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Insights into the mechanism and activation of nonclassical monocyte patrolling in murine models of atherosclerosis and metastatic melanoma

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Paola Marie Marcovecchio is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
ApoE	apolipoprotein E
BHT	butylated hydroxytoluene
BM	bone marrow
BMT	bone marrow transplant
BrdU	bromodeoxyuridine
CAD	coronary artery disease
CDP	common dendritic cell progenitor
CM or C	classical monocytes
cMoP	common monocyte progenitor
DAP12	DNAX Activating Protein of 12KDa
DC	dendritic cell
EDTA	ethylenediaminetetraacetate
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
FOV	field of view
GFP	green flourescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
gMFI or MFI	geometric mean fluorescence intensity or median fluorescent intensity
GPCR	G-coupled protein receptor
ICAM	intercellular adhesion molecule
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
i.v.	intravascular
i.p.	intraperitoneal
K-KO	Kindlin-3 knockout
KO	knockout
LFA-1	lymphoyte function antigen-1
M-CSF	macrophage-colony stimulating factor
MDP	macrophage/dendritic cell progenitor
mmLDL	minimally modified low-density lipoprotein
MPS	mononuclear phagocyte system
Msr1	macrophage scavenger receptor 1
NCM or NC	nonclassical monocytes
NK	natural killer cell
nLDL	native low-density lipoprotein
OxLDL	oxidized low-density lipoprotein
PBS	phosphate buffered saline

poly I:C, pIC	poly inosinic acid:cytidylic acid
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RBC	red blood cell
RFP	red fluorescent protein
SD	standard deviation
SEM	standard error of means
SFK	src family kinase
SR-A	scavenger receptor-class A
Syk	spleen tyrosine kinase
TLR	toll-like receptor
TNF	tumor necrosis factor
t-SNE	t-distributed stochastic neighbor embedding
VCAM	vascular cell adhesion molecule
VLA-4	very late antigen-4
VLDL	very low-density lipoprotein
WD	western diet
WT	wild-type

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FIELDS OF STUDY

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ABSTRACT OF THE DISSERTATION

Insights into the mechanism and activation of nonclassical monocyte patrolling in murine models of atherosclerosis and metastatic melanoma

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2018

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Patrolling is a unique surveillance phenotype carried out by a subset of monocytes (nonclassical; Ly6C- mouse or CD16+ human) on the endothelium of blood vessels and interstitium. It is characterized by slow crawling for long periods of time under high shear stress or even against blood flow through the interactions of LFA-1 integrin and its ligands ICAM-1 and ICAM-2. Additionally, patrolling does not necessarily terminate with extravasation into local

tissues as other immune cells do after short-term crawling in response to damage or infection. These patrolling monocytes have high levels of the $G\alpha_i$ chemokine receptor CX3CR1, which is necessary for their homeostasis in the periphery. The CX_3CR1 ligand, fractalkine (CX_3CL1), also helps to signal nonclassical patrolling monocytes to the endothelium and to tissues, but is not necessarily required to execute the patrolling behavior. This suggests that whatever extracellular signals are activating the patrolling mechanism in nonclassical monocytes, it is distinct from homing and homeostatic maintenance of these monocytes. When this monocyte subset was first defined, it was thought that their sole purpose was as an accessory cell to the endothelium, scavenging endothelial debris and resolving vascular inflammation in coordination with other innate immune cells. Subsequent reports have focused on recruitment of nonclassical monocytes to tissues, especially in the context of sterile wound healing and inflammatory diseases, but few studies have looked at the mechanism of patrolling, what induces that particular behavior in the context of a disease, and what the functional consequences are of the patrolling phenotype. In order to begin to address these questions, we employed intravital microscopy, fluorescence-activated cell sorting, RNA sequencing and dimensionality reduction algorithms. The aim of this thesis is to understand what activates patrolling in the setting of atherosclerosis and metastatic cancer and how patrolling contributes to the function of nonclassical monocytes in these disease states. The following studies elucidate the ability of scavenger receptors to induce patrolling by nonclassical monocytes and the intracellular proteins that contribute to mediating the patrolling behavior in two murine models of vascular inflammation: atherosclerosis and metastatic cancer.

INTRODUCTION

Monocyte development and differentiation

Monocytes have been reported in the literature for well over a hundred years. Exudate studies of rabbits, rodents and human tissue yielded evidence of phagocytic mononuclear cells capable of taking up pathogens and debris (Murray et al., 1926; Ebert and Florey, 1939). The experiments published by van Furth and Cohn in the 1930s are often cited as the definitive papers on the mononuclear phagocyte system (MPS; van Furth and Cohn, 1968; van Furth et al., 1972). However, this system of promonocytes in the bone marrow → monocytes in circulation → macrophages in tissue has been rewritten in the past 30 years. A more detailed and accurate picture of monocyte development has been achieved with sophisticated mouse models, confocal microscopy, RNA and transcriptome sequencing, and high dimensional analysis of cell surface markers. During this time, researchers have been able to investigate the complexities of monocyte lineage and function in the periphery in vivo.

Transcriptional control of monocyte lineage

The lineage and differentiation of monocytes is a major focus of immune research. The ability to identify and isolate progenitor cells that give rise to unique and distinct subsets via flow cytometry allows for more refined immunological questions about development and disease. Monocyte differentiation from hematopoietic stem cells takes place in the bone marrow through a stepwise process of progressively more committed progenitors until they become one of two major subsets: classical and nonclassical (Fogg et al.,, 2006; Auffray et al., 2009; Hettinger et al., 2013). Both of these subsets, as well as their progenitors, rely on colony stimulating factor 1 (Csf-1) for survival (Dai et al., 2002). The human counterpart of the previously identified mouse

common monocyte progenitor (cMoP), which gives rise to Ly6C^{hi} and possibly Ly6C^{lo} monocytes, was recently found using CLEC12A and CD64 markers (Kawamura et al., 2017). This unipotent progenitor differentiated into both CD14^{+} and CD16^{+} monocytes. However, these human cMoPs also expressed CD135, unlike mouse cMoPs. Functional assays showed that these cells made for poor antigen presenting cells (APCs), secreted inflammatory cytokines in response to LPS and interferon stimulation, and could phagocytose dextran particles equal to peripheral blood monocytes.

IRF8 and KLF4 transcription factor interactions are crucial for classical (Ly6C^{+}CCR2^{+}) monocyte development from the common monocyte progenitor (cMoP) as well as to prevent neutrophil differentiation from the monocyte-dendritic cell progenitor (MDP)(Kurotaki et al., 2013, 2014), but dispensable for maintenance in the periphery (Sichien et al., 2016). Further downstream, the nuclear receptor Nr4a1 has been shown to control the differentiation of nonclassical (Ly6C^{lo}CCR2^{-}) monocytes in the periphery and bone marrow (Hanna et al., 2011). These studies in mice and human tissue begin to illuminate the heterogeneity of myeloid progenitors and the intricate transcriptional programming necessary to generate a myriad of monocyte subsets.

There is now substantial evidence that Ly6C^{lo} monocytes arise from Ly6C^{hi} monocytes in the periphery (Patel et al., 2017; Yona et al., 2013; Varol et al., 2009, 2007). Recently, however, Satoh et al demonstrated that a separate Ly6C^{lo} monocyte subset precursor, SMP (SatM precursor), arises from GMPs in the bone marrow and differentiates into segregated- nucleus-containing atypical monocytes (SatM) by the transcription factor C/EBPβ (Satoh et al., 2017). These Ceacam1^{+}Msr1^{+}Ly6C^{-}F4/80^{-}Mac1^{+} monocytes, which were found in the lung, are prominent during the fibrotic phase of bleomycin-treated mice and contributed indirectly to the

initiation of fibrosis through increased levels of TGF-β and Spp1. What is particularly interesting about this finding is that Ceacam1 is expressed on these cells since it is typically a marker associated with granulocytes. Mildner et al picks up where Satoh et al leaves off, by examining the peripheral blood monocyte subsets in C/EBPβ-deficient mice(Mildner et al., 2017). While MDP and cMoP cells in the bone marrow are relatively unaffected, more mature monocyte subsets were drastically reduced upon ablation of C/EBPβ, with the greatest effect seen in the $Ly6C^{lo}$ nonclassical subset, suggesting an interaction with Nr4a1. The authors showed that while C/EBPβ could bind to a part of the Nr4a1 promoter region, there is still the possibility of independent as well as dependent interactions between the two transcription factors for control over $Ly6C^{lo}$ differentiation, with possible overlap by other C/EBP members. Lastly, PU.1 transcription factor levels have been shown to dynamically regulate conversion of $Ly6C^{hi}$ monocytes to highly varied myeloid effector subsets (Menezes et al., 2016). With the clever use of mouse models, researchers showed a dose-dependent effect of PU.1 on monocyte differentiation into inflammatory iNOS-producing macrophages, or a more DC-like $PD\text{-}L2^{+}$ $209a^{+}$ subset, upon stimulation with LPS or GM-CSF (Menezes et al., 2016).

Gene regulation of nonclassical monocytes has expanded beyond transcription factor binding. An enhancer region upstream of Nr4a1 gene has been shown to be critical to induce Nr4a1 expression through KLF2 binding in order to generate $Ly6C^{lo}$ monocytes (Thomas et al., 2016). Deletion of the E2 super-enhancer region upstream of Nr4a1 proved essential for maintaining $Ly6C^{-}MHCII^{-}$ subsets specifically without affecting macrophage polarization or the lymphoid compartment. This region is also found upstream of human $CD16^{+}$ monocytes, showing evolutionary conservation between mouse and human. The exact specificity of this

deletion to generate a Ly6C^{lo} monocyte mouse model will prove useful to dissect the exact role of nonclassical monocytes in disease states.

Fate, longevity and survival in the periphery

Thus far it has been shown that monocytes exist as 2 major subsets in bone marrow, which egress into the periphery through the vasculature. For classical monocytes, CCR2 is the crucial receptor responsible for their migration out of bone marrow is CCR2 following the binding of CCL2 (Serbina and Pamer, 2006). For nonclassical monocytes, the receptor necessary for their migration out of the bone marrow appears to be an S1P receptor, S1PR5 (Debien et al., 2013). S1PR5$^{-/-}$ mice lacked Ly6C^{lo} monocytes in the periphery but maintained normal numbers in bone marrow. Interestingly, S1P disruption did not alter migration of Ly6C^{lo} monocytes, nor did it decrease Ly6C^{lo} survival in the periphery, suggesting that S1PR5 acts through an unidentified ligand. Although both monocyte subsets express the fractalkine receptor (CX_3CR1), it appears that nonclassical monocytes particularly depend on CX_3CL1 while in circulation for homeostasis and survival (Panek et al., 2015; Tanaka et al., 2005; Landsman et al., 2009).

As mentioned before, there is a paradigm that nonclassical monocytes originate from Ly6C^{hi} monocytes in the circulation, or return to the bone marrow to differentiate into Ly6C^{lo} monocytes (Sunderkotter et al., 2004; Patel et al., 2017; Varol et al., 2007). New evidence has emerged about the survival of monocytes once they egress out of the bone marrow. Within vascular niches of mouse bone marrow and the marginal zone of spleen,Ly6C^{hi} monocytes bind to endothelial cell ligand DLL1 via the Notch2 receptor and induce conversion to Ly6C^{lo} monocytes (Gamrekelashvili et al., 2016). Adoptive transfers of Notch2$^{-/-}$ Ly6C^{hi} monocytes show reduced conversion to Ly6C^{lo} monocytes, while in vitro co-culture of Ly6C^{hi} monocytes

with DLL1 ligand shows increased conversion to Ly6C^{lo} monocytes. However, it remains to be seen if this Notch2-DLL1 axis can be manipulated in vivo to yield higher levels of Ly6C^{lo} monocytes, as this would present a beneficial tool for studying effects of Ly6C^{lo} monocytes populations in various diseases. Additionally, conversion of classical monocytes to Ly6C^{lo} patrolling monocytes during bacterial infections can be induced by NOD2 receptors (Lessard et al., 2017). These receptors bind to fragments of bacterial peptidoglycans, which can then increase intermediate and nonclassical monocyte populations for both human and mouse monocyte cultures, although the mechanism is not clear. When thioglycolate was used to induce an inflammatory response, activation of NOD2 receptors through binding of muramyl dipeptide (MDP) increased the nonclassical monocyte population and decreased markers of inflammation such as IL-6 and TNFa. Most interesting was the ability of MDP to elicit an increase in Ly6C^{lo} monocytes in Nr4a1$^{-/-}$ mice, suggesting either the Ly6C^{lo} monocyte population is genetically diverse, or that another key transcription factor is involved with nonclassical monocyte differentiation. This work demonstrates a potential in vivo mechanism for mouse and human monocyte subset regulation.

It has been shown in both human and mouse that monocytes require fractalkine (CX_3CL1) for survival through binding of its cognate receptor CX_3CR1 on monocytes by inhibiting apoptotic pathways (Landsman et al., 2009; White et al., 2014). More recently, tumor necrosis factor (TNF) has been shown to play a crucial role in regulating monocyte survival and function in the periphery. In an adoptive transfer with wild-type and TNF receptor 1 or 2 (TNFR1 or TNFR2) knockout monocytes, only monocytes with TNF receptors showed an ability to persist in the periphery by signalling through autonomous TNF production (Wolf et al., 2017).

By 2 days post transfer, these deficient monocytes had increased levels of apoptosis as measured by annexin V and propidium iodide staining with little effect on monocyte progenitors.

Investigating human monocytes in vivo presents a significant challenge that often leads to ex vivo or in vitro experiments. However, in an elegant study by Patel et al, the kinetics of peripheral human monocytes in steady-state and during inflammation were quantified using in vivo deuterium glucose labeling of human subjects (Patel et al., 2017). All three monocyte subsets, classical ($CD14^+$), intermediate ($CD14^+CD16^+$), and nonclassical ($CD16^+$), displayed similar generation kinetics to those observed in rodent studies: bone marrow → classical monocytes → intermediate monocytes → nonclassical monocytes, with a post-mitotic bone marrow residence of ~1.6 days and circulation for 1 day (classical), 3 days (intermediate), and 7 days (nonclassical). When challenged with endotoxemia, human monocytes were depleted from circulation and repopulated by classical monocytes released from the bone marrow, thus recapitulating mouse studies and establishing kinetic generation of monocytes into the periphery.

In the past 18 years, it has become increasingly clear that monocytes constitute a variety of subsets and can survey tissue during homeostasis as well as respond to infection, inflammation and cancer (Serbina et al., 2009; Hanna et al., 2015a; Jakubzick et al., 2013; Auffray et al., 2007; Shi and Pamer, 2011). Both subsets possess unique effector functions and differentiation capacity to replenish macrophages and dendritic cell (DC) populations in certain tissues such as lung, gut, heart, spleen and skin (Jakubzick et al., 2008; Randolph et al., 1999; Swirski et al., 2009; Tacke et al., 2006; Yona et al., 2013; Patel et al., 2017). Depending on the context, i.e. infection, autoimmunity, wound healing, etc..., each subset has been reported to have inflammatory or anti-inflammatory properties, making their programming malleable to the microenvironment and type of stimulus (Bain et al., 2013; Rivollier et al., 2012; Mukherjee et al., 2015; Serbina et al., 2003; Olingy et al., 2017; Cros et al., 2010). One important study of note examined the diversity of

human blood myeloid cells on a single cell level (Villani et al., 2017). By investigating the RNA transcripts of individually sorted blood cells, coupled with unbiased gating algorithms, researchers were able to break down traditional classification of monocytes, dendritic cells and hematopoietic progenitors in the circulation to refine their subtype. While the identification of monocytes and monocytic progenitors has funneled into more precise and narrow definitions of what cell type gives rise to peripheral monocytes, the heterogeneous phenotypes and functions of circulating monocytes has broadened well beyond simply being macrophage precursors.

The discussion of what monocytes do after receiving a signal depends heavily on the type of ligand:receptor interaction, the tissue involved, and the experimental conditions used to assess the outcome. There is some consensus in the literature that classical monocytes are less mature than nonclassical monocytes and can therefore differentiate into effector macrophages and dendritic cells when there is inflammation. Additionally, classical and nonclassical monocytes have different potential for becoming tissue APCs or phagocytes depending on the tissue as well. The literature for the differential capacity for differentiation between subsets is substantial enough that it exceeds the scope of this thesis. Suffice to say, the question of what nonclassical monocytes can differentiate into, especially in the context of atherosclerosis and cancer is still not well defined or explored.

Patrolling in the vasculature

Because monocytes are circulating cells, there is a question of whether crawling, homing/recruitment, and patrolling are of a similar mechanism. The study of leukocyte migration has been investigated and described for at least 100 years. At its most basic level, leukocytes circulate through the vasculature and when tissues become inflamed, or when leukocyte programming requires returning to a particular tissue, these circulating cells will enter into a

series of steps that culminates in extravasating out of the blood vessel. These steps can be collectively referred to as the leukocyte adhesion cascade. In recent years, the leukocyte adhesion cascade has been revised to reflect the growing understanding of mechanical and biochemical forces which enable the trafficking of leukocytes between the circulation and tissues (Ley et al., 2007). The following sections will examine what is known about cell motility and patrolling.

Canonical Model of Cell Motility

Rolling

Rolling is considered the first step in the leukocyte adhesion cascade. This is the process by which leukocytes utilize selectins, both on their own cell surface as well as on the endothelial cell surface, to tether themselves to the vascular wall in an attempt to "brake" or slow down before diapedesis. Selectins come in several flavors, with L-selectin being found on leukocytes, and E- and P-selectin endothelial cells. Ligands to these selectins include P-selectin glycosylated ligand-1 (PSGL-1) and CD44 (Rose et al., 2007). Nonclassical monocytes have little to no expression of CD62L, but some expression of CD44 and PSGL-1 or in some human nonclassical subsets, the carbohydrate modified version of PSGL-1, Slan (An et al., 2008; Hofer et al., 2015). Engagement of PSGL-1 to E-selectin can activate integrins through ITAM adapter proteins DAP12 and/or FcRγ and Src family kinases (Herter and Zarbock, 2013). In addition, integrins are capable of mediating rolling through intermediate-affinity conformation, such as α4β1, α4β7, and $\alpha_L\beta2$, which depend on the tissue and inflammatory state. Downstream of capture by leukocyte rolling, signaling can occur via two pathways: PLCγ2 or PI3Kγ. It is not clear whether patrolling requires rolling, or if monocytes can enter into patrolling directly from circulation.

Adhesion/Arrest

The next important step in leukocyte motility is firm adhesion. It is this step in the cascade where integrins play a vital role in mediating leukocyte recruitment to the endothelium and eventual extravasation. Integrins are heterodimeric cell surface proteins that have 8 promiscuous beta subunits binding to 18 alpha subunits, generating 24 distinct integrins (Chigaev and Sklar, 2012; Herter and Zarbock, 2013). Monocytes predominantly express β1 (i.e. $\alpha_4\beta1$, VLA-4) and β2 integrins (i.e. $\alpha_L\beta2$, LFA-1; $\alpha_M\beta2$, Mac-1). Integrins are often cell type and context dependent, making them analogous to a signature of a cell type or state. Integrins can be found in various conformations, which are indicative of their activation and affinity, from resting (bent, low-affinity) to activated (extended, high-affinity) with several states in between (Chigaev and Sklar, 2012). These changes are mediated by inside-out signaling, whereby G-coupled protein receptors (GPCRs) and adaptor proteins (such as RIAM and Rap1) initiate activation of high-affinity integrin conformation through phosphorylation and recruitment of adaptor proteins like talin, kindlin, and paxillin (Banno and Ginsberg, 2008; Rose et al., 2007; Rognoni et al., 2016). It is this high-affinity conformation which mediates firm adhesion to the endothelium, which can withstand the shear stress in the circulation and particularly large blood vessels so that leukocytes can eventually extravasate through an optimal path through the endothelium. Upon firm adhesion to the endothelium, integrins can also signal back into the cell via outside-in signaling, to initiate focal adhesions and clustering of integrins (Schmidt et al., 2013).

Crawling

Upon clustering of integrins and focal adhesions, cytoskeletal rearrangements occur to enable leukocyte movement along the endothelium. This is also mediated by integrin binding to

ligands ICAM and, in some cases, VCAM (Sumagin et al., 2010; Collison et al., 2015). As microscopy techniques for visualizing leukocyte crawling has improved significantly over the years, more accurate descriptions of leukocyte trafficking and kinetics have been reported. For example, using intravital microscopy of the cremaster muscle in mice and antibody blockade, researchers showed that neutrophils and monocytes have inherently different crawling patterns based on the use of LFA-1 (monocytes) or Mac-1 (predominantly neutrophils and also monocytes)(Sumagin et al., 2010). Specifically, LFA-1 allowed monocytes to travel further and for longer periods of time, whereas neutrophil crawling was characterized by short, faster bursts of endothelial crawling prior to extravasation. In another study using intravital imaging, blockade of CD11b (Mac-1) during TLR-induced inflammation did not interfere with patrolling monocytes on the endothelium (Imhof et al., 2016). Conversely, during homeostasis Mac-1 antibody blockade was able to knock off patrolling monocytes from the endothelium, suggesting that this integrin could play a role in homeostatic patrolling, at least in the mesenteric vein. Additionally, human monocyte subsets were passed over cultured endothelial cells with antibody blockade against ICAM-1, VCAM, and CX_3CL1 (Collison et al., 2015). Although these experiments were done in vitro, they showed possible modalities for human monocyte subsets, such that classical and nonclassical monocytes were able to patrol for longer periods of time compared to intermediate monocytes, and classical monocytes preferred macrovascular endothelial cell crawling whereas nonclassical and intermediate monocytes preferred microvascular endothelial cells. Lastly, nonclassical monocytes required the use of ICAM, VCAM and fractalkine, whereas classical monocytes only required ICAM. Additionally, intravital imaging of the internal carotid artery in Western diet fed mice showed that CX_3CR1-GFP+ cells were shown to utilize both LFA-1 and α_4 integrins for patrolling over atherosclerotic plaques (Quintar et al., 2017). These studies confirm the dependence of patrolling on LFA-1,

with possible disease or inflammation-dependent use of VLA-4.

The actual mechanism of crawling is complex and highly regulated, and involve rearrangement of the cytoskeleton in a rearward projection with intracellular recycling of integrins (Van Haastert and Devreotes, 2004; Takenawa and Suetsugu, 2007; Frame et al., 2002; Mattila and Lappalainen, 2008). The activation of integrins results in changes to the cell membrane and cytoskeleton. These morphological changes are classified as pseudopodia. At the leading edge of a migrating cell is the lamellipodia, which are broad, flat protrusions that depend on actin polymerization induced by Rho GTPase Rac1 and the formation of large protein complex called the adhesome. Another type of membrane protrusions used in cell motility is the filopodium, which is a long, thin protrusion, usually branching off of the lamellipodium and induced by another Rho GTPase CDC42. At the rear of a migrating cell is a thin trailing structure called a uropod. The sequential process for these changes in a cell about to undergo migration is pseudopod formation, polarization (leading edge and rear edge), and directional sensing, which is usually induced by a chemotactic gradient. Intracellularly, actin polymerization, as well as de-polymerization in a directional path, is crucial for leukocytes to migrate. Many scaffolding proteins and adaptor proteins come together to rearrange actin into filamentous actin (F-actin), such as the Wiskott-Aldrich syndrome protein (WASP), the WASP-family verprolin homologous protein (WAVE), ENA/VASP and Arp2/3, which culminates in the branching actin present in lamellipodia. The maturation of these complexes involve major structural proteins such as vinculin, paxillin, talin, and focal adhesion kinase (FAK) under the regulation of other proteins such as myosin II and Rho-associated protein kinase (ROCK). With the contraction and depolymerization of these structures, actin undergoes retrograde flow, which is necessary for the cell to propel forward. Understanding the complex and immense protein networks that regulate cell migration requires use of model organisms and in vitro culture, therefore much of the

mechanism for what regulates patrolling must be applied from non-monocyte cell experiments. However, intravital imaging has shown that nonclassical monocytes develop lamellipodia as well as uropods, therefor the action of forward migration is assumed to be similar if not exactly as reported by these in vitro experiments.

Transendothelial migration

Ultimately, leukocytes that have initiated the adhesion cascade will likely extravasate into tissues. At this point they have been captured on the endothelium, probably receiving a chemokine signal or some other receptor capable of inside-out signaling, and is moving to find a portal through which to extravasate (Ley et al., 2007). Most extravasation takes place in post-capillary venules with the exception being in the lung, where leukocytes can exit the bloodstream in capillaries that are smaller than the diameter of a leukocyte so the adhesion cascade doesn't come into play(Muller, 2016). Monocytes, with rare exception, are one of the few types of cells that can reverse migrate, as evidenced by a clever use of junctional adhesion molecule C (JAM-C) antibody blockade and human monocytes under shear flow over cultured endothelial cells (Bradfield et al., 2007). Using this in vitro system, researchers were able to show that monocytes could not reverse migrate back through the endothelial layer to the luminal side when JAM-C was functionally blocked, indicating this was a phenomenon that could occur normally with JAM-C. Extravasation can take place either between endothelial cells, or through endothelial cells, as long as two critical events happen: movement of VE-cadherin away from the site of transendothelial migration, and the enrichment of positive regulators of transendothelial migration such as PECAM, CD99, and JAMs (Bradfield et al., 2007; Muller, 2016). Extravasation through the endothelium, past the basement membrane and pericytes and into the interstitium of the tissue changes the activation of migrating leukocytes such as upregulation of

β1, β2, and β3 integrins (Ley et al., 2007) to enable matrix crawling in the interstitium. One interesting observation from several papers indicate that patrolling monocytes do not often extravasate into the tissue, even during inflammation, suggesting that patrolling is indeed a separate phenomenon from the leukocyte adhesion cascade (Imhof et al., 2016; Marcovecchio et al., 2017; Auffray et al., 2007).

Kindlin-3

The integrin adaptor protein Kindlin-3 was first shown in the 2000's to be the culprit behind leukocyte adhesion deficiency III (LAD-III), which was originally thought to be caused by CALDAGGEF1 (Harris et al., 2013). These patients suffer from severe bleeding, osteopetrosis and recurrent infection and inflammation due to defects in beta integrin activation in platelets and neutrophils (Harris et al., 2013; Meves et al., 2009). Kindlin-3 is the third isoform of the Kindlin family of focal adhesion proteins, almost exclusively expressed in hematopoietic cells, although some evidence suggests that endothelial cells have low expression of the Kindlin-3 gene, fermt3 (Rognoni et al., 2016). In the past decade, Kindlin proteins have been shown to be the missing link in the course of integrin activation, both in inside-out and outside-in signaling. Depending on the cell type under investigation, as well as whether the experiments were performed in vitro or in vivo, the Kindlin proteins can perform assistant duties to talin-mediated activation of integrins to achieve high affinity conformations, displace inhibitors keeping integrins in a low affinity bent conformation, recruit other adaptor or scaffolding proteins to assist with integrin activation, or increase avidity of integrin:ligand binding through clustering talin-activated integrins (Calderwood et al., 2013). Kindlin-3 is perhaps the least well studied of the Kindlins, and the newest to be identified as an integrin adaptor protein. Therefore, questions still remain about its involvement in various aspects of cell-

specific migration, and as of yet, no studies have shown whether Kindlin-3 is involved in patrolling.

Many of the recent studies on the role of Kindlin-3 in cell migration have investigated primary neutrophils, platelets and T cells, or monocytic cell lines, but not primary nonclassical monocytes. As such, the following data is suggestive of what could be happening within nonclassical monocytes during patrolling, but certainly requires testing. Several studies in platelets have confirmed that Kindlin-3 binds to the cytoplasmic tails of beta integrins to aid talin-mediated integrin activation (Moser et al., 2008) and clusters integrins after activation has occurred (Ye et al., 2013), although there is some discrepancy about whether Kindlin-3 is necessary for initial activation at the monomeric state. This leads to how Kindlin-3 is mediating conformational changes in monomeric integrins during activation. In a neutrophil study of LFA-1 activation, Kindlin-3 was found to be necessary for high-affinity conformation of LFA-1, whereas Talin-1 mediated the initial activation from bent (low affinity) to an extended conformation (intermediate affinity)(Lefort et al., 2012). Functionally, this translates to Kindlin-3 being able to stabilize talin-induced $\alpha_4\beta1$ activation and ligand binding under high shear flow in granulocytic/monocytic cell lines, but not through GPCR induced affinity regulation (Hyduk et al., 2011; Lu et al., 2016). In a series of T cell experiments, homing to lymph nodes or the central nervous system was impeded by Kindlin-3 deficiencies, and when endothelial cells express low levels of cell surface integrin ligands, cannot extravasate (Moretti et al., 2013; Morrison et al., 2013). However, when ligands are abundant, as during inflammation, T cells can enter inflamed tissues, illustrating that Kindlin-3 is not necessary for diapedesis (Moretti et al., 2013; Cohen et al., 2013). Lastly, T cell interactions with APCs were also not affected by Kindlin-3, suggesting that it is dispensable for cell-cell interactions when there is no high shear flow stress (Morrison et al., 2013).

Another series of experiments examined the role of Kinlind-3 in outside-in signaling. This is the process by which integrin:ligand binding induces signal transduction back down the integrin into the cell. Initially LFA-1 outside-in signaling was found to be mediated by Kindlin-3 through interactions with the scaffold protein RACK1 (receptor for activated C kinase 1) through the pleckstrin homology (PH) domain on Kindlin-3 (Feng et al., 2012). Again, the granulocytic cell line K562 was used, along with human T cell line SKW3 using FRET and immunoprecipitation. This same group found that Kindlin-3 also mediated outside-in signaling through Mac-1 integrin and is required for cell spreading/adhesion by detecting phosphorylation of Syk and Vav1, which utilized Rac1 and CDC42 downstream (Feng et al., 2012; Xue et al., 2013). Lastly, a recent study illustrated that Kindlin-3 was required for microglial, but not necessarily monocyte-derived macrophage, motility in vivo through both inside-out and outside-in signaling pathways (Meller et al., 2017).

Kindlin isoforms possess differences in their ability to interact with scaffolding proteins and adhesome complex proteins in the course of cytoskeletal rearrangement. For example, Kindlin-2 and Kindlin-3 do not equally bind the integrin-linked kinase (ILK) complex contained within focal adhesions (Meller et al., 2017; Huet-Calderwood et al., 2014). It was found that Kindlin-3 did not bind ILK and did not localize to focal adhesions in a model of β1 activation in fibroblasts. More recently, a putative interaction was reported between Kindlin-3 and clustered inositol phosphates (PtdIns(3,4,5)P_3) using the PH domain of Kindlin-3 as a mechanism for recruitment to activate integrins. Thus, the data suggests not only that Kindlin-3 has undefined roles in integrin activation that requires investigation, but also plays an extensive role in cell migration, regardless of cell-type, although how extensive a role depends on the specific cell and the environment. It is possible that Kindlin-3 would play an important role in patrolling in order to withstand the high shear stress of the circulation, and possibly for integrin clustering.

Activation of patrolling

Fractalkine

The use of this chemokine receptor appears to be controversial in the literature. In one instance, genetic knockin/knockout of the GFP gene into the CX_3CR1 site reduced patrolling in numbers as well as the path length (Auffray et al., 2007). However, the remaining patrolling cells maintained the same velocity. In another mouse model of patrolling, $CX_3CR1^{gfp/gfp}$ (knockin-knockout) mice were assayed for their ability to patrol in the presence of pertussis toxin (PT) or an antibody blocking CCN1, a extracellular matrix associated molecule that is secreted by endothelial cells (Imhof et al., 2016). This study suggests that chemokine signalling through CX_3CR1 is required for recruitment to the mesenteric endothelium, and in the absence of the receptor, patrolling is reduced in both steady-state as well as inflammatory conditions. However, the authors also showed through flow cytometry that the ligand for CX_3CR1 was not highly expressed on mesenteric vessels, raising the question of whether the patrolling observed was inside or outside of the circulation. More importantly, these data suggest a novel binding ligand for patrolling monocytes, CCN1, which when blocked, reduced the slow patrolling of Ly6C- monocytes. Interestingly, is was suggested that platelets play a role mediating patrolling through binding monocytes and recruiting them to the endothelium. This interaction was not dependent on CCN1. Several studies using human monocytes in a flow chamber over cultured endothelial cells showed that nonclassical monocytes required the presence of fractalkine, ICAM and VCAM in order to patrol, while classical monocytes only required ICAM (Collison et al., 2015), and in addition to fractalkine, stromal derived factor 1 (SDF-1, CXCL12) was required for transendothelial migration (Ancuta et al., 2003). These studies suggest that the recruitment of patrolling monocytes to the endothelium and extravasation requires fractalkine, especially during

inflammation. Only the first study, performed in mice, concluded that fractalkine was required for actual patrolling, a key distinction since it is not clear that patrolling is part of the leukocyte adhesion cascade.

Other studies have shown that the fractalkine receptor is not necessary for patrolling to occur. In one setting, fractalkine receptor was not required for patrolling monocytes to patrol the lumen off microvessels during focal necrosis of endothelial cells, but rather was required for extravasation into inflamed tissue (Carlin et al., 2013). Another facet of this study was that smaller vessels in the kidney and ear dermis displayed an order of magnitude more patrolling monocytes compared to peripheral larger blood vessels. In chapter 1 of this thesis, one experiment using $CX_3CR1^{gfp/gfp}$ mice in large blood vessels of the femoral vasculature showed that patrolling was unaltered compared to wild-type $CX_3CR1^{gfp/+}$ mice (Marcovecchio et al., 2017). Lastly, in a study of recruitment to developing plaques during atherosclerosis, researchers showed that recruitment and entrance into the plaque did not require CX_3CR1 by nonclassical monocytes (Tacke et al., 2007). Thus it seems that the use of fractalkine for patrolling versus recruitment of monocytes to the endothelium is still under debate. This also illustrates the lack of knowledge in the field about whether patrolling is subsequent to recruitment, or if patrolling is a process independent of the canonical leukocyte adhesion cascade.

TLRs

It has been shown that TLR7/8 agonist R848 (Resiquimod) induces patrolling in mesenteric, dermal, carotid, and kidney vasculature (Buscher et al., 2017). However, upon transfer of wild-type bone marrow into TLR7 knockout mice, patrolling was abrogated in capillaries of the skin and kidnsey and nonclassical monocytes could not recruit neutrophils in a model of sterile necrosis (Carlin et al., 2013). In another recent study, mice were treated

systemically with TLR agonists and intravital imaging revealed that TLRs 2, 3, 4, and 7/8 were able to induce meticulous patrolling of nonclassical monocytes on the mesenteric vein. One confounder in the experimental design is that endothelial cells including those in the mesentery, express many TLRs, so it is highly probable that these agonists were inducing inflammation on the vasculature as well as in other cells that could be secreting other factors that activated patrolling (Salvador et al., 2016). A BMT of TLR-specific knockout bone marrow into a wild-type recipient is required for obtaining more specific results before concluding that TLRs play a role in monocyte patrolling. It is possible that TLR signaling through MyD88 or TRIF induces patrolling, however as yet no in vivo experiment has shown such a mechanism upon careful examination of the data. Thus the contribution of TLR stimulation on patrolling induction remains an unresolved question. Studies looking at MyD88 or TRIF ablation would be useful for examining their role in patrolling.

Western Diet

Although many previous studies have looked at arrest of monocytes on inflamed endothelium during atherosclerosis, primarily using VLA-4 integrin (Dansky et al., 2001; Chan et al., 2001), only one study to date has looked at live imaging of patrolling monocytes on the surface of atheroma in carotid arteries. This is partly due to the high degree of difficulty in imaging a blood vessel so close to the beating heart, which contributes substantial artifactual motion which can only be overcome through the use of intravital live cell triggered imaging system (ILTIS)(McArdle et al., 2015; Quintar et al., 2017). By imaging large atherosclerotic vessels, researchers showed that a high fat diet such as a Western diet could induce patrolling along the endothelium, at even greater frequency than induction with R848 (Quintar et al., 2017). Although in this study, no conclusion could be drawn about whether patrolling monocytes were

entering the plaque or what the monocytes were doing to the endothelium, it showed for the first time that patrolling monocytes could withstand high shear stress in large blood vessels with significant increase during atherosclerosis.

Another study that contributed to the understanding of how nonclassical monocytes behave during atherosclerotic inflammation examined the use of receptors to migrate to and enter plaques (Tacke et al., 2007). Although this study did not directly examine patrolling by intravital imaging, the authors did show through the use of genetic knockout mice that only classical monocytes required the use of chemokine receptors CCR2, CCR5 and CX_3CR1, whereas nonclassical monocytes only required the use of CCR5 to enter plaques, albeit infrequently. With more advanced imaging techniques and widespread use of the $CX_3CR1^{gfp/+}$ mouse, it is anticipated that additional studies will emerge to define the trafficking of patrolling monocytes during atherosclerosis and chronic vascular inflammation.

Monocytes in Disease

The development and differentiation of monocytes serves as a basis for how monocytes behave during homeostasis; their progression through the bone marrow, exit into the periphery and what they are capable of becoming when there is a stimulus. However, monocytes come in many flavors and depending on the context of the disease, the response between subsets may be drastically different. Here we will discuss two diseases that contribute to vascular inflammation and the monocyte response to them. Previous work elucidated 2 important functions of nonclassical monocytes: anti-atherosclerotic and anti-tumoral activity (Hanna et al., 2015b, 2012). Without the presence of nonclassical monocytes, atheroma progression and tumor cell

metastasis were exacerbated. Two questions that arose from both of these studies was whether patrolling was affected by onset of the disease and whether patrolling was necessary for nonclassical monocyte function.

Atherosclerosis

Atherosclerosis is the development of lipid accumulation within the subendothelial layer of blood vessels that develop into fatty streaks (M et al., 2016). These fatty streaks can progress into fibrous, unstable lesions and ultimately rupture, causing fatal cardiovascular events. Monocytes and resident myeloid cells drive the progression of lipid accumulation, foam cell formation, and cell necrosis through dysfunctional inflammation resolution, yielding a chronic inflammatory state with a core of necrotic cells at the center of the lesion (Robbins et al., 2013; Zhu et al., 2009; Paulson et al., 2010; Dutta et al., 2012). This process can begin happening as early in life as 11-12 years of age with fibrous streaks seen at 15-30 years of age(M et al., 2016).

In order to study the pathogenesis of the disease, two key mouse models were developed for furthering our understanding of atherosclerosis: $ApoE^{-/-}$ and $Ldlr^{-/-}$. Bred to the plaque susceptible strain C57Bl6/J, each of these mouse models promotes atherosclerosis through increased circulating levels of low density lipoproteins (LDL) by either deleting the apoprotein necessary for cholesterol uptake ($apoe^{-/-}$) or by deleting the receptor necessary for removing cholesterol from the circulation ($ldlr^{-/-}$)(Getz and Reardon, 2016).

Two important receptors have been implicated in the development and progression of atheromata. Both are members of the scavenger receptor family and possess differential capacities for taking up lipid particles. Msr-1, or SR-A, is the archetypical class A receptor and can bind modified LDL (Platt and Gordon, 2001). CD36 is a member of class B receptors and preferentially binds oxidized phospholipids such as oxLDL, oxPS and fatty acids (FA)(Jay et al.,

2015). Genetic deletions of these receptors in mouse models of atherosclerosis show that lipid loading is significantly reduced in lesional macrophages, and lesions are less complex with fewer inflammatory markers, although not entirely eliminated (Kunjathoor et al., 2002; Manning-Tobin et al., 2009). Both mouse and human monocytes possess these receptors, although studying the effects of these receptors on monocytes during atherosclerosis is made difficult by the fact that receptor expression is switched between mouse and human subsets (Ingersoll M et al., 2010).

Monocyte recruitment to nascent plaques has been questioned, but substantial evidence indicates that monocytes do migrate and enter lesions, most of which has been shown in mice (Tacke et al., 2007; Herbin et al., 2016; Jaipersad et al., 2014a). In humans, there are mostly correlative studies examining the frequencies of monocyte subsets in patients with coronary artery disease (CAD). Although there is some discrepancy in the reporting, the majority of human peripheral blood monocyte studies indicate that the intermediate CD14+ CD16+ and/or the classical monocyte subsets correlate with severe peripheral artery disease and are predictive of severe carotid stenosis, intima media thickness and neovascularization (Wildgruber et al., 2016; Jaipersad et al., 2014b). All these measurements are hallmarks of unstable plaques and advanced atherosclerosis. When classical monocytes in peripheral blood of patients with CAD were examined by transcriptome analysis, one major upregulated gene was ARID5B (Liu et al., 2017). Transcription of this gene is associated with adipogenesis and smooth muscle cell proliferation, which the authors believe dysregulates immunometabolism in classical monocytes during atherosclerotic progression in humans, leading to chronic inflammation.

In terms of mobilizing monocytes to the endothelium or lesions during dyslipidemia, recent studies have shown that cholesterol affects monocyte endothelial rolling due to CD44 membrane distribution on monocytes (Saha et al., 2017). Monocytes that were depleted of intracellular cholesterol were able to roll in a smoother fashion with less stopping. This suggests

that the leukocyte adhesion cascade is dysfunctional during dyslipidemia, with monocytes more frequently arresting near plaques. Monocyte arrest on or near plaques appears to be mediated by VCAM upregulation on aortic plaques in mice, which contributes to foamy monocyte development (Foster et al., 2015; Gower et al., 2011; Wu et al., 2009; Xu et al., 2015). These studies not only confirm previous data implicating VLA-4 and VCAM binding in the recruitment of monocytes to nascent lesions, but that nonclassical monocytes might be the culprit cell early in the process. Additionally, very low density lipoproteins (VLDLs) can disrupt chemotaxis of migrating monocytes and cytoskeletal rearrangement through inhibition of RhoA signaling, although patrolling, phagocytosis and transendothelial migration did not seem to be affected in this study (Jackson et al., 2016). In a previous report by the same group, Ly6C- monocytes were induced to extravasate into surrounding tissues from the vasculature through CCL4 signaling, which then developed into CD68+ macrophages, during hypertriglyceridemia (Saja et al., 2015). Similarly in humans, LDL has been shown to increase chemokinesis, as opposed to directed chemotaxis, in activated monocytes during hypercholesterolemia through dysregulation of DUSP3/p38 MAPK (Tjaden et al., 2018).

Since it is thought that atherosclerosis is driven by dysregulation of inflammatory signals, researchers investigated the function of the NLRP3 inflammasome during dyslipidemia (Christ et al., 2018). NLRP3 knockout mice showed significantly less Western diet (WD) induced systemic inflammation, but more importantly, myeloid progenitors in mice fed a WD were irrevocably altered in their ability to respond to inname immune cues in which they were hyper-inflammatory. In the presence of cholesterol efflux transporters ABCA1 and ABCG1, atherosclerosis is milder than in $ABCA1^{-/-}ABCG1^{-/-}$ mice, less netosis by neutrophils is seen, and atherosclerotic plaque development is decreased (Westerterp et al., 2018). This suggests that cholesterol efflux helps to modulate the inflammatory response in innate immune cells, which

would otherwise be hyperactivated during a high fat diet feeding. Clearly there are mechanisms of monocyte involvement in atherosclerosis that have not been identified yet, with definite implications for therapeutic applications.

Cancer and Metastasis

The field of oncoimmunology is a vast and ever-increasing list of publications. This section will simply focus on the involvement of monocytes, in particular nonclassical monocytes, in metastatic cancer to the lungs.

Lungs are a common site for metastatic cancer to seed itself due to the amount of contact circulating cells have with lung tissue, as much as 100 m^2 of vascular surface (Weidle et al., 2016). In addition, circulating tumor cells often have upregulated different receptors on their cell surface, such that they are able to home to new tissues such as the lung (seed and soil hypothesis), while also being physically larger than the capillaries in the lung, which leads to seeding of the lung tissue by default since they are stuck there. Unfortunately, lung metastasis is often a determinant of mortality for most advanced cancers (Nguyen et al., 2009; Paku et al., 2017; Gómez-Cuadrado et al., 2017).

In studying tumor metastasis, many mouse models and cell lines have been generated (Gómez-Cuadrado et al., 2017). The simplest and perhaps most artificial model for investigating metastasis is intravenous injection of cultured tumor cell lines. Although this method bypasses the primary tumor epithelial to mesenchymal transition, it does allow rapid and consistent investigation of how immune cells respond to a controlled dose of tumor cells over a controlled period of time after initiating metastasis. B16F10 is a mouse melanoma cell line that has been passed through live mice to select for aggressive metastatic features. While this has many similarities with human melanoma, researchers have begun opting for more humanized mouse

models, or if possible, tissues obtained from patients through biopsy.

Monocytes have recently begun to take the spotlight in immunotherapy investigations. Although their role in primary tumor progression or regression is still unclear, their ability to rapidly home to metastatic cancers, especially in the lung, have been reported (Qian et al., 2011; Headley et al., 2016). Monocyte subsets can differentially affect the invading tumor cell by aiding extravasation through secretion of classical monocyte-derived vascular endothelial growth factor (VEGF), or through nonclassical monocyte recruitment of tumor killing cells such as NK cells to the metastatic niche (Plebanek et al., 2017). Recently, new evidence has suggested that Ly6C+ monocytes can differentiate into anti-tumoral CD103+ dendritic cells that are Batf3+ but co-express monocytic markers(Sharma et al., 2018). This profoundly altered the dogma in the myeloid field that CD103+ dendritic cells are independent from monocytic precursors, which also mediate anti-tumoral responses (Headley et al., 2016). In another seminal study done with human lung carcinoma and healthy lung biopsies, researchers showed that in early-stage lung tumors, CD16+ monocytes are excluded from the tumor site in progressive adenocarcinoma, with a concomitant decrease and impairment of NK cells (Lavin et al., 2017). More recently, a study examining patient responses to anti-PD-1 immunotherapy with metastatic melanoma showed that activated classical monocytes (CD33+ CD14+ CD16-) had higher amounts of migration and activation markers such as ICAM-1 and HLA-DR in responsive patients with increased long-term survival. This is similar to a study investigating nonclassical monocytes using anti-CTLA-4 immunotherapy, which reported a similar result (Lavin et al., 2017; Romano et al., 2015). Thus, the role of monocytes in promoting or repelling tumor metastases is dependent on the therapy and microenvironment for a given cancer type.

By understanding the mechanism of how patrolling is activated and what enables nonclassical monocytes to patrol, we can potentially target this behavior in diseases that would

benefit from increasing or abrogating this behavior. The first chapter will look at whether western-diet induced atherosclerosis affects nonclassical monocyte patrolling and what cellular components the patrolling mechanism uses. The second chapter will examine the role of an integrin adapter protein, Kindlin-3, in patrolling and whether deletion of this gene renders nonclassical monocytes non-functional. With this animal model we examine the effects of patrolling on lung endothelium and the consequences of non-functional nonclassical monocytes.

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CHAPTER 1

Scavenger receptor CD36 directs nonclassical monocyte patrolling along the endothelium during early atherogenesis.

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Abstract

Objective - Nonclassical monocytes function to maintain vascular homeostasis by crawling or patrolling along the vessel wall. This subset of monocytes responds to viruses, tumor cells, and other pathogens to aid in protection of the host. In this study, we wished to determine how early atherogenesis impacts nonclassical monocyte patrolling in the vasculature.

Approach and Results - To study the role of nonclassical monocytes in early atherogenesis, we quantified the patrolling behaviors of nonclassical monocytes in $ApoE^{-/-}$ and C57Bl/6J mice fed a Western diet. Using intravital imaging, we found that nonclassical monocytes from Western diet fed mice display a 4-fold increase in patrolling activity within large peripheral blood vessels. Both human and mouse nonclassical monocytes preferentially engulfed oxidized LDL in the vasculature, and we observed that oxidized LDL selectively induced nonclassical monocyte patrolling in vivo. Induction of patrolling during early atherogenesis required scavenger receptor CD36, as $CD36^{-/-}$ mice revealed a significant reduction in patrolling activity along the femoral vasculature. Mechanistically, we found that CD36-regulated patrolling was mediated by a Src family kinase (SFK) through DAP12 adaptor protein.

Conclusions - Our studies show a novel pathway for induction of nonclassical monocyte patrolling along the vascular wall during early atherogenesis. Mice fed a Western diet showed increased nonclassical monocyte patrolling activity with a concurrent increase in SFK phosphorylation. This patrolling activity was lost in the absence of either CD36 or Dap12. These data suggest that nonclassical monocytes function in an atheroprotective manner through sensing

and responding to oxidized lipoprotein moieties via scavenger receptor engagement during early atherogenesis.

Introduction

Monocytes circulate in blood as predominantly 2 subsets: $Ly6C^{+}CCR2^{high}$ and $Ly6C^{-}CX_3CR1^{high}$ in mice, and $CD14^{high}CD16^{dim}$ and $CD14^{dim}CD16^{high}$ in humans, respectively(Geissmann et al., 2003a). Key experiments by several laboratories have shown that nonclassical $Ly6C^{-}CX_3CR1^{high}$ monocytes (NCM) remain in circulation longer than classical $Ly6C^{+}CCR2^{high}$ monocytes (CM), aid in vascular maintenance, and migrate to the sites of plaque formation during atherosclerosis (Yona et al., 2013; Auffray et al., 2007a; Tacke et al., 2007). Although other leukocytes can bind to and crawl along the endothelium, NCM appear to play the primary role of sentinels by patrolling the endothelium over long distances, often traveling against directional blood flow. A previous study by our group found that NCM are regulated by the transcription factor Nr4a1, and with the deletion of this gene, $Ly6C^{-}$ monocytes are absent from blood and bone marrow (Hanna et al., 2011). $Nr4a1^{-/-}$ mice that were bred onto atherosclerosis-prone $Ldlr^{-/-}$ or $ApoE^{-/-}$ mice and fed a Western diet (WD) showed a 3-fold increase in atherosclerotic plaque formation and a greater inflammatory phenotype of macrophages in atherosclerotic lesions (Hanna et al., 2012; Hamers et al., 2012). This study suggests that NCM likely play a role in the resolution of vessel inflammation during the onset of atherosclerosis. However, the effect of an atherogenic diet on vascular patrolling of NCM has not been examined in detail.

During atherogenesis, LDLs circulate in the bloodstream and eventually enter the subendothelial layer of the vessel wall, where they become trapped and oxidized to form modified forms of LDL (Tabas et al., 2015). These modified forms of LDL, such as minimally-

modified LDL (mmLDL) and oxidized LDL (OxLDL), are considered immunogenic as well as atherogenic and can trigger leukocyte recruitment to the vascular wall (Tsimikas and Miller, 2011; Miller et al., 2011). Intimal cell proliferation and Ly6C$^+$ monocyte recruitment to nascent lesions are markedly increased after 2 weeks of WD-feeding(Zhu et al., 2009). Scavenger receptors such as SR-A (class A), and CD36 (class B), recognize and bind to specific oxidized lipid moieties present on OxLDL (Febbraio et al., 1999; Nicholson et al., 1995). SR-A appears to signal in conjunction with Toll-like receptors (TLRs), as well as with Src family kinases (SFK) to mediate cell adhesion, possibly through $G_{i/o}$ signalling pathways (Post, 2002; Nikolic et al., 2006; Yu et al., 2012; Miki et al., 1996). On the other hand, CD36 can utilize DAP12 and/or FcRɣ adaptor proteins to further signal to Syk and Src family kinases (SFK)(Heit et al., 2013; Stuart et al., 2007). As part of our goal to study NCM in early atherogenesis, we asked which key intracellular proteins are involved in the induction of NCM patrolling. The role of scavenger receptors and the intracellular signaling downstream of these receptors in NCM as they relate to patrolling has not been investigated.

Using a novel method of intravital imaging of the mouse femoral vasculature during atherogenesis, we show that NCM preferentially internalize OxLDL through a scavenger receptor-mediated process involving DAP12 and SFK that induces their patrolling along the endothelium.

Results

Increased Ly6C$^-$ nonclassical monocyte patrolling along large peripheral blood vessel endothelium during early atherogenesis.

We hypothesized that vascular inflammation during atherogenesis in large blood vessels would lead to changes in nonclassical monocyte (NCM) patrolling activity. In order to address this, we used an established a mouse model of early atherosclerosis by feeding a high fat

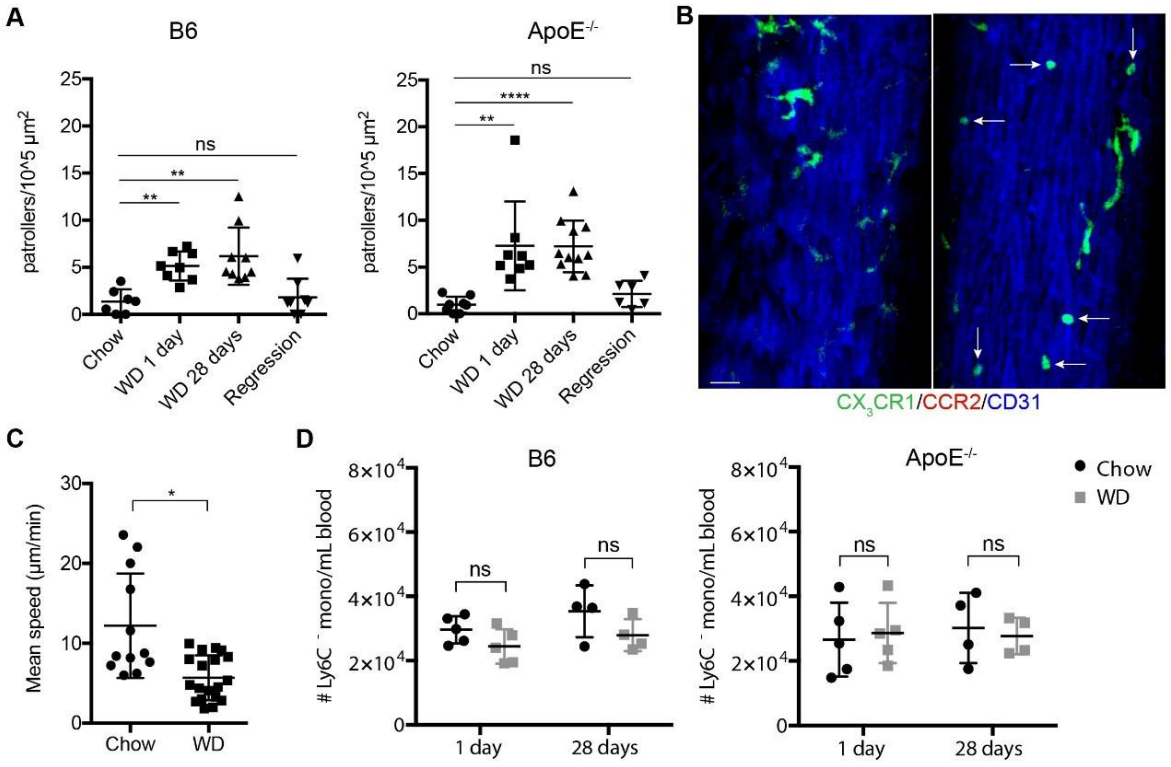


Figure 1.1 Nonclassical monocyte patrolling activity increases in large peripheral blood vessels of mice fed a Western Diet. Age and sex-matched ApoE$^{-/-}$ and B6 mice were fed a WD for the times indicated and assessed for patrolling activity and monocyte frequency A, The number of CX_3CR1^{high} patrolling monocytes per surface area of blood vessel was determined for each recording (n=3 videos per mouse). Each point represents the total number of patrolling monocytes per total surface area of blood vessel for each mouse. n=6-11 mice per group. A Kruskal-Wallis test with Dunn's multiple comparison correction was used to analyze WD and Regression groups using Chow as the control group. B, Representative images of patrolling monocytes (highlighted by white arrows) in large blood vessels of ApoE$^{-/-}$ mice: CX_3CR1 (Ly6C$^-$ NCM), CCR2 (Ly6C$^+$ CM) and injected with anti-CD31 (endothelium). scale bars: 30 µM C, Patrolling speeds for NCM from ApoE$^{-/-}$ mice fed a Western diet (28 days) or chow. D, NCM numbers in blood were obtained by flow cytometry from chow and WD-fed mice. n=4-5 mice per group. Error bars represent mean ± SEM for all graphs. *P < 0.05, **P < 0.01, ****P < 0.0001, ns indicates not significant.

Western diet (0.2% cholesterol, 42% calories from fat) to ApoE$^{-/-}$ mice or C57BL/6J (B6) mice for up to 28 days (Jongstra-Bilen et al., 2006; Paigen et al., 1987; Zhang et al., 1992; Plump et al., 1992). Age and sex-matched groups of B6 or ApoE$^{-/-}$ mice crossed to CX$_3$CR1$^{gfp/+}$CCR2$^{rfp/+}$ reporter mice were fed a WD for either 1 day or 28 days and imaged for patrolling activity of CX$_3$CR1^{high}CCR2^{low} monocytes using a novel femoral artery imaging method (described in Supplemental Figure I). These mice enable us to visualize both NCM (CX$_3$CR1$^{gfp/+}$) and classical monocytes (CM) (CCR2$^{rfp/+}$) by confocal microscopy as expression of CX$_3$CR1 is higher on NCM and expression of CCR2 is higher on CM (Geissmann et al., 2003b). NCM from WD-fed mice showed a significant 4-fold increase in patrolling activity along the vascular endothelium in vivo compared to NCM from chow-fed mice (Figure 1, A and B). This increase was observed even after one day of WD-feeding and is sustained through 4 weeks of WD-feeding. When either B6 or ApoE$^{-/-}$ mice were placed on WD for 2 weeks and subsequently put back on chow for an equal amount of time, the NCM showed reduced patrolling activity, almost reverting to the levels of pre-WD-feeding (Figure 1A, Regression). The overall speed monocyte patrolling was also affected by WD-feeding (Figure 1C), as chow-fed mice exhibited the expected average of 12 μm/minute (ranging from 6-24 μm/minute)(Auffray et al., 2007b), but NCM from WD-fed mice had reduced patrolling speeds, down to 5 μm/minute (ranging from 2-10 μm/minute). Additional characteristics such as track length, track displacement (distance from start point to end point of track), and confinement (displacement/length, 0 = circular track, 1 = straight track), were not significantly different (Supplemental Figure IIA).

Earlier studies have reported that ApoE$^{-/-}$ mice fed a WD develop monocytosis in the Ly6C^{+} compartment (Murphy et al., 2011; Tacke et al., 2007; Swirski et al., 2007). We wanted to confirm that the observed increase in patrolling activity was not due simply to increased numbers of NCM present in the blood during atherogenesis. We measured the frequency of Ly6C^{-}

monocytes in chow-fed mice and WD-fed mice by flow cytometry. In both B6 and ApoE$^{-/-}$ mice, we observed an increase in the frequency of classical Ly6C^{+} monocytes (Supplemental Figure IIB), but not Ly6C^{-} monocytes (Figure 1D). Additionally, we measured monocyte proliferation using BrdU incorporation. We found that Ly6C^{+} monocytes incorporated BrdU in 50-70% of cells by 48 hours in WD-fed mice compared to only 0.5-1% incorporation into Ly6C^{-} monocytes (Supplemental Figure IIC). Importantly, these data indicate that during early atherogenesis, the increase in patrolling of Ly6C^{-} monocytes on blood vessel walls is due to changes in activation of NCM and not the result of increased numbers of NCM circulating in the blood.

Sequencing analysis of nonclassical monocytes in early atherogenesis shows increased expression of genes involved in cell migration, cholesterol efflux, and vascular repair.

To determine if there were gene expression changes in monocyte subsets during atherogenesis that were related to functional changes in patrolling activity, we sorted CM and NCM from 8 week WD-fed mice and cohort chow-fed mice (gating strategy shown in Supplemental Figure IIIA) and performed RNA-seq analysis. Genes that are involved in cell migration (Rab GTPase activation, kinases, integrin activation, and lamellipodia components)(Stenmark, 2009; Rottner et al., 1999; Moser et al., 2008) and resolution of inflammation/vascular repair were more highly expressed in Ly6C^{-} monocytes from WD-fed mice than in Ly6C^{-} monocytes fed chow or in Ly6C^{+} monocytes (Figure 2A)(Michelucci et al., 2013).

Based on our earlier studies using global Nr4a1$^{-/-}$ mice (Hanna et al., 2012), we hypothesized that an absence of patrolling by Ly6C^{-} monocytes would increase atherosclerotic progression. We generated mice selectively lacking Ly6C^{-} monocytes (E2-def) with no

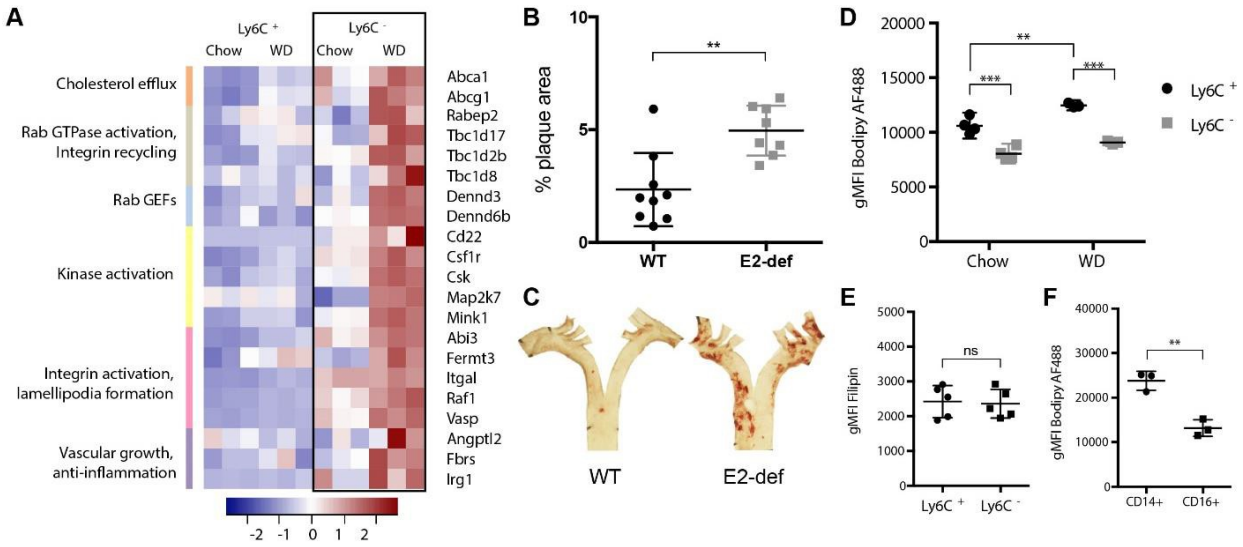


Figure 1.2 Nonclassical monocytes from WD-fed mice are atheroprotective. A, Differential gene expression of RNA transcripts between monocyte subsets of WD or chow-fed mice. Ly6C^{+} and Ly6C^{-} monocyte subsets were sorted from chow or WD-fed mice. Genes of interest related to lipid metabolism, cell migration, and inflammation resolution that were uniquely upregulated in nonclassical monocytes from WD-fed mice are listed. n=30 mice (3 groups of 10 mice pooled). Statistical significance of upregulated genes was determined by False Discovery Rate (FDR) correction (Benjamini-Hochburg) with filtering of variance outliers by tagwise dispersion (>99.9%), P>0.001. B, Comparison of atherosclerotic plaque formation in B6 or E2 mice by en face Oil Red O (ORO) staining of aortas from mice fed a 15-week Western diet. Quantification of plaque area as a percentage of total aortic area. n=8-9 mice per group. C, Representative images of aortic root ORO staining. D, Bodipy staining of neutral lipids in blood monocyte subsets from chow and WD-fed mice by flow cytometry. n=4 mice per group. E, Filipin III staining of free cholesterol in blood monocyte subsets from chow-fed mice. n=5 mice. F, Bodipy staining of neutral lipids of blood monocyte subsets from healthy human donors by FACS. n=3 individuals. Error bars represent means ± SEM. **P < 0.01, ***P < 0.001, ns indicates not significant.

observable changes in any other immune cell type in vivo(Thomas et al., 2016), and transplanted bone marrow from either WT or E2-def mice into $Ldlr^{-/-}$ recipients. After checking for successful bone marrow reconstitution (Supplemental Figure IIIB) and feeding a high cholesterol diet for 15 weeks, we assessed plaque formation by Oil Red O staining in WT-$Ldlr^{-/-}$ and E2-def-$Ldlr^{-/-}$ mice. We found a 2-fold increase in plaque formation in E2-deficient mice selectively lacking NCM (Figure 2B and 2C), indicating that nonclassical monocytes are functionally atheroprotective.

We next examined whether NCM showed differences in free or esterified cholesterol accumulation. Blood monocytes from mice and humans (gating strategies shown in Supplemental Figure IIIA and C, respectively) were stained with either Bodipy (identifies esterified cholesterol/lipid droplets) or filipin (identifies free cholesterol). Mouse NCM had lower cholesteryl ester content (Figure 2D) than CM, even after 4 weeks of WD feeding. We observed no changes in filipin staining (Figure 2E). Human nonclassical $CD16^{+}$ monocytes also had lower cholesteryl ester content than $CD14^{+}$ classical monocytes (Figure 2F). This correlates with our RNA-seq findings of increased mRNA expression of both of the key cholesterol transporters, ABCG1 and ABCA1, in NCM isolated from WD-fed mice (Figure 2A), suggesting that these transporters regulate the cholesterol content of NCM.

F-actin formation is increased in NCM from WD-fed mice

Leukocyte migration involves high-affinity conformation integrin activation, allowing the cell to bind to endothelial cell surface ligands and form lamellipodia for patrolling, which involves the polymerization and depolymerization of filamentous-actin (F-actin) in a front-to-rear wave-like pattern. Many of the genes that were specifically upregulated in $Ly6C^{-}$ monocytes from WD-fed mice are involved in either integrin activation or in forming cellular cytoskeletal

structures necessary for cell crawling along the vasculature. Initially we asked whether Gαi chemokine receptors were involved in inducing patrolling during atherogenesis. We measured patrolling NCM in WD-fed mice before and after pertussis toxin injections and could not find a significant decrease in patrolling frequency or any change in patrolling speeds (Supplemental Figure IVA). We then measured integrin expression in both monocytes from WD-fed mice (Supplemental Figure IVB,C) as well as from human subjects with documented CAD (Supplemental Figure IVD), but found no significant changes.

We next measured F-actin formation in blood monocyte subsets from chow-fed or WD-fed mice by confocal microscopy using phalloidin-AF488, a phallotoxin that binds to and stabilizes filamentous actin. Monocytes sorted from WD-fed mice exhibited greater F-actin content than monocytes from chow-fed mice (Figure 3A). When F-actin intensity was quantified for each cell within each condition, the $Ly6C^{-}$ monocytes from WD-fed mice had increased F-actin content compared to the $Ly6C^{+}$ subset (Figure 3B). These data suggest that $Ly6C^{-}$ NCM more readily form the machinery necessary for cell motility within blood vessels, particularly in WD-fed mice.

Oxidized lipoprotein moieties present in early atherogenesis induce NCM patrolling.

Previous studies have reported that human and mouse NCM do not ingest native LDL (LDL) or very low-density lipoproteins (VLDL) as much as do CM(Jackson et al., 2016), so we tested whether blood NCM would preferentially uptake modified forms of LDL. $CX_3CR1^{gfp/+}CCR2^{rfp/+}ApoE^{-/-}$ mice were injected intravenously with fluorescently-labeled LDL, native LDL protected from oxidation by BHT (BHT-LDL), minimally modified LDL (mmLDL)(Liao et al., 1991; Steinbrecher et al., 1984) and copper-oxidized LDL (OxLDL). Flow cytometry analysis showed that OxLDL was preferentially taken up by NCM in the

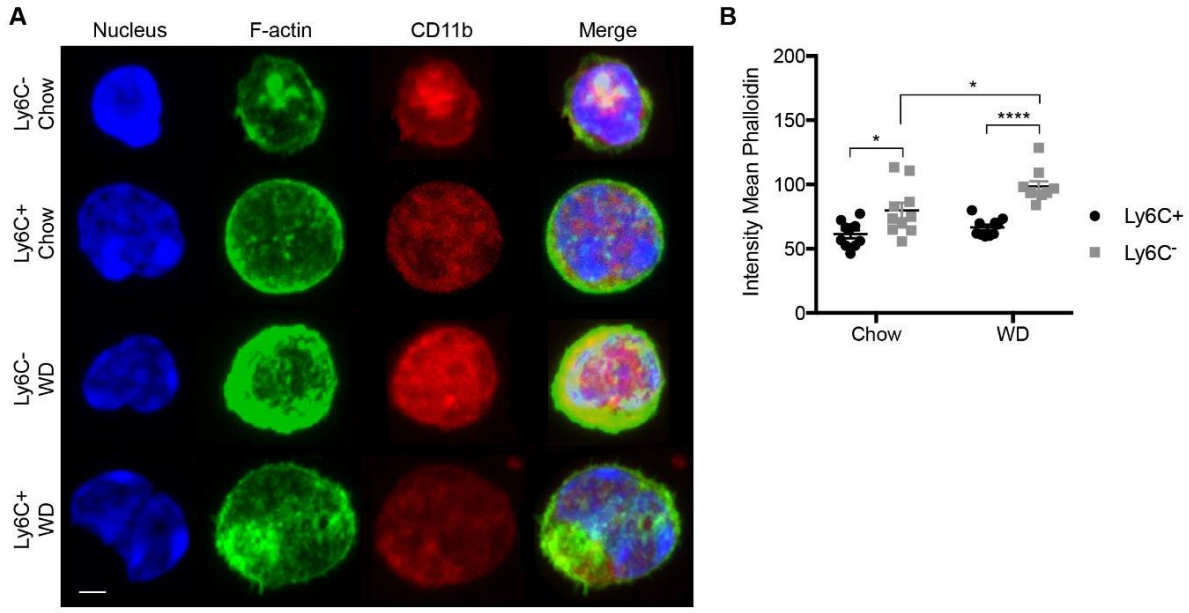


Figure 1.3 Increased F-actin formation in nonclassical monocytes from mice fed a Western diet. A, Representative images of Ly6C^{+} (classical) and Ly6C^{-} (nonclassical) monocytes stained for F-actin formation using phalloidin-AF488 by confocal microscopy. scale bar = 2μM B, Mean intensity of F-actin staining (phalloidin-AF488) of monocyte subsets sorted from chow or WD-fed mice by confocal microscopy. n=10 cells per group. Error bars represent mean ± SEM for all graphs. *P < 0.05, ****P < 0.0001

circulation (Figure 4A). Compared to CM, NCM showed 2-fold increased uptake of OxLDL (Figure 4B). We next looked at F-actin formation by confocal microscopy in NCM when OxLDL was added (Figure 4C). Again we saw a significant increase in F-actin intensity in the presence of OxLDL (Figure 4D), suggesting that OxLDL can mobilize NCM to adhere to the endothelium and patrol.

To determine whether OxLDL could directly increase patrolling monocyte activity, either OxLDL, nLDL, or vehicle (PBS) was injected intravenously into $CX_3CR1^{gfp/+}CCR2^{rfp/+}ApoE^{-/-}$ mice (Figure 4E). Patrolling monocytes were quantified pre- and post-injection by imaging an area of the femoral vasculature over time. We found that OxLDL injection significantly induced CX_3CR1^{high} nonclassical monocyte patrolling along the endothelium (Figure 4E, lower panels), while PBS (Figure 4E, upper panel) and LDL (data not shown) did not.

We next characterized the patrolling behavior of NCM that had ingested OxLDL. Within 10 minutes post-injection, CX_3CR1^{high} NCM had taken up DiI-OxLDL, which localized to the rear cell body and not in the leading edge of the lamellipodia (Figure 4F). At no point did we observe extravasation of the monocytes into the surrounding leg muscle tissue in mice fed either chow or WD. However, we did notice a "slingshot" behavior of CX_3CR1^{high} nonclassical monocytes that rapidly attached to the endothelium and immediately transitioned into patrolling (video not shown). These results suggest that oxidized lipoprotein moieties present in the vasculature during early atherogenesis bind to scavenger receptors on NCM and induce an activation cascade that triggers nonclassical monocyte patrolling.

Nonclassical monocytes lacking CD36 fail to patrol the vasculature in early atherogenesis

CD36 and Msr1 (SR-A) are known scavenger receptors involved in the uptake of oxidized LDL (Miller et al., 2011). Based on our data that illustrate that NCM preferentially uptake OxLDL and that this OxLDL uptake changes the F-actin content of NCM (Figure 3), we examined monocyte patrolling in mice lacking either the class A scavenger receptor, Msr1 or the class B scavenger receptor CD36, or both. $Msr1^{-/-}$, $CD36^{-/-}$, or $Msr1^{-/-}CD36^{-/-}$ double knockout (DKO) mice were fed a WD for 28 days prior to imaging. Circulating NCM in these mice were labeled with CD115-AF488 and FcγRIV(CD16.2)-PE (Ingersoll et al., 2010) for imaging. We found that monocytes from WD- fed $CD36^{-/-}$ or DKO mice showed significantly reduced patrolling in response to WD, and in some of the $CD36^{-/-}$ or DKO mice, patrolling was completely abolished (Figure 5A). Fewer $CD36^{-/-}$ NCM patrolled the endothelium (Figure 5B), although the numbers of NCM present in blood were similar in WT vs $CD36^{-/-}$ mice (Figure 5C). Of the NCM that did patrol in the $CD36^{-/-}$ and $Msr1^{-/-}$ or DKO mice, mean speeds of patrolling were significantly reduced (Figure 5D). Interestingly, the patrolling characteristics of the NCM were different in the $Msr1^{-/-}$ mice (Supplemental Figure VA), such that they uniformly patrolled slower and straighter over shorter distances relative to WT or $CD36^{-/-}$ mice, suggesting loss of directional control in absence of OxLDL sensing. NCM from mice transplanted with DKO bone marrow predominantly exhibited patrolling characteristics very similar to that of the global $CD36^{-/-}$ model (Figure 5A,D and Supplemental Figure VA,B). $CD36^{-/-}$ NCM showed significantly reduced uptake of OxLDL (Figure 5E). We also observed a slight reduction in OxLDL uptake of $Msr1^{-/-}$ mice, but not to the significant extent of $CD36^{-/-}$ mice (Supplemental Figure VC).

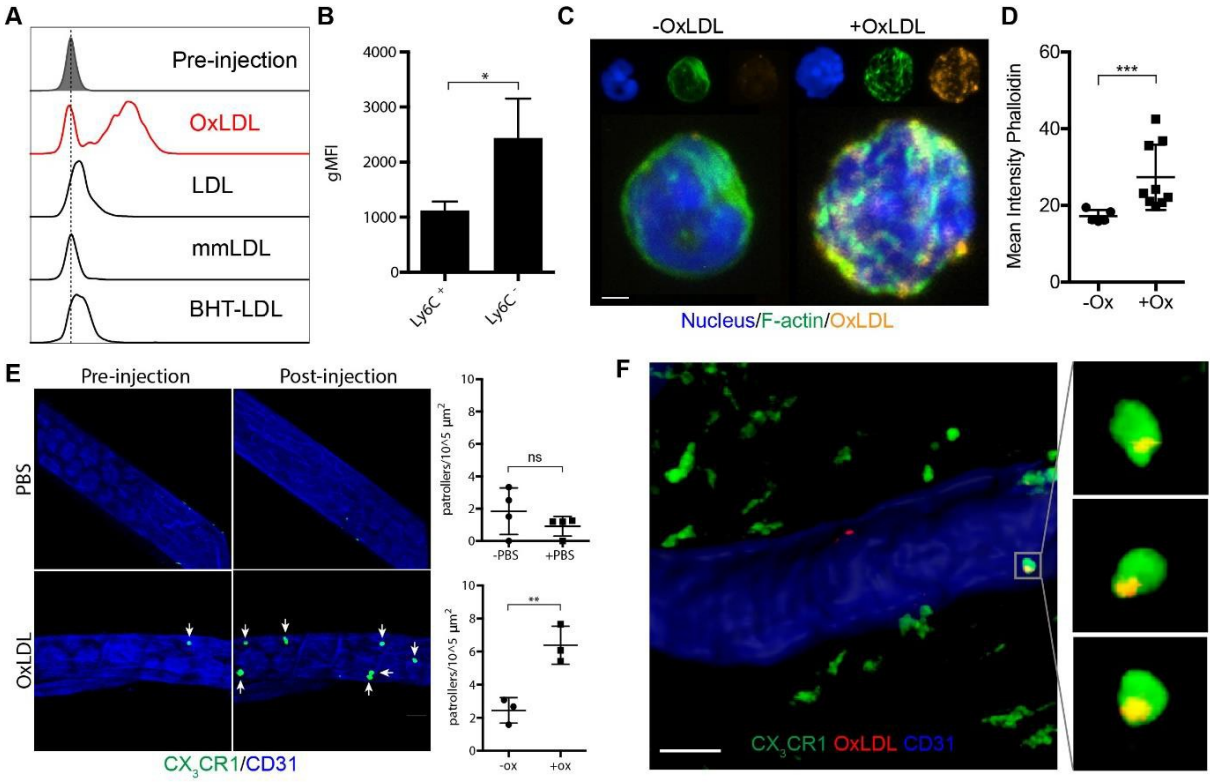


Figure 1.4 OxLDL is preferentially taken up by nonclassical monocytes and induces NCM patrolling. A, Chow-fed ApoE$^{-/-}$ mice were injected with AF633-labeled OxLDL, LDL, mmLDL, or BHT-LDL. 15 minutes after injection, mice were analyzed for LDL uptake by flow cytometry (n=1 mouse per injection). Mice were anesthetized and bled prior to injection to obtain a baseline (Pre-injection). B, Comparison of AF633-labeled OxLDL uptake in Ly6C^{+} (CM) and Ly6C^{-} (NCM) blood monocyte subsets ex vivo by flow cytometry. n=3 mice. C, Representative images of F-actin staining in Ly6C^{-} monocytes incubated with or without OxLDL. Scale bar: 2 µM D, Quantification of F-actin mean fluorescent intensity of Ly6C^{-} monocytes with or without DiI-OxLDL. E, CX$_3$CR1$^{gfp/+}$ApoE$^{-/-}$ mice were imaged to obtain a baseline of patrolling frequency in a large blood vessel of a mouse leg (left column). PBS (100 µL) or OxLDL (100 µg) solutions were injected during imaging after obtaining a baseline.
Timelapse images were obtained post-injection from the same pre-injected areas (right column). White arrows highlight patrolling NCM. Quantification of patrolling frequency before and after injection of PBS (upper graph) or OxLDL (lower graph) were graphed for each condition. n=3 independent experiments, one representative experiment shown. scale bar: 30 µM F, Representative figure of CX$_3$CR1$^{gfp/+}$ApoE$^{-/-}$ mice injected with DiI-OxLDL during imaging to observe OxLDL uptake by nonclassical monocytes while patrolling. n=2 independent experiments. scale bar: 30 µM. Error bars represent mean ± SEM for all graphs. *P < 0.05, **P < 0.01, ***P < 0.001.

To determine if CD36 expression on the NCM rather than on vascular endothelium was critical in directing patrolling behavior, we performed a BMT where we transplanted WT bone marrow into either WT or $CD36^{-/-}$ recipients. We found that patrolling behavior was similar in WT and $CD36^{-/-}$ recipients, confirming that deletion of $CD36^{-/-}$ on the endothelium plays an insignificant role in directing NCM patrolling (Figure 5F and Supplemental Figure VD). Additionally, we tested NCM from $TLR7^{-/-}$ or $CX_3CR1^{-/-}$ mice for patrolling activity based on our previous reports that illustrated that these receptors played functional roles in NCM(Auffray et al., 2007a; Carlin et al., 2013). Neither receptor played a role in orchestrating the patrolling behavior of NCM in response to WD feeding (Supplemental Figure VE).

Human $CD16^{+}$ nonclassical monocytes also possess scavenger receptors capable of binding and internalizing OxLDL, including CD163 (scavenger receptor class I), SR-A (CD204), and to a lesser extent CD36 (Supplemental Figure VIA). They also possess high levels of tetraspanin CD81 (Supplemental Figure VIA), which has been shown to associate with CD36 and assists in internalizing the receptor upon binding to OxLDL (Heit et al., 2013). Although expression is much higher in mouse NCM, transcript and protein levels of CD36 in human NCM are detectable by qPCR (Supplemental Figure VIB) and flow cytometry (Figure 5G). Moreover, when whole blood from healthy donors was incubated with various forms of LDL, similar to the in vivo injections in mouse, OxLDL was preferentially taken up by $CD16^{+}$ monocytes (Figure 5H).

$Ly6C^{-}$ monocytes utilize Src family kinases and DAP12 to patrol during early atherosclerosis

CD36 lacks a cytoplasmic tail that can propagate signaling cascades on its own and must rely on adapter proteins (Heit et al., 2013). We examined the effect of deleting DAP12, a known

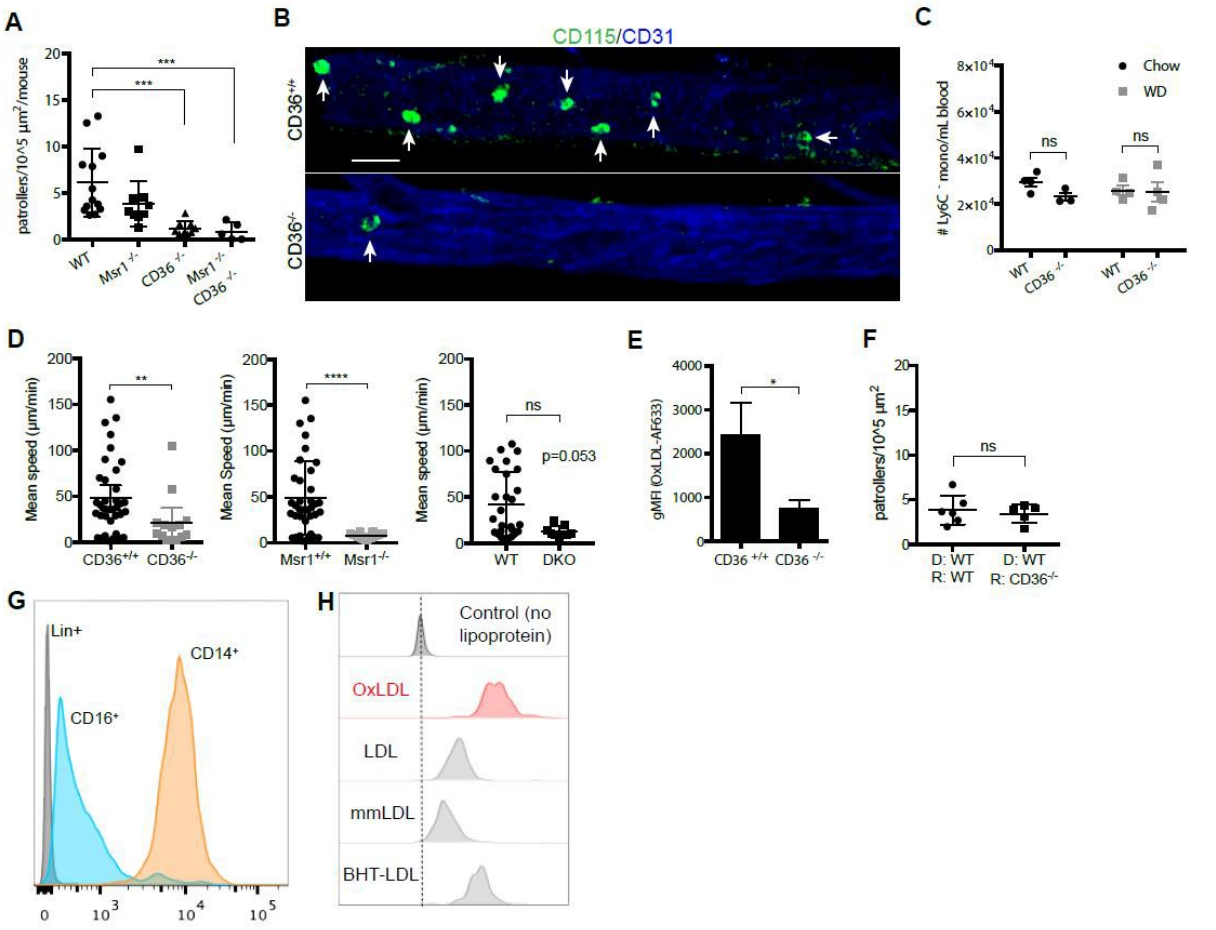


Figure 1.5 CD36 is critical for nonclassical monocytes to patrol the vasculature in WD-fed mice. A, Mice were fed a WD for 28 days and imaged for patrolling activity. CD115 (shown in green) and FcγRIV antibodies were used to label nonclassical monocytes in vivo, blood vessels were labeled with CD31 antibody. Ratios of patrolling monocytes per surface area of each blood vessel recording (n=3 per mouse) were summed and reported per mouse. n=5-11 mice per group. B, Representative images of patrolling NCM (highlighted with white arrows) in mouse femoral vasculature obtained by intravital confocal microscopy. scale bar: 30 µM. C, Mean speeds of NCM for each imaging experiment shown in graph A. D, Frequencies of Ly6C$^-$ monocytes from retro-orbital bleeds of B6 or CD36$^{-/-}$ mice measured by FACS. n=3-4 mice per group. E, Blood monocytes were incubated ex vivo with AF633-labeled OxLDL and analyzed for uptake by flow cytometry. n=3 mice per group. F, WT CX$_3$CR1$^{gfp/+}$ bone marrow was transplanted into either WT recipients or CD36$^{-/-}$ recipients. Graph represents ratios of patrolling NCM per surface area of each blood vessel recording (n=3 per mouse) that were summed and reported per mouse. n=5-6 mice per group. G, Representative histogram of CD36 expression on CD16$^+$ monocytes from healthy human donors determined by flow cytometry. n=4 donors. H, Healthy human blood samples were incubated for 20 minutes at 37°C with AF633-labeled OxLDL, LDL, mmLDL, or BHT-LDL (and lineage antibodies) and measured by FACS for LDL uptake. Representative histogram of n=3 donors. Error bars represent mean ± SEM for all graphs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns indicates not significant.

CD36-associated adapter protein, on NCM patrolling in WD-fed mice. We transplanted DAP12$^{-/-}$ bone marrow into WT recipients, let mice recover and then fed the mice WD for 28 days. After WD feeding, we observed a significant 2-fold reduction in the number of NCM patrolling the vasculature (Figure 6A). Moreover, the mean speed of NCM patrolling along the vasculature was higher in the absence of DAP12 (Figure 6B), suggesting that the NCM lacking DAP12 could not adequately respond to the presence of OxLDL in vivo. There was no significant difference in the frequency of NCM in blood of DAP12$^{-/-}$ mice (Supplemental Figure VIIA).

Next, we examined which kinases might be acting downstream of CD36. We inhibited phosphorylation of Syk and SFK, two kinases known to transduce signals between receptors and integrins. We injected either PP1, a reversible cell permeable SFK inhibitor, or Piceatannol, a cell permeable Syk phosphorylation inhibitor, intravenously into WD-fed $CX_3CR1^{gfp/+}CCR2^{rfp/+}ApoE^{-/-}$ mice and imaged NCM patrolling (Figure 6C). Within 20 minutes of injection, NCM that had been actively patrolling either arrested or detached from the endothelium (Figure 6C). When vehicle (equal amount DMSO diluted with PBS) was injected by the same route, patrolling monocytes continued to crawl along the vasculature undisturbed (Figure 6C). In contrast, injection of Piceatannol had no effect on patrolling (Figure 6C and Supplemental Figure VIIB).

We next performed immunoblotting for active-site phosphorylation of SFKs in NCM from chow or WD-fed WT or CD36$^{-/-}$ mice or from WT WD-fed mice treated with PP1. We used the pan-pSFK Y416 antibody, which recognizes SFK phosphorylation at the Y416 active site. NCM from WD-fed mice showed activation of SFKs as measured by Y416 phosphorylation (Figure 6D). Phosphorylation was abrogated with addition of the PP1 inhibitor (Figure 6D). Importantly, NCM from CD36$^{-/-}$ mice showed no activated pSFK (Figure 6D), supporting the

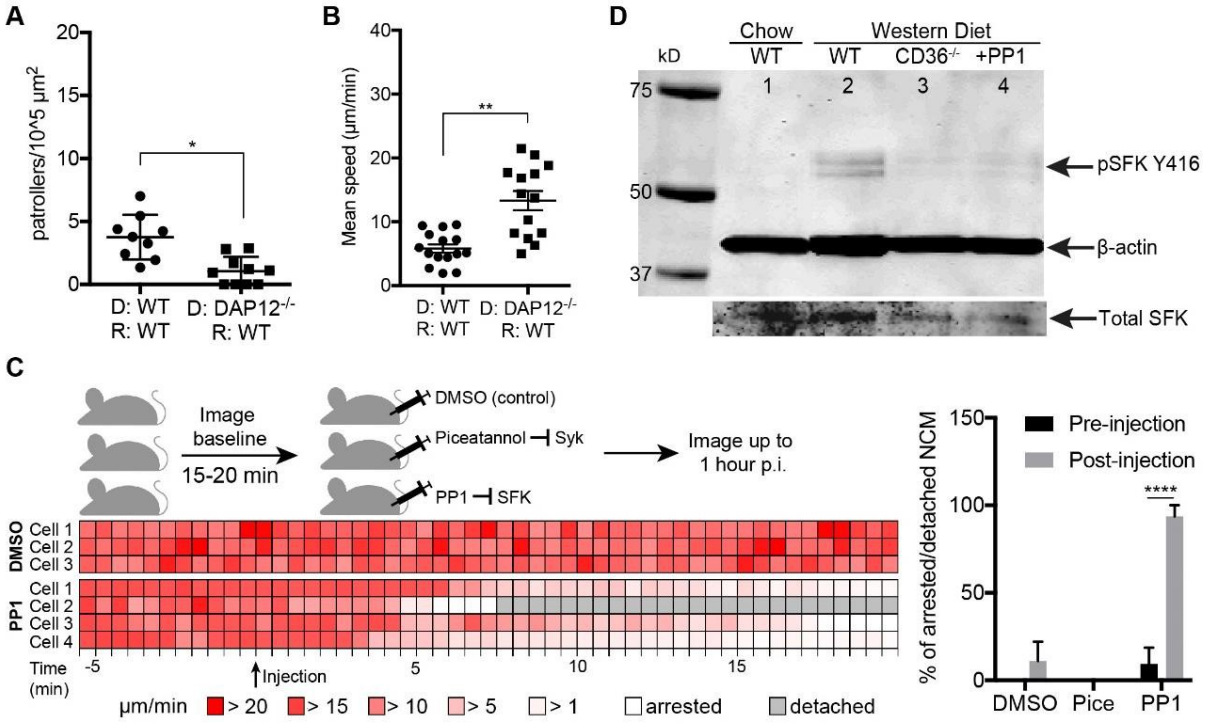


Figure 1.6 Src family kinases mediates signaling downstream of CD36 through Dap12. A, DAP12$^{-/-}$ and B6 bone marrow chimeras were fed a WD and imaged by intravital microscopy for patrolling. Graph represents ratios of patrolling monocytes per surface area of each blood vessel recording (n=3 per mouse) that were summed and reported per mouse. n=9-10 mice per group. B, Mean speed of patrolling monocytes from mice fed a WD for 28 days. n=10 mice per group C, Representative western blot of phosphorylated Src family kinases (SFK) at Y416 with β-actin loading control. Blood monocytes were pooled and sorted from B6 mice fed chow (lane 1), WD (lane 2), CD36$^{-/-}$ WD-fed mice (lane 3), or monocytes from WD-fed mice incubated with PP1 (lane 4). Blots were stripped and reprobed for total protein for Src protein. D, $CX_3CR1^{gfp/+}CCR2^{rfp/+}ApoE^{-/-}$ mice on WD were imaged before (baseline) and after (post injection, p.i) injections of DMSO (vehicle, DMSO+PBS), Piceatannol (Syk inhibitor), or PP1 (SFK inhibitor). Each mouse was injected with 1 reagent and imaged intravitally to obtain patrolling speeds and behavior (arrest or detachment). Patrolling speed heat map represents each mouse injected with either DMSO or PP1 and plotted as each cell in the field of view in each mouse over time. Quantification of patrolling speeds were obtained with Imaris software and plotted in Excel. Bar graph shows what percent of NCM were arrested or detached after at 20 minutes post-injection listed on X-axis. n=2-3 mice per group. Error bars represent mean ± SEM for all graphs. *P < 0.05, **P < 0.01

notion that ligand binding of CD36 requires downstream activation of SFKs to induce patrolling. Thus, NCM require CD36-ligand binding and signalling through DAP12 to activate SFKs for inducing patrolling along blood vessel walls in WD-fed mice.

Discussion

In this study, we examine how NCM patrolling behavior is changed in early atherogenesis. We found that patrolling by NCM is significantly increased in the vasculature during early atherogenesis, and that patrolling in this setting was triggered by scavenger receptor recognition and uptake of oxidized LDL, primarily through CD36, and to a lesser extent, SR-A. This signal is transmitted through DAP12 and SFKs, culminating in changes in F-actin formation in NCM, leading to patrolling along the vascular endothelium. Our data suggest that SFK and DAP12 are working downstream of CD36 to promote integrin activation and dynamic changes in F-actin that lead to patrolling.

We also examined patrolling activity of NCM in $Msr1^{-/-}$ mice. We did not observe a significant difference in patrolling frequency in the global $Msr1^{-/-}$ mice, nor did we detect a significant change in OxLDL uptake by $Msr1^{-/-}$ NCM. It is possible that changes in the patrolling characteristics of NCM in $Msr1^{-/-}$ mice may be contributed by the endothelium. In the $Msr1^{-/-}$ $CD36^{-/-}$ double knockout bone marrow transfer experiment that we performed, CD36 behaved as the dominant receptor on NCM, as the patrolling frequency and characteristics of these monocytes looked similar to $CD36^{-/-}$ mice. We further examined $CD36^{-/-}$ recipient mice that had been transplanted with WT bone marrow to explore how selective endothelial loss of CD36 would impact patrolling. We found no changes in patrolling behavior in the absence of endothelial CD36, suggesting that endothelial CD36 had little impact on regulation of NCM patrolling in WD-fed mice. Further, we examined the role of DAP12, a CD36-adaptor protein,

and found that absence of DAP12 in NCM significantly reduced, but did not completely abolish, patrolling. Thus, other pathways, in addition to CD36, likely play a role in regulating NCM patrolling in response to WD-feeding during early atherogenesis.

Previous studies (Auffray et al., 2007b; Quintar et al., 2017) have shown that LFA-1 and VLA-4 integrins mediate patrolling, particularly in atherosclerotic mice. It has been proposed that $G_{\alpha i}$ chemokine receptors are necessary for inducing patrolling in NCM during inflammation(Carlin et al., 2013). However, we did not observe significant reductions in NCM patrolling after pertussis toxin injections of WD-fed mice. We propose that integrin changes in affinity conformation are responsible for the increase in patrolling, and that this is due to CD36 activation in response to OxLDL sensing in early atherogenesis. Due to the lack of integrin conformation-specific antibodies available for mice, and because the human CAD patient samples used in our study were processed using EDTA (which prevents integrins from reaching high-affinity conformations), we could not directly link CD36 with integrin conformational changes. However, we have shown that F-actin formation, which is necessary for cell motility, is indeed increased in NCM from WD-fed mice, as well as when OxLDL is bound to NCM.

As a scavenger receptor, CD36 can bind to multiple ligands, including OxLDL and apoptotic cells via recognition of oxidized phosphatidylserine and oxidized phospholipids (Greenberg et al., 2007). Studies from our laboratory and others have shown than NCM scavenge apoptotic endothelial cells, and tumor debris in the vasculature(Carlin et al., 2013). Further, NCM can migrate to and enter sites of atherosclerotic plaque formation in mice (Tacke et al., 2007). Whether the trigger for NCM patrolling in early atherogenesis is OxLDL itself or some oxidized moiety present in the OxLDL that is a ligand for scavenger receptors is unclear. Thus, we cannot rule out that other ligands or other oxidized LDL moieties

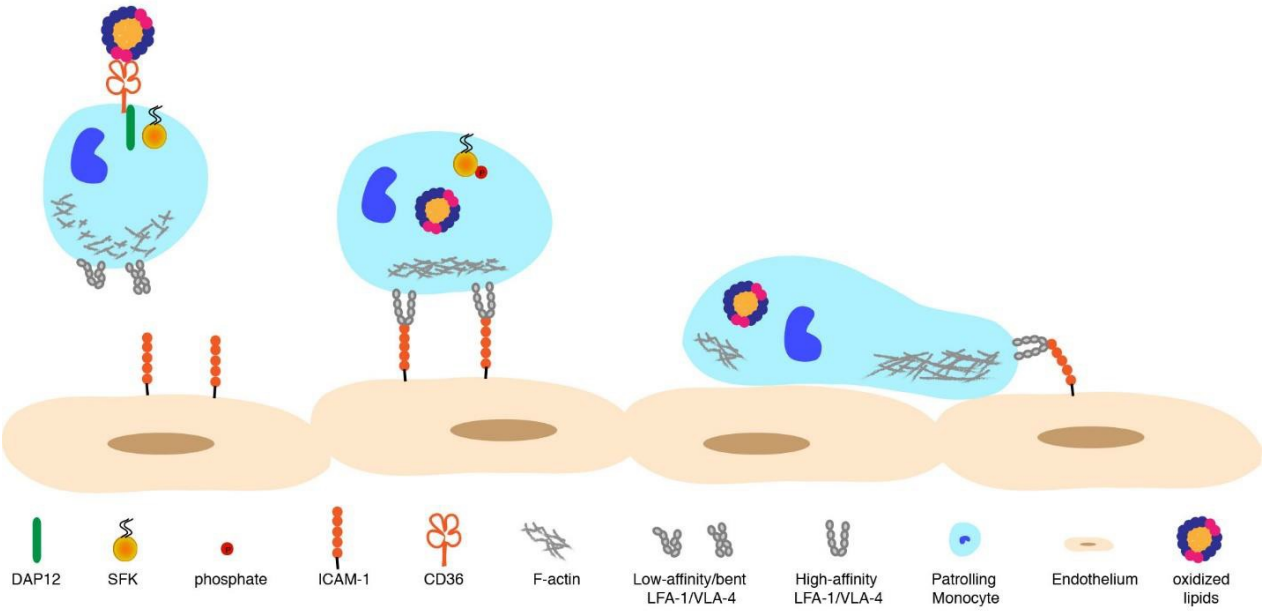
are binding to CD36 on NCM during atherosclerosis and triggering patrolling. It is clear that the absence of NCM increases plaque formation in mice, but the exact roles of CD36 on NCM as it pertains to atherosclerosis progression remains a question for future studies.

SFKs consist of several members shown to be involved in signal transduction that are present in monocytes including Src, Lyn, and Fyn. We found that NCM from WD-fed mice have higher levels of phosphorylated SFK at the activation site Y416 using a pan-SFK antibody. Further, in vivo administration of the SFK inhibitor PP1 abrogated patrolling. In support of this, we have observed that Csk, a negative regulator of SFK, is increased in NCM in WD-fed mice (Figure 2A), further suggesting that SFKs are activated in NCM during WD-feeding(Obergfell et al., 2002). Conversely, in vivo administration of Piceatannol (Syk inhibitor) failed to abolish patrolling in WD-fed mice, suggesting that Syk is not involved in regulation of CD36-mediated NCM patrolling in atherogenesis. We have not yet definitively determined which SFK contributes to CD36-mediated NCM patrolling.

In contrast to mouse monocytes, human $CD14^{dim}CD16^{+}$ monocytes have low levels of CD36 expression compared to the classical subset. However, human $CD14^{dim}CD16^{+}$ monocytes do express some low levels of CD36 and higher levels of the CD36-associated tetraspanin CD81. In addition, they possess relatively abundant levels of other scavenger receptors that are capable of taking up OxLDL, such as SR-A and CD163. We found that OxLDL is indeed preferentially taken up by human NCM, which may be bound by CD36 or one of the other more abundant scavenger receptors that can bind OxLDL.

In summary, by using intravital imaging in vivo to examine nonclassical monocyte function in large peripheral blood vessels during early stages of atherosclerosis, we have discovered that nonclassical monocytes respond to oxidized LDL and show increased patrolling

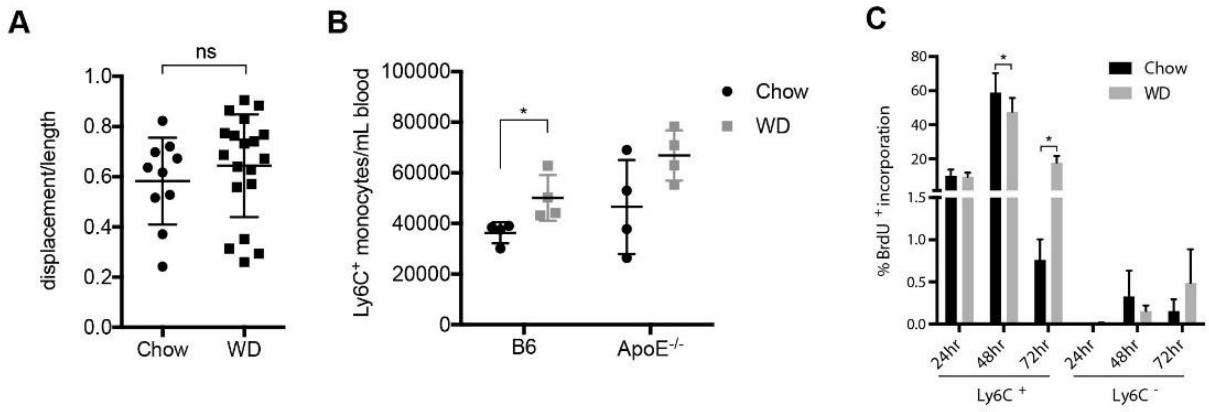
along the vascular endothelium. In mice, this appears to be mediated by CD36, and recognition of OxLDL by CD36 causes downstream activation of Src family kinases, which promote cytoskeletal changes, leading to increased patrolling. Further studies to dissect the role of CD36 in nonclassical monocyte activation during atherogenesis will enhance our understanding of how the innate immune system controls inflammation and atherosclerotic plaque formation.



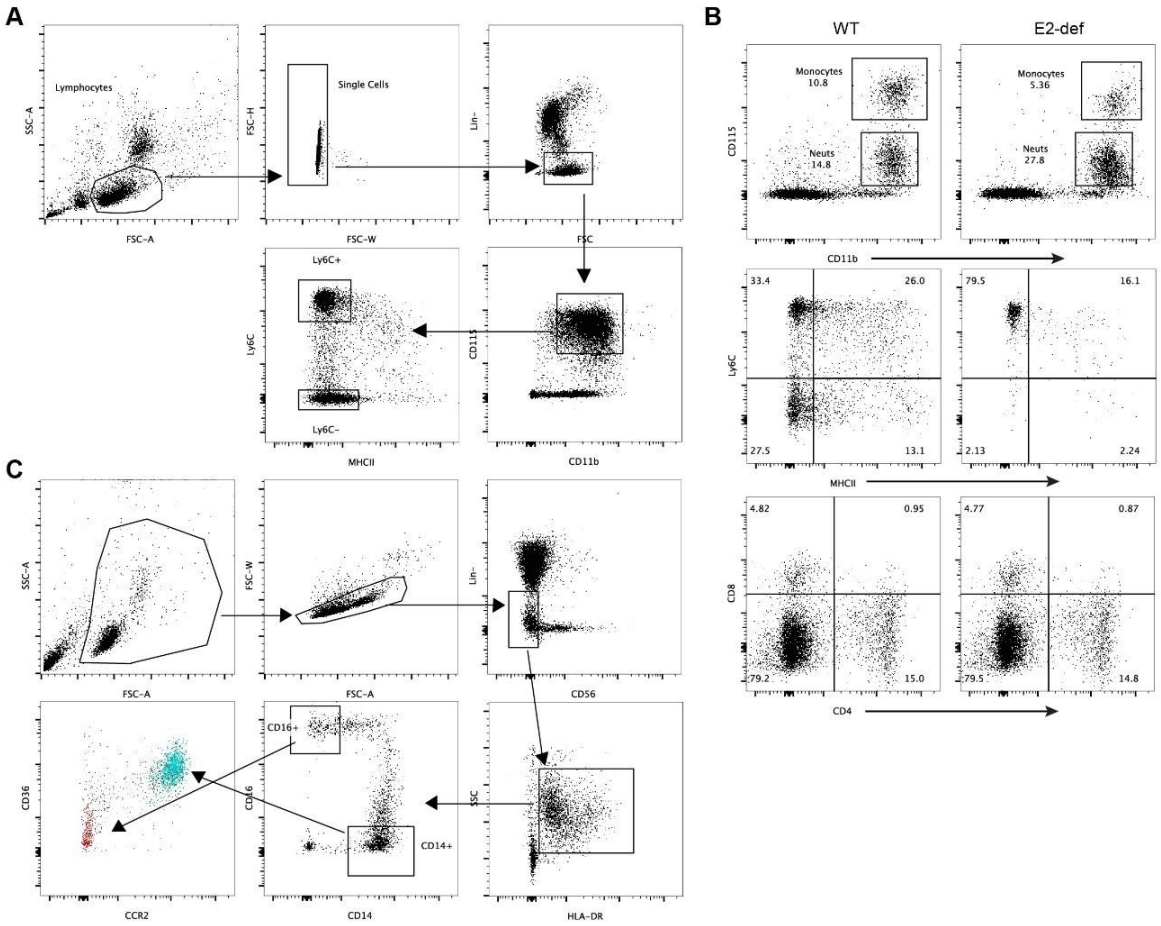
Scheme 1.1: Activation of nonclassical monocyte patrolling occurs through binding of oxidized lipids to scavenger receptor CD36 which leads to F-actin formation and recruitment to the endothelium.

Supplemental Figures

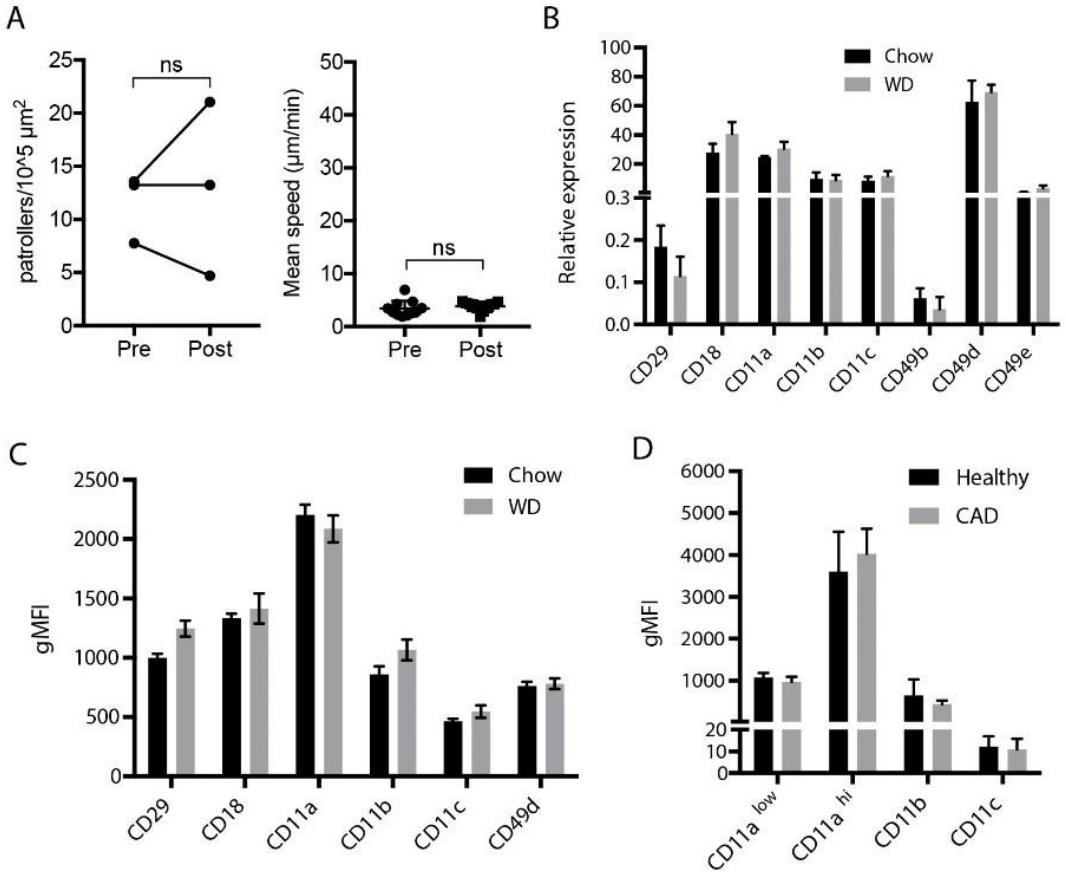
Supplemental Figure 1.1 Experimental procedure for imaging femoral vasculature. Mice were anesthetized and leg hair was removed with depilatory cream. Antibodies to fluorescently label patrolling monocytes or endothelium were injected retro-orbitally prior to superficial removal of skin. Vacuum grease was layered on a piece of rectangular styrofoam (with leg-shaped cutout in styrofoam) to hold leg in place. Mice were then laid on the heating pad and the leg was positioned on the greased styrofoam (left picture). Surgical removal of the skin above the fremoral vasculature was done by pulling the skin away from the leg with sterile tweezers and cutting down the midline of the leg. Vacuum grease was also applied to either side of the cutout to hold the glass coverslip in place. PBS was added under the coverslip to keep the tissue moist.



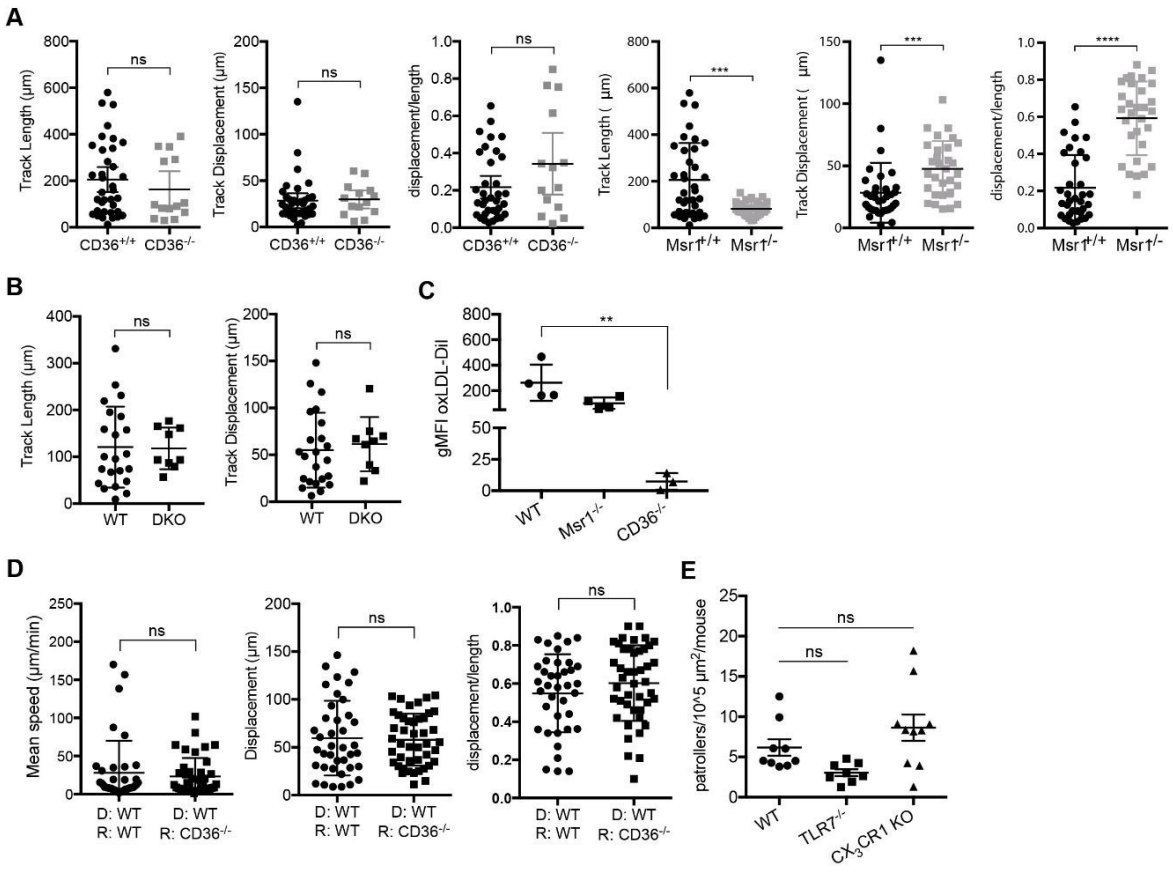
Supplemental Figure 1.2 Changes in monocyte proliferation and patrolling behavior during Western diet feeding. A, The confinement ratio (displacement divided by length) of patrolling NCM in WD_fed mice. B, Ly6C CM concentrations in blood from B6 or ApoE$^{-/-}$ WD-fed mice (28 days) were measured by flow cytometry. N=4 mice per group. C, BrdU staining of blood monocyte subsets from 3 WD-fed mice (28 days) and 3 choe fed mice (all age/sex matched). Blood was obtained for 24 hr and 48 hr time points. Error bars represent mean ± SEM for all graphs. *P<0.05



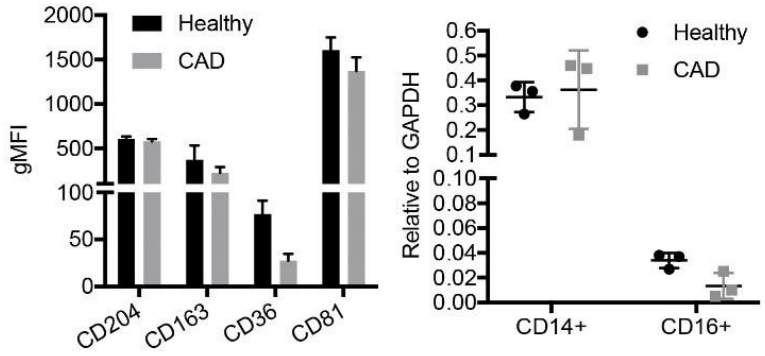
Supplemental Figure 1.3 General gating scheme for FACS analysis and sorting monocytes from mouse and human blood. A, Mouse blood: CD115 and CD11b were used to identify total monocytes then gated for either Ly6C^{+} or Ly6C^{-} subsets. B, Ldlr$^{-/-}$ mice were reconstituted with WT or E2-def bone marrow and tested for leukocyte populations 8 weeks after BMT. C, Human blood: after lineage gate, HLA-DR^{+} cells were used to identify blood monocytes, then gated for either CD16^{+} or CD14^{+} subsets and verified using CD36 and CCR2 antibody staining.



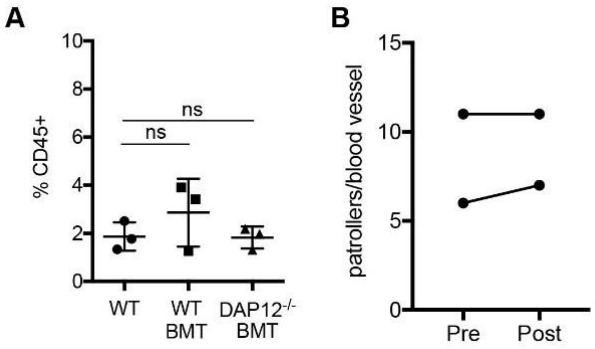
Supplemental Figure 1.4 Integrin expression in human and mouse monocytes. A, WD-fed mice (6 weeks) $CX_3CR1^{gfp/+}$ mice were imaged before (Pre) and after (Post) pertussis toxin (PTx) injections (0.16mg/kg body weight). The frequency of patrolling NCM for the surface area of the blood vessel imaged in each mouse is reported. Graph of mean speeds for each cell imaged. N=3 independent experiments. B, Blood was collected from 3 individual choe and WD-fed mice and sorted for $Ly6C^-$ monocytes directly into Trizol LS. qPCR was performed in duplicate for each mouse, using GAPDH as a reference. C, Flow cytometry analysis of integrin cell surface expression by antibody staining of blood $Ly6C^-$ monocytes from 3 individual chow and WD-fed mice. Fluoroscence-minus-one was used to determine positive staining. D, Flow cytometry analysis of integrin cell surface expression by antibody staining of $CD16^+$ monocytes in 3 individual CAD patients or healthy donors. Fluoroscence-minus-one was used to determine positive staining. ns indicates not significant. Error bars represent mean ± SEM for all graphs.



Supplemental Figure 1.5 Monocyte patrolling characteristics of scavenger receptor-deficient mice fed a Western diet. A-B Analysis of patrolling characteristics between WD-fed (28 days) WT and $CD36^{-/-}$, $Msr^{-/-}$, or double knockout (DKO) mice. Each point represents a patrolling monocyte. n=9-11 mice per group. C, Comparison of ex vivo DiI-OxLDL uptake by WT (B6), $Msr^{-/-}$, or $CD36^{-/-}$ nonclassical monocytes. n=3-4 mice per group. D, Characteristics of patrolling monocytes in B6 mice receiving either WT or $CD36^{-/-}$ bone marrow. Each point represents a patrolling monocytes. n=5-6 mice per group. E, WT, $TLR7^{-/-}$, and CX_3CR1 KO mice were fed a WD for 28 days. Each point represents the total number of patrolling monocytes per total surface area of blood vessel for each mouse. n=8-10 mice per group. Error bars represent mean ± SEM for all graphs. ns = not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$



Supplemental Figure 1.6 Human monocyte expression of scavenger receptors. A, Expression levels of scavenger receptors and associated proteins on human blood $CD16^+$ monocytes from healthy donors and CAD patients were assessed by flow cytometry. N=4 donors per group. B, CD36 expression in $CD14^+$ and $CD16^+$ monocytes from healthy and CAD blood donors was assessed by qPCR. Each sample was run in duplicate and ΔΔCt values are reported relative to GAPDH. n=3 donors



Supplemental Figure 1.7 Monocyte frequency in DAP12$^{-/-}$ in peripheral blood and patrolling after piceatannol injection. A, After bone marrow reconstitution, WT and DAP12$^{-/-}$ recipients were fed a WD for 28 days. The frequency of Ly6C^{-} monocytes in each mouse (including WT without irradiation for comparison) was assessed by flow cytometry. N=3 mice per group. B, Number of patrolling monocytes within a continuously imaged blood vessel before and after Piceatannol injection. n=2 independent experiments. Error bars represent mean ± SEM for all graphs. ns = not significant.

Acknowledgements

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Disclosures

None.

Materials and Methods Mice

All C57BL/6J (B6), E2-deficient, $ApoE^{-/-}$, $CX_3CR1^{gfp/+}CCR2^{rfp/+}$, $CX_3CR1^{gfp/+}CCR2^{rfp/+}ApoE^{-/-}$, $TLR7^{-/-}CX_3CR1^{gfp/+}$, $CX_3CR1^{gfp/gfp}$ male and female mice were bred in-house. Knockout mice for CD36 (019006) and Msr1 (006096) were purchased from The Jackson Laboratory along with appropriate age and sex matched B6 controls. $Msr1^{-/-}CD36^{-/-}$ bone marrow was generously sent to us by Dr. Ronit Shiri-Sverdlov, Maastricht University,

Netherlands. Dap12$^{-/-}$ bone marrow was generously sent to us by Dr. Jessica Hamerman, Benaroya Institute. All experiments followed guidelines of the La Jolla Institute for Allergy and Immunology (LJI) Animal Care and Use Committee, and approval for use of rodents was obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were euthanized by CO_2 inhalation and subsequent cervical dislocation.

Bone Marrow Transplantation

Bone marrow chimeras were generated by injecting 10^6-10^7 bone marrow cells into 8-12 week old B6 recipient mice irradiated with two doses of 500 rads each (total 1000 rads) 4 hours apart. Recipients were fed a Western diet (Harlan TD.88137) at 5 weeks after transplantation for 28 days. All other mice were fed a Western diet for the indicated amount of time starting between 8-10 weeks of age.

Blood Collection

Blood was obtained either by retro-orbital bleeds or by cardiac puncture. Retro-orbital bleeds were carried out under anesthesia using micro-hematocrit capillary tubes (Fisherbrand 22-362-566) and 2 mM EDTA (final concentration) to prevent clotting. Cardiac punctures were performed post-mortem using 25G needles loaded with 100 units of heparin (Fresenius Kabi USA 504201) per mouse. Blood was processed by red blood cell (RBC) lysis (NH4Cl 150 mM, NaHCO3 10 mM, EDTA 2 mM) for 10 minutes at 4°C and resuspended in FACS buffer (PBS, 1% BSA, 2mM EDTA).

Antibodies and reagents

Mouse blood monocytes were stained by Fc block (anti-CD16/32, Tonbo Biosciences, 2.4G2), CD3 (1452C11), CD19 (6D5), NK1.1 (PK136), Ly6G (1A8), CD115 (AFS98), CD11b (M1/70), Ly6C (HK1.4), MHCII (M5/114.15.2), FcγRIV (9-E9), CD11a (M17/4), CD11c (N418), CD29 (HMb1-1), CD18 (M18/2), CD49d (9C10) (all from Biolegend). Human blood monocytes were identified using CD3 (HIT3a), CD19 (HIB19), NK1.1 (MEM-188), CD86 (IT2.2), HLA-DR (L243), CD16 (3G8), CD14 (63D3), CD36 (5-271), CCR2 (K036C2), CD11a (HI111), CD11b (M1/70), CD11c (3.9) (all from Biolegend). Anti-mouse CD31 (Biolegend, MEC13.3 or 390) was used for intravital imaging of the endothelium. Human DiI-labeled and unlabeled Cu^{2+}-oxidized LDL and LDL (Kalen Biomedical) were used for imaging uptake in vivo.

Human BHT-LDL, mmLDL, LDL and Cu^{2+}-oxidized LDL for uptake experiments were kindly provided by Dr Yury Miller from UC San Diego. Filipin III, PP1 and Piceatannol inhibitors (all from Cayman Chemical) were reconstituted according to manufacturer specifications. Bodipy 493/503 (Life Technologies) was reconstituted according to manufacturer specifications. Phalloidin-AF488 for staining F-actin formation came ready to use (Life Technologies A12379). Pertussis toxin (Tocris 3097) was prepared according to manufacturer's recommendation.

Intravital Imaging

All intravital imaging was performed on the Leica SP5 confocal microscope with resonant scanner. Mice were imaged either as $CX_3CR1^{gfp/+}$ or antibody labeled with CD115 and FcγR4. Mice were anesthetized using a ketamine/xylazine cocktail previously described(Auffray

et al., 2007a). Mice were positioned on a WPI ATC2000 heating pad ventral side up and kept at 37°C. Vacuum grease (Dow Corning) smeared over a styrofoam cutout was used to imbed the leg for further surgical removal of the skin to expose the leg vasculature. Additional vacuum grease was applied on either side of the leg and a coverslip (22x50-1.5 Fisher Scientific) was laid over the exposed vasculature. PBS was added between the leg and coverslip to keep the tissue moist. Large (diameter >30 microns) blood vessels in mouse legs, including the femoral artery/vein and primary branches, were imaged at 25x for 12-16 minutes per recording and acquired using Leica Application Suite. For measuring patrolling frequency, the number of patrolling monocytes and the surface area of the blood vessel were determined for each recording, then totaled and divided (total # of patrolling monocytes for all 3 videos/total surface area for all 3 videos = average # of patrolling monocytes per mouse). No gamma adjustments or deconvolution processes were applied. Statistics (mean speed, track length, track displacement, confinement ratio), surface rendering, and migration graphs for each patrolling monocyte cell was generated using Imaris software (Bitplane version 7.2) and plotted using Prism (Graphpad version 6) or Excel (Microsoft 2011). Labeling of the vasculature was performed by injecting 10 µL of anti-CD31 AF647 prior to surgical preparation of mice immediately before all imaging experiments. All antibodies and reagents were injected retro-orbitally. Reagents were injected 30 minutes (approximately 2 recordings) after baseline imaging. Labeled and unlabeled LDL forms were injected at 100 µg/mL of blood assuming a 25g mouse has 1.46 mL of blood. PP1 inhibitor was injected at a final concentration of 67 µM (1 mg/kg). Piceatannol inhibitor was injected at a final concentration of 120 µM (2.5 mg/kg). Control mice received equal volumes of DMSO (stock diluent) as inhibitor with recommended PBS dilution according to manufacturer. Pertussis toxin was injected at 0.16mg/kg body weight.

Imaging of F-actin formation

For monocytes that were prepared from Western diet-fed mice, blood was collected and stained as indicated above, and fixed with 1% paraformaldehyde (PFA, no methanol) for 30 minutes at 4°C before being sorted. After sorting, cells were plated on an 8-chamber cell culture slide (MatTek, CCS-8), spun down at 350xg for 1 minute, and stained with phalloidin-AF488 (1:200, Thermo) and Hoechst (1:2500, Sigma) for 30 minutes at 4°C. For oxLDL uptake, monocytes were sorted into warm DMEM (2mM L-glutamine, 1% Pen/Strep), plated in a U-bottom 96-well culture plate and incubated with or without DiI-labeled OxLDL for 30 minutes. Cells were then washed with cold PBS, fixed with 1% PFA for 30 minutes at 4°C, plated on an 8-chamber cell culture slide (MatTek, CCS-8), spun down at 350xg for 1 minute, and stained with phalloidin-AF488 (1:200, Thermo) and Hoechst (1:2500, Sigma) for 30 minutes at 4°C. All slides were mounted with Prolong Gold (Thermo) and imaged with a Zeiss LSM780 confocal scanning microscope. Settings were set with control samples and used throughout the experiment.

Flow cytometry and sorting

Sorting was performed by the LJI core facility on a FACSAria cytometer (BD Biosciences). Fc block was added before all staining and primary conjugated antibodies were incubated with cells in FACS buffer (PBS, 1% BSA, 2mM EDTA, 0.05% NaN_3) for 15 minutes at 4°C in the dark unless otherwise stated. FACS experiments were performed on a LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar version 10.7).

BrdU assay

ApoE$^{-/-}$ mice were injected with 1mg of BrdU (Sigma) in 200 μL sterile PBS intraperitoneal. Water bottles containing 0.8 mg/mL BrdU were given to the mice until the end of the experiment. Cells were stained normally for surface markers, then fixed/permeabilized, DNase treated, and stained with anti-BrdU-APC according to manufacturer specifications (APC BrdU Flow Kit, BD Biosciences).

RNAseq

Cohorts of 10 mice on chow or WD were exsanguinated by cardiac puncture, RBC-lysed (Biolegend) and pooled before sorting directly into Trizol LS (Life Technologies) in order to obtain a minimum of 50 ng purified RNA per subset. 45 ng of each experimental group was sent to the UCSD Genomics Core for Tru-seq library preparation and Hiseq4000 mRNA single-read sequencing (Illumina). Raw FASTQ data were obtained from the UCSD sequencing core facility and mapped to the mouse mm10 reference genome compiled with Ensembl v73 gene annotations using STAR (v2.3.0). Gene expression levels were quantified using featureCounts (v1.4.3) and differential expression analysis performed with edgeR in the R/Bioconductor environment. Differentially expressed genes were identified as those with a significance P value >0.001 after FDR correction (Benjamini-Hochberg), with post filtering for variance outliers defined as a tagwise dispersion >99.9% of all genes. Heatmap analysis was performed using the gplots package in R.

Aortic atherosclerosis quantification

Atherosclerosis was assessed by en face analysis as we have reported previously(Hanna et al., 2012). Aortas were harvested and immersed in paraformaldehyde, opened longitudinally

and pinned. and stained with Oil Red O, Images were scanned, and the percent surface areas occupied by lesions were determined with ImageJ software.

Quantitative real-time PCR

Blood monocytes were sorted by subset ($Ly6C^+/Ly6C^-$, $CD16^+/CD14^+$) from chow or WD-fed mice into Trizol LS. All RNA from sorted monocytes (mouse and human) was obtained using Direct-zol (Zymo Research) and quantified on a Tapestation 2200 (Agilent), then stored at -80°C until all biological replicates were completed. iScript cDNA Synthesis Kit (Bio-Rad) was used to obtain cDNA, then run on a MyIQ Single-Color Real-Time PCR detection system (Bio-Rad) using SYBR Green Master Mix (Thermo Fisher) with integrin and GAPDH probes (GeneCopoeia): ItgaL (MQP027460), ItgaM (MQP026401), ItgaX (MQP032499), Itga2 (MQP027456), Itga4 (MQP029479), Itga5 (MQP029480), Itgb1 (MQP029481), Itgb2 (MQP027464), Itgb3 (MQP031443), GAPDH (MQP078891). Melting curves were run to validate specificity of probes. Human samples used CD36 (Hs00169627_m1) and GAPDH (Hs02758991_g1) Taqman probes with Taqman Universal Master Mix II (all from Thermo Fisher).

LDL uptake assays

Fresh whole blood from either human or mouse was obtained as described above and co-incubated with native or oxidized LDLs at 100 µg/mL and lineage antibodies for 20 minutes at 37°C. Samples were immediately lysed and fixed using Phosflow Lyse/Fix buffer (BD Biosciences) and run on a LSRII cytometer. In vivo uptake assays were performed by anesthetizing the animals with ketamine/xylazine, injecting the indicated form of LDL, then

exsanguinating the animal 15 minutes later. FACS antibodies were added to the blood collection and processed as described above.

Western blot

Monocytes were sorted by subset into cold sterile PBS, pelleted at 350xg for 5 minutes, then lysed with RIPA buffer (Abcam) supplemented with protease inhibitor (Thermo, Halt Protease Inhibitor Single-Use Cocktail). A 30G needle and syringe was used to shear the cell pellet, then sonicated for 2 minutes (Fisher, FS20H). After BCA quantification and loading equal amounts of cell lysates, primary antibodies were added to PBS overnight at 4°C. Primary rabbit-anti-mouse antibodies were used at 1:1000: phospho-Src Y419 (Cell Signaling Technologies) and 1:5000 β-actin (Cell Signaling Technologies). For PP1-inhibited monocytes from WD-fed mice, blood was collected from all WD-fed mice, lysed, then divided in half and either incubated with PP1 inhibitor or nothing until the sort time.

Human studies

CAD and healthy study subject samples were collected as previously described(Hanna et al., 2012; Manichaikul et al., 2014). PBMCs were obtained from the Coronary Assessment in Virginia cohort (CAVA) cohort, frozen in FBS and 10% DMSO, stored in liquid nitrogen and shipped on dry ice to LJI for analysis. This cohort includes patients between the ages of 30 and 80 undergoing a medically necessary cardiac catheterization. We used samples from patients who had a Gensini score of >30, indicating severe CAD, as quantified by coronary angiography. Patients were excluded if they had any of the following: a current acute coronary syndrome, cancer of any type, autoimmune disease of any type, anemia, pregnancy, HIV infection,

immunosuppressive therapy, or prior organ transplantation. All protocols and procedures were approved by the Institutional Review Board at the University of Virginia (IRB HSR #15328).

Blood donors from LJI for western blotting and LDL uptake assays were obtained from the LJI Normal Blood Donor Program in accordance with the Department of Health and Human Services Policy for Protection of Human Research Subjects (45 CFR 46). Blood was collected in heparin tubes. Whole blood was immediately used for either LD uptake assays, or immediately processed by Histopaque-1077 (Sigma Aldrich) isolation to obtain PBMCs (excluding RBCs and granulocytes) before blocking with human serum and staining for flow cytometry.

Statistics

All statistics in this study were performed using Graphpad Prism 6.0. Error bars represent the mean ± SEM for at least 3 independent experiments, or as indicated by the N listed in each figure legend. For grouped analyses, we used a nonparametric Kruskal-Wallis test with Dunn's test for multiple comparisons, or an ordinary 1way or 2way ANOVA if the data sets had equal variances. Multiple t-tests using Holm-Sidak method was performed for surface marker data sets between chow and WD groups. Student's t-test was used for analyzing LDL injections and F-actin content. Pairwise t-test was used for comparison of $Ly6C^+$ and $Ly6C^-$ F-actin content from each mouse. For all analyses, statistical significance was set at alpha=5%.

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CHAPTER 2

Kindlin-3 is required for the patrolling function of nonclassical monocytes during cancer surveillance.

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Conflict of Interest Disclosures

The authors declare no potential conflicts of interest.

Abstract

Nonclassical monocytes patrol the vasculature in order to maintain vascular homeostasis and to respond to inflammatory signals. During cancer metastasis, nonclassical monocytes are effective in preventing metastatic tumor cells from seeding new tissues, particularly in lung. Whether patrolling is required for nonclassical monocytes to prevent tumor metastasis is unknown. Using an inducible Mx-1 promoter cre-lox mouse model and intravital microscopy, we show that Kindlin-3, an intracellular adaptor protein capable of activating integrins to their high-affinity conformation, is essential for driving the patrolling activity of nonclassical monocytes. Without Kindlin-3, nonclassical monocytes cannot patrol along the endothelium in peripheral blood vessels or in the lung vasculature. When B16F10 murine melanoma tumor cells were injected intravenously into mice that possessed Kindlin-3 deficient nonclassical monocytes, there was a significant 4-fold increase in tumor metastases in the lungs compared to wild-type mice. Kindlin- $3^{-/-}$ nonclassical monocytes showed defective patrolling and defective migration into the lung tissue and failed to take up tumor material. Both migration and tumor uptake required the integrin LFA-1. We conclude that the adaptor protein Kindlin-3 is essential for nonclassical monocyte patrolling, which in turn is necessary for endothelial homeostasis and for preventing tumor metastasis to the lung.

Introduction

Nonclassical monocytes are a distinct subset of monocytes in both humans ($CD16^{+}CD14^{low}$) and mice ($CX_3CR1^{high}Ly6C^{-}$) that can be found predominantly in bone marrow, blood and highly vascularized tissues such as lungs, with the ability to patrol along the endothelium (Auffray et al., 2007; Geissmann et al., 2003). As such, they play an important role in mediating endothelial cell turnover and repair (Carlin et al., 2013), anti-viral immunity (Cros et al., 2010) and immune surveillance during cancer metastasis (Hanna et al., 2015; Plebanek et al., 2017). LFA-1 (αLβ2) has been identified as the dominant integrin for mediating contact between nonclassical monocytes and the endothelium to enable patrolling (Auffray et al., 2007), but how patrolling is mediated and whether it is essential for preventing cancer metastasis by nonclassical monocytes is unclear.

Cancer is the second leading cause of death in the United states, and the metastasis of primary cancer cells to distant organs through the lymphatics or circulation is a major cause of morbidity for cancer patients (Steeg, 2016). The lungs are often a site of metastatic colonization for many types of tumors (Nguyen et al., 2009; Weidle et al., 2016). Arrest and invasion of the circulating tumor cells into the lung tissue (interstitium) is met by waves of immune cells, such as neutrophils, monocytes and DCs, that can influence the expansion or repulsion of newly seeded cancer cells (Sagiv et al., 2015; Qian et al., 2011; Headley et al., 2016; Hanna et al., 2015). In particular, patrolling monocytes can encounter particles secreted by cancer in the lung microvasculature and become enriched, which results in reduction of tumor metastasis through the recruitment of NK cells (Plebanek et al., 2017).

Integrin activation and clustering are essential processes for monocyte adherence and motility (Hakkert et al., 1991; Sumagin et al., 2010). In our previous report, RNAseq analysis of

nonclassical monocytes in Western-diet vs chow fed mice showed upregulation of the gene encoding Kindlin-3 (*fermt3*)(Marcovecchio et al., 2017). Kindlin-3 is a leukocyte specific adapter protein that binds to the cytoplasmic tails of β1, β2 and β3 integrins to promote high-affinity conformation and adhesion of leukocytes to the endothelium (Moser et al., 2009; Mory et al., 2008). Disruption of this gene expression leads to leukocyte adhesion deficiency (LAD-III), characterized by bleeding disorder and increased infections (Svensson et al., 2009). In particular, Kindlin-3 is necessary for LFA-1 high-affinity conformation (Manevich-Mendelson et al., 2009; Lefort et al., 2012). In vivo investigations of Kindlin-3 have shown it to be involved with neutrophil motility and function (Xu et al., 2015, 2018; Lefort et al., 2012), adaptive immune responses (Morrison et al., 2015; Feigelson et al., 2011), homing and retention of hematopoietic stem cell progenitors to the bone marrow (Ruppert et al., 2015) and NK activation (Gruda et al., 2012). Yet in other contexts it has shown to be dispensable, such as with T-cell diapedesis into inflamed tissues (Cohen et al., 2013). A functional role for Kindlin-3 in nonclassical monocytes in vivo has not yet been reported.

In this study, we investigated whether patrolling by nonclassical monocytes requires Kindlin-3, and whether Kindlin-3-mediated patrolling is necessary for surveillance of metastatic cancer cells to the lung. Understanding how patrolling by nonclassical monocytes aids in preventing metastasis and whether this is dependent on nonclassical monocytes having cell-cell contact may offer a new therapeutic pathway to target.

Results

Kindlin-3 is necessary for patrolling behavior of nonclassical monocytes in the periphery.

In order to observe whether Kindlin-3 is necessary for nonclassical monocyte patrolling, we first bred Kindlin-$3^{fl/fl}$Mx1-cre+ (K-KO) or Kindlin-$3^{fl/fl}$Mx1-cre- (WT) mice to CX_3CR1 GFP reporter mice(Jung et al., 2000). These mice can express cre recombinase when the Mx1 promoter is stimulated by poly(I:C), which results in *fermt3* gene excision. Since these mice also express GFP under the CX_3CR1 promotor, nonclassical monocytes are fluorescently labeled, which allows us to track the patrolling patterns of WT and K-KO nonclassical monocytes using fluorescence intravital microscopy. At approximately 8 weeks of age, cohort mice were injected with poly(I:C) to induce *fermt3* gene excision. qRT-PCR of *fermt3* using sorted blood monocytes from wild-type (WT) and Kindlin-3 knockout (K-KO) mice 3 weeks after poly(I:C) injection confirmed the complete (K-KO) or near-complete ablation of *fermt3* compared to the WT mouse lacking cre-recombinase (Fig. 1 A).

Using this mouse model, we assessed whether Kindlin-$3^{-/-}$ nonclassical monocytes could patrol the vasculature. When mice were injected with TNFa to create an inflammatory environment, nonclassical monocytes patrolled the femoral vasculature normally in control mice that lacked cre-recombinase or did not receive poly(I:C) (Fig. 1 B left panel, 1 C). However, in the K-KO mouse that received poly(I:C), all nonclassical monocytes within the field of view (FOV) failed to patrol the vasculature (Fig. 1 B right panel, 1 C). Although not as common, we were able to find an occasional classical monocyte ($Ly6C^+GFP^+$) or a $Ly6C^+$ cell briefly crawling along the femoral endothelium in a cre+ mouse injected with poly(I:C) (video not shown). Since all immune cells lack Kindlin-3 in this inducible mouse model, we concluded that not only is Kindlin-3 necessary for nonclassical monocyte patrolling, it is also specifically essential to the patrolling mechanism as opposed to other types of immune cell crawling.

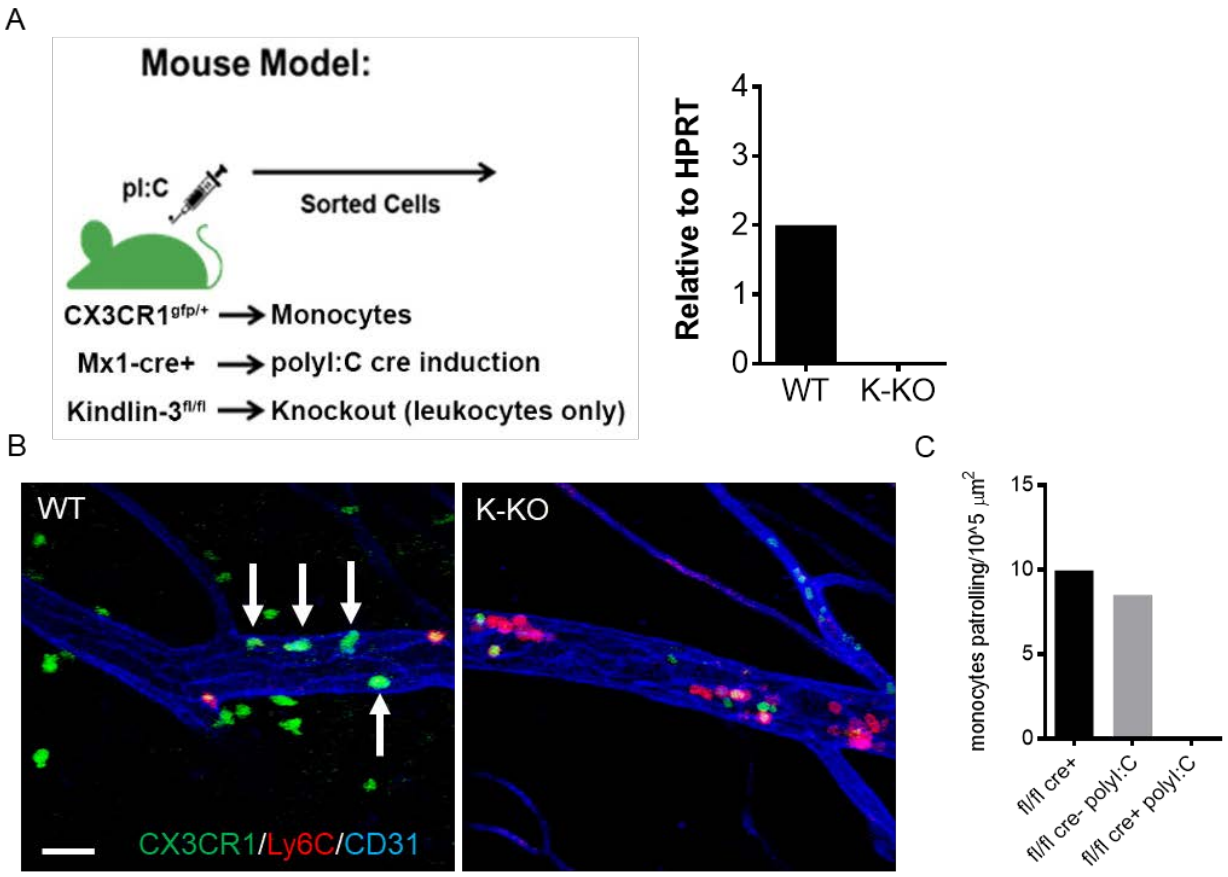


Figure 2.1: Kindlin-3 is necessary for patrolling behavior of nonclassical monocytes in the periphery. (A) 11 week old male mice from the same cohort were used to confirm the Kindlin-3 gene knockdown (Fermt3). Kindlin-$3^{fl/fl}CX_3CR1^{gfp/+}$Mx1-cre+ and Mx1-cre- mice were injected with 200ug poly I:C in PBS 3x in 5 days and assayed for reduced transcript expression of Fermt3 3 weeks later. (B) Timelapse images were acquired using intravital confocal microscopy from the leg vasculature of cohort 12-week old female mice. Mice were injected intraperitoneal with 500 ng recombinant mouse TNFa in PBS 1 hour before imaging. Snapshot images of cre- (Kindlin-3-WT) and cre+ (Kindlin-3-KO) with patrolling monocytes highlighted by white arrows. Scale bar = 30 uM. (C) Number of patrolling nonclassical monocytes (CX_3CR1-GFP+) per 10^5 μm surface area of blood vessel. Graph is representative of 3 biological replicates.

Kindlin-3 is required by nonclassical monocytes to home to lung tissue and patrol in situ and in the lung vasculature.

Kindlin-3 is known to mediate high-affinity conformation of integrins, which in turn mediates cell migration and trafficking between tissues (Svensson et al., 2009). We hypothesized that Kindlin-3 deficiency would disrupt the migration of nonclassical monocytes out of bone marrow and blood into peripheral tissues. We generated a mixed bone marrow chimera wherein all WT monocytes express DsRed and all K-KO monocytes express GFP under the CX_3CR1 promoter (Fig. 2 A). No significant differences were found between the frequencies or numbers of red and green monocyte subsets (Fig. S1 A and B), although there was a significant decrease in the median fluorescence intensity (MFI) of LFA-1 integrin on K-KO $Ly6C^-$ monocytes (Fig. S1 C). In bone marrow, for both $Ly6C^+$ and $Ly6C^-$ monocytes, there was a significant decrease in the frequency of K-KO monocytes (Fig S2 A). We again found that the MFI of LFA-1 on these cells, as well as the homing receptor CXCR4, was either significantly reduced or absent (Fig. S2 B). These data indicate that Kindlin-$3^{-/-}$ monocytes require integrin activation for proper homing or retention in the bone marrow compartment.

Next we examined monocyte frequencies in the lungs of DsRed:Kindlin-$3^{-/-}$ mixed BMT mice. Monocyte subsets in lung were gated based on $CD115^+CD11b^+$ $Ly6C^+$ and $Ly6C^-$ markers (Fig. 2 B). Total monocyte frequencies (Fig. 2 C) as well as the number of total monocytes per lung (Fig. 2 D) were significantly decreased in the K-KO monocyte population compared to the WT monocytes within the same mice. After refining monocyte subsets using the Ly6C marker, nonclassical monocyte numbers were significantly decreased per lung while the $Ly6C^+$ monocyte subset remained unchanged (Fig. 2 E). Using intravital microscopy, we observed

nonclassical monocytes in the lung vasculature and interstitium failed to patrol during homeostasis (video not shown).

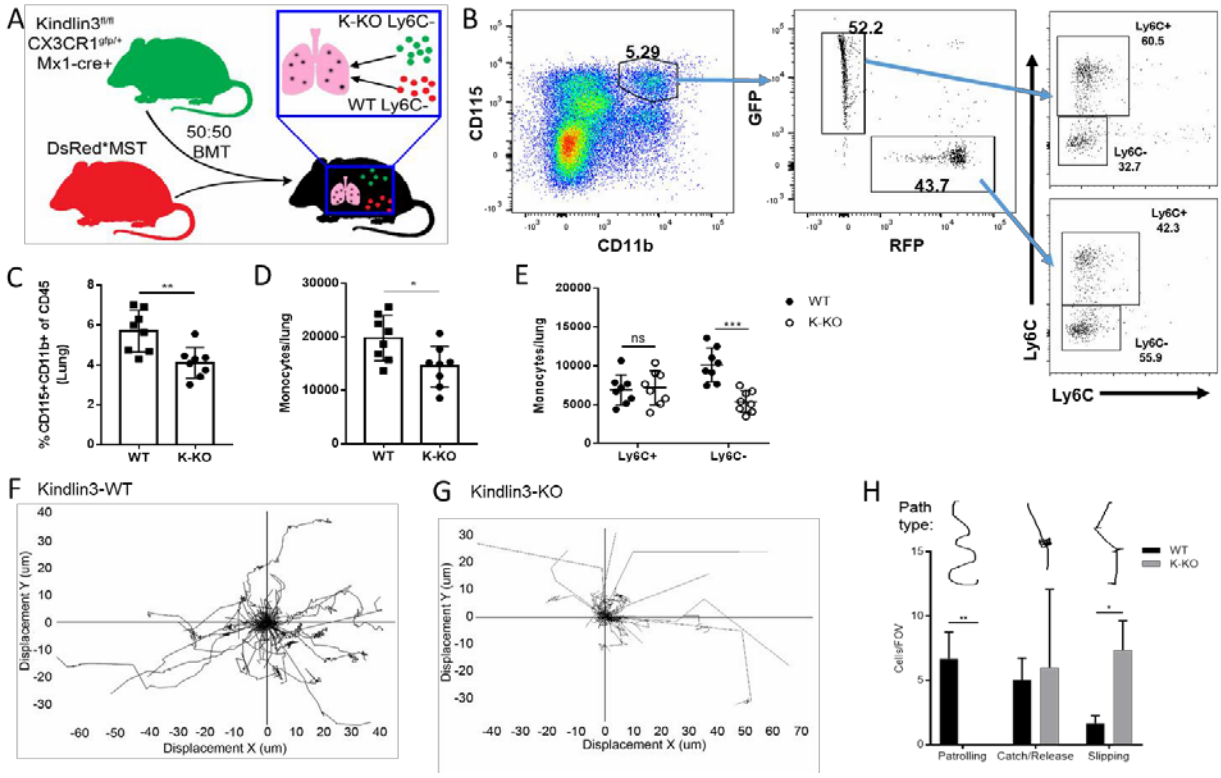


Figure 2.2: Nonclassical monocytes lacking Kindlin-3 fail to home to lung tissue and patrol the lung vasculature. (A) Schematic of BMT using Kindlin-3$^{fl/fl}$CX$_3$CR1$^{gfp/+}$Mx1-cre positive or negative bone marrow and DsRed reporter bone marrow at a 50:50 ratio. (B-E) Perfused lungs were collected from cre- (WT) and cre+ (KO) BMT mice, weighed for the entire lung as well as the left lobe, and then the left lobes were mechanically dissociated for FACS staining. The entire sample was then run on a LSR-II to collect all events. (B) Gating scheme for wild-type (WT; DsRed+) and Kindlin-3 knockout (K-KO; GFP+) monocytes within each mouse lung. (C) The frequency of total monocytes (CD115+CD11b+) out of CD45+ cells per lung. n=8 mice per group. (D) The number of total monocytes per mouse lung calculated by multiplying the number of monocytes (CD115+CD11b+) by the ratio of total lung weight/left lobe weight. n=8 mice per group. (E) Monocyte subset numbers per mouse lung, as gated in (B) and calculated in (D). n=8 mice per group. (F-G) Spider plots of cre- (F) and cre+ (G) nonclassical monocyte tracks in mouse lung under homeostasis (CX$_3$CR1-GFP+, Ly6C- or Gr-1-). (H) Nonclassical monocyte tracks were binned into 3 groups: patrolling, catch and release (exiting or re-entering blood flow and stopping for more than 2 frames on the endothelium without crawling), and slipping (dragging or tripping along endothelium with arrest). *p<0.05, **p<0.01, ***p<0.001

When cell tracks were plotted on an XY graph, WT nonclassical monocytes exhibited characteristic patrolling tracks both in the vasculature and in situ (Fig. 2 F), while K-KO exhibited straight tracks with few contact points on the endothelium (Fig. 2 G). After tracking nonclassical monocytes within each FOV, we categorized their motility based on how long they were able to cling to the vasculature by the number of frames. In WT mice, nonclassical monocytes could patrol as defined by nonlinear crawling for at least 1 minute. The K-KO nonclassical monocytes could not crawl through lung blood vessels or tissue for long periods of time or distances, but rather temporarily arrested without crawling before re-entering the circulation (catch/release, >2 frames), or very briefly touched the endothelium repeatedly in a slipping or skidding behavior (slipping, 1-2 frames). When these 3 types of paths were graphed per FOV, there were no $GFP^{+}Gr1^{-}$ cells patrolling, while the numbers of cells that slipped were significantly increased in K-KO mice (Fig. 2 H). However, the cells that were capable of arresting for more than 2 frames (approximately 14+ seconds) did not change significantly between WT and K-KO, indicating that some level of adhesion is possible for Kindlin-$3^{-/-}$ nonclassical monocytes, and that firm adhesion precedes patrolling.

Mice that lack functional patrolling monocytes show endothelial cell dysfunction in lung

Patrolling monocytes scavenge endothelial microparticles and are needed to recruit neutrophils to remove debris from focal necrosis of endothelial cells (Carlin et al., 2013). We hypothesized that when nonclassical monocytes fail to patrol, endothelial homeostasis would be disturbed. To investigate, we used a mouse model recently generated by Thomas et al, which specifically ablates the nonclassical monocyte population through a super-enhancer deletion (E2-def)(Thomas et al., 2016). Since this mouse has all other immune cells present except for

nonclassical monocytes, we filled this void by mixing bone marrow from Kindlin-3$^{-/-}$ mice or a wild-type donor as control. Thus, half of the mixed chimera bone marrow donated by the E2-def mouse would possess all normal immune cells except for nonclassical monocytes, and the other half of the bone marrow donated by a Kindlin-3$^{-/-}$ mouse would occupy that niche with Kindlin-3$^{-/-}$ nonclassical monocytes. Even though other immune cells derived from the Kindlin-3$^{-/-}$ bone marrow would be abnormal, it is compensated for by the rest of the wild-type immune cells coming from the E2-def bone marrow (Fig. 3 A, Fig. S3 A). Therefore, any functional defects observed in the E2:Kindlin-3$^{-/-}$ BMT mice would be attributed to Kindlin-3$^{-/-}$ nonclassical monocytes.

CD31^{+} endothelial cells (EC) were sorted from lungs of mixed BMT mice and cytokine transcript levels were measured by qRT-PCR. IL-1β, CX_3CL1, and TNFα (Fig. 3 C, top row) mRNA were increased in E2:K-KO EC. In addition, ligands for integrins LFA-1 and VLA-4 are upregulated in E2:K-KO EC compared to E2:WT EC (Fig. 3 C, bottom row). Upregulation of integrin ligands and inflammatory cytokines are hallmarks of EC activation and inflammation(Szmitko, 2003). Thus, E2:K-KO mice were unable to patrol the endothelium, and consequently, these mice had inflamed lung endothelium. We conclude from these data that patrolling monocytes are responsible for maintaining endothelial health through their patrolling function.

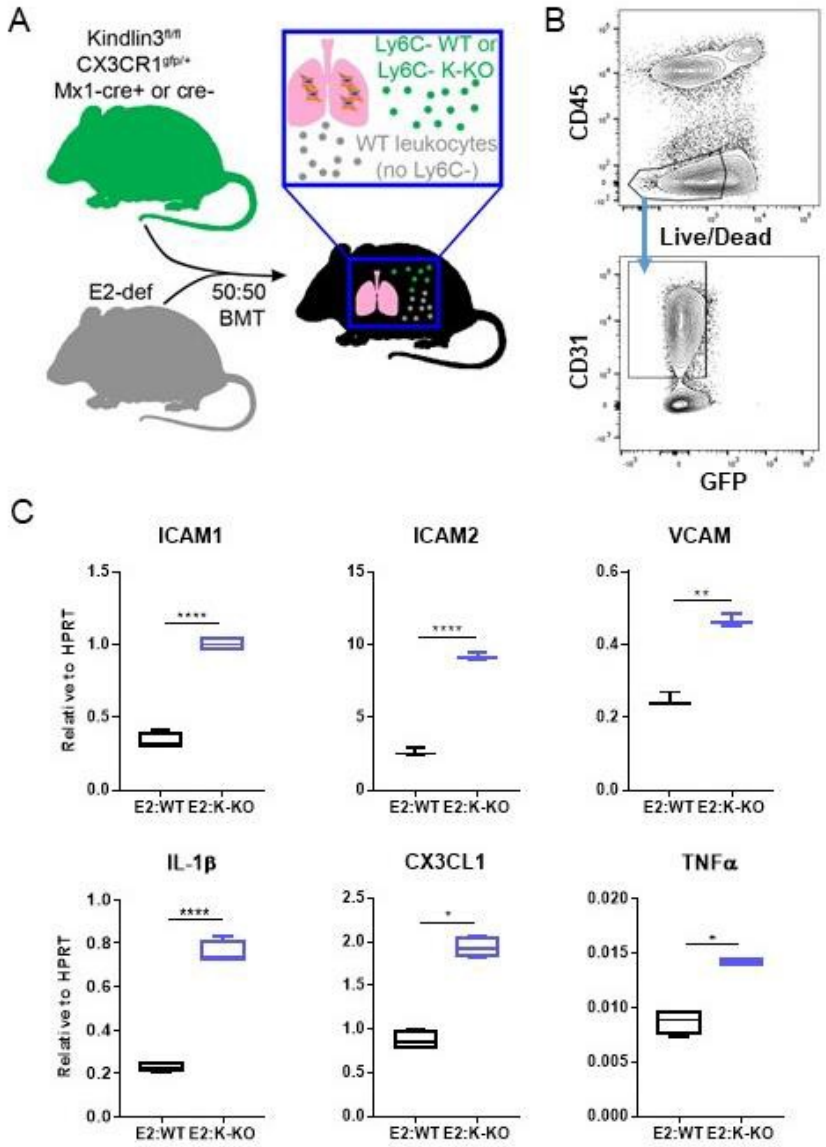


Figure 2.3. Mice that lack functional patrolling monocytes exhibit lung endothelial cell dysfunction. (A) Schematic of E2-def bone marrow (absent nonclassical monocytes) mixed with Kindlin-3fl/flCX3CR1gfp/+Mx1-cre+ or cre- bone marrow (all leukocytes, including nonclassical monocytes) as a model to study the effects of Kindlin-3-/- specifically in nonclassical monocytes. (B) Gating scheme for sorting out CD31+ endothelial cells from digested lungs of E2:WT and E2:K-KO BMT mice. (C) qRT-PCR of transcripts for genes reported to be expressed by endothelial cells from E2:WT or E2:K-KO BMT mice. Each point represents 1 mouse. n=3-4 mice. *p<0.05, **p<0.01, ****p<0.0001

Kindlin-3 is required for nonclassical monocyte recruitment to sites of invading melanoma metastases in the lung and for cancer particle uptake.

Monocyte subsets are differentially recruited early on to sites of metastatic colonization in the lung (Headley et al., 2016; Hanna et al., 2015). Therefore, we hypothesized that Kindlin-3$^{-/-}$ nonclassical monocyte recruitment to metastatic tumor cells in the lung would be stunted. WT and K-KO mice were injected with 2.5x10^5 B16F10-RFP cells and lungs were imaged (Fig. 4 A). When we calculated the number of GFP$^+$Gr-1$^-$ cells within a 30 μm distance of the B16F10-RFP cells we found that WT mice had more GFP$^+$Gr-1$^-$ cells associated with the B16F10-RFP cells compared to K-KO mice (Fig. 4 B), suggesting a defect in recruitment of Kindlin-3$^{-/-}$ nonclassical monocytes to the sites of invading tumor cells in the lung. To validate that this effect was due to the Kindlin-3 ablation, we validated that the frequency of all GFP$^+$CD11b$^+$ cells after poly(I:C) injection but prior to B16F10 injection was not significantly different between WT and K-KO mice (Fig. S3 B). When we examined the lungs of K-KO mice after cancer injection, we did not observe any patrolling monocytes within the FOV (Fig. 4 C). These data show that Kindlin-3-mediated firm adhesion and patrolling are necessary for proper sensing and migration of nonclassical monocytes toward the newly seeded cancer cells.

Next, we investigated if there was a difference in the number of monocytes recruited to the interstitium of the lung after B16F10 cells were injected. To differentiate between immune cells in the vasculature versus immune cells within the lung tissue, CD45-BV650 was injected 1 minute before sacrificing the mice and harvesting the lungs (Fig. 4 D). We then stained for total CD45$^+$ cells with CD45-APC to detect which cells were in the microvasculature (BV650$^+$APC$^+$) versus the interstitium (APC$^+$). There was a significant decrease in the number of Kindlin-3$^{-/-}$ CD115$^+$ monocytes compared to Kindlin-3$^{+/+}$CD115$^+$ monocytes within the lung interstitium

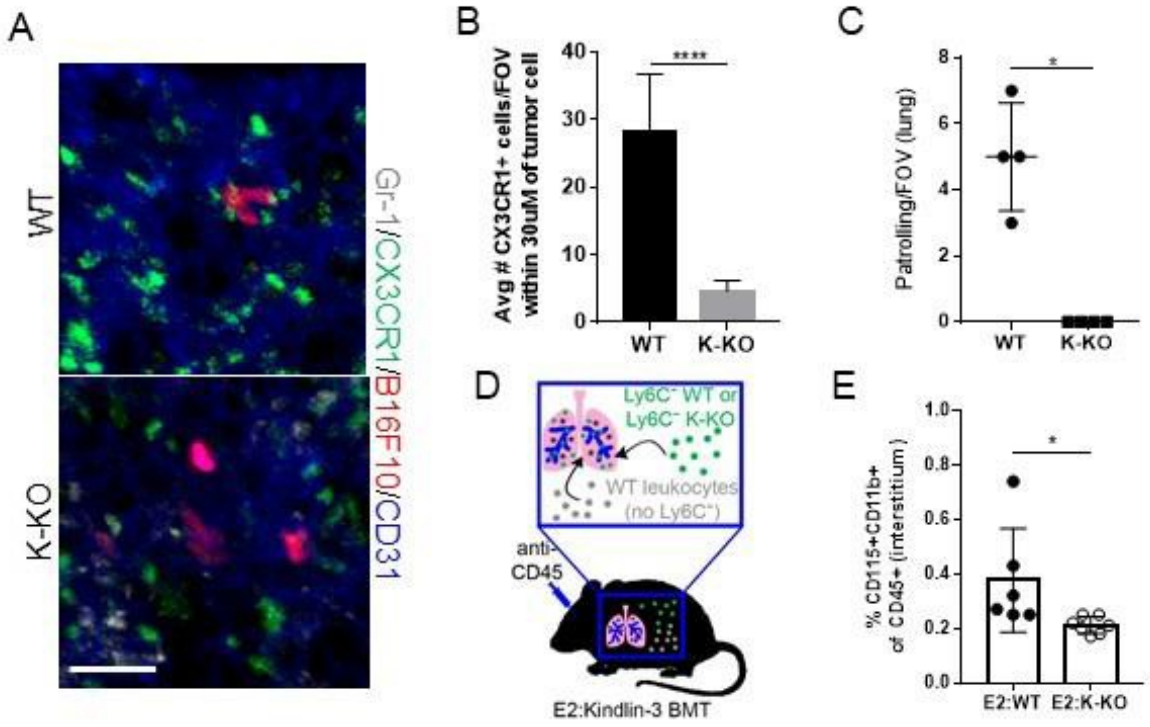


Figure 2.4. Kindlin-3 is required for nonclassical monocyte recruitment to sites of invading melanoma metastases in the lung and cancer particle uptake. (A) Snapshots of lung intravital imaging (Leica SP8, 25x 0.8 NA objective) in CX3CR1gfp/+Kindlin-3fl/flMx1-cre- (WT) or cre+ (K-KO) mice 2 hours after i.v. injection of 2.5x105 B16F10-RFP cells. Scale bar = 50 uM. (B) Analysis of intravital imaging from (A) using Imaris software to detect the average number of GFP+ monocytes per timepoint within 30 µM of B16F10-RFP cells. n=3 mice per group, independent experiments. (C) Number of patrolling nonclassical monocytes per FOV in mice from (A). n=4 mice per group, independent experiments. (D) Scheme of E2:Kindlin-3 BMT mice injected with 2 µg of BV650-labeled CD45 antibody 1 minute before mice were sacrificed, 16 hours after CellTrace Violet-labeled B16F10 were i.v. injected. Lungs were perfused with PBS, weighed, and the left lobe was mechanically dissociated and stained for leukocytes as well as APC-labeled CD45 antibody in order to detect how many monocytes are in the vasculature vs interstitial lung. (E) The frequency of total monocytes in the interstitial space of lungs from E2:Kindlin-3 BMT mice. n=6-7 mice per group. *p<0.05, ****p<0.0001

(Fig. 4 E), but not in the circulating vasculature (Fig. S3 C). We performed a similar experiment using LFA-1 integrin (αLβ2) knockout mice in place of Kindlin-3$^{-/-}$ bone marrow (Fig. S4 A), and found that E2:αL$^{-/-}$ and E2:β2$^{-/-}$ BMT mice mimicked the Kindlin-3$^{-/-}$ phenotype regarding nonclassical monocyte homing and cancer uptake. (Fig. S4 B, C). Overall the data suggest that trafficking to the sites of invading cancer cells in the lung is the limiting factor for monocytes to be able to take up cancer particles. In addition, Ly6C^{+} monocyte uptake of B16F10 particles were not affected by the lack of Kindlin-3 or high-affinity LFA-1 conformation, suggesting that this is unique to nonclassical monocytes and their patrolling behavior.

Effects of non-functional Ly6C^{-} monocytes on lung endothelial cells during cancer metastasis

Endothelial cells are capable of making transcripts that are involved in immune cell activation proliferation, and recruitment (Carson et al., 1994; Berard et al., 2003; Powell et al., 2017; Shi et al., 2006; Hamilton, 2008). Therefore, we measured the relative abundance of IL-6, CXCL1, IL-15, M-CSF, GM-CSF and VEGFR to ask if ECs in the lung responded aberrantly to invading B16F10 cells in the absence of functional patrolling monocytes. IL-6, IL-15, M-CSF and GM-CSF were all significantly decreased in E2:K-KO lung endothelial cells, while VEGF receptor was decreased but not significantly (Figure 5A and 5B). Not surprisingly, when we examined the immune cell population in the lung by flow cytometry, NK cells were reduced in lungs from E2:K-KO mice (Fig. S5 A). Interestingly, CXCL1, a neutrophil recruitment chemokine, was upregulated in E2:K-KO endothelial cells 2-fold above E2:WT cells. However, neutrophil frequency was not significantly altered at 16 hours post injection, although this could be a suboptimal time point to examine neutrophil recruitment (Fig. S5 B). These data suggest

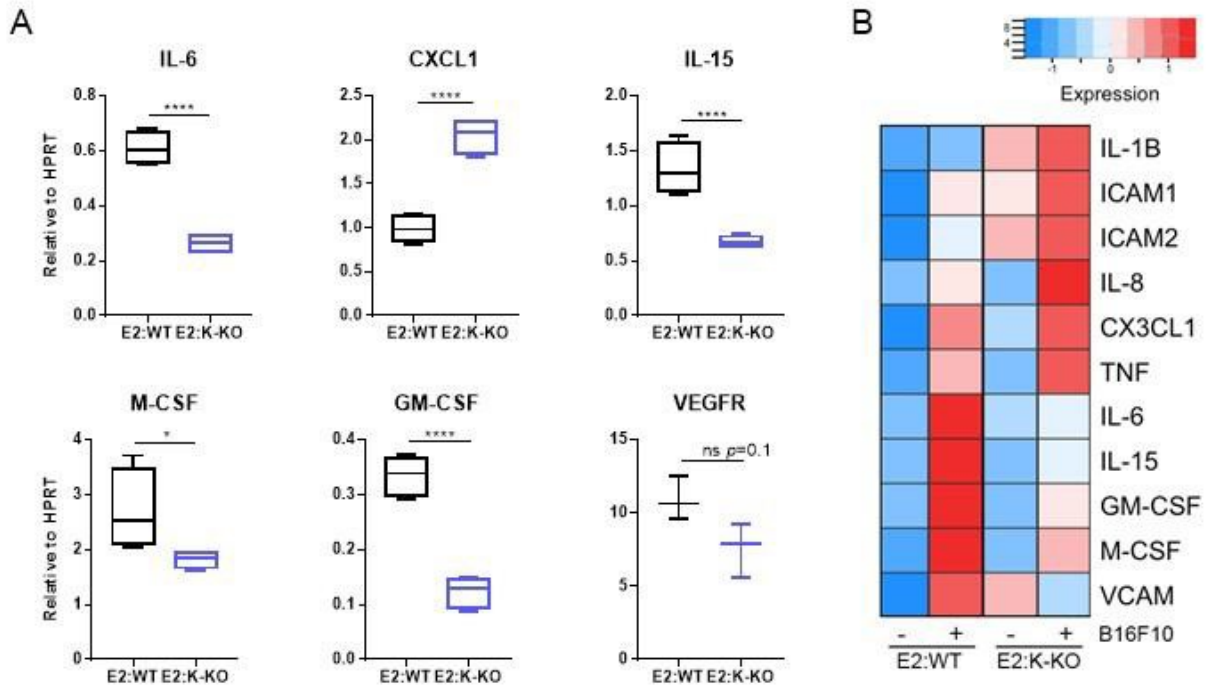


Figure 2.5: Lung endothelial cells in mice with dysfunctional nonclassical monocytes fail to make NK, T cell, macrophage and DC attracting cytokine and chemokine transcripts while increasing neutrophil recruitment transcripts. (A) Endothelial cells were digested out of lungs harvested from E2:Kindlin-3 BMT mice 16 hours after i.v. injection with 5x105 B16F10 cells, then sorted and purified for mRNA. qRT-PCR for inflammatory cytokines, integrin ligands and other signaling cytokines were run in duplicate with HPRT (housekeeping gene). n=3-4 mice per group. (B) Heat map of transcript signatures for each group of mixed BMT mice with and without B16F10 cancer cells injected. Each gene expression value was rescaled to reflect the range of expression for each individual gene. *p<0.05, ****p<0.0001, ns=not significant.

that patrolling directs how ECs respond to invading cancer cells. It is possible that in the absence of patrolling, pro-tumoral neutrophils are recruited to sites of metastasis (Mishalian et al., 2014; Sagiv et al., 2015), while other anti-tumoral populations may fail to be activated early on in metastasis.

Metastatic cancer is better controlled when nonclassical monocytes are able to patrol and take up cancer particles.

In the absence of patrolling, we asked whether nonclassical monocytes could still control metastasis to the lung. When B16F10 cells were labeled and injected into the DsRed:Kindlin-3$^{-/-}$ BMT mice, uptake of B16F10 particles in the Kindlin-3$^{-/-}$ nonclassical monocyte population was significantly decreased compared to WT nonclassical monocytes in the same mice (Fig. 6 A). To investigate the consequences of having non-functional Ly6C^{-} monocytes, we returned to the E2:Kindlin-3 BMT model. To test if these animals developed more metastases in the lung when nonclassical monocytes could not patrol, we injected 5x10^{5} B16F10 cells and assayed for metastatic sites by hematoxylin and eosin staining 2 weeks later (Fig. 6 B). Mice with WT nonclassical monocytes presented fewer areas of metastases compared to mice with K-KO nonclassical monocytes and the number of metastases per mm^{2} of lung was 4-5 fold higher on average in E2:K-KO mice (Fig. 6 C), indicating that patrolling is necessary for prevention of metastasis to the lung. The significant reduction of cancer particle uptake by Ly6C^{-} monocytes and the decrease in trafficking to the newly seeded tumors suggest that nonclassical monocytes exert their anti-tumoral function through their patrolling behavior, either through direct contact with endothelial cells that are exposed to metastatic cancer cells, or through recruitment of other immune cells.

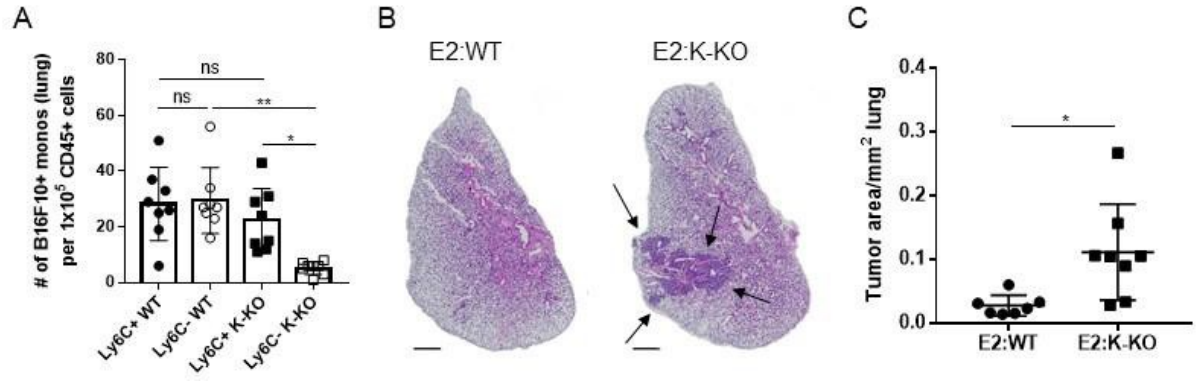


Figure 2.6. Metastatic cancer is better controlled when nonclassical monocytes are able to patrol and take up cancer particles. (A) 2.5x105 B16F10 cells were labeled with CellTrace Violet and injected i.v. into DsRed:Kindlin3-/- BMT mice. 16 hours later, classical (Ly6C+) and nonclassical (Ly6C-) monocytes from WT donors or K-KO donors within the same mice were assessed for uptake of B16F10-violet particles per 1x105 CD45+ cells in the lung. (B) Hematoxylin and eosin stained lungs were prepared from E2:Kindlin-3 BMT mice i.v. injected with 5x105 B16F10 cells 2 weeks before harvesting. Images represent biological triplicate. Scale bar 1000 μM. (C) Number of B16F10 metastases quantified per section of H&E stained lungs from E2:Kindlin-3 BMT mice. Each point is an individual section from a total of 3 biological replicates. *p<0.05, **p<0.01

Discussion

In this study we find that Kindlin-3 is essential for the patrolling behavior of nonclassical monocytes, and that firm adhesion is required prior to patrolling. We designated the inability to patrol due to lack of firm adherence as “slipping”, which suggests that high-affinity conformation and clustering of integrins is required to initiate patrolling. Furthermore, Kindlin-3 is necessary for patrolling monocytes to take up metastasizing cancer particles and to recruit NK cells to the lung. Despite nonclassical monocytes circulating through the lung vasculature, without firm adhesion and patrolling, they were unable to reduce tumor metastases to the lung.

During metastasis, both E2:WT and E2:K-KO mice showed elevated levels of several inflammatory transcripts, in addition to other cytokines and chemokines which recruit and regulate activation and proliferation of various immune cells. It is surprising that patrolling

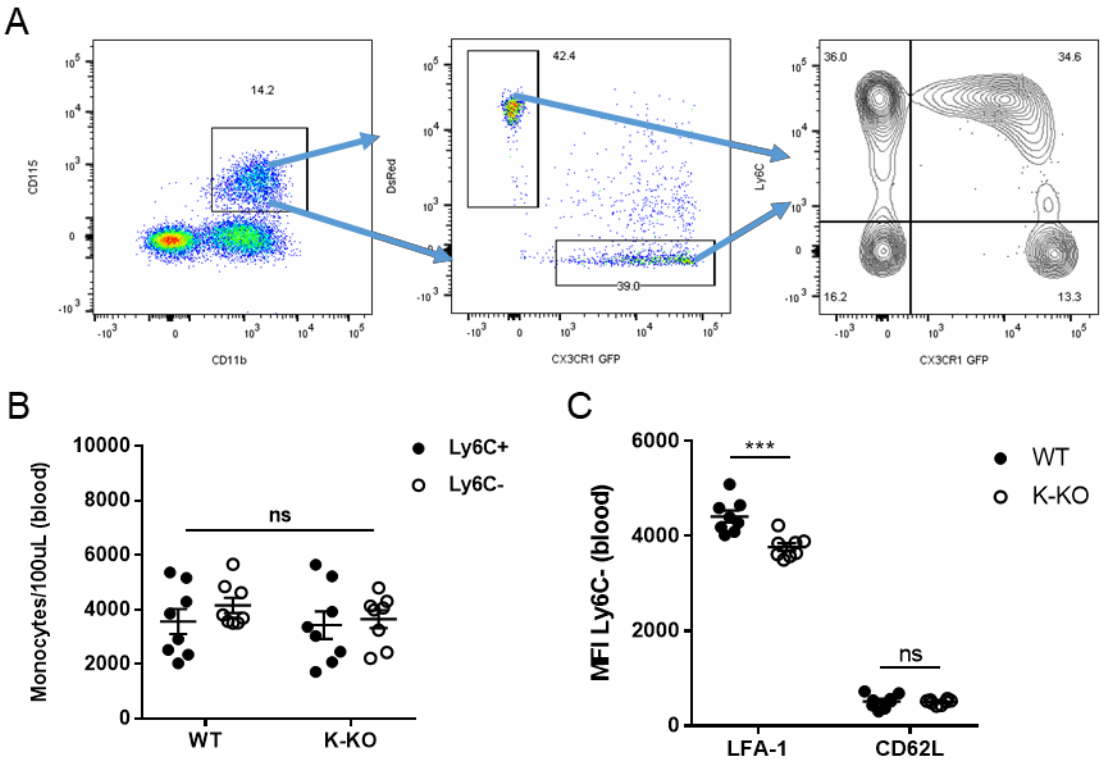
monocytes could affect such a wide range of output from endothelial cells, but these findings reinforce the notion that nonclassical monocytes communicate with the endothelium via a cell-cell contact basis. Despite CXCL1 being elevated in E2:K-KO mice, we did not find increased neutrophil frequencies in the lung or vasculature, but due to the timing of neutrophils arriving minutes to hours after metastasis is initiated, it is possible that 16 hours post B16F10 injection might have been too long to observe neutrophil recruitment(Headley et al., 2016; Qian et al., 2011).

The E2:Kindlin-3 mixed BMT mouse model uses wild-type immune cells to subsidize the immune cells that are Kindlin-$3^{-/-}$, except for the nonclassical monocyte population. This BMT was generated to look at the consequences of having Kindlin-$3^{-/-}$ nonclassical monocytes, since there is no mouse model at present that is able to examine specific knockouts within that subset. While we expect the normal half of the immune system to function identically to a healthy wild-type mouse, it is possible that simply having Kindlin-$3^{-/-}$ counterpart immune cells present in the same mice could affect the biological outcomes.

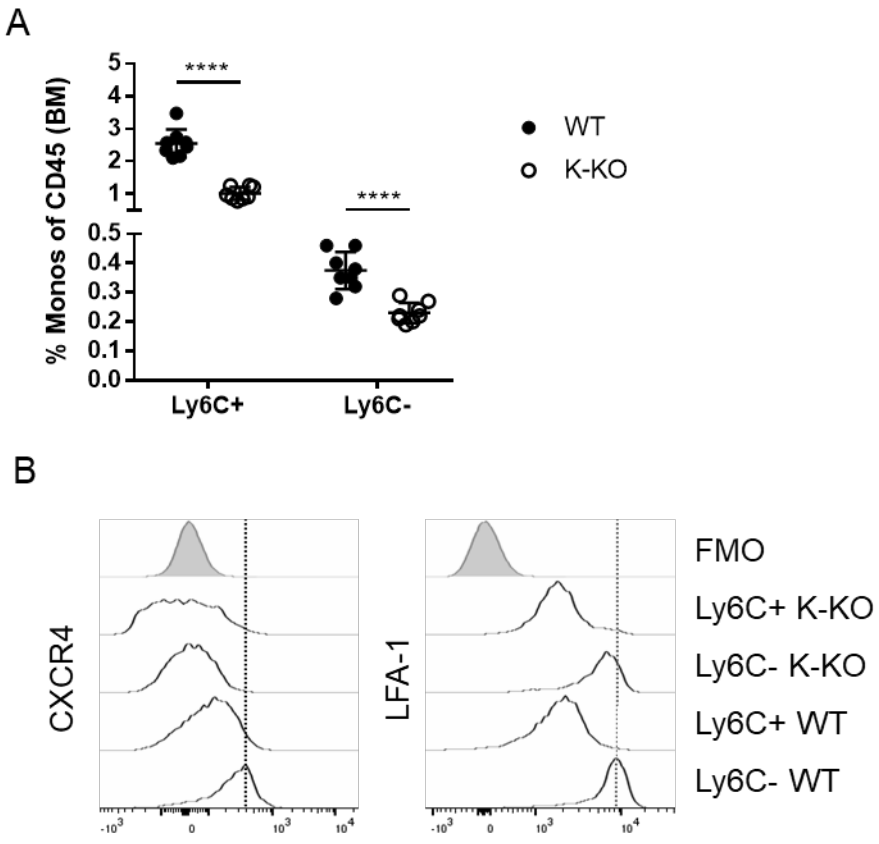
In this report, we have established an experimental system that maintains nonclassical monocytes in the periphery but renders them non-functional through the deletion of Kindlin-3. Our data show that patrolling is necessary not only for the anti-tumoral response, but also for maintaining endothelial health by cell-cell contact. Whether non-patrolling nonclassical monocytes can still secrete chemokines for anti-tumoral immune cell recruitment, or whether they can recognize tumor cells through integrin interactions are future questions that could help us better understand the myeloid immune microenvironment during metastasis. In summary, nonclassical monocyte patrolling is essential for carrying out their biological functions, and the

more this pathway is understood, the greater chance for finding a therapeutic target in this pathway.

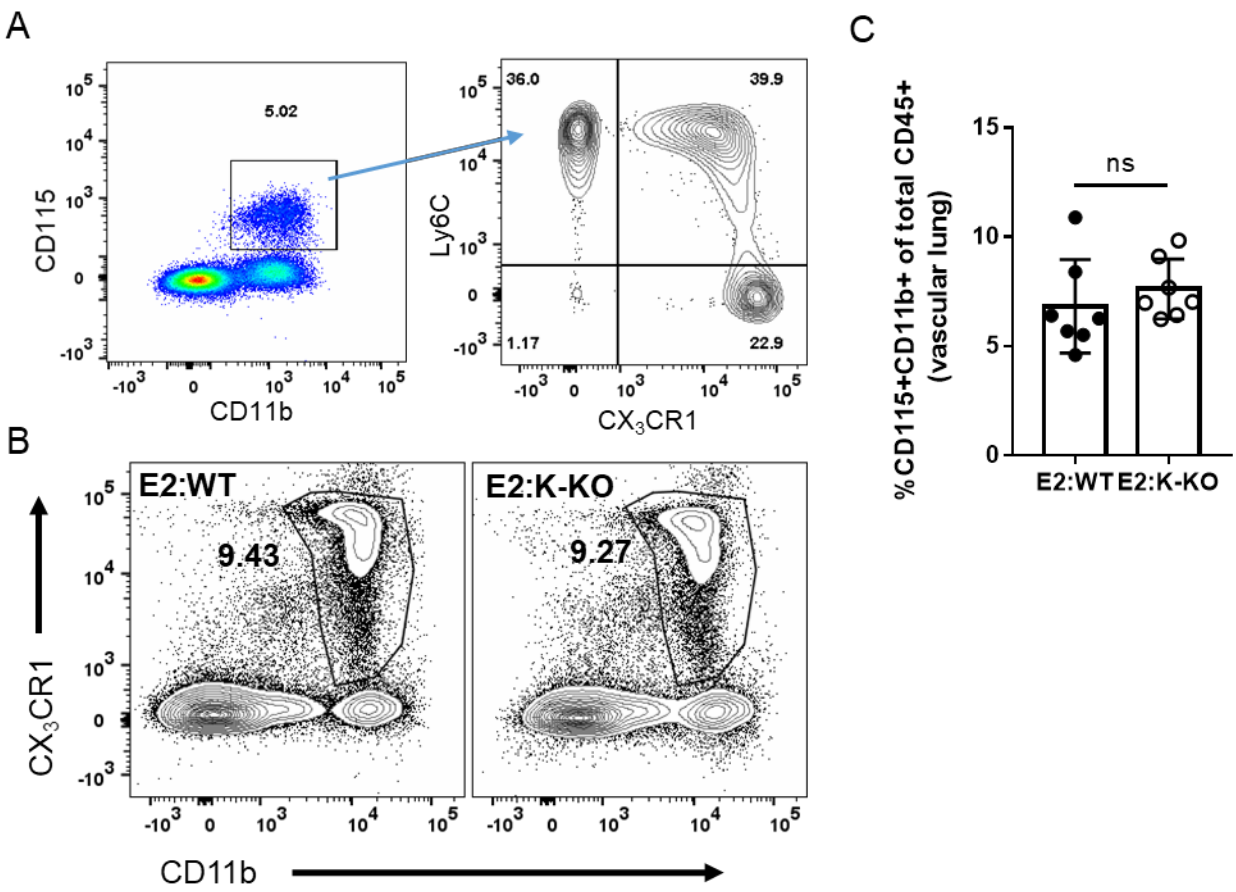
Supplemental Information



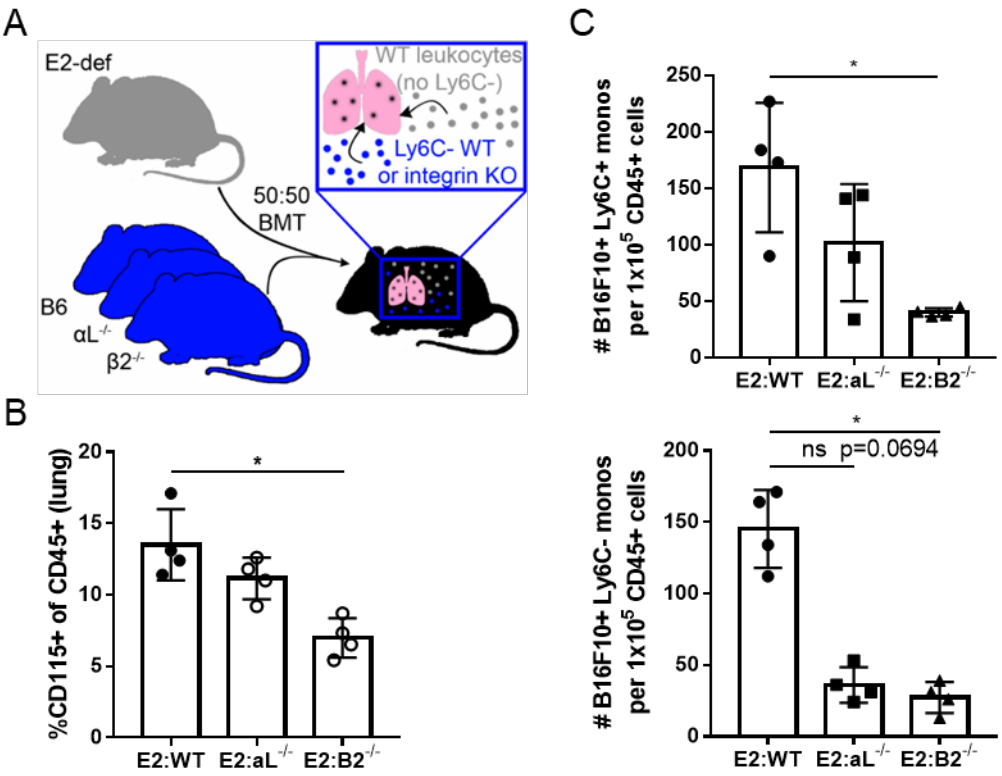
Supplemental Figure 2.1. DsRed:Kindlin-3 BMT mice mice have equal numbers of monocytes in the periphery, but decreased levels of LFA-1. (A) Gating scheme for monocyte subsets in DsRed:Kindlin-3 BMT mice. (B) Blood was collected from WT and K-KO mice by cardiac puncture and a 100 µL aliquot was stained for $CD45^{+}$ $CD11b^{+}CD115^{+}Ly6C^{+/-}$ monocytes and run on a LSRII flow cytometer until the entire sample was acquired. n=8 mice per group. (C) Median fluorescent intensity for LFA-1 (Kindlin-3 binding) and selectin CD62L (non-Kindlin-3 binding) for wild-type and Kindlin-$3^{-/-}$ $Ly6C^{-}$ monocytes in blood of the same recipient. n=8 mice per group ***p<0.001, ns=not significant.



Supplemental Figure 2.2. Monocytes show decreased frequency in the bone marrow of DsRed:Kindlin-3 BMT mice and increased necrosis in the periphery. (A) The frequency of $CD115^{+}CD11b^{+}CD117^{-}$ monocytes in the bone marrow of $CD45^{+}$ cells. (B) Representative histogram distribution of CXCR4 and LFA-1 on $Ly6C^{+}$ and $Ly6C^{-}$ monocytes in the bone marrow, compared to FMO (fluorescence minus one). Dotted line marks highest expression. Representative of n=7 mice per group. ****p<0.0001



Supplemental Figure 2.3. Monocyte frequencies and CX_3CR1^+ myeloid cells from lungs of E2:Kindlin-3 BMT mice. (A) Gating scheme for E2:Kindlin-3 BMT mice from blood collected by cardiac puncture. (B) Frequencies of myeloid cells that are GFP^+CD11b^+ in E2:Kindlin-3 BMT mice. (C) Monocyte frequencies in the vascular bed of the lungs in mice out of total $CD45^+$ cells in E2:Kindlin-3 cre- (E2:WT) or cre+ (E2:K-KO) BMT mice. ns=not significant. (D) Frequency of $NK1.1^+$ cells out of $CD45^+$ cells in the lungs of E2:Kindlin-3 BMT mice. n=7 mice per group. (E) Frequency of $Ly6G^+$ cells out of $CD45^+$ cells in the lungs of E2:Kindlin-3 BMT mice. n=7 mice per group. ***$p<0.001$, ns=not significant.



Supplemental Figure 2.4. LFA-1 knockout mimics Kindlin-3 knockout phenotype during cancer metastasis to the lung. (A) Scheme of bone marrow transplantation for integrin knockout chimeras with E2 bone marrow donors at a 50:50 ratio. (B) Frequency of $CD115^{+}$ monocytes in the lungs of BMT mice. n=4 mice per group. (C) Numbers of $Ly6C^{+}$ (top panel) or $Ly6C^{-}$ (bottom panel) that are positive for B16F10 cell particles (CellTrace Violet labeled) 16 hours after i.v. injection in either E2:$\alpha L^{-/-}$, E2:$\beta 2^{-/-}$ or E2:WT bone marrow chimeric mice. n=4 mice per group. *p<0.05, ns=not significant

Methods

Mice and Reagents

C57Bl/6J, E2-def, and $CX_3CR1^{gfp/+}$Kindlin-$3^{fl/fl}$Mx1-cre(+/-) were bred inhouse. CD45.1 (002014) and DsRed*MST (006051) mice were purchased from the Jackson Laboratory. B16F10-RFP (AntiCancer Incorporated) and B16F10 cells (ATCC CRL-6475) were cultured according to ATCC protocols. Poly(I:C) (Sigma P1530) was used to induce cre expression in Mx-1 cre+ mice by injecting 200uL i.p. every other day for 5 days. B16F10 were labeled with CellTrace Violet (Thermo Fisher C34571) according to manufacturer instructions. B16F10 injections were performed retro-orbitally for overnight (16 hour) experiments, or by tail-vein for longer experiments. All injections were 0.4 μM filtered and resuspended in 100 μL sterile PBS.

Bone marrow transplants

Bone marrow donors were sacrificed and bones were separated from marrow by sterile centrifugation (8000 x g 30 seconds). Cells were resuspended in sterile PBS, washed and filtered twice and counted with a BD Coulter Counter. CD45.1 recipients were irradiated with 900 rads and rehoused in sterile cages with sterile food, a gel cup and water (supplemented with 0.5mL/8oz). Mice were anesthetized with isoflurane immediately after irradiation and retro-orbitally injected with 10^7 bone marrow cells from mixed bone marrow donors (50:50 ratio).

Flow Cytometry

Blood was obtained either by retro-orbital bleeds or by cardiac puncture. Retro-orbital bleedsc were carried out under anesthesia using micro-hematocrit capillary tubes (Fisherbrand 22-362-566) and 2 mM EDTA (final concentration) to prevent clotting. Cardiac punctures were performed post-mortem using 25G needles loaded with 2mM EDTA. Blood samples were first

lysed of red blood cells with 1x lysis buffer (0.15M NH_4Cl, 10mM $NaHCO_3$, 1.1mM EDTA-disodium in sterile H_20) for 10 minutes at 4C on a rotator. Lungs were first weighed (total lungs and left lobe), then either mechanically dissociated (left lobe) with a 70 μM cell strainer and syringe plunger, or diced and enzymatically digested (remainder of lung lobes) in 1 mL of Liberase TM and DNaseI in HBSS at 37C shaker for 30 minutes in a microtube shaker (all Thermo Fisher). Bone marrow was centrifuged out of tibias (1 per mouse) and RBC lysed for 5 minutes at room temperature. All samples were resuspended in FACS buffer (1x PBS, 1% BSA, 2 mM EDTA) with 1:200 Fc blocker (Biolegend; 93) and stained with antibody cocktails for 10 minutes on ice. CXCR4 (Biolegend; L276F12) staining was done at 37C for 15 minutes before adding the remaining antibodies and incubating on ice. The following antibodies were used for flow cytometry: CD45 (30-F11), CD115 (AFS98), CD11b (M1/70), Ly6G (1A8), Ly6C (HK1.4), CD3 (17A2), CD19 (6D5), B220 (RA3-6B2), NK1.1 (PK136), MHCII (M5/114.15.2), CD11c (3.9), F4/80 (BM8), CD54 (YN1/1.7.4), CD11a (I21/7), CD18 (1B4/CD18), LFA-1 (H155-78), CD62L (MEL-14), CD117 (2B8), all from Biolegend; Live/Dead (Tonbo; 13-0870). FACS experiments were performed on a LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar version 10.7).

FACS Sorting

Sorting was performed by the LJI core facility on a FACSAria (BD Biosciences). Fc block was added before all staining and primary conjugated antibodies were incubated with cells in FACS buffer (PBS, 1% BSA, 2 mM EDTA, 0.05% NaN_3) for 15 minutes covered at 4C.

Intravital Imaging

All intravital imaging was performed on a Leica SP8 confocal microscope with a 25x 0.95 NA objective using LAF X software. Mice were surgically prepared as previously described for

imaging of the femoral vasculature (Marcovecchio et al., 2017). Antibody labeling of Ly6C (HK1.4; Biolegend) and CD31 (390; Biolegend) was done by injecting 2-5 μg of each antibody 5 minutes prior to imaging the animal. For stimulation of monocyte patrolling, 500 ng of recombinant mouse TNFα in PBS was injected i.p. 1 hour before imaging the animal. Lung imaging was prepared as described previously (Hanna et al., 2015; Looney et al., 2011; Headley et al., 2016). Antibodies to label CD31 (Biolegend; 390), Gr-1 (Biolegend; RB68C5) or Ly6C (Biolegend; HK1.4) were injected as described above. B16F10-RFP cells were injected retro-orbitally. Mice were imaged for approximately 1 hour after surgery. Timelapse images were analyzed using Imaris (Bitplane) 9.1 software. Images were drift-corrected and $GFP^{+}Gr\text{-}1^{-}$ (or $Ly6C^{-}$) monocytes were tracked using spot selection. For binning path types: monocytes that moved at least 5 μm (track displacement) in the lung tissue or endothelium for more than 60 seconds (track duration) were counted as patrolling; monocytes that stopped momentarily (1-2 frames at a time) were counted as slipping; monocytes that stopped on the endothelium for more than 2 frames, regardless of whether they remained or entered the circulation again were counted as arrested.

H&E staining of lung sections

Histology services were provided by the La Jolla Institute for Allergy and Immunology Histology Core Facility. 5 μm sections were deparaffinized with ProPar and rehydrated in a series of graded alcohols, stained with hematoxylin and eosin using a regressive staining protocol, dehydrated, and embedded with #1.5 coverglass (Fisherbrand). Sections were imaged on an AxioScan Z1 automated slide scanner (Zeiss) with 20x 0.8 NA objective.

Quantitative RT-PCR

E2:Kindlin-3 BMT mice were injected with $3x10^{5}$ B16F10-RFP cells approximately 16

hours prior to harvesting. Lungs were perfused with 10 mL of PBS with 2 mM EDTA. Lungs were diced and enzymatically digested as described in Flow Cytometry. Cells were stained with Live/Dead (Tonbo; Ghost Dye Violet 510) , CD31 (Biolegend; 390), and CD45 (Biolegend; 30-F11). Endothelial cells were identified as Live/Dead^{-}CD45^{-}CD31^{+} and sorted into PBS with 2% FBS. Cells were resuspended in Trizol (Thermo Fisher 15596026) to obtain purified RNA using Zymo Research Direct-zol kit (R2052). cDNA was made using iScript Reverse Transcription Supermix (Bio-Rad 1708840) from equal amounts of RNA input and 20 μL reaction volumes were run on a Roche 480 Lightcycler for 50 cycles. Samples from 3-4 mice per group were run in duplicate. The following Taqman probes from Thermo Fisher were used to quantify gene expression: HPRT (Mm03024075_m1), Il-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), CXCL1 (Mm04207460_m1), IL-15 (Mm00434210_m1), CX3CL1 (Mm00436454_m1), TNFα (Mm00443258_m1), VEGFR (Mm00438980_m1), ICAM-1 (Mm00516023_m1), ICAM-2 (Mm00494862_m1), VCAM (Mm01320970_m1), M-CSF (Mm00432686_m1), GM-CSF (Mm01290062_m1). Ct values were averaged per sample and gene expression was normalized to HPRT expression using delta delta Ct calculations.

Statistics

Monocyte frequencies and numbers were analyzed using a Kolmogorov-Smirnov test without assuming consistent SD. Grouped analyses (monocyte subsets and path types) was done using multiple t-tests with Holm-Sidak method for multiple comparisons. B16F10 uptake by monocyte subsets was analyzed with a Kruskal-Wallis test. qRT-PCRs were analyzed by 2-way ANOVA (WT, WT+B16, K-KO, K-KO+B16) or Kolmogorov-Smirnov without assuming consistent SD.

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Authorship

P.M.M and C.C.H. conceived and designed the study. P.M.M. performed all the experiments and analyses except where acknowledged in the Acknowledgements. R.N.H. and S.M. provided expertise in lung imaging and aided imaging experiments. R.W. provided assistance with H&E sample preparation. S.R. assisted with animal experiments and qRT-PCR. K.L. provided invaluable discussions and manuscript editing as well as Kindlin-3$^{fl/fl}$Mx-1-cre mice. H.Q.D. performed helpful analysis of flow cytometry data using t-SNE and clustering methods.

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CONCLUSION

From the data generated by these studies, we propose the following model for nonclassical monocyte patrolling: receptor binding (non-GPCR), ITAM-SFK activation/phosphorylation, Kindlin-3 recruitment to β2 cytoplasmic tail, LFA-1 clustering, patrolling. Many of the details within this proposed mechanism are still unclear, such as how Kindlin-3 is recruited after ITAM-SFK activation, and what other accessory proteins are required during patrolling. Much of the work involving the role of SFKs and Kindlin-3 in cell migration has been done in other immune cell types, such as neutrophils, macrophages and T-cells, or in cell lines from in vitro experiments. However, to our knowledge, this is the first set of experiments to look at the in vivo intracellular molecules involved in patrolling.

The ability to respond within minutes to inflammatory cues and to survey tissues during homeostasis are the two essential functions of nonclassical monocytes. Their preference for residing in the circulation and ability to interact with the endothelium in the periphery as well as the tissue microvasculature puts them in a position to rapidly respond to damage and inflammation. How they respond once they are within the microenvironment of the stimulus is not always predictable. In some cases, they can respond with additional inflammatory cytokines, while in other situations they appear to be mediators of inflammation resolution. The data from our studies suggest that effector functions of nonclassical monocytes occur after activation of patrolling, and certainly the ability to survey the vasculature would be the first effector function to be hindered. In the context of different diseases, for example atherosclerosis, it would depend on nonclassical monocytes having the appropriate receptors to react to the danger signal. Subsequently, patrolling would be initiated, theoretically to search for the source of distress, and once the area of damage or inflammation is encountered, carry out their function based on the

environmental stimulus. We typically think of cytokines, chemokines and receptor/ligands to recruit or activate immune cells as different types of signals: “danger”, “eat me/don’t eat me”, self/non-self. In regards to nonclassical monocyte responses to inflammation, patrolling appears to be initiated or induced by “help me” signals. Atherosclerosis and metastatic cancer are progressive diseases in which host derived factors (cholesterol or host cells) induce vascular inflammation which leads to tissue damage and more fatal downstream events. Without patrolling monocytes, both of these diseases are exacerbated, i.e. more plaque formation or more metastases).

In atherosclerosis and dyslipidemia there is some evidence to suggest that nonclassical monocytes in mice can accumulate LDL or triglyceride particles and extravasate into atheromata or tissues where they presumably differentiate further into CD11c+ cells (Saja et al., 2015; Tacke et al., 2007). Several papers now have shown that patrolling is increased during atherosclerosis and that in the absence of nonclassical monocytes the aortic endothelium possess greater plaque per surface area (Hanna et al., 2012; Marcovecchio et al., 2017; Quintar et al., 2017). Although we did not observe any extravasation into the surrounding interstitium while imaging the femoral vasculature during Western-diet feeding, we did find that nonclassical monocytes readily took up fluorescently labeled OxLDL. However, these particles did not seem to significantly accumulate within nonclassical monocytes as assessed by staining of free cholesterol (filipin) and cholesterol esters/lipid droplets (bodipy), both of which were far less abundant when compared to classical monocytes. One approach to answer whether patrolling monocytes enter the plaque and reside there would be to look at adoptively transferred fluorescently labeled cells into an atherosclerotic mouse. Although technically difficult and reagent consuming, this would allow for a very controlled look at nonclassical monocytes homing to plaques, without the complications of

ontogeny. Previous models to look at extravasation of nonclassical monocytes have used dextran particle uptake or the CX_3CR1-GFP expressing mouse, but both methods come with drawbacks. As yet there is no clear understanding of how patrolling monocytes protect the endothelium from damage during atherosclerosis. Further investigation of whether nonclassical monocytes enter the atheromata/plaque area and what they do downstream of entering the site is required.

An interesting hypothesis would be if the patrolling function of nonclassical monocytes during atherosclerosis acted as a sponge or mop in order to shuttle excess cholesterol in the form of LDL to the liver. There is already evidence that nonclassical monocytes migrate to the liver sinusoids for wound healing and resolution of inflammation in mice (Brempelis and Crispe, 2016). In addition, there is RNAseq evidence of cholesterol efflux transporters ABCA1 and ABCG1 being upregulated in the patrolling monocyte subset, raising the question of why these cells would be taking up and effluxing cholesterol (Marcovecchio et al., 2017). Based on the evidence, it appears that patrolling monocytes vacuum up harmful particles to prevent endothelial dysfunction during atherosclerosis, but it is possible these cells are carrying out additional biological activities outside the blood vessels.

Patrolling has now been shown to occur in large blood vessels with high shear stress. As mentioned before, not only are there differences between microvessels and macrovessels, the interactions between leukocytes and endothelial cells also differ between microvasculature compared to macrovasculature. The frequency of patrolling in the femoral vasculature during homeostasis is dramatically reduced than the frequency of patrolling in microvessels during homeostasis. However, during vascular inflammation, such as with Western diet feeding, patrolling monocytes are found not just at the sites of plaque deposition (i.e. near the aorta), but are found even more distally, at sites where plaque is thought to never develop. This suggests

that homeostatic patrolling is not random or unsolicited. Rather, even during homeostasis, a signal or cue must be received by the nonclassical monocyte for recruitment to the endothelium for patrolling.

Many of the initial studies on patrolling were performed in microvessels or intra-organ vasculature, such as in the skin and kidney, which possess diverse molecular and physiological interactions with leukocytes that traverse them (Auffray et al., 2007; Cros et al., 2010; Carlin et al., 2013; Tacke et al., 2007; Butcher et al., 1986; Belloni and Tressler, 1990). As such, differences in what receptors might be necessary for homing and recruitment, as opposed to what molecules are necessary for the patrolling mechanism, would be illustrated by the tissue type, shear stress, and biochemical signal. For example, homing to a site of endothelial distress, or extravasation into a tissue type might be mediated by a chemokine receptor such as CX_3CR1. It has been shown with classical monocytes that C-C and C-X-C receptors are crucial for entering the circulation from bone marrow, entering atheromata, and extravasating into kidneys. It has also been shown that TLR signaling induces patrolling, although in part this is due to the endothelium becoming activated using TLR7 agonist R848. We tested several mutant mice with ablations in TLR7, CX_3CR1, and CCR5 (not shown in paper) during a Western diet feeding, and none of these deterred nonclassical monocytes from patrolling despite some decreases in frequencies. This could be due to the type of stimulus (high fat diet) and differences in location of imaging the patrolling, but we conclude that these receptors are not directly contributing to the patrolling mechanism, which we consider to be distinct from homing, extravasation, or even traditional crawling. In the case of chemokine receptors or TLRs, survival and extravasation into tissues are likely mediated by these receptors being activated.

The results of previous studies using antibody blockade and genetic deletions have shown that LFA-1 is the predominant integrin used by nonclassical monocytes to patrol the vasculature in mice (Auffray et al., 2007).Two categories have been assigned to the type of signalling occurring through integrins: inside-out and outside-in. They concisely describe the mode of signal transduction, either initiating from a receptor and traversing adaptor proteins and kinases until integrins become fully activated (inside-out), or, once they bind extracellular macromolecules and ligands, transmit signals back down into the cell to carry out further functions like cytoskeleton rearrangement, cell division and gene expression, and cell motility(outside-in)(Clark and Brugge, 1995; Qin et al., 2004). The archetype of inside-out signaling to activated integrins is through a G-coupled protein receptor (GPCR), such as a chemokine receptor. Therefore, when we studied the effects of knocking out CD36, a receptor that requires adaptor proteins due to a truncated cytoplasmic tail, it was surprising that we saw lack of patrolling. If indeed CD36 is directly responsible for activating integrins through DAP12 and SFK, it would be a deviation from the canonical pathway of GPCR stimulation. Although this is not unheard of in the literature, it requires more detailed and molecular studies to prove that CD36 is activating integrins in a sequential process from ligand binding to integrin binding. In addition, CD36 might not be the only receptor involved with inducing patrolling during Western diet feeding, as CD36 has been shown to associate with other cell surface receptors. Since the mechanisms involved in patrolling have not been elucidated and by all accounts is distinct from the traditional mode of leukocyte crawling, it is possible that patrolling could be categorized as a function of nonclassical monocytes, rather than a part of cell motility, such that the CD36 receptor could reasonably be involved with its activation.

Essentially, whether in homeostasis or during vascular inflammation, patrolling is a response to a “help me” signal by the endothelium. We showed that patrolling is increased in large vessels, not just at sites of plaque formation, but distal to traditional vulnerable or susceptible sites on the endothelium such as the aorta. This suggests when there is systemic vascular inflammation, patrolling is not site specific in its role of endothelial repair. It is probably that nonclassical monocytes follow a gradient to where there is the greatest endothelial inflammation, but as vascular inflammation is by nature systemic, all endothelial cells have the potential to be dysregulated. Indeed, despite the distance from the aorta, we still found a 4-fold increase in the numbers of patrolling monocytes during Western diet feeding. Although we tested for apoptotic signals within the endothelium with intravital imaging (data not shown), we did not see any significant increases in cells that were being patrolled versus cells that did not have patrolling monocytes on them. This also suggests that patrolling can be induced early during dysregulation of endothelial cells, at the first hint of trouble, before endothelial cells enter apoptosis or even necrosis. The next question remains, what are patrolling monocytes doing while they are patrolling? Is it just cell-cell receptor:ligand contact that is inducing pathways in the endothelium, or are patrolling monocytes actively taking up molecules that are deposited on the endothelium? Such investigations require a more detailed molecular or superresolution approach to answer, but would useful in predicting their role in other diseases.

During cancer metastasis, patrolling again serves as a scavenger and responder to endothelial distress, this time due to invading tumor cells in the lung microvasculature. In both mice and humans, nonclassical monocytes play an important role in mediating a proper anti-tumoral response by taking up tumor particles and recruiting NK cells through CCL3/4/5, which allow for cytolytic tumor killing and stimulation of adaptive responses (Hanna et al., 2015; Lavin

et al., 2017; Morvan and Lanier, 2016). Again, the presence of patrolling monocytes is necessary for an anti-tumoral response, but is the patrolling function itself involved? To study this, we first looked at the role of an integrin adaptor gene, Kindlin-3, in patrolling. Again to our surprise, patrolling in the periphery was completely eliminated after knocking down Kindlin-3 with an inducible cre/lox system. Prior studies have shown that Kindlin-3 has a very specific role of changing integrin conformation to a high-affinity state. Often paired with Talin-1, Kindlin-3 can modulate the binding affinity of integrins through the binding of beta integrin tails. Since the Kindlin-3 isoform is found predominantly in leukocytes, it has been shown to play an important role in LFA-1-mediated motility, although to some extent it also is involved with VLA-4. Therefore, our focus was on the role of Kindlin-3 acting through LFA-1 to mediate patrolling.

It has been shown that while Kindlin-3 is not necessarily required for diapedesis or certain types of integrin binding, it has been shown to be necessary for certain types of immune cell and platelet function. Thus, if patrolling is a cellular function of nonclassical monocytes that requires the use of integrins, it stands to reason that Kindlin-3 would fit into this model of regulating the patrolling behavior. Kindlin-3 has been reported in humans as necessary for proper blood clotting and immune response to infection, namely due to platelet and neutrophil dysfunction. The shortened lifespan of leukocyte adhesion deficiency (LAD-III) patients carrying mutations in their fermt3 gene can be attributed to the dysregulation of hematopoietic progenitor activation and proliferation. However, it has not been reported extensively in the literature what the phenotype of dysregulation in monocytes would be in these patients. With the evidence from our mouse models showing that nonclassical monocytes are unable to patrol, have increased endothelial inflammation, and have less capacity for preventing lung metastasis, it is possible that monocytes are also defective in humans with similar physiological dysfunctions.

Although it is very clear that Kindlin-3 binds to the N-P-X-Y motif, which overlaps with beta integrin cytoplasmic tail binding sites of negative regulators of integrin activation (filamin) or regulators cell spreading (14-3-3) (Moser et al., 2009; Morrison et al., 2013; Takala et al., 2008; Fagerholm et al., 2014), additional binding sites of Kindlin-3 are being explored. Not only are new putative binding sites for Kindlin-3 being discovered, but also other protein binding sites onto Kindlin-3 itself are still undetermined. Therefore, it is entirely possible that in the course of patrolling activation, Kindlin-3 may be binding to non-canonical sites, or have interactions with non-canonical mediators of Kindlin-3 recruitment.

Lastly, our model highlights a possible pathway for nonclassical monocytes to differentiate into antigen presenting cells during metastasis. Although further work is needed to identify exactly what population of APC nonclassical monocytes are differentiating into, we have shown that it is possible patrolling monocytes play a role in managing the microenvironment after metastasis to the lung occurs. In wild-type mice, nonclassical monocytes are able to extravasate into the tissue at a higher rate than Kindlin-$3^{-/-}$ monocytes, and in the lungs of these mice after 16 hours with B16F10 melanoma cells there is a population missing when Kindlin-3 is ablated. Part of the uncertainty is that the field of monocyte research is lacking a nonclassical monocyte-specific knockout mouse model, whereby genes may be conditionally knocked out solely in the Ly6C- monocyte population. We have tried to overcome this by utilizing a mixed chimera in which half the leukocytes are wild-type but deficient for nonclassical monocytes, while the other half of the leukocyte population is deficient in Kindlin-3, including a nonclassical monocyte population. With this scheme, we conducted our metastatic cancer experiments with the conclusion that any differences we found were due to the nonclassical monocyte population being deficient for Kindlin-3, since it is the only leukocyte

population in the mouse which is not compensated for by a wild-type counterpart. In theory, all other leukocytes should behave normally, even at 50% frequency, but as we did not conduct extensive comparisons at baseline, it is possible there are consequences that we could not detect using this chimeric bone marrow model. Therefore, whether the abnormalities detected in the lungs of tumor injected mice are a direct link to nonclassical monocytes simply not being able to get into the lung tissue, or if patrolling monocytes indirectly influence the myeloid repertoire in the lung after metastasis through cytokine secretion or some other aspect of their presence, remains to be clarified.

While these are the first studies to look at intracellular activation and mediation of the patrolling behavior in nonclassical monocytes, there is still much more detail about the mechanism of patrolling to be elucidated with the potential for new protein interactions in the pathway of integrin activation, recycling, or clustering. Patrolling by nonclassical monocytes could be categorized as a new cellular function, as opposed to an altered form of cell motility, as nonclassical monocytes seem to use this process exclusively in the endothelium, compared to other leukocytes in the bloodstream, for the sole purpose of surveying and repairing the endothelium. As for that matter, the actual mechanism of homeostatic maintenance has yet to be shown, and only experimental data during induced necrosis has been offered to illustrate the sentinel duty of nonclassical monocytes. In addition, new discoveries of patrolling being effective at controlling amyloid plaque deposits and viral infections (Lüdtke et al., 2016; Michaud et al., 2013) encourage further studies of this monocyte subset for use in various therapeutic treatments.

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