vitro* assay. The result suggests major neutrophil-cancer communication may occur via soluble mediators secreted in the microenvironment. We focus our attention on the higher miR-34a level found in Tollip^{-/-} neutrophils compared to WT neutrophils. MiR-34a is one of the best studied miRNAs and have been shown to be downregulated in many cancers. Addition of miR-34a inhibitor to co-culture assay diminished the enhanced suppression capability found in Tollip^{-/-} neutrophils, which lead us to believe miR-34a is responsible, if partially, for neutrophil suppression in cell line growth. We need further study to identify the exact association behind Tollip signaling pathway and miR-34a.

The findings in this study have the potential to dramatically change the original view of transient and limited nature of neutrophils since data implies that neutrophils can also confer long term effects and be harnessed for therapeutic means. Furthermore, modifying miR-34 generation and production offer multiple points of entry to intervene for future immune cell-based cancer therapy.

Neutrophil modulation in the host may render phenomenal value to medicine previously unexplored.