Focal Adhesion Kinase (FAK) Family Members Regulate Monocyte Survival at Homeostasis and Macrophage-mediated Control of Breast Tumor Progression

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Abstract

Monocytes and macrophages are innate immune effector cells that perform a diverse set of activities essential for the maintenance of tissue homeostasis and the process of disease resolution. Hallmarks of these cells include substantial functional plasticity and the capacity to migrate. These attributes are strongly influenced by extra-cellular stimuli, and the ability to properly integrate environmental signals is critical for monocytes and macrophages to perform their effector functions. The FAK family of kinases consists of two closely related non-receptor protein tyrosine kinases, FAK and Pyk2, which function as important regulators of integrin-mediated signaling. My thesis research has focused on establishing the expression and function of FAK kinases in monocyte and macrophage populations. In this thesis, we demonstrate that FAK is not abundantly expressed in monocytes, but is highly expressed in macrophages. In contrast, Pyk2 could be detected in both monocytes and macrophages; however, Pyk2 protein expression is significantly upregulated in Ly6C^{neg} monocytes and macrophages compared with less differentiated monocyte subsets. We go on to show that the regulated expression of FAK kinases in monocyte and macrophage populations also has functional implications. Our data strongly suggest that Pyk2 limits the accumulation of Ly6C^{neg} monocytes at homeostasis by promoting apoptosis in these cells. We also show that FAK activity in macrophages restricts the outgrowth of breast carcinomas in a murine model of cancer. The studies presented here reveal novel functions for FAK kinases in monocytes and macrophages, and contribute to a more comprehensive understanding of the

molecular signaling that regulates the activities of these cells during steady-state and pathogenic conditions.

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List of Abbreviations

ACK	Ammonium/chloride/potassium
AGO2	Argonaute 2
AKT	Protein kinase B
Bcl2	B-cell lymphoma 2
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C57Bl/6	C57 black 6
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CEBP	CCAAT/enhancer binding protein
CM	Complete media
cMoP	Committed monocyte progenitor
CMP	Committed myeloid progenitor
CNS	Central nervous system
Cre	Cre recombinase
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride

DC	Dendritic cell
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
ETOH	Ethanol
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FasL	Fas Ligand
FAT	Focal adhesion targetting
FBS	Fetal bovine serum
FERM	Four-point-one, ezrin, radixin, moesin
FLICA	Fluorochrome-Labeled Inhibitors of Caspases
FLT3	FMS-like tyrosine kinase-3
FMO	Fluorescence minus one
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor

GMP	Granulocyte-macrophage progenitor
H&E	Hematoxylin and eosin
HBSS	Hank's buffered saline solution
HIF	Hypoxia inducible factor
HSC	Hematopoietic stem cell
I.P.	Intra-peritoneal
ICAM	Intercellular adhesion molecule
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF8	Interferon regulatory factor 8
KLF4	Kruppel-like factor 4
LFA-1	Leukocyte function-associated antigen-1
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
M-CSF	Macrophage Colony stimulating factor
M-CSFR	Macrophage Colony stimulating factor receptor
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase

MDP	Monocyte dendritic cell progenitor
MDSC	Myeloid-derived suppressor cell
MFG-E8	Milk-fat globule-epidermal growth factor–VIII
MG1	Mammary gland 1
MHCII	Major histocompatibility complex II
MI	Myocardial infarction
MIN	Mammary intraepithelial neoplasia
MMP	Matrix metalloproteinase
MPS	Mononuclear phagocyte system
NF-κB	Nuclear Factor-Kappa Beta
NK	Natural killer
NR4A1	Nuclear receptor subfamily 4 group A member 1
op/op	*Osteopetrotic/osteopetrotic*
PB	Peripheral blood
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEA3	Polyoma enhancer activator 3
PI3K	Phosphatidyl-inositol 3 kinase
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PRR	Proline-rich region

Pyk2	Proline-rich tyrosine kinase 2
PyVmT	Polyoma virus middle T antigen
RBPJ	NOTCH/recombination signal-binding protein for the IgKJ region
RIPA	Radioimmunoprecipitation assay buffer
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
S.E.M.	Standard error of the mean
S1P	Sphingosine 1-phosphate
Sca1	Stem cells antigen-1
SF	Serum-free
SFK	Src family kinase
SGR	Specific growth rate
SH2	Src homology 2
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophage
TCR	T cell receptor
TGF	Transforming growth factor
T_H	T helper
TLR	Toll like receptor
TNF-α	Tumor Necrosis Factor-α
T_{regs}	Regulatory T cell

VEGF	Vascular endothelial growth factor
WT	Wild-type
Y	Tyrosine
YS	Yolk sac

Chapter 1: Introduction

1.1 Perspective on the mononuclear phagocyte system (MPS)

Evidence for innate immune function in tissue homeostasis and defense against pathogens can be found across the plant and animal kingdoms, and specialized cells known as “phagocytes” are critical for these processes [1]. The evolutionarily conserved nature of the innate immune system allowed for the early work of Ilya Metchnikoff to first identify and describe the activity of phagocytes in the late 1800s [2]. Using starfish larvae and water fleas as model organisms, Metchnikoff observed the engulfment of particulate matter by amoeboid cells after non-sterile injury. He went on to postulate that these cells played an active role in protection against pathogens [3-5]. In subsequent work, he hypothesized that these phagocytes were also important for the maintenance and repair of tissues [5, 6]. These pioneering discoveries laid the groundwork for succeeding studies that described the generation of macrophages from blood precursors both *in vivo* [7, 8] and *in vitro* [9, 10].

Together, these and other findings supported a linear model of macrophage development whereby monocytes are generated from immature precursors in the bone marrow (BM) and released into the peripheral blood (PB) where they function to continually replenish tissue-resident macrophages. Van Furth formalized this concept in 1968, when he coined the term “mononuclear

phagocyte system" (MPS) in describing it [11, 12], and this model has persisted as a fundamental doctrine in the field of innate immune development. The identification and functional characterization of dendritic cells (DCs) in the 1970s led to their incorporation into the MPS as well [13], although the legitimacy of this inclusion has come into question after the discovery of a DC lineage-specific precursor in the BM [14, 15]. Recent studies have also challenged the paradigm of the MPS as a linear system, revealing that the vast majority of tissue-resident macrophages are embryonically derived under steady-state conditions [16, 17]. While our understanding of MPS cellular ontogeny and functionality continues to evolve, this model has remained useful for classifying a network of innate immune cellular constituents dedicated to tissue surveillance and the initiation of adaptive immune responses.

1.2 Monocyte subset development and function at steady-state

Monocytes represent a subpopulation of leukocytes traditionally defined by their location, function, morphology, and gene expression signatures [18-21]. Together with macrophages, neutrophils, and DCs, they constitute the cells of the myeloid lineage. Initially described merely as transient precursors to macrophages and DCs, recent discoveries have elucidated additional functional roles for these cells in the maintenance of the vasculature and peripheral tissues [22-25]. Furthermore, the recent detection of previously unidentified heterogeneity within the peripheral monocyte pool has enriched the study of monocyte differentiation

and homeostatic maintenance [26, 27]. Many of these discoveries were facilitated by mouse models, which allow the use of genetic alterations, as well as *in vivo* and *ex vivo* methods of monocyte lineage tracing and subsetting. Therefore, the majority of this thesis will focus on murine monocyte and macrophage populations unless otherwise noted.

1.2.1 Monopoiesis

Monocytes derive from lineage committed progenitor cells that reside in the fetal liver during embryonic hematopoiesis [28], and subsequently the bone marrow during adult hematopoiesis [29]. The classical model of monopoiesis asserts that hematopoietic stem cells (HSCs) give rise to these committed progenitors through progressive rounds of proliferation and differentiation in which progeny become more restricted to the monocyte lineage whilst concurrently losing their capacity for self-renewal [30-32]. The mechanistic control over the differentiation of HSCs to lineage committed progenitors is still an area of extensive research, but it is thought that the process is stochastic and driven by the coordination of growth factor and cytokine signaling together with antagonism of transcription factors within HSCs [33-36]. Analytical techniques using *in vitro* clonal assays and population-based tracking *in vivo* have allowed the identification of progressively more lineage-committed intermediaries that have been classified based on their clonogenic ability and distinct surface marker profiles [37-39]. This model of monopoiesis is summarized in Figure 1.1; however, linear models such as these should be interpreted with caution. Newly developed “cellular barcoding”

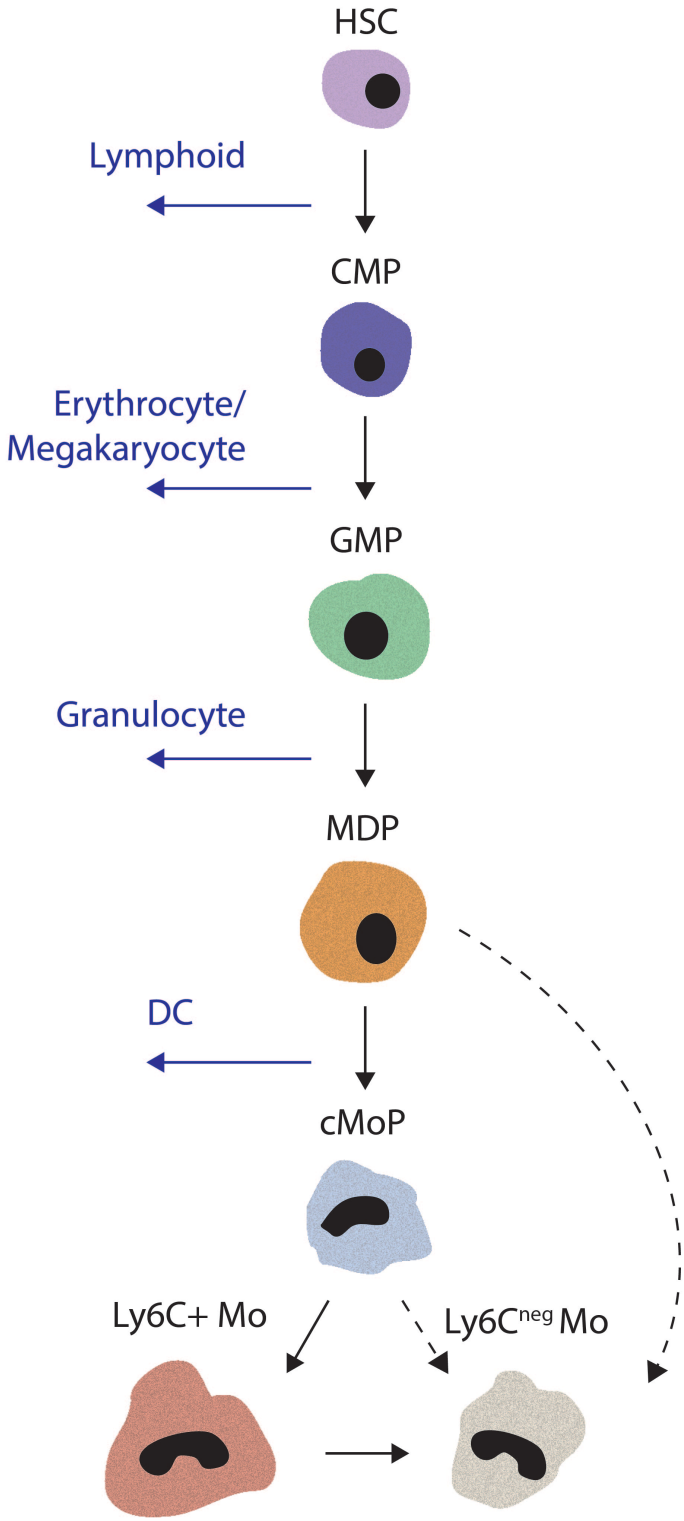

HSC
Lymphoid
CMP
Erythrocyte/
Megakaryocyte
GMP
Granulocyte
MDP
DC
cMoP
Ly6C+ Mo
Ly6C^neg Mo

Figure 1.1 Murine monocyte lineage development in the bone marrow. Schematic depicts the traditional, linear model of monocyte lineage commitment from hematopoietic stem cell (HSC) to common myeloid progenitor (CMP) to granulocyte-macrophage progenitor (GMP) to macrophage- dendritic cell progenitor (MDP) to committed monocyte progenitor (cMoP). Cells of the lymphoid lineage, erythrocytes and megakaryocytes, granulocytes, and dendritic cells (DCs) are excluded as cells commit to cMoP. Ly6C+ monocytes develop from cMoP. Ly6C^{neg} monocytes develop from Ly6C+ monocytes and may also derive from MDP and/or cMoP under certain conditions. Figure adapted from [20, 311].

techniques that trace the fate of HCSs at a single-cell level *in vivo* suggest that these populations are likely more heterogeneous than originally described and should be viewed within the framework of a developmental continuum based on "graded commitment" to the monocyte lineage [14, 40].

A significant advancement in our understanding of monocyte lineage development was made with the identification of the "macrophage and dendritic cell precursor" (MDP) in 2006 [41]. The Geissmann group demonstrated that this population of highly proliferative BM cells gave rise to monocytes and DCs *in vivo*, but had lost granulocyte potential. They went on to show that MDPs could be identified using distinctive cell surface markers, including CD117 (c-kit), CD115 (M-CSFR), CD135 (FLT3), and the chemokine receptor CX_3CR1. In fact, use of the CX_3CR1^{GFP} knock-in mouse [42] was instrumental for the identification of this population, as this marker is restricted to MDPs and their progeny.

The next breakthrough in the field of monocyte development came shortly thereafter with the identification of a progenitor population that gave rise to monocytes but had lost DC potential [29]. The authors showed that this population shared common surface markers with the MDP, but did not express CD135. Additionally, this monocyte-restricted progenitor had gained expression of the glycoprotein Ly6C. They termed this population "committed monocyte progenitors" (cMoPs), and our current understanding of monopoiesis places

cMoPs directly downstream of the MDPs and immediately preceding the establishment of mature monocytes.

1.2.2 The establishment of distinct Ly6C+ and Ly6C^{neg} monocyte subsets in mice

Pioneering work by Geissmann and others first identified heterogeneity among peripheral murine monocytes, again using the CX_3CR1^{GFP} knock-in mouse [26, 27, 42]. Geissmann noted that distinct expression patterns of CX_3CR1, as well as other surface markers, allowed the identification of two major monocyte subsets. CX_3CR1^{mid} cells expressed high levels of Ly6C and the chemokine receptor CCR2, whereas CX_3CR1^{high} cells were negative for these markers. These observations led to further characterization of these subsets, and the subsequent classification of Ly6C+ “classical” and Ly6C^{neg} “nonclassical” monocyte subpopulations. These subsets can be distinguished by their surface marker expression (Table 1.1), function (see section 1.2.4), half-life, and differentiation potential.

Murine monocytes constitute ~4% of nucleated peripheral blood cells [43], with relatively equal numbers of Ly6C+ and Ly6C^{neg} monocytes [26]. In contrast, Ly6C^{neg} monocytes make up a small fraction of the total monocytes found in the BM [44]. Both subsets have also been shown to accumulate in the spleen, where together with BM monocytes, these cells are thought to represent a reservoir that can be mobilized in response to inflammatory cues [45]. While monocytes as a

Table 1.1 Monocyte Lineage Subsets

Mouse monocyte subset	Surface marker profile	Human counterpart	Anatomical location(s)	Main functions	Half-life	Survival factors
cMoP	CD115+ CD117+ Ly6C+ CD11b^{neg} CX_3CR1+ CCR2^{neg} CD11c^{neg}	Unknown	BM	• Highly proliferative • Monocyte lineage committed • Produce Ly6C+ and Ly6C^{neg} monocytes	Unknown	Unknown
Ly6C+ Mo	CD115+ CD117^{neg} Ly6C+ CD11b+ CX_3CR1^{low} CCR2+ CD11c^{neg}	CD14+ CD16^{neg}	BM PB Spleen	• Classical "Inflammatory" monocytes • Respond to inflammatory cues • Differentiate into DCs and macrophages • Steady-state precursors for Ly6C^{neg} mo	18 hours	Unknown
Ly6C^{neg} Mo	CD115+ CD117^{neg} Ly6C^{neg} CD11b+ CX_3CR1^{hi} CCR2^{neg} CD11c+	CD14^{neg} CD16+	BM PB Spleen	• Nonclassical "Patrolling" monocytes • Patrol microvasculature • Survey for danger signals • Scavenge microparticles	2.3 Days	M-CSF CX_3CL1 NR4A1

Adapted from Ginhoux, 2014; Italiani, 2014.

whole are characteristically short-lived cells, Ly6C^{neg} monocytes persist considerably longer than the more transient Ly6C+ population, exhibiting half-lives of 2.3 days and 18 hours, respectively [46].

There is considerable controversy regarding the precise developmental relationship between Ly6C+ and Ly6C^{neg} monocytes. Adoptively transferred Ly6C+ monocytes spontaneously give rise to Ly6C^{neg} monocytes that can be found in the BM and PB of recipient mice [46, 47]. Additionally, grafted MDPs and cMoPs differentiate to Ly6C+ monocytes prior to the appearance of congenically-labeled Ly6C^{neg} monocytes [29, 47]. Similarly, BrdU administration results in tracer-labeled Ly6C+ monocytes prior to the appearance of labeled Ly6C^{neg} monocytes [46]. These results support a model in which Ly6C+ monocytes serve as precursors for the Ly6C^{neg} fraction of monocytes. However, there is also evidence to suggest that Ly6C^{neg} monocytes can be derived independently from Ly6C+ monocytes. Interferon regulatory factor 8 (IRF8)-deficient mice exhibit a dramatic paucity of Ly6C+ monocytes [48]. However, Ly6C^{neg} monocytes persist in the BM and PB of IRF8-/- animals, albeit in reduced numbers compared with WT mice [48]. Additional studies will be necessary to determine whether Ly6C^{neg} monocytes can develop directly from hematopoietic progenitors or if Ly6C+ monocytes serve as their obligatory precursors. There is also debate over whether the differentiation from Ly6C+ to Ly6C^{neg} monocytes can occur in the peripheral blood, or whether this process is confined to the BM [44, 46, 47].

1.2.3 Molecular control of monocyte development

Monopoiesis is a tightly regulated process that is controlled by both intrinsic and extrinsic factors. While monocyte development is incompletely understood, disruption of myeloid-specific genes in mouse models has provided a powerful tool for identifying a number of the cellular factors required for monocyte development. For example, *op/op* mice that harbor a naturally occurring mutation in the coding region of the *Csf1* gene, rendering them devoid of M-CSF, completely lack circulating monocytes [49, 50]. These animals also exhibit severe osteopetrosis due to a failure in osteoclast development that results in bone remodeling defects. Notably, *CSF1R*-deficient mice also show similar defects in monopoiesis and bone development [51].

While M-CSFR signaling is required for the development of all monocyte lineage populations, several transcription factors have been identified that regulate the development of specific monocyte subsets. Feinberg *et al.* showed that the Kruppel-like factor 4 (KLF4) functions as a critical regulator of Ly6C+ monocyte differentiation. KLF4-deficiency resulted in the absence of Ly6C+ monocytes and significantly reduced $Ly6C^{neg}$ monocyte numbers, concomitant with increased granulocyte numbers [52, 53]. This phenocopies the aforementioned monocytopenia observed in IRF8-/- mice. In fact, Kurotaki *et al.* showed that KLF4 expression is directly induced by IRF8 activity [48]. Thus, Ly6C+ monocyte development is dependent upon a KLF4-IRF8 axis.

Recently, the transcription factor NR4A1 (Nur77) was identified as necessary for the development and survival of Ly6C^{neg} monocytes. The Hedrick group demonstrated that NR4A1-/- mice exhibit a significant reduction in Ly6C^{neg} monocyte numbers in the BM, PB, and spleen [44]. The few remaining Ly6C^{neg} monocytes accumulated in S phase and displayed several hallmarks of apoptosis [44]. Notably, MDP and Ly6C+ monocyte numbers were unaffected by loss of Nr4A1 in these mice, and a subsequent study showed that protein expression of NR4A1 is restricted to the Ly6C^{neg} fraction of monocytes [29].

1.2.4 Monocyte subset effector functions

The classical model of the MPS dictates that circulating monocytes function exclusively as the obligatory precursors for tissue-resident macrophages, and that this process is amplified in response to inflammatory insults. As previously mentioned, aspects of this model required revision following the discovery that most tissue-resident macrophages found at homeostasis are embryonically-derived [16, 17]. Nevertheless, early studies clearly established the robust recruitment of monocytes during inflammation and their ability to differentiate to macrophages in peripheral tissues. As early as 1939, Ebert and Flory described the step-wise process of monocyte extravasation, differentiation, and phagocytic activity using a chamber to observe acute tissue damage in rabbit ears [7]. These and other studies provided the initial evidence for monocyte effector function *in vivo*.

For decades, monocytes continued to be considered merely a homogeneous population of highly mobile and transient precursors that functioned to seed peripheral macrophages. Work from the early 2000s, however, revitalized the field of monocyte biology. Geissmann's seminal paper describing discrete monocyte subsets in the PB of mice prompted the question of whether these subsets were functionally distinct [26]. Ensuing studies have indeed illuminated a dichotomy of function for these subpopulations in the periphery. Ly6C+ monocytes fit the classical paradigm of non-terminally differentiated cells that are highly responsive to inflammatory cues and readily extravasate into peripheral tissues in response to these signals; Ly6C^{neg} monocytes, on the other hand, fulfill previously unappreciated roles in the surveying and maintenance of vascular integrity. The nomenclature for these cells has begun to reflect these distinct functions, with Ly6C+ and Ly6C^{neg} subsets being classified as "inflammatory" and "patrolling" monocytes, respectively. Below, these functions are further detailed.

1.2.4.1 Ly6C+ monocyte function at steady-state

As mentioned previously, Ly6C+ monocytes circulate throughout the PB and are recruited to peripheral tissues by inflammatory cues, including the chemokine CCL2 [20]. The subsequent differentiation of these cells to monocyte-derived DCs and macrophages is a pivotal step in immunity against pathogens and response to injury. The recruitment, differentiation, and function of monocyte-derived macrophages during inflammation will be detailed further in section 1.4.1.2. At steady-state, however, it is theorized that circulating Ly6C+

monocytes fulfill a dual role: to survey the PB for inflammatory cues, and to serve as precursors to Ly6C^{neg} monocytes in the absence of these inflammatory signals.

Intriguingly, one recent study reported minimal differentiation of Ly6C+ monocytes that were isolated from lung and skin extravascular tissue [23]. These cells persisted as Ly6C+MHCII+ cells and were also capable of acquiring antigen and subsequently migrating to lymph nodes via the lymphatics. The authors described these cells as “tissue monocytes” since this population retained a monocytic gene expression profile and never acquired phenotypic qualities consistent with macrophages or DCs. However, given that these cells lose the potential to re-circulate through the PB, others have questioned whether these cells should truly be classified as “monocytes”, as this trait has traditionally been used in defining *bona fide* monocytes [20]. The functional contribution of minimally differentiated Ly6C+MHCII+ cells has not been addressed.

1.2.4.2 Ly6C^{neg} monocyte function at steady-state

While a dichotomy of PB monocytes was established in 2003 following the identification of a $CX_3CR1^{hi}Ly6C^{neg}$ population, the functional contributions of this population proved elusive. In 2007, pioneering intravital microscopy studies by Auffray *et al.* first provided evidence for the distinct behavioral characteristics of Ly6C^{neg} monocytes *in vivo* [24]. Ly6C^{neg} monocytes localized to the luminal vasculature and appeared to “crawl” in a distinctive pattern, often against the

direction of blood flow. This work went on to show that Ly6C^{neg} monocyte "patrolling" of the vasculature was dependent on LFA-1 and CX_3CR1 signaling, and that these cells rarely extravasated during steady-state conditions. Furthermore, unlike Ly6C+ monocytes, Ly6C^{neg} monocytes did not differentiate to macrophages or DCs in response to *Listeria* infection.

Evidence for functional contributions associated with the "patrolling" behavior of Ly6C^{neg} monocytes was provided from an elegant study by Carlin *et al.* They demonstrated that Ly6C^{neg} monocytes extend filipodia-like structures and scavenge microparticles from the surface of epithelial cells lining the kidney capillaries [25]. In response to Toll-like receptor 7 (TLR7)-specific nucleic acid danger signals, Ly6C^{neg} monocytes were recruited to the kidney vasculature and extended their average dwell time on the endothelial surface. The authors went on to describe a CX_3CR1- and CD11b-dependent process in which Ly6C^{neg} monocytes subsequently recruited neutrophils, which triggered endothelial necrosis, and the Ly6C^{neg} monocytes then cleared the resultant debris. Thus, Ly6C^{neg} monocytes constantly patrol the vasculature at steady-state and coordinate intraluminal stress responses after exposure to local danger signals.

1.2.5 Human monocyte subpopulations

Mouse models have proven invaluable in advancing our understanding of monocyte biology, but the question arises as to whether these findings are applicable to humans as well. Monocytes represent approximately 10% of

peripheral blood cells in humans, and two principle subsets have also been identified [54]. These subsets are defined as CD14+ $CD16^{neg}$ "classical" monocytes and the $CD14^{low}$ CD16+ "nonclassical" monocytes [55]. Whereas Ly6C+ classical monocytes constitute roughly 50% of blood monocytes in mice, CD14+ $CD16^{neg}$ classical monocytes make up approximately 85% of human PB monocytes [18, 56]. A high degree of homology between the parallel human and mouse monocyte subsets has been described via gene expression profiling [19], suggesting evolutionary conservation within each subset. Based on protein expression profiles, functional similarity between the parallel subsets seems likely, although this has not been definitively proven. In support of this theory, however, intravital imaging demonstrated that $CD14^{low}$ CD16+ nonclassical monocytes exhibit patrolling behavior in the vasculature when adoptively transferred to mice [18].

1.3 The homeostatic maintenance of peripheral monocyte subpopulations

1.3.1 Monocyte egress from the bone marrow

In order to perform their effector functions, monocytes must exit the BM where they are produced, and travel throughout the PB. However, monocyte subpopulations utilize different signaling pathways to accomplish this task. Ly6C+ monocyte extravasation from the BM is CCR2-dependent [57, 58]. Thus, *Ccr2-/-* mice display drastically reduced Ly6C+ monocytes in the periphery, concomitant with slightly increased Ly6C+ monocyte numbers in the BM [57]. CCR2-deficient

mice also have impaired Ly6C+ monocyte mobilization in response to inflammatory stimuli, including infection with *Listeria monocytogenes* [57, 58]. Additionally, mice deficient for the chemokines CCL1 or CCL7 exhibit improper egress of Ly6C+ monocytes from the BM during inflammation [57].

Conversely, Ly6C^{neg} monocyte egress is dependent upon the sphingosine-1 phosphate receptor 5 (S1PR5) [59]. *S1pr5-/-* mice exhibited normal numbers of Ly6C^{neg} monocytes in the BM but significantly reduced frequencies of this subset in the PB. Debien *et al*. showed that survival of adoptively transferred S1PR5-deficient Ly6C^{neg} monocytes in the periphery was comparable to WT controls, and therefore attributed the paucity of PB Ly6C^{neg} monocytes in the knock-out animals to a cell-intrinsic defect in their emigration from the BM. Notably, Ly6C+ monocyte numbers were normal in the BM and PB of these mice, and Ly6C^{neg} monocyte numbers are relatively unaffected in CCR2-/- animals [57, 58]. These findings argue against, but do not definitively disprove, the current model of monocyte development wherein Ly6C+ monocyte differentiation to Ly6C^{neg} monocytes occurs in the PB.

1.3.2 Monocyte survival signaling

Circulating Ly6C+ and Ly6C^{neg} monocytes exhibit characteristically short half-lives of 18 hours and 2.3 days, respectively [46]. However, the mechanisms promoting the survival and cell death of these populations are only beginning to be elucidated. M-CSFR signaling is essential for the development of

mononuclear phagocyte populations [20], leading to the widespread assumption that this factor is also necessary for the survival of mature monocyte populations. However, several groups have reported that use of neutralizing antibodies to M-CSF, as well as blocking antibodies to the M-CSFR, leads only to a selective reduction in the Ly6C^{neg} fraction of monocytes during steady-state conditions *in vivo*, with little to no effect on Ly6C+ numbers [60-62]. In contrast, administration of recombinant M-CSF acted as a pro-survival factor for both Ly6C+ and Ly6C^{neg} monocytes *in vitro* [60]. The authors of this study speculated that the already short half-life of Ly6C+ monocytes in the PB may not allow sufficient time to observe survival defects after M-CSF neutralization *in vivo*.

The chemokine receptor CX_3CR1 is expressed at variable levels on peripheral monocyte subsets [26], and its cognate ligand, CX_3CL1 (fractalkine), has an established role in promoting the survival of multiple cell lines [63-65]. In 2009, Landsman *et al.* demonstrated a functional role for monocytic CX_3CR1 when they reported that both CX_3CL1- and CX_3CR1-deficient mice displayed a specific reduction in circulating Ly6C^{neg} monocytes under steady-state conditions [66]. Similar to M-CSF and M-CSFR neutralization, the numbers of Ly6C+ monocytes were unaffected in these mice. This phenotype could be reverted by enforced expression of the anti-apoptotic factor Bcl2, providing evidence that CX_3CL1 signaling promotes Ly6C^{neg} monocyte survival.

While our knowledge of the exogenous factors that control monocyte homeostasis is limited, the molecular signaling that mediates murine monocyte subset survival downstream of these factors is even less understood. The cellular signaling that stimulates Ly6C^{neg} monocyte survival downstream of CX_3CR1 activation has not been addressed; however, several studies have implicated the PI3K/AKT pathway downstream of M-CSF signaling in promoting human monocyte cell survival [67-69]. Following M-CSF-induced tyrosine phosphorylation of the M-CSFR, PI3K-dependent AKT activation promoted monocyte survival *in vitro* [67]. Furthermore, PI3K inhibitors blocked the M-CSF-induced activation of AKT and suppressed survival in this system, and co-administration of caspase-9 inhibitors reversed this phenomenon. A subsequent study by the same group showed that caspase-3, which is activated downstream of caspase-9, is also suppressed via the same pathway [69].

These findings implicate caspase activation in promoting monocyte turnover. Indeed, freshly isolated human monocytes spontaneously activate caspase-3, but not caspase-1, and subsequently undergo apoptosis when placed into culture [70]. This spontaneous activation of caspase-3 was shown to be partly dependent on PKCδ activity in these cells [71]. More recent studies that distinguish human monocyte subsets have revealed higher PKC*δ* kinase activity, earlier caspase cleavage, and greater susceptibility to apoptosis in CD16+ non-classical monocytes compared with their CD16^{neg} classical counterparts [72]. *In vivo* evidence for caspase-mediated control of monocyte cell death was shown in

Fas-deficient (*lpr/lpr*) mice [73], which display elevated numbers of PB Ly6C+ and Ly6C^{neg} monocytes compared with WT control mice. Importantly, canonical fas death receptor signaling promotes apoptosis via a caspase-8/caspase-3 axis [74]. Together, these findings support a model in which monocytes undergo spontaneous cell death in the absence of exogenous survival signals via a caspase-dependent mechanism. However, the cellular factors controlling this process are incompletely understood. In Chapter 2 of this thesis, I will present evidence supporting a role for the tyrosine kinase Pyk2 in promoting caspase-mediated apoptosis in Ly6C^{neg} monocytes.

1.4 Macrophages

Within the context of the MPS, macrophages constitute a heterogeneous population of terminally-differentiated phagocytes that reside in essentially every tissue. Early studies describing the development of macrophages used animal models incorporating inflammatory conditions. Because recruited monocytes and monocyte-derived macrophages dominate the pool of mononuclear phagocyte cells in peripheral tissues during inflammatory settings [75], this led to the longstanding belief that PB monocytes served as the obligatory precursors for all tissue-resident macrophages [76-78]. However, increasing evidence has developed in recent years demonstrating that macrophages develop embryonically prior to the emergence of hematopoietic stem cells, disseminate to peripheral tissues, and persist locally during steady-state conditions throughout

adulthood [79-82]. Thus, a more nuanced interpretation of MPS development, maintenance, and function has emerged, and the research that underlies these advances in our understanding is outlined below.

1.4.1 Macrophage origins

1.4.1.1 Tissue-resident macrophage development

Macrophages first appear in mice at E8.5, during the first wave of definitive hematopoiesis that occurs in the yolk sac (YS) [83, 84]. In contrast, HSCs are not observed in mice until they colonize the fetal liver at E10.5 [84], indicating that macrophages can be derived independently of HSCs. Moreover, unlike BM HSC-derived progeny, macrophage development in the YS is independent of the transcription factor c-Myb [79]. Using a tamoxifen inducible *Csf1rMer-iCre-Mer* mouse model, Schulz *et al*. pulse labeled M-CSFR-expressing cells at E8.5. This allowed the authors to selectively label YS macrophages and trace their progeny into adulthood. Results from this study showed that adult tissue-resident macrophages, including microglia, Langerhans cells, and Kupffer cells, were derived from YS macrophages. Furthermore, selective labeling of BM HSCs and their progeny using *Tie2Cre* or *Flt3Cre* mouse models demonstrated that these cells were not the main contributor to tissue-resident macrophage populations [79, 85]. Additional studies using parabiotic mice and other fate-mapping systems have also corroborated these conclusions [46, 79, 82, 86, 87].

1.4.1.2 Monocyte-derived macrophages

Although most tissue-resident macrophages are maintained independently of monocytes at steady state, notable exceptions exist in some highly specialized macrophage populations. Several studies have demonstrated that CX_3CR1^{hi} macrophages of the *lamina propria* in the gut, for example, are colonized by Ly6C+ monocytes at the point of weaning and depend on constant replenishment from circulating Ly6C+ monocytes [88-90]. Notably, these gut macrophages exhibit a short half-life of around three weeks, compared with most other tissue-resident macrophage populations that are longer lived [89]. Furthermore, the recruited Ly6C+ monocytes undergo considerable clonal expansion in the gut and acquire a gene expression profile associated with an anti-inflammatory phenotype [91]. Additional studies indicate that a small proportion of macrophages found in the skin, heart, and spleen also require monocyte input to maintain their numbers, and their representation may increase with age [85, 92-95]. Thus, subpopulations of monocyte-derived macrophages coexist with embryonically-derived tissue-resident macrophages within several tissues of healthy animals.

While Ly6C+ monocytes are required to maintain some steady-state macrophage populations in particular tissues, Ly6C+ monocytes are abundantly recruited to peripheral tissues and subsequently differentiate to macrophages and DCs during inflammatory conditions [96]. Multiple studies have demonstrated that inflammation-dependent trafficking of BM, PB, and spleen Ly6C+ monocytes is

largely mediated by CCL2 production by stromal cells in response to microbial products or inflammatory cytokines [56, 57, 97]. The importance of the CCL2-CCR2 signaling axis for Ly6C+ monocyte recruitment during pathological conditions is illustrated in CCR2-deficient mice, as ablation of CCR2 results in significantly reduced accumulation of Ly6C+ monocytes at the site of tissue damage in acute models of skeletal muscle injury, skin injury, and myocardial infarction [98, 99]. Once Ly6C+ monocytes have extravasated to inflamed tissue, they respond to local cues that dictate their function as either pro-inflammatory or anti-inflammatory mediators. These opposing activities of monocyte-derived macrophages likely depend on consecutive waves of recruited Ly6C+ monocytes that acquire different phenotypes in response to the dynamically changing tissue microenvironment [100].

1.4.2 The maintenance of macrophage populations in peripheral tissues

Given that most tissue-resident macrophage populations are derived during embryonic development, a mechanism of long-term maintenance is required for these populations to persist into adulthood. Macrophage populations in peripheral tissues were initially characterized as largely quiescent, and this contributed to the misconception that macrophages required constant replenishment from bone marrow-derived monocytes. However, low-level proliferation of resident macrophages has been reported at steady-state in many tissues, including populations in the skin, brain, liver, lungs, spleen, and peritoneum [82, 87, 101-105]. Furthermore, some tissue-resident macrophages

have the capacity to replenish their populations via a proliferative burst immediately following inflammation-associated depletion [106]. It is currently unclear whether all resident macrophages have the capacity to self-renew, or if proliferation is restricted to subsets of “stem cell-like” macrophage populations within tissues.

In contrast to YS- and fetal liver-derived tissue-resident macrophages, monocyte-derived macrophages are relatively short-lived [20, 89]. Furthermore, monocyte-derived macrophages are largely non-proliferative, but instead rely on “waves” of recruitment from the circulating pool of Ly6C+ monocytes during inflammatory settings [43]. These observations have led to a revised view of the MPS wherein circulating monocytes seed a population of transient effector macrophages that aids in the resolution of acute inflammatory events, and this population is distinct from long-lived tissue-resident macrophages that are responsible for tissue homeostasis and repair during steady-state conditions [107]. While the majority of experimental models of inflammation support this theory, it should be noted that recruited monocytes have been reported to proliferate and persist as resident macrophages under some experimental conditions [29, 46, 108-110]. Thus, a more nuanced view of tissue-resident macrophage ontogeny following inflammatory insult may be necessary to account for some tissue- and insult-specific influences.

1.4.2.1 Colony-stimulating factors

The molecular and cellular mechanisms responsible for the maintenance and proliferation of peripheral macrophage populations is currently an area of active research. Multiple studies implicate colony stimulating factors as crucial mediators of these processes. The M-CSFR is expressed by all macrophage populations, including YS populations during embryogenesis [79]. Under homeostatic conditions, M-CSF is constitutively produced by mesenchymal cells within peripheral tissues [111, 112], and macrophages are responsible for clearing the circulating M-CSF in a negative feedback loop that maintains appropriate levels of mononuclear phagocyte numbers both locally and systemically [107, 113, 114]. M-CSFR signaling in turn promotes tissue-resident macrophage survival and proliferation [108, 115], and administration of neutralizing antibody to M-CSF significantly reduces the ability of these macrophages to self-renew [108].

Indeed, the importance of the M-CSF/M-CSFR signaling axis to macrophage development and survival is further demonstrated in genetically altered mice. In addition to the aforementioned deficiencies in the production of osteoclast and circulating monocyte populations, mice that have a homozygous null mutation in the *Csf1* gene locus (*op/op)* also show striking losses of gut, kidney, and peritoneal macrophage numbers, with more mild impacts on liver and splenic macrophage populations [82, 116]. M-CSFR-deficient mice present with all of the defects observed in *op/op* mice; however, brain and skin macrophages are also

absent in these animals [51]. The subsequent identification of IL-34, which functions as a second ligand for the M-CSFR, accounted for the more drastic phenotype observed in M-CSFR-deficient mice [117, 118]. IL-34 is selectively produced by keratinocytes and neurons, and supports the steady-state maintenance and proliferation of Langerhans cells and microglia [119, 120].

In contrast to the ubiquitously expressed M-CSF, the granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed at low to negligible levels during homeostasis [121, 122]. Loss of GM-CSF therefore does not affect the majority of tissue-resident macrophage populations at steady-state [123]. Notable exceptions include the alveolar and peritoneal macrophage pools, which are compromised in GM-CSF-deficient mice [123-125]. GM-CSF is synthesized in response to microbial challenge [126] and appears to play a prominent role in driving monopoiesis and granulopoiesis during inflammatory conditions [121, 122].

1.4.3 Macrophage function

Macrophage activities are indispensible to a myriad of biological processes integral to proper tissue homeostasis and immune response. Macrophages are probably best known as the “first responders” of the innate immune system, reflective of their fundamental contributions to the surveillance of peripheral tissues for physiological changes as well as pathogenic challenge. After exposure to these stimuli, macrophages facilitate the initiation of immune

responses, including the recruitment of other leukocyte populations, and are also critical for the resolution of inflammatory reactions and the promotion of wound healing processes [108, 127-131]. In addition, tissue-resident macrophages play integral roles in shaping tissue architecture during development [28]. Furthermore, macrophages maintain tissue homeostasis in adults by clearing apoptotic and dead cells and repairing damaged tissues [132, 133]. These examples merely highlight the broad array of biological activities that macrophages support; an exhaustive description of the studies that have advanced our understanding of macrophage functional heterogeneity, as well as the cellular and molecular mechanisms that control their activities, is beyond the scope of this thesis. Rather, I will specifically focus on macrophage activities in the context of tumor development and progression in Chapter 3 of this document. For a more comprehensive description of macrophage functions during homeostasis and other pathological settings, we refer the reader to the following review articles: [28, 134].

Given the vast diversity of functions that macrophages perform, it is not surprising that these cells display marked heterogeneity in transcriptional profiles from organ to organ [135, 136]. Indeed, the highly tissue-specific nature of macrophage function is reflected in the commonly used nomenclature that classifies macrophages by their anatomical location (microglia as macrophages of the central nervous system, Kupffer cells as macrophages of the liver, etc.). Moreover, increasingly sophisticated technologies, including intravital imaging

techniques, have revealed phenotypically distinct “subclasses” of macrophages within microanatomical niches of peripheral organs [137, 138]. The intracellular and extracellular factors responsible for maintaining distinct subsets of macrophages within a given organ are largely uncharacterized to this point, and the regulation of gene expression in these populations is an area of active research [139]. Furthermore, studies from the majority of disease models have not distinguished the functional contribution of embryonically-derived tissue-resident macrophages from monocyte-derived macrophages. Thus, the relative contributions of tissue microenvironmental factors [137, 138] versus “hard-wired” differentiation programs based on cellular origins [86, 102] toward macrophage activities remain unclear. Altogether, phenotypic plasticity and diversity of function are hallmarks of terminally differentiated macrophages found in peripheral tissues; however, careful studies incorporating fate-mapping systems and attention to microanatomical milieus will be required to determine precisely how the interplay between transcriptional control elements and local extracellular factors influences unique macrophage phenotypes during homeostasis and pathogenic states.

1.4.3.1 Macrophage polarization states

Given the extensive functional diversity that macrophages exhibit, a simplified operational concept has developed to broadly classify macrophages according to their phenotype. Within this model, macrophages are polarized to an “M1” state by exposure to IFN-γ and/or TLR activation, and are associated with aiding a T_H1

response and generally promoting inflammation [140, 141]. M1 macrophages are characterized by increased antigen presentation and production of inflammatory cytokines including IL-1β, IL-6 and TNF-α [142, 143]. “M2” polarized macrophages, conversely, respond to restorative mediators including TGF-β, glucocorticoids, and M-CSF, and are typically associated with the resolution of inflammation and tissue repair processes [144, 145]. The M2 designation also incorporates “alternatively activated macrophages”, which were initially described during parasitic infections. Alternatively activated macrophages respond to IL-4 and IL-13, and produce IL-4 and IL-10 to support T_H2-polarized responses [146]. Tissue-resident macrophages generally fulfill M2-like functions during steady-state conditions, concomitant with their established role in the maintenance of tissue homeostasis [144, 145]. Furthermore, M-CSF signaling is known to potentiate a more restorative, growth-oriented genetic signature in cultured macrophages, compared with a pronounced inflammatory phenotype that results from GM-CSF treatment [147]. Indeed, these observations correlate with the aforementioned expression patterns for these colony-stimulating factors, wherein M-CSF is ubiquitously present at homeostasis, as opposed to GM-CSF, which is synthesized during inflammatory reactions. As a word of caution, the M1/M2 binary classification scheme should not be over-interpreted as a rigid dichotomy. The M1/M2 paradigm does not accurately reflect the spectrum and plasticity of macrophage functionality that is governed by the highly complex microenvironment found *in vivo* [148].

1.5 The MPS and tumor progression

Although the M1/M2 archetype may portray extreme and distinct activation states, it also depicts the incredible functional diversity that macrophages are capable of displaying. Furthermore, cells of the MPS readily adapt their transcriptional programs in response to the external stimuli they encounter. As a result, macrophages are ideally positioned to identify changes in tissue physiology, initiate inflammatory responses, and mediate the resolution of these responses. However, macrophage functions can also be subverted during pathogenic conditions, particularly under circumstances of chronic inflammation, and these cells will instead adapt phenotypes that facilitate disease progression [28].

As early as the mid-1800s, the pathologist Rudolph Virchow linked cancer to an inflammatory response when he noted the presence of phagocytic cells alongside transformed cells in tumor specimens [149]. Numerous studies have since implicated the MPS in influencing virtually every stage of tumor initiation and progression (reviewed in [150, 151]), and clinical evidence generally correlates the accumulation of tumor MPS populations with poor patient prognosis [152, 153]. As detailed below, MPS cell populations can be found at various stages of differentiation within the tumor, and while unique effector functions have been ascribed to subclasses of monocyte-derived cells, together these populations predominantly contribute to an immunosuppressive environment that generally favors cancer growth and metastasis [154].

1.5.1 Tumor associated macrophages (TAMs)

Stromal components constitute a significant proportion of solid tumors, of which tumor associated macrophages (TAMs) often represent the major infiltrating leukocyte population [155]. Monocyte- and embryonically-derived macrophages coexist within tumors; however the ontogenic origin of these TAMs does not seem to have an appreciable effect on the activities that these cells perform [156]. Rather, Van de Laar *et al.* reported that local tumor-derived factors primarily dictate TAM phenotype [156]. Several recent reports indicate that an increasing majority of TAMs derive from recruited Ly6C+ inflammatory monocytes as tumors progress, rather than the local proliferation of tissue-resident macrophages [157-159]. The abundant recruitment of Ly6C+ monocytes to the tumor is facilitated by tumor-derived soluble factors that are released into the periphery. This process is chiefly mediated through canonical M-CSF/M-CSFR and CCL2/CCR2 signaling axes [157, 158, 160], as has been described in other pathological settings. Additional tumor-derived chemoattractants involved in monocyte recruitment to neoplastic tissue include IL-34, VEGF, CCL5, CXCL12, TGF-β, and the complement component C5a [159, 161-164], although the involvement of particular signaling modalities is likely to be highly dependent upon tumor type and stage of progression.

Once Ly6C+ monocytes extravasate from the PB into the tumor mass, local cues prompt their differentiation to a macrophage phenotype, and they adapt a surface marker profile commonly associated with this cell type

(Ly6C^{neg}CD11b^{low}MHCII+F4/80+VCAM1+). This process is reportedly dependent upon the transcriptional regulator of NOTCH signaling, NOTCH/recombination signal-binding protein for the IgKJ region (RBPJ), as RBPJ-deficient mice display a reduction in both TAM accumulation and tumor growth in a mouse model of breast cancer [157]. The subsequent localization and activity of macrophages within the tumor is dictated by the stimuli they encounter [163]; thus, it should be noted that distinct surface marker signatures and gene expression profiles are associated with discrete anatomical locations. In support of this notion, phenotypically discrete populations of TAMs have been described in hypoxic and necrotic tumor regions [165-168]. Moreover, one recent study reported that fully differentiated macrophages could reprogram both their chromatin landscape and cellular transcriptome when adoptively transferred into alternate tissues [138], highlighting the importance of the microenvironment in influencing macrophage phenotype.

Thus, TAM phenotype and function are highly dependent on the tumor microenvironment, which changes dynamically according to the stage of tumor progression [166]. In nascent tumors, activated TAMs have been shown to produce soluble factors that assist T cells in the elimination of transformed cells, thereby contributing to “immunoediting” processes [169]. Furthermore, TAMs have also been shown to exhibit direct anti-tumor activity via phagocytosis of cancerous cells [170-172]. These activities reflect what has classically been associated with an “M1-like” inflammatory phenotype. Moreover, TAMs display

gene expression profiles typically associated with a T_H1 response and the promotion of inflammation during the early stages of tumor development [173].

Unfortunately, the inflammatory responses that initially constrain tumor growth seem to come at a cost. Accumulation of free-radical compounds and the resultant tissue damage incite mutagenic pressure on neoplastic cells, escalating the genetic instability that underlies cancer progression [174, 175]. Furthermore, as observed in other non-resolving pathological conditions, TAMs eventually switch to an “M2-like” immunosuppressive phenotype in most established tumors, presumably as a method to limit excessive tissue damage that is associated with prolonged states of chronic inflammation. This switch is stimulated by stromal- and tumor cell-derived factors, including soluble IL-4, IL-13, IL-10, M-CSF, TGF-β, CCL2, CCL18, CCL17, and CXCL4 [159, 176-184]. TAM activities characteristically associated with this M2-like state generally resemble embryonically-derived tissue-resident macrophages involved in tissue growth and repair processes [158]. As previously described, substantial phenotypic heterogeneity is evident among subpopulations of TAMs based on tumor type, as well as anatomical localization within a tumor. The M2-like designation is therefore meant to encompass the spectrum of macrophage activation states that share the functional consequence of suppressing adaptive immunity and promoting tumor growth.

1.5.1.1 TAM 'pro-tumor' activities

TAMs support tumor progression and metastasis via a number of mechanisms (Figure 1.2). Mitogenic factors secreted by TAMs can directly stimulate the proliferation of tumor cells. Several studies have revealed that TAM-derived EGF supports proliferation in murine breast carcinoma cells [159, 172, 185]. TAM-derived milk-fat globule-epidermal growth factor–VIII (MFG-E8) also promotes cancer cell proliferation via activation of the STAT3 pathway [186]. Other TAM-secreted factors that sustain tumor proliferation include TNF-α and IL-6, which act on the NF-κB and STAT3 signaling pathways [187, 188].

TAMs also contribute to the pervasive tolerogenic environment found in tumors by suppressing adaptive immunity. This is achieved through a variety of mechanisms that generally block T cell anti-tumor functions and proliferation in the tumor. M2-like TAMs alter T cell metabolism by producing arginase and IDO1/2 pathway metabolites, leading to T cell proliferation arrest and functional inhibition [148]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released into the tumor microenvironment as a byproduct of TAM activity and metabolism; these species have well established roles in inhibiting T cell activation and suppressing T cell receptor (TCR) signaling mechanisms [189-191]. Additionally, prostaglandins produced by TAM metabolism of arachidonic acids have immunosuppressive effects on T cells [192]. Furthermore, TAMs have been reported to express the cell surface ligands PD-L1 and PD-L2 [193-195]. Engagement of these ligands with their cognate receptor (PD-1) on T cells

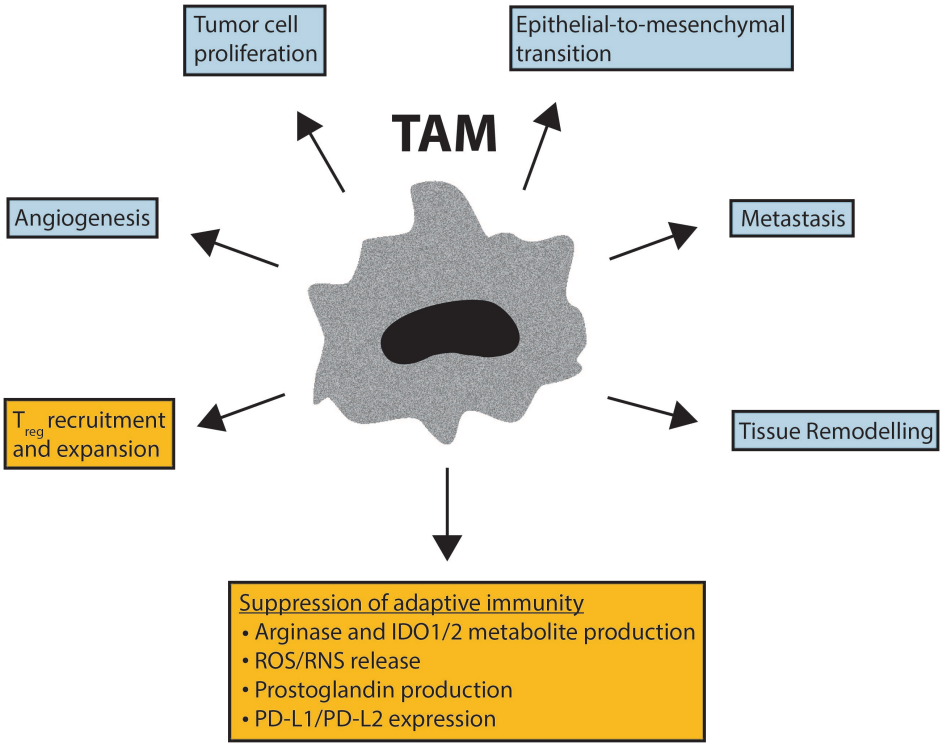

Tumor cell proliferation
Epithelial-to-mesenchymal transition
TAM
Angiogenesis
Metastasis
T_{reg} recruitment and expansion
Tissue Remodelling
Suppression of adaptive immunity
• Arginase and IDO1/2 metabolite production
• ROS/RNS release
• Prostoglandin production
• PD-L1/PD-L2 expression

Figure 1.2 Tumor associated macrophage (TAM) 'pro-tumor' functions. TAMs influence numerous aspects of tumor biology and can promote cancer progression by a number of mechanisms, including: supporting angiogenesis and tissue remodeling; inducing EMT and metastasis; stimulating tumor cell proliferation; directly and indirectly suppressing the adaptive immune system. Figure adapted from [214, 312].

triggers inhibitory immune checkpoint blockade. TAMs also indirectly inhibit cytotoxic T cell activity by supporting the recruitment of regulatory T cells (T_{regs}) to the tumor via the production of CCR5 and CCR6 [196-198]. T_{reg} expansion and immunosuppressive activity at the tumor site is further supported by TAM production of TGF-β, IFN-γ, and IL-10 [196, 197, 199].

Adequate oxygen levels are essential for the continued growth of primary tumors, and TAMs play crucial roles in regulating the development of tumor-associated vasculature that is required for progression to malignancy. This is evidenced by the significant inhibition of Polyoma Virus Middle T (PyVmT) breast tumor outgrowth in M-CSF-deficient animals, which lack TAMs and thus fail to undergo the so-called “angiogenic switch”, identified as the formation of a high-density blood vessel network [200]. The authors of this study went on to show that restoration of TAMs in PyVmT tumors rescued the angiogenic switch and tumor progression. Furthermore, TAM infiltration into premalignant lesions initiated the premature onset of blood vessel development [200]. A subset of particularly angiogenic TAMs that express the angiopoietin receptor TIE2 have also been described, and ablation of this population inhibits vessel formation and tumor growth in a several mouse models [201-203]. In response to hypoxia, TAMs stimulate angiogenesis by releasing soluble VEGF, CXCL8, IL-8, TNF-α, as well as other glycolytic enzymes [171, 204, 205]. Proteases released by TAMs, such as cathepsins and MMP9, act to remodel the tumor microenvironment by cleaving extracellular matrix proteins and also promote angiogenesis by freeing

heparin-bound growth factors, such as VEGF-A [206-208].

In addition to roles in promoting primary tumor growth, the angiogenic switch and ECM remodeling are also essential for tumor invasion and metastasis. Additionally, TAMs have been shown to directly induce tumor cell migration. In a seminal study, the Pollard/Condeelis groups described a paracrine loop between breast cancer cells and TAMs that is established in PyVmT tumors [209]. PyVmT tumor cells secreted M-CSF, which supported the recruitment, development, and M2 polarization of TAMs. In turn, TAMs synthesized EGF, which promoted the motility and eventual metastasis of transformed epithelial cells. In a follow-up report by Wyckoff *et al*., intravital imaging revealed that tumor cells and TAMs localize to blood vessels, and TAMs directly assist tumor cell intravasation into the PB [210]. TAM-produced TGF-β and WNT7-β also reportedly induce the epithelial to mesenchymal transition (EMT) of cancer cells in several models [211, 212].

1.5.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) can be broadly defined as a heterogeneous population of immature myeloid cells with immunosuppressive capacities that accumulate during chronic pathological conditions, including cancer [213]. MDSC expansion is associated with advanced tumor progression in patients, and multiple studies have correlated high numbers of MDSCs with poor prognosis [214]. Furthermore, numerous murine models of cancer have

demonstrated increased levels of MDSCs in advanced stages of the disease [213, 215]. MDSCs can be divided into two main subgroups, classified as M-MDSCs and PMN-MDSCs, which are morphologically and phenotypically similar to monocytes and neutrophils, respectively [216]. M-MDSCs share many common features with Ly6C+ monocytes, including developmental pathways and a dependence on CCR2-signaling for trafficking [217]. In fact, a defining surface marker that distinguishes M-MDSCs from monocytes has not been identified to date, although this is currently an area of intensive research [213]; rather, the distinction between Ly6C+ inflammatory monocytes and M-MDSCs is based on functionality. Ly6C+ monocytes typically promote inflammatory reactions important for restricting tumor progression, whereas MDSCs characteristically perform immunosuppressive activities that favor tumor growth and metastasis.

MDSCs initially gained interest in the cancer biology field for their potent ability to suppress CD8+ T cell responses [218, 219]. One early report by Gallina *et al*. demonstrated direct inhibition of T cell activation and proliferation *in vitro* by MDSCs isolated from the spleen of tumor-bearing mice [220]. The authors went on to show that this process was dependent on IFN-γ and IL-13 release by MDSCs downstream of IL-4Rα signaling. Subsequent studies have described various additional mechanisms whereby MDSCs suppress both adaptive and innate immunity *in vitro* and *in vivo*. For example, MDSCs produce soluble factors that favor T cell inhibition, including arginase, iNOS, ROS and RNS species, as well as the immunosuppressive cytokines IL-1β, IL-6, IL-10, and

TGF-β [216, 221-226]. MDSCs can also recruit and induce the *de novo* development of T_{regs} in the tumor [227]. Moreover, MDSCs have been shown to directly interact with TAMs via a cell contact-dependent mechanism that results in impaired synthesis of TAM-produced IL-12 [218]. In turn, TAMs stimulate increased IL-10 release by MDSCs. Thus a high IL-10 to IL-12 balance is perpetuated in the tumor milieu, which inhibits the priming of T cell responses. MDSCs also support tumor progression through tissue remodeling of the tumor microenvironment. MDSC-derived VEGF and MMP9 have been shown to prompt angiogenesis and ECM degradation, processes necessary for the development of metastases [228-230]. Yan *et al.* showed that myeloid-specific ablation of MMP9 resulted in significantly diminished lung metastasis in mice bearing mammary adenocarcinomas [231].

MDSCs have been shown to suppress anti-tumor immunity both within the tumor itself and in peripheral lymphatic tissues [154, 232], suggesting that a systemic milieu of tumor-derived factors potentiates MDSC development and activity. Some of the identified factors that promote MDSC expansion include prostaglandins, stem-cell factor (SCF), M-CSF, GM-CSF, VEGF, and IL-6 [233-237]. However, ablation or blockade of individual proteins has not been successful in completely eliminating MDSCs in cancer models, suggesting the concurrent participation of multiple factors in MDSC accumulation [154]. The signaling pathways downstream of tumor-derived factors that promote the expansion, persistence, and immunosuppressive activity of MDSCs are not

completely understood [213]; however, STAT transcription factors have been identified as important mediators of these processes. STAT3 stimulates myelopoiesis and inhibits myeloid differentiation [219]. MDSCs isolated from tumor-bearing mice exhibit increased phosphorylation of STAT3 compared with immature myeloid cells from naive mice [238], and myeloid-specific deletion of STAT3 or the use of selective STAT3 inhibitors significantly reduced MDSC expansion [238, 239]. STAT3 activity in MDSCs is facilitated at least in part by CEBPβ and IRF8 signaling [240-242]. Additionally, STAT6 is activated in MDSCs by IL-4 and IL-13 signaling following IL-4Rα ligation [243]. Both STAT3 and STAT6 can directly bind to the ARG1 promoter and stimulate arginase production [243, 244]. The activation of STAT1 and STAT6 can also lead to iNOS and TGF-β production in MDSCs [219]. Of note, the HIF1α signaling pathway has also been implicated in several MDSC-mediated T cell suppressive activities, including the upregulation of arginase and iNOS production, as well as the induction of PD-L1 cell surface expression [195, 245].

It should be noted that many of the aforementioned activities of MDSCs overlap substantially with the mechanisms employed by TAMs to support tumor growth and metastasis. Although MDSCs and TAMs are considered separate entities, the boundaries that distinguish these MPS populations are not well defined. Indeed, numerous studies have reported that tumor-infiltrating M-MDSCs can differentiate into TAMs in a process driven by tumor-derived factors that include M-CSF [28, 214, 222, 245-248]. This conversion can also be mediated by the

HIF1α signaling pathway [245]. Thus, the phenotype and differentiation state of mononuclear phagocytes are driven by exposure to microenvironmental stimuli that vary according to tumor type, tumor stage, and anatomical location within the organism [213]. Altogether, these observations are consistent with what has emerged as a fundamental feature of MPS biology, wherein mononuclear phagocytes dynamically alter their function in response to the external cues that they encounter, and conversely transform the tissue microenvironment in which they reside.

1.6 Focal adhesion kinase (FAK) family kinases

The marked phenotypic plasticity displayed by mononuclear phagocytes during immune responses is dependent on the ability of these cells to properly integrate the complex array of environmental cues they encounter. Monocytes, for example, are produced in the BM and must travel great distances from their site of origin during inflammatory reactions. During their migration to inflamed sites, monocytes are exposed to various microenvironmental signals, including chemokines, cytokines, growth factors, and ECM components. Biological responses to these stimuli (including proliferation, survival, directional motility, and differentiation) must therefore be carefully orchestrated in order to facilitate the proper resolution of inflammatory reactions. Intracellular signaling molecules that are activated by diverse extracellular cues and consequently regulate multiple downstream signaling pathways, known as “integrators”, are therefore

necessary for the coordinated regulation of cellular processes. The focal adhesion kinase (FAK) family of tyrosine kinases have been identified as important intracellular integrators of integrin-mediated adhesion and growth factor signals [249]. FAK family kinase activity has been shown to regulate cell motility, survival, and proliferation in many cell types [250]; however, their role specifically in MPS populations has not been thoroughly studied.

1.6.1 Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) was originally identified as a highly tyrosine-phosphorylated v-Src substrate that localized to focal contacts in cells plated on fibronectin [251, 252]. A separate report published shortly thereafter revealed that FAK phosphorylation resulted from integrin clustering or adhesion to fibronectin, collagen IV, and laminin [253]. These early discoveries laid the groundwork for ensuing studies that explored the nature of FAK localization to integrin complexes. FAK-deficient mice display defective developmental morphogenesis and do not survive beyond embryonic d8.5 [254, 255]. FAK-/- fibroblasts isolated from these mice at d7.5 exhibited defective cell migration, which has been attributed to the prolonged formation of stable adhesion structures [255, 256]. Furthermore, FAK-/- cells had reduced numbers of focal adhesions at the cell periphery, but greater numbers of adhesion structures overall. These observations indicate that one of the functions of FAK is to mediate the disassembly of focal adhesion complexes [257, 258]. Numerous other studies have provided evidence implicating FAK in the regulation of

directional migration in many cell types [259-263]. More recently, FAK-mediated integrin signaling has been linked to proliferation and survival signaling via the ERK/MAPK pathway and through its ability to function as a mechanosensor [264-266].

FAK is a 125 kDa protein comprised of an N-terminal FERM domain, a central kinase domain, and a C-terminal domain that contains proline-rich regions and a focal adhesion targeting (FAT) domain (Figure 1.3). Each structural component contributes distinct traits necessary for FAK localization and function. The FERM domain has been shown to mediate interactions with integrins, as well as growth factor receptors, including the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) [260, 267]. The central kinase domain of FAK shares sequence similarity to other canonical protein tyrosine kinases. Autophosphorylation at the Y397 site is a particularly important event that allows for the binding of various Src homology 2 (SH2)-domain containing proteins, including Src family kinases (SFKs) [268]. Association with FAK induces conformational changes in Src that result in its activation, which in turn potentiates maximal activation of FAK via phosphorylation at the Y861 and Y925 sites [269]. Furthermore, the Src-FAK complex activates other proteins involved in focal adhesion dynamics, including p130Cas and paxillin [264, 270, 271]. The proline rich regions of FAK have been shown to bind Src homology 3 (SH3)-containing proteins, several of which have been implicated in processes related to cytoskeletal dynamics and focal adhesion formation [264]. Finally, the FAT

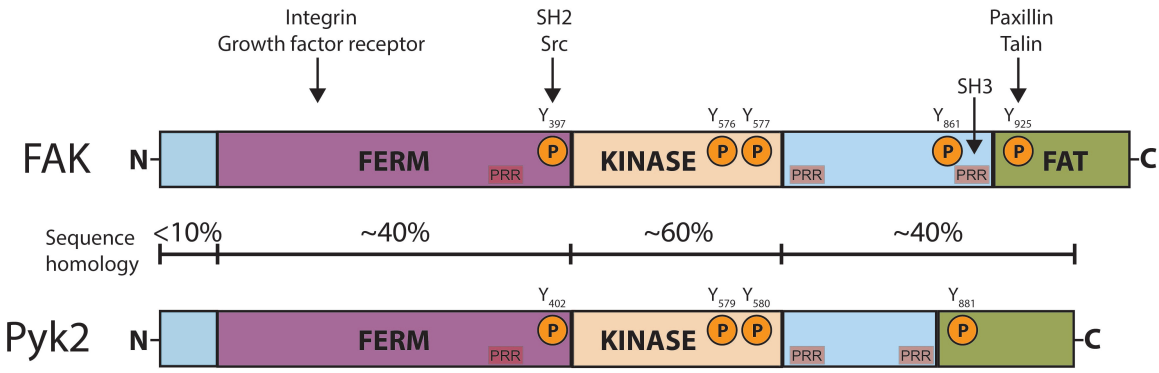

Integrin
Growth factor receptor
SH2
Src
Paxillin
Talin
SH3
FAK
N
FERM
PRR
KINASE
PRR
PRR
FAT
C
Y397
Y576
Y577
Y861
Y925
Sequence homology
<10%
~40%
~60%
~40%
Pyk2
N
FERM
PRR
KINASE
PRR
PRR
C
Y402
Y579
Y580
Y881

Figure 1.3 Sequence homology and structural organization of FAK family kinases. Focal adhesion kinase (FAK) and Proline-rich tyrosine kinase 2 (Pyk2) share high sequence homology and are both comprised of an N-terminal FERM domain, a central catalytic domain, and a C-terminal focal adhesion targeting (FAT) domain. Both kinases also display conservation of proline-rich regions (PRRs) and four tyrosine (Y) phosphorylation sites: FAK (Y)397, (Y)576, (Y)577, and (Y)925 correspond to Pyk2 (Y)402, (Y)579, (Y)580, and (Y)881, respectively. Auto-phosphorylation of FAK on (Y)397 and Pyk2 on (Y)402 generates a binding site for Src family kinases (SFKs). Figure adapted from [249, 268, 313].

domain is responsible for recruiting FAK to focal contacts through associations with integrin-binding proteins, including paxillin and talin [272]. Thus, FAK is well positioned as both a kinase and an adaptor protein to modulate intracellular signaling pathways that regulate cell adhesion dynamics and motility.

1.6.2 Proline-rich tyrosine kinase 2 (Pyk2)

Proline-rich tyrosine kinase 2 (Pyk2) represents the only other member of the FAK family kinases besides FAK itself. As such, Pyk2 shares several common structural features with FAK, including an N-terminal FERM domain, a central catalytic domain, and a C-terminal FAT domain (Figure 1.3) [249]. FAK and Pyk2 share approximately 45% sequence identity and 65% sequence similarity [273], and several phosphorylation sites are conserved at analogous positions in both family members [274]. However, FAK and Pyk2 share the least amount of sequence homology in their respective FAT domains, leading to distinct localization patterns [275, 276]. While FAK is targeted to focal complexes, Pyk2 is mainly localized to perinuclear regions of the cell [277]. As a result, Pyk2 and FAK interact with different target proteins, although both proteins are activated by SFKs [249].

The differential localization of Pyk2 and FAK may account for distinct intracellular signaling and modes of activation that have been reported for Pyk2. Furthermore, in contrast to the relatively ubiquitous expression pattern of FAK, Pyk2 is preferentially expressed in cells of the central nervous system, endothelium, and

hematopoietic lineages [278-280]. Pyk2 is phosphorylated by a diverse range of stimuli in different cell types, including G protein-coupled receptor agonists, stress signals, inflammatory cytokines, and SFKs downstream of integrin ligation [281-285]. Extracellular signals that elevate cytoplasmic Ca+ levels have also been shown to activate Pyk2 in multiple cell types, including after T cell receptor ligation [278, 279, 283, 286, 287]. However, the molecular mechanisms controlling Pyk2 activation by Ca+ are not well understood.

Less is known about the functional contributions of Pyk2 compared with FAK. Both FAK family kinases bind to proteins that interact with the cytoskeleton, suggesting a role in the control of directional migration or cellular morphology [277, 281, 288, 289]. However, overexpression of Pyk2 in FAK-deficient fibroblasts did not fully rescue the migratory defect of these cells, indicating that Pyk2 cannot completely compensate for FAK in promoting directional motility in these cells [261]. Studies using Pyk2-deficient mice have illuminated several functions of Pyk2 in hematopoietic lineage cells. Unlike FAK-deficient animals, Pyk2-/- mice are viable and exhibit no gross anatomical abnormalities [273]. They do, however, display mild osteopetrosis that has been attributed to a defect in osteoclast resorption of bone and/or increased bone formation by osteoblasts [290-292]. Macrophages isolated from Pyk2-/- animals exhibited defects in chemotaxis owing to a failure to detach from substrate at the trailing edge of the cells [259, 273]. Pyk2-deficient macrophages also showed morphological abnormalities in cellular spreading and lamellapodial formation. A subsequent

study in Pyk2-/- mice revealed a paucity of marginal zone B cells in the spleen of Pyk2-/- mice [293]. Pyk2-deficient B cells also exhibited diminished chemotaxis and reduced directional motility.

In addition to the aforementioned functions in regulating directional migration and cytoskeletal dynamics, several reports also implicate Pyk2 in the control of cell survival or apoptosis. Xiong *et al.* demonstrated that exogenous overexpression of Pyk2, but not FAK, led to apoptotic cell death in several fibroblast and epithelial cell lines [294]. The induction of apoptosis was dependent on Pyk2 kinase activity and could be suppressed by co-overexpression of catalytically active PI3K, v-Src, c-Src, or AKT. Transient overexpression of Pyk2 subsequently was shown to induce apoptosis in other cell types, including multiple myeloma cells, osteocytes, and neonatal cardiomyocyte cells [295-297]. However, other studies have associated Pyk2 upregulation in cancer cells with inhibition of apoptosis and prolonged survival [298-300]. Given these seemingly contradictory results, a role for Pyk2 in regulating cell survival is likely to be context-specific. Further clarification is therefore necessary to determine the function of Pyk2 in regulating the cell death under conditions in which it is normally expressed.

1.6.3 FAK family kinase expression and function in the MPS

While the studies highlighted above raise intriguing questions regarding the importance of FAK family kinase activity in monocyte and macrophage functions,

much of the previous work was performed on non-hematopoietic cells, overexpression systems, or immortalized cell lines. In fact, prior to the initiation of the current study, FAK expression in monocyte lineage cells was controversial. Several early reports failed to visualize FAK in monocytes or macrophages [301-307]. It should be noted, however, that these studies did not account for the monocyte heterogeneity that is appreciated today, and thus these findings did not preclude the possibility that FAK may be expressed in some subpopulations of monocytes. Other studies clearly showed FAK expression in mature macrophages [259, 308-310]. Pyk2 was also expressed in macrophages [259, 273], but its expression in monocyte subsets had not been assessed. Moreover, while both FAK and Pyk2 were shown to be activated in mature macrophages in response to integrin ligation, and to regulate migration and cytoskeletal dynamics [259, 273], their functions in MPS cell subsets and contribution to homeostasis and pathological conditions were relatively unexplored.

1.7 Research objectives and overview

As critical components of the innate immune system, MPS constituents act as sentinels of tissue homeostasis and initiators of immunological defenses against pathogens and other physiological perturbations. Mononuclear phagocytes must therefore be equipped to sense inflammatory cues, migrate to regions of danger, integrate signals from the microenvironment, and adapt their phenotype appropriately. Disruptions to this delicate balance can result in the initiation and

persistence of pathological conditions. Thus, the molecular mechanisms controlling these biological processes require further elucidation. FAK and Pyk2 are well-established mediators of integrin signaling, cytoskeletal dynamics, and directional migration in many cell types. Other studies have implicated these molecules in the regulation of cell proliferation and survival. As integrators of a diverse array of environmental stimuli, studies in this thesis were developed to test the hypothesis that FAK family kinases promote fundamental activities necessary for proper immunological responses in MPS cells.

The research presented in this thesis addresses several questions regarding the nature of FAK family kinases in MPS biology. First, using recently established lineage markers to identify distinct MPS subpopulations, we assessed the expression patterns of FAK family kinases in mononuclear phagocyte populations at homeostasis (**Chapter 2**). We demonstrate that FAK and Pyk2 expression is distinct and developmentally regulated in these cell populations. The second goal of this research was to determine whether FAK family kinases regulate aspects of monocyte and macrophage functionality during homeostasis and pathological settings. To this end, the use of genetically altered mouse models was indispensible to our studies. Our data indicate that Pyk2 functions to promote the turnover of Ly6C^{neg} monocytes during homeostatic conditions in a cell-intrinsic manner (**Chapter 2**). Using the MMTV-PyVmT mouse model of breast carcinogenesis, we also investigated the role of FAK expression in macrophage-mediated control over tumor progression (**Chapter 3**). Although

preliminary, our data indicate that FAK expression in myeloid lineage cells is necessary to restrict primary breast tumor outgrowth. Finally, in **Chapter 4**, we discuss the relevance of our findings to what has been established in the literature and propose future experiments that may further elucidate the role of FAK family kinases in MPS functions.

Chapter 2: Pyk2 promotes the turnover of monocytes at steady-state

(Adapted from Llewellyn *et al.,* In Press - Journal of Leukocyte Biology)

2.1 Introduction

Monocytes comprise a heterogeneous population of short-lived mononuclear phagocytes that contribute to tissue homeostasis and protective immunity [340]. Committed monocyte progenitors (cMoP) continually replenish cells of this lineage through the tightly regulated process of bone marrow (BM) monopoiesis [29, 107, 312]. Subsequent egress of BM monocytes to the vasculature results in two main subpopulations of peripheral blood (PB) monocytes that have been classified based on distinct phenotypic markers [23, 26, 27, 42]. In mice, Ly6C+ "classical" monocytes circulate throughout the PB and are poised to extravasate into peripheral sites in response to inflammatory cues. Subsequent terminal differentiation of these cells into monocyte-derived macrophages and dendritic cells (DCs) is integral for the proper resolution of tissue damage and infection [98, 109, 313-315]. Recent evidence indicates that during homeostatic conditions, most tissue resident macrophages are self-maintaining and are not derived from monocytes [79-82]. Thus, in the absence of inflammatory signals, the Ly6C+ monocytes instead function as short-lived precursor cells that can terminally differentiate into the second major population of blood monocytes, $Ly6C^{neg}$ "nonclassical" monocytes [46, 47]. At steady-state, it is believed that these cells function to maintain vascular integrity by "crawling" along the luminal

surface of capillaries in a patrolling and scavenging capacity [24, 25]. Ly6C^{neg} monocytes also constitute a relatively small percentage of BM monocytes, although their function and cellular origin in this tissue are currently under debate [29, 44, 46]. Both subsets of monocytes also accumulate in the spleen, constituting a reservoir that can be mobilized in response to inflammatory cues [45].

While our understanding of monocyte differentiation and function has advanced considerably, the molecular mechanisms responsible for homeostatic maintenance of these subpopulations are still unclear. Ly6C+ and Ly6C^{neg} monocytes exhibit a relatively short half-life of approximately 18 hours and 2.3 days, respectively [46]. The continuous production and rapid turnover of these cells suggest a carefully balanced orchestration of pro-survival and pro-death signaling in order to maintain proper subset representation. To date, multiple factors that support prolonged survival of Ly6C+ and/or Ly6C^{neg} monocytes have been identified, including M-CSF, CX3CL1, and the transcription factor NR4A1 [44, 49, 51, 66, 316]. In the absence of these stimuli, peripheral monocytes have been shown to spontaneously undergo cell death [70, 71, 317]. Thus, it has been theorized that pro-survival signals may act to impede a continually active apoptotic program existing in monocytes at steady-state. However, the specific molecular signaling pathways that drive constitutive monocyte turnover are relatively unexplored.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that is localized to focal complexes and facilitates signaling downstream of integrin and growth factor receptor activation [260, 318]. Proline-rich tyrosine kinase 2 (Pyk2) comprises the only other member of the FAK family and shares significant amino acid sequence homology to FAK. While FAK is expressed in most cell types, Pyk2 is predominantly expressed in hematopoietic and neuronal cells [274]. Pyk2 can be activated by a variety of stimuli, including integrin engagement, growth factor signaling, intracellular calcium influx, and antigen receptor engagement on lymphocytes [274, 319, 320]. Functionally, FAK and Pyk2 have both been shown to regulate numerous cellular processes, including adhesion signaling, directional motility, immune cell activation, and proliferation (Reviewed in [274, 319]). Several studies have also revealed a role for Pyk2 in controlling cell death [294-297, 321], while others have linked Pyk2 upregulation to prolonged survival of transformed cells [298-300]. Given these discrepancies, it appears that the role of Pyk2 in regulating cell survival is likely to be context-specific. Notably, several of these overexpression studies were performed *in vitro* using immortalized cell lines or cell types that normally express low levels of endogenous Pyk2. Further clarification is therefore necessary to determine the role of Pyk2 in regulating the survival of cells under conditions in which it is normally expressed.

Previous studies have reported that FAK and Pyk2 are expressed at variable levels in cells of the mononuclear phagocyte system [259, 273, 310, 322]. However, a close examination of the expression and function of these molecules

across the continuum of monocyte development has not been performed. In this study, we investigated the expression of FAK and Pyk2 in monocyte lineage subsets from the BM, PB, and splenic reservoirs. We show that FAK expression is low to negligible in monocyte subsets, whereas Pyk2 expression is detectable in Ly6C+ monocytes and significantly elevated in the Ly6C^{neg} fraction of monocytes. We therefore focused on exploring the role of Pyk2 in the differentiation and accumulation of monocyte subpopulations at steady-state. Data from these studies demonstrate that mice deficient for Pyk2 exhibit a greater representation of Ly6C^{neg} monocytes in the BM and periphery. Studies using chimeric mice indicate that this phenotype is cell-autonomous. Additional evidence suggests that Ly6C^{neg} monocytes exhibit a survival benefit in the absence of Pyk2. Given these results, we propose that Pyk2 plays a role during homeostasis in maintaining appropriate Ly6C^{neg} monocyte levels by promoting the turnover of these characteristically short-lived cells.

2.2 Materials and Methods

2.2.1 Mice. Wild-type (WT) C57BL/6 mice were bred and housed on site. The Pyk2-/- mouse model has been previously described [273, 323]. C57BL/6-Ly5.1 (CD45.1+) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Male and female mice (8-14 wk. of age) were age- and sex-matched for experiments. All studies were performed in accordance with University of Virginia Animal Care and Use Committee guidelines.

2.2.2 Harvest and preparation of cell suspensions from mouse tissues. Mice were euthanized and tissues harvested in the following order. Blood (300-700µl/mouse) was drawn through cardiac puncture and placed in 1ml of 5mM EDTA/Hank's balanced saline solution (-Mg,-Ca) (HBSS). Spleens were excised, pushed through a 70µm filter, and washed in MACS buffer (0.5% BSA, 250mM EDTA in PBS). BM was flushed from both femora and tibiae with MACS buffer, washed in MACS buffer, and filtered through a 30µm filter. To remove erythrocytes, the tissues were incubated in ammonium/chloride/potassium (ACK) lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2 EDTA $2H_2O$ in H_2O) for 10 mins. on ice, quenched with complete media (10% FBS/DMEM), and washed in MACS buffer.

2.2.3 Flow cytometry. BM, PB, and spleen cell suspensions containing approximately $1x10^6$ cells in 100µl MACS buffer were incubated with the F_C blocking antibody anti-CD16/32 (eBioscience, San Diego, CA, USA) for 10 mins. on ice. The cells were subsequently incubated with primary monoclonal antibodies to cell surface antigens for 25 mins. on ice. Antibodies were purchased from the following companies and used at the concentration suggested by the manufacturer: AbD Serotec, Oxford, UK: anti-F4/80 (Cl:A3-1[clone]); BD Biosciences, San Jose, CA, USA: anti-F4/80 (T45-2342), anti-CD11a (2D7); Biolegend, San Diego, CA, USA: anti-CD115 (AFS98), anti-CD117 (ACK2), anti-Ly6C (HK1.4), anti-CD11b (M1/70), anti-Ly6G (1A8), anti-CD117 (2B8), anti-CD45.1 (A20), anti-CD192 (SA203G11); eBioscience, San Diego, CA,

USA: anti-CD115 (AFS98), anti-CD11c (N418), anti-CD45.2 (104), anti-CD3e (145-2C11), anti-CD49b (DX5), anti-CD19 (MB19.1). Samples were stained concurrently with fluorescence minus one (FMO) antibody panels. Samples were washed in MACS buffer, and incubated with fixable live/dead cell stain (Invitrogen, Carlsbad, CA, USA) for 30 mins. on ice. Samples were then washed in MACS buffer and fixed for 20 mins. on ice with Cytofix (BD Biosciences, San Jose, CA, USA), resuspended in MACS buffer, and data were acquired on the Cyan ADP LX (Beckman Coulter, Brea, CA, USA) or LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA). FlowJo software (Tree Star Inc., v.10, Ashland, OR, USA) was used for data analysis. Forward- and side-scatter parameters were used for exclusion of doublets from analysis. Absolute numbers were calculated using Accucount beads (Spherotech, Lake Forest, IL, USA) according to the manufacturer's instructions. BM absolute numbers reflect counts from both femora and tibiae per mouse.

To measure cells undergoing DNA synthesis, and for BrdU lineage-tracing analyses, the BD BrdU Flow Kit (BD Pharmingen, San Jose, CA, USA) was used. Mice received a single 2mg BrdU pulse by intraperitoneal injection. At the indicated time points, single cell preparations of tissues were prepared and surface antigens were stained, followed by fixable live/dead staining, as described above. The cells were subsequently permeabilized, DNase treated, and stained with anti-BrdU-FITC according to the manufacturer's instructions. The percentages and absolute numbers of BrdU+ cells were determined by flow cytometry.

For *ex vivo* detection of annexin V+ and dead monocytes, cell suspensions derived from BM were first stained for surface antigens to identify monocyte subpopulations. The suspensions were then incubated with annexin V-FITC (BD Pharmingen) and 0.1µg/ml DAPI (Life Technologies, Carlsbad, CA, USA) in annexin V staining buffer (BD Pharmingen) for 15 mins. at room temperature immediately prior to analysis by flow cytometry. Dead cells were identified as annexin V+/DAPI+. Apoptotic cells were identified as annexin V+ after dead cell exclusion.

For *ex vivo* detection of active caspase, the Vybrant FAM Poly Caspases Assay Kit (Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's instructions. Briefly, cell suspensions derived from BM were first stained for surface antigens to identify monocyte subpopulations. The suspensions were then incubated with FLICA reagent (1:30 from working solution) in MACS buffer for 60 mins. at 37^{o}C, followed by multiple washes in wash buffer. Immediately prior to analysis by flow cytometry, cells were resuspended in wash buffer/DAPI (0.1µg/ml). Dead cells were excluded based on DAPI incorporation, and the percentage of monocyte subsets expressing active caspases was assessed by FLICA staining.

2.2.4 Immunomagnetic column separation of cell populations. CD115+ monocytes were isolated using the mouse MACS CD115 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Briefly, single cell suspensions were first prepared as described above. F_C

receptors were blocked with anti-CD16/32 (eBioscience, San Diego, CA, USA) for 10 mins. on ice, and the cells were subsequently incubated with anti-CD115-biotin. The CD115+ population was isolated by magnetic column following incubation with anti-biotin magnetic beads. Purity of the isolated population was assessed by flow cytometry.

2.2.5 Fluorescence activated cell sorting (FACS). Single cell preparations of tissues were prepared and enriched for CD115+ monocytes by immunomagnetic column separation, and cells were then stained for cell surface markers, as described above. Immediately prior to FACS sorting, cells were resuspended in MACS buffer/DAPI [0.1µg/ml] (Life Technologies). FACS sorting of monocyte subpopulations was performed on the Influx Cell Sorter (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2.6 *In vitro* generation of macrophages from BM (BMDMs). Whole BM was collected as described above, enumerated by hemacytometer, and added to media preparations at 4-6 x 10^6 cells per 10 cm. plate. Base media (αMEM with 10% FBS and penicillin/streptomycin) was supplemented with 10% CMG 14-12 conditioned media (source of M-CSF). After 7 days in culture, the adherent cells were washed 3 times with PBS, incubated with trypsin/EDTA for 15 min., quenched with complete media, washed with MACS buffer, and resuspended in MACS buffer.

2.2.7 Immunoblotting. Cell suspensions were washed in PBS, pelleted, and incubated in RIPA lysis buffer as described previously [259]. Immunoblotting was

performed as described previously [259]. The following antibodies were utilized: anti-Pyk2 (Upstate, Lake Placid, NY, USA), anti-FAK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK p44/42 (Cell Signaling, Danvers, MA, USA), and anti-AKT (Cell Signaling).

2.2.8 BM chimera generation. CD45.1+ WT recipient mice were depleted of endogenous lymphoid tissue by irradiation (2 doses of 550 cGy at 3-hour intervals), and 1 day later were intravenously injected with $5x10^6$ BM cells isolated from CD45.2+ WT or Pyk2-/- donor femora and tibiae. BM reconstitution was allowed to proceed in recipients for 8 weeks before analysis. Recipient mice were provided oral sulfamethoxazole and trimethoprim for the duration of the experiment.

2.2.9 *In vitro* culture of BM for monocyte viability assays. Whole BM was collected as described above, enumerated by hemocytometer, and added to serum-free RPMI1640 or RPMI1640 supplemented with 10% FBS and penicillin/streptomycin at $5x10^6$ cells per 60 mm plate. After 4 hours at 37^{o}C, non-adherent cells were collected and placed on ice. Adherent cells were washed with PBS, incubated with trypsin/EDTA for 15 mins., quenched with complete media, and collected. Non-adherent and adherent cells were combined, washed with MACS buffer, and prepared for flow cytometry, as described above. Cell death was determined by DAPI incorporation.

2.2.10 Statistical analysis. Comparisons between groups were made using an unpaired two-tailed student t-test , Mann-Whitney test, or one-way ANOVA. P

values ≤ .05 (*), ≤ .01 (**), and ≤ .001 (***) were considered statistically significant.

2.3 Results

2.3.1 Pyk2 protein expression is elevated in Ly6C^{neg} monocytes.

In order to assess protein expression of FAK and Pyk2 in monocytes during steady-state conditions, CD115+ (also known as the M-CSF receptor) cells isolated from the BM, PB, and spleen of wild-type (WT) mice were enriched via magnetic bead selection (Figure 2.1A). Lysates were prepared, and western blotting was performed on the extracts (Figure 2.1B). FAK and Pyk2 expression was markedly greater in monocytes isolated from the PB and spleen compared with BM monocytes (Figure 2.1B, compare lanes 2 and 3 to lane 1). Of note, the PB and spleen have been found to contain a higher proportion of Ly6C^{neg} monocytes than the BM [26, 45].

Given these results, we hypothesized that FAK family kinase expression may be developmentally regulated during monocyte differentiation. To address this question, we refined our analysis by isolating monocyte subpopulations from BM, PB, and spleen by fluorescence-activated cell sorting (FACS). CD115+ monocytes were magnetically enriched and FACS-sorted into cMoP, Ly6C+ and Ly6C^{neg} subsets based on established lineage markers [26, 29, 44, 45] (Figure 2.2A). Backgating analyses of these monocyte subpopulations using additional

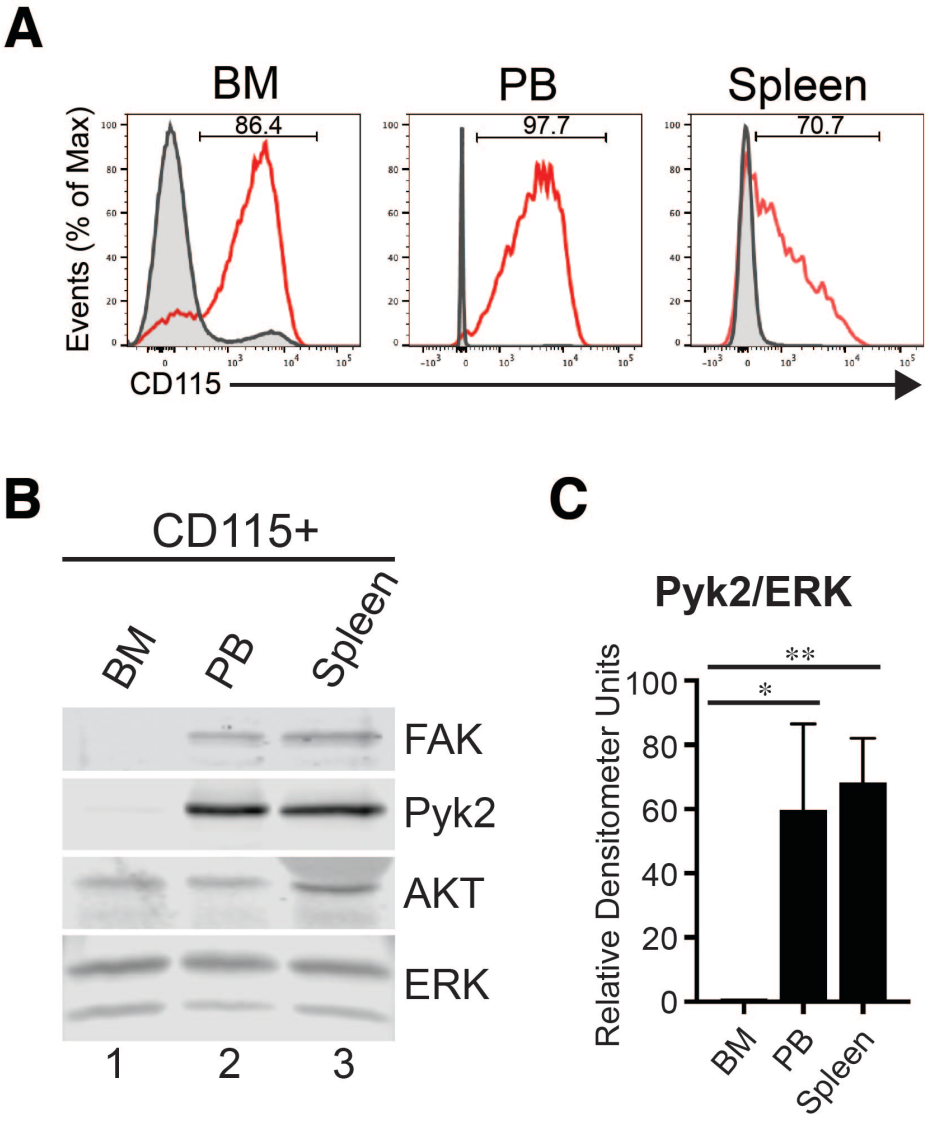

A
BM
PB
Spleen
86.4
97.7
70.7
Events (% of Max)
CD115
B
CD115+
BM
PB
Spleen
FAK
Pyk2
AKT
ERK
1
2
3
C
Pyk2/ERK
Relative Densitometer Units
**
*
100
80
60
40
20
0
BM
PB
Spleen

Figure 2.1 FAK and Pyk2 protein expression is elevated in peripheral monocytes. (A) Flow cytometry histogram plots depicting efficiency of CD115+ magnetic bead enrichment from the BM, PB, and spleen of WT mice. Plots display non-enriched cell preparations (shaded curves) and enriched fractions (red lines). Numbers above the gated regions represent percent CD115+ of the enriched cell populations. (B) Representative immunoblot showing FAK and Pyk2 expression in cell lysates of CD115-enriched fractions from the indicated tissues. AKT and ERK are presented as loading controls. (C) Quantification of relative Pyk2 expression normalized to ERK is presented. Data shown are the mean of 3 independent experiments ± S.E.M. * $p < 0.05$, ** $p < 0.01$, compared to all other groups (One-way ANOVA). The immunoblotting from panel B was performed by Keena Thomas.

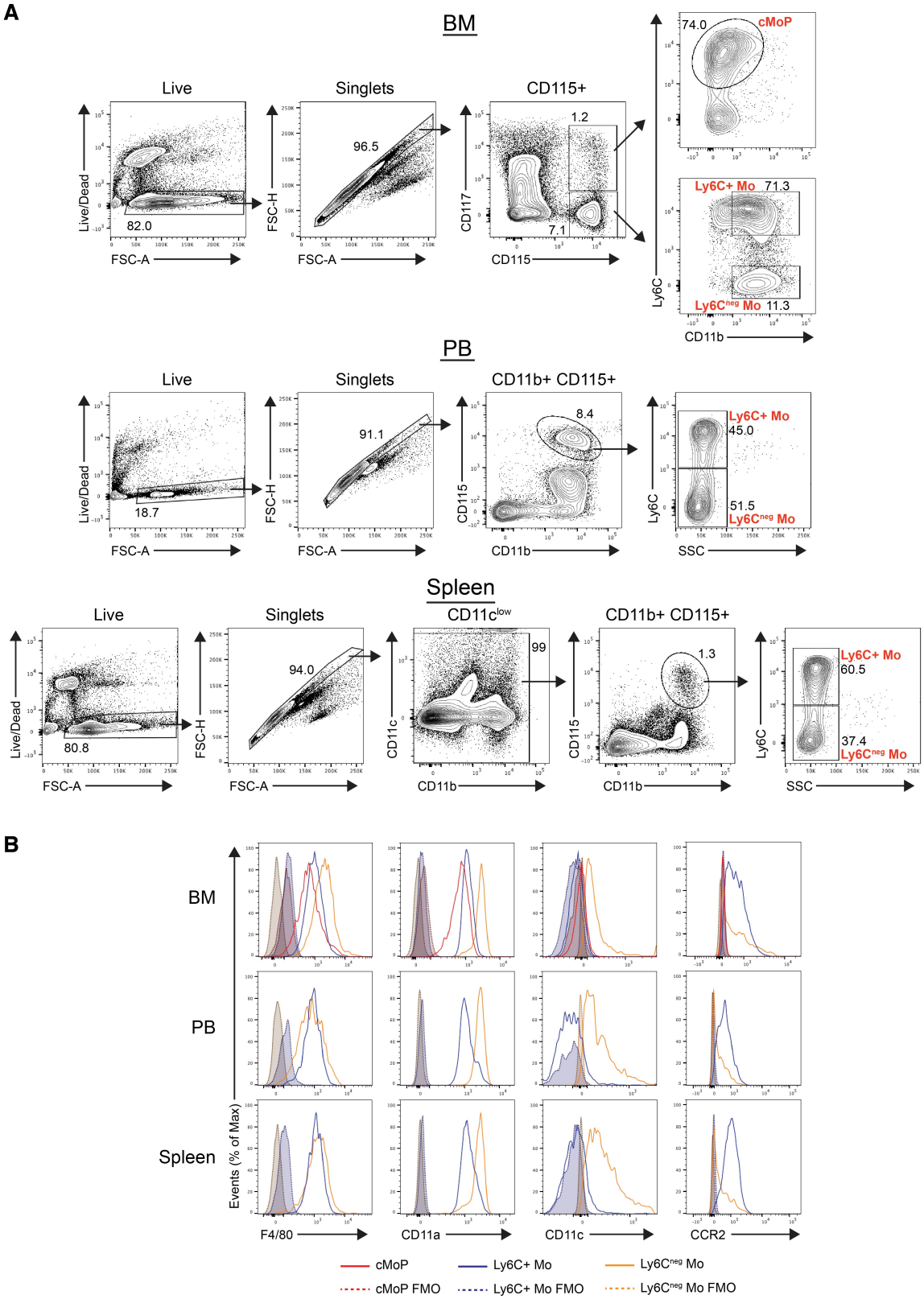
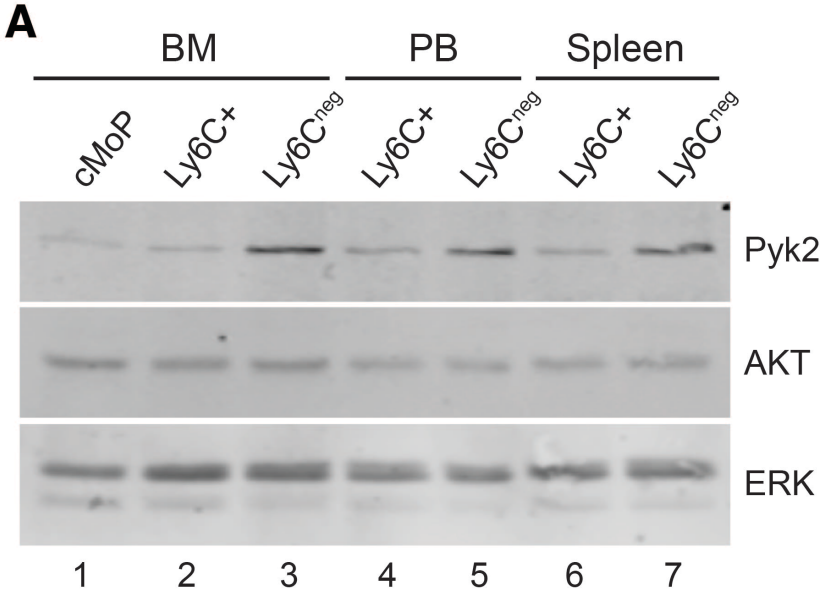

A
BM
Live
Singlets
CD115+
Live/Dead
FSC-A
FSC-H
CD117
CD115
82.0
96.5
1.2
7.1
74.0
cMoP
Ly6C+ Mo 71.3
Ly6C^neg Mo 11.3
Ly6C
CD11b
PB
CD11b+ CD115+
18.7
91.1
8.4
Ly6C+ Mo
45.0
51.5
Ly6C^neg Mo
SSC
Spleen
CD11c^low
80.8
94.0
99
1.3
Ly6C+ Mo
60.5
37.4
Ly6C^neg Mo
CD11c
B
BM
PB
Spleen
Events (% of Max)
F4/80
CD11a
CD11c
CCR2
cMoP
Ly6C+ Mo
Ly6C^neg Mo
cMoP FMO
Ly6C+ Mo FMO
Ly6C^neg Mo FMO

Figure 2.2 Gating strategy and analysis of surface marker expression for monocyte subpopulations. (A) Flow cytometry gating strategy for identification of monocyte populations from the indicated organs. Numbers adjacent to the outlined areas indicate percent of the gated cell populations. (B) Monocyte populations from the indicated tissues were further examined for a variety of surface proteins by flow cytometry. Histograms depicting surface marker expression and complementary fluorescent minus one (FMO) control staining are presented as solid and dotted/shaded lines, respectively.

cell-surface markers (F4/80, CD11a, CD11c, CCR2; negative for the lineage markers CD135, Ly6G, CD49b, CD3e, CD19) confirmed expression profiles characteristically associated with the respective subsets (Figure 2.2B; data not shown) [29, 44, 45]. Lysates from the sorted subsets were then immunoblotted for FAK and Pyk2 (Figure 2.3). Pyk2 expression was significantly elevated in Ly6C^{neg} monocytes from the BM compared with the cMoP and Ly6C+ subsets (compare lane 3 with lanes 1 and 2). A similar increase in Pyk2 expression was observed in Ly6C^{neg} monocytes isolated from the PB (compare lanes 4 and 5) and spleen (compare lanes 6 and 7) compared with Ly6C+ monocytes isolated from the same sites. In contrast to Pyk2, we were consistently unable to visualize FAK in any of the sorted monocyte populations (data not shown, n=3).

FAK and Pyk2 expression has previously been characterized in mouse macrophages, but not in monocyte subsets [273]. Therefore, we directly compared FAK and Pyk2 expression between BM Ly6C^{neg} monocytes and bone marrow-derived macrophages (BMDMs) (Figure 2.4A), and between splenic Ly6C^{neg} monocytes and resident macrophages (defined as CD11b^{low}F4/80^{hi}) (Figure 2.4B). In both cases, Pyk2 expression was similar between the Ly6C^{neg} monocytes and the macrophages. FAK, on the other hand, was not detectible in Ly6C^{neg} monocytes, but was clearly expressed in the macrophage populations. These results may seem incongruent with the data presented in Figure 2.1B, in which FAK expression was detected in magnetically isolated CD115+ monocytes from the PB and spleen. However, due to technical limitations, fewer sorted


A
BM
PB
Spleen
cMoP
Ly6C+
Ly6Cneg
Ly6C+
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Pyk2
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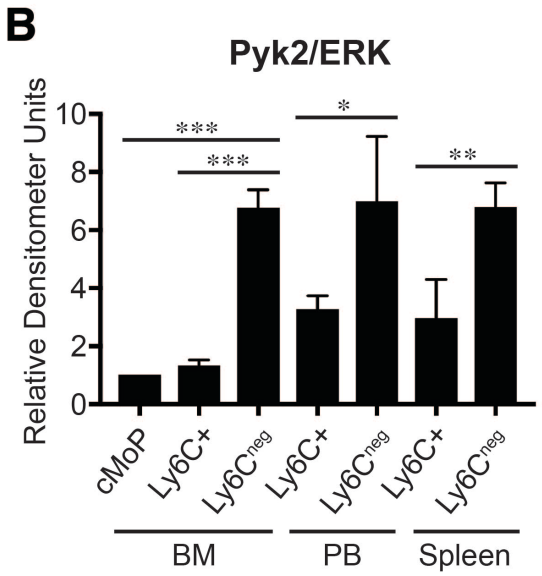

B
Pyk2/ERK
Relative Densitometer Units
10
8
6
4
2
0
***
***
*
**
cMoP
Ly6C+
Ly6Cneg
Ly6C+
Ly6Cneg
Ly6C+
Ly6Cneg
BM
PB
Spleen

Figure 2.3 Pyk2 protein expression is elevated in Ly6C^{neg} monocytes. (A) Representative immunoblot showing Pyk2, ERK, and AKT expression in cell lysates generated from $5x10^4$ FACS-sorted monocyte subsets isolated from the indicated tissues. (B) Quantification of relative Pyk2 expression normalized to ERK. Data shown are the mean of 3 independent experiments ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to all other groups (One-way ANOVA). The immunoblotting from panel A was performed by Keena Thomas.

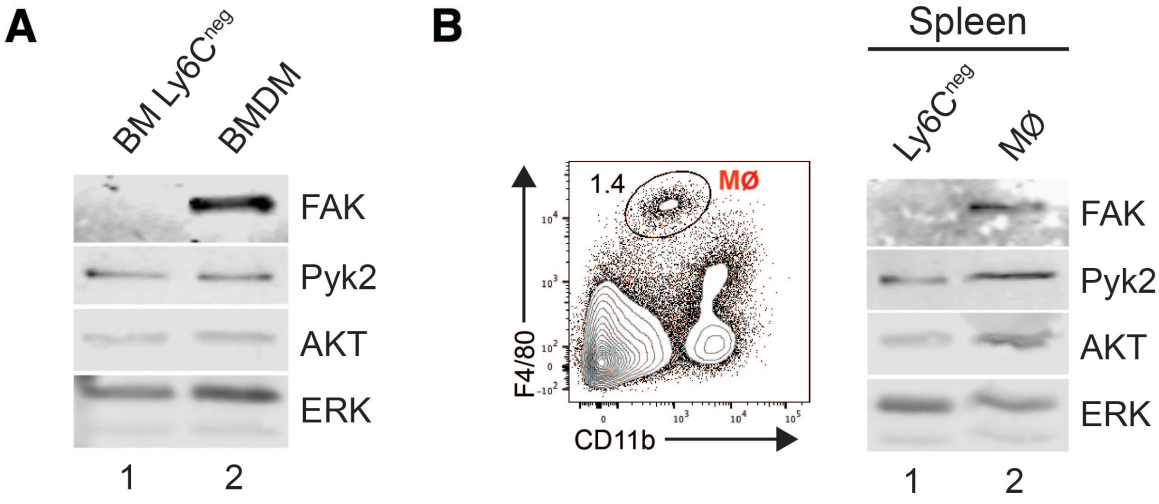

A
BM Ly6C^neg
BMDM
FAK
Pyk2
AKT
ERK
1
2
B
1.4
MØ
F4/80
CD11b
Spleen
Ly6C^neg
MØ
FAK
Pyk2
AKT
ERK
1
2

Figure 2.4 FAK and Pyk2 expression in macrophage populations. (A) Representative immunoblot of FAK, Pyk2, AKT and ERK expression in cell lysates from Ly6C^{neg} monocytes isolated from the BM (lane 1) or adherent bone marrow-derived macrophages (BMDMs) after 7 days in culture with M-CSF (lane 2). (B) Left, flow cytometry gating strategy to identify tissue-resident macrophages in the spleen. Dead cells and doublets were excluded prior to the displayed gating. Right, representative immunoblot comparing FAK, Pyk2, AKT and ERK expression in cell lysates from Ly6C^{neg} monocytes (lane 1) or macrophages (lane 2) isolated from the spleen. The immunoblotting was performed by Keena Thomas.

monocytes were available to be analyzed compared with the magnetically enriched monocyte samples. Therefore, we suggest that FAK may be expressed at very low levels in one or more monocyte subsets from the PB and spleen.

2.3.2 Loss of Pyk2 alters the relative proportion of monocyte subpopulations.

Given the data above showing that FAK is poorly expressed in monocytes, and that Pyk2 expression is elevated in Ly6C^{neg} monocyte populations, we hypothesized that Pyk2 may be important for regulating the differentiation and/or maintenance of monocyte subpopulations. While previous reports have described the overall immune cell populations in lymphoid tissues of Pyk2-/- mice as similar to those of WT mice [273], the distribution of monocyte subsets in BM and peripheral organs of these mice has not been previously determined.

Pyk2-/- mice have been described by our group and others as being mildly osteopetrotic due to a defect in osteoclast resorption of bone and/or increased bone formation by osteoblasts [290-292]. Thus it was not surprising that the Pyk2-/- mice exhibited significantly reduced total BM cellularity (Figure 2.5A), particularly since other mouse models of osteopetrosis exhibit a similar, albeit more drastic, reduction in BM cellularity that has been attributed to a smaller BM cavity [49, 51]. However, to our knowledge this is the first time this phenotype had been reported in Pyk2-/- mice.

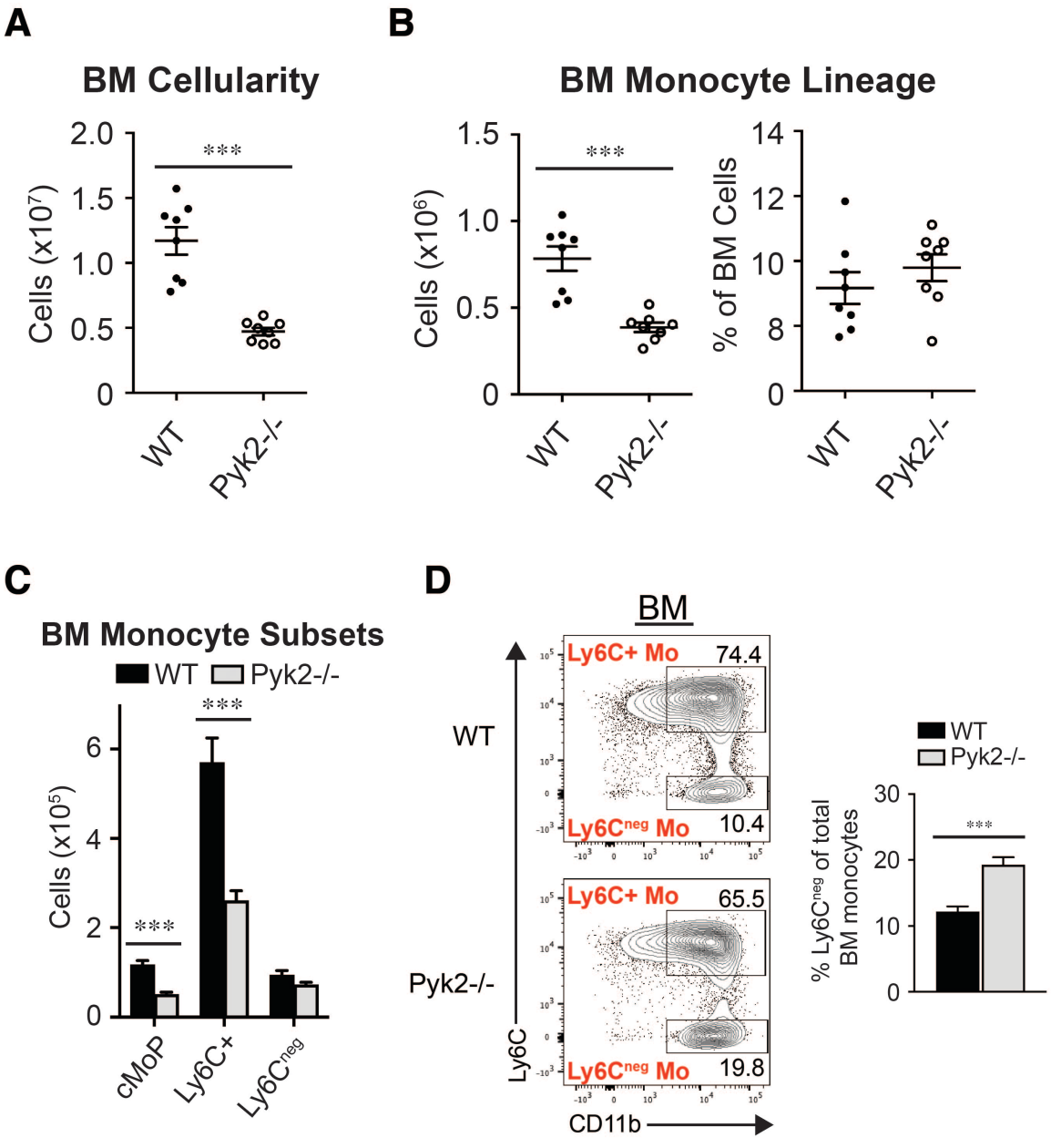

A
BM Cellularity
Cells (x10^7)
2.0
1.5
1.0
0.5
0
***
WT
Pyk2-/-
B
BM Monocyte Lineage
Cells (x10^6)
1.5
1.0
0.5
0
***
WT
Pyk2-/-
% of BM Cells
14
12
10
8
0
WT
Pyk2-/-
C
BM Monocyte Subsets
WT
Pyk2-/-
Cells (x10^5)
6
4
2
0
***
***
cMoP
Ly6C+
Ly6C^neg
D
BM
WT
Pyk2-/-
Ly6C+ Mo
74.4
Ly6C^neg Mo
10.4
Ly6C+ Mo
65.5
Ly6C^neg Mo
19.8
Ly6C
CD11b
WT
Pyk2-/-
% Ly6C^neg of total BM monocytes
30
20
10
0
***

Figure 2.5 Pyk2-/- mice exhibit altered BM monocyte subset representation. Single cell suspensions from the indicated tissues were analyzed by flow cytometry and monocyte subsets were identified as described in Figure 2.2. (A) Comparison of the absolute number of live cells in the BM of WT and Pyk2-/- mice. Each circle represents an individual mouse. (B) Left, total numbers of BM monocytes, defined as CD115+ cells. Right, percentage of monocyte lineage cells among total live BM cells. (C) Total numbers of BM monocyte subpopulations per mouse (WT n=8, Pyk2-/- n=8). (D) Left, representative FACS contour plots displaying Ly6C+ and $Ly6C^{neg}$ monocyte subpopulations from WT and Pyk2-/- BM. Right, data from panel C presented as the percentage of $Ly6C^{neg}$ monocytes among total BM monocyte lineage cells.

Data are acquired from at least 2 independent experiments. *** $p < 0.001$ (unpaired Student's t-test).

The decrease in total BM cellularity was accompanied by a corresponding reduction in the absolute number of monocyte lineage cells (as defined by CD115 expression) in the BM of Pyk2-/- mice (Figure 2.5B). However, despite this reduction in total monocyte numbers, Pyk2-deficient monocytes made up a similar proportion of the total BM present in these animals (Figure 2.5B). As expected given the decreased number of CD115+ monocyte lineage cells present in Pyk2-/- BM, the absolute numbers of cMoP and Ly6C+ monocyte subsets in the BM of Pyk2-/- mice were significantly reduced compared to WT controls (Figure 2.5C). Surprisingly though, the total number of Ly6C^{neg} monocytes was similar between WT and Pyk2-/- mice (Figure 2.5C), leading to a disproportionate enrichment of these cells within the monocyte compartment of the BM in Pyk2-/- mice (Figure 2.5D).

Unlike the BM, no reductions in total cellularity were observed in the PB and spleen of Pyk2-/- mice (data not shown). Nevertheless, Ly6C^{neg} monocytes were also proportionally overrepresented in the monocyte compartments of both the spleen and PB (Figure 2.6A). This was driven by a significant increase in Ly6C^{neg} monocytes in the spleen, with no appreciable changes to Ly6C+ monocyte numbers (Figure 2.6B). A similar increase in Pyk2-deficient Ly6C^{neg} monocyte numbers was not seen in the PB (Figure 2.6B), possibly due to the inherent variability in absolute monocyte counts in this organ. Taken together, these data suggest that Pyk2 plays a role in maintaining the proper balance of monocyte populations under homeostatic conditions.

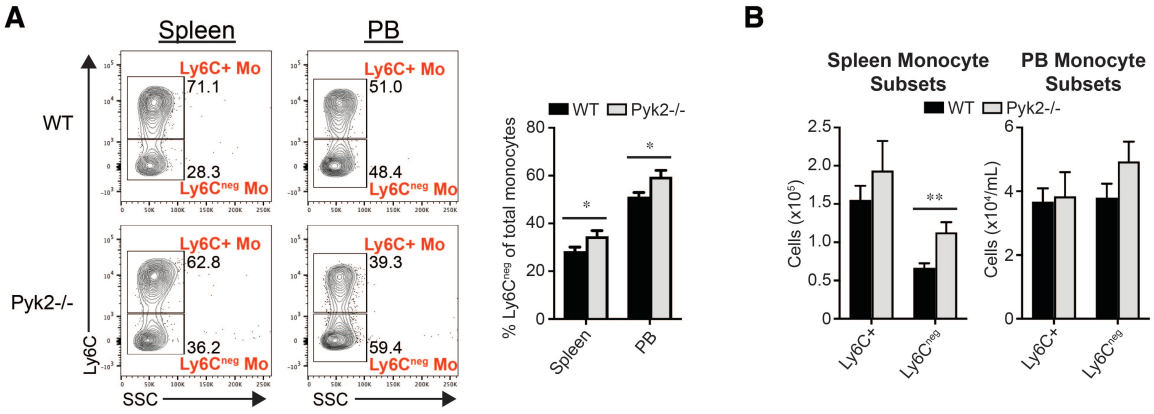

A
Spleen
PB
WT
Pyk2-/-
Ly6C+ Mo
71.1
28.3
Ly6C^neg Mo
Ly6C+ Mo
51.0
48.4
Ly6C^neg Mo
Ly6C+ Mo
62.8
36.2
Ly6C^neg Mo
Ly6C+ Mo
39.3
59.4
Ly6C^neg Mo
Ly6C
SSC
SSC
WT
Pyk2-/-
% Ly6C^neg of total monocytes
Spleen
PB
B
Spleen Monocyte Subsets
PB Monocyte Subsets
WT
Pyk2-/-
Cells (x10^5)
Cells (x10^4/mL)
Ly6C+
Ly6C^neg
Ly6C+
Ly6C^neg

Figure 2.6 Pyk2-/- mice exhibit altered peripheral monocyte subset representation. Single cell suspensions from the indicated tissues were analyzed by flow cytometry and monocyte subsets were identified as described in Figure 2.2. (A) Left, representative FACS contour plots displaying Ly6C+ and Ly6C^{neg} monocyte subpopulations from the PB and spleen of WT and Pyk2-/- mice. Right, percentage of Ly6C^{neg} monocytes among total monocytes in the indicated tissues. (B) Absolute number of monocyte subpopulations per spleen (left) or per ml of PB (right); (Spleen: WT n=8, Pyk2-/- n=8; PB: WT n=21, Pyk2-/- n=22).

Data are acquired from at least 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ (unpaired Student's t-test).

2.3.3 Loss of Pyk2 alters the representation of Ly6C^{neg} monocytes via a cell-autonomous mechanism.

The altered representation of monocyte subsets in Pyk2-/- mice could be the result of a loss of Pyk2-dependent activities in the monocytes (cell-intrinsic) or in the stromal microenvironment that supports the development and/or survival of these cells (monocyte-extrinsic). To discriminate between these possibilities, reciprocal transfers of WT or Pyk2-/- BM were performed into lethally irradiated WT hosts (Figure 2.7A). Efficient reconstitution of CD45.2+ donor BM was observed 8 weeks after the transfer, concomitant with complete ablation of native CD45.1+ BM in the lethally irradiated recipient mice (Figure 2.7B).

Within the confines of a WT host harboring a normal skeletal structure, Pyk2-deficient BM was able to reconstitute to the same level as WT BM (Figure 2.7C). Thus, the deficiency in BM hematopoiesis observed in Pyk2-/- mice (Figure 2.5A) likely results from the smaller BM cavity associated with osteopetrosis. In line with this possibility, the total number and percentage of monocyte lineage cells in the BM of chimeric mice was unaltered by loss of Pyk2 (Figure 2.7D) and the pools of cMoP and Ly6C+ monocytes were unchanged (Figure 2.7E). This indicates that the development of cMoP and Ly6C+ monocytes in the BM does not require Pyk2 activity in these cells. However, a modest but statistically significant increase in Ly6C^{neg} monocytes was observed in the mice receiving Pyk2-/- BM (Figure 2.7E), and this was accompanied by a greater representation of Ly6C^{neg} monocytes in the BM and spleen of these mice (Figure 2.7F). These

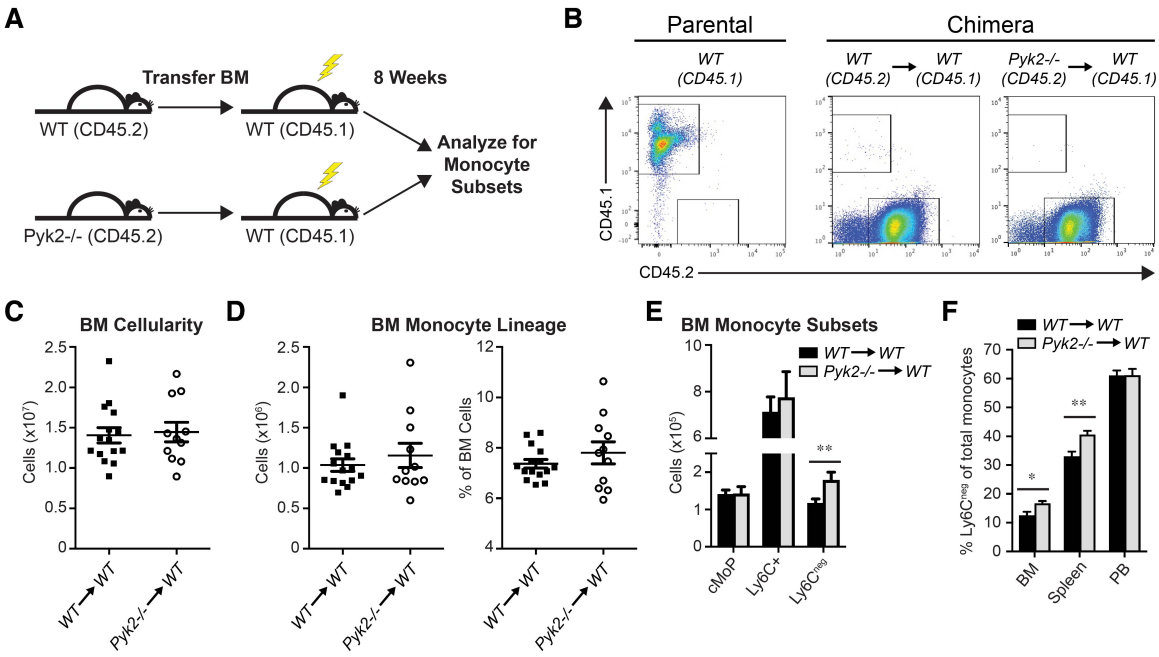

A
Transfer BM
8 Weeks
WT (CD45.2)
WT (CD45.1)
Analyze for Monocyte Subsets
Pyk2-/- (CD45.2)
WT (CD45.1)
B
Parental
Chimera
WT (CD45.1)
WT (CD45.2) → WT (CD45.1)
Pyk2-/- (CD45.2) → WT (CD45.1)
CD45.1
CD45.2
C
BM Cellularity
Cells (x10⁷)
WT → WT
Pyk2-/- → WT
D
BM Monocyte Lineage
Cells (x10⁶)
% of BM Cells
E
BM Monocyte Subsets
WT → WT
Pyk2-/- → WT
Cells (x10⁵)
cMoP
Ly6C+
Ly6C^neg
F
% Ly6C^neg of total monocytes
BM
Spleen
PB

Figure 2.7 Loss of Pyk2 alters the representation of Ly6C^{neg} BM and spleen monocytes via a cell-autonomous mechanism. (A) Schematic showing experimental design for reciprocal BM transfers. Lethally irradiated CD45.1+ WT mice received CD45.2+ WT or CD45.2+ Pyk2-/- whole bone marrow ($5x10^6$ cells), followed by reconstitution for 8 weeks. Single cell preparations of BM, PB, and spleen were analyzed by flow cytometry. (B) Representative flow cytometry dot plots show CD45.1+ native bone marrow in a non-irradiated mouse (left), compared with lethally irradiated chimeric animals eight weeks after receiving CD45.2+ WT BM (middle) or CD45.2+ Pyk2-/- BM (right). Over 99% of native bone marrow was ablated in chimeric mice. (C) Total numbers of live cells in the BM of WT→WT and Pyk2-/-→WT mice, assessed by flow cytometry. (D) Left, total numbers of CD115-expressing BM monocytes. Right, Percentage of monocyte lineage cells among total live BM cells. (E) Total numbers of BM monocyte subpopulations per mouse (WT→WT n=15, Pyk2-/-→WT n=11). (F) Percentage of Ly6C^{neg} monocytes among total monocytes in the indicated tissues (BM: WT→WT n=15, Pyk2-/-→WT n=11; spleen: WT→WT n=18, Pyk2-/-→WT n=14; PB: WT→WT n=18, Pyk2-/-→WT n=14). Data shown are acquired from 2 independent experiments. * $p < 0.05$, ** $p < 0.01$, (unpaired Student's t-test).

results, which recapitulate the disproportionate representation of Ly6C^{neg} monocytes observed in the BM and spleen of Pyk2-/- mice (Figure 2.5D, Figure 2.6A), establish that environmental (monocyte cell-extrinsic) factors are not solely responsible for these changes in monocyte subset distribution. Rather, these data indicate that loss of Pyk2 activity in Ly6C^{neg} monocytes leads to the increased accumulation of this subpopulation.

In contrast to the BM and spleen, the Ly6C^{neg} population in the PB of chimeric mice receiving Pyk2-deficient BM was identical to those receiving WT BM (Figure 2.7F). This suggests that loss of Pyk2 in non-monocytic cells may contribute to the relative representation of PB monocyte subsets observed in Pyk2-/- mice (Figure 2.6A). Unfortunately, it was not possible to generate the reciprocal chimeras in Pyk2-/- hosts due to radiosensitivity of these mice (data not shown), thus precluding our ability to directly test for environmental (non-autonomous) effects on monocyte subset accrual. Nonetheless, the data presented above indicate that the accumulation of Ly6C^{neg} monocytes in the BM and spleen is controlled at least in part by cell-intrinsic activities of Pyk2 in these populations. Given that Pyk2 is most highly expressed in these cells (Figure 2.3A), we suggest that it functions to maintain a proper balance of Ly6C^{neg} monocytes at steady-state.

To further investigate the contribution of Pyk2 to the development and maintenance of monocyte subpopulations, we performed competitive

reconstitution experiments (Figure 2.8A). Bone marrow from CD45.2+ Pyk2-/- mice and CD45.1+ WT control mice were mixed 1:1 (Figure 2.8B, left panel) and transplanted into irradiated CD45.1+ WT recipients. Strikingly, eight weeks after bone marrow transfer, nearly 80% of the BM CD45+ cells were derived from Pyk2-/- BM (Figure 2.8B, right panel, and Figure 2.8C). Accordingly, significantly more Pyk2-deficient cMoP, Ly6C+, and Ly6C^{neg} monocytes were observed after competitive reconstitution in the BM compared with their WT counterparts (Figure 2.8D). These data indicate that loss of Pyk2 confers a competitive advantage to the establishment, proliferation, and/or survival of several hematopoietic lineages, including monocyte lineage cells. It is also worth noting that, compared with WT cells, Pyk2-deficient BM cells were more efficient at generating Ly6C^{neg} monocytes in particular, as Ly6C^{neg} monocytes comprised a significantly greater proportion of the monocyte compartment (defined as total CD115+ cells) present in the BM (Figure 2.8E). These results are consistent with the disproportionate over-representation of Ly6C^{neg} monocytes observed in the BM of Pyk2-/- mice (Figure 2.5D) and reciprocal chimeric mice that received Pyk2-/- BM (Figure 2.7F).

2.3.4 Pyk2 may promote apoptosis of BM monocytes.

Ly6C^{neg} monocytes are derived from Ly6C+ monocytes and/or cMoPs [46, 47, 324], and are non-proliferating at steady state [29, 44, 46]. Therefore, the disproportionate accumulation of Ly6C^{neg} monocytes observed in the absence of Pyk2 could result from an increase in the proliferation rate of the precursor cells

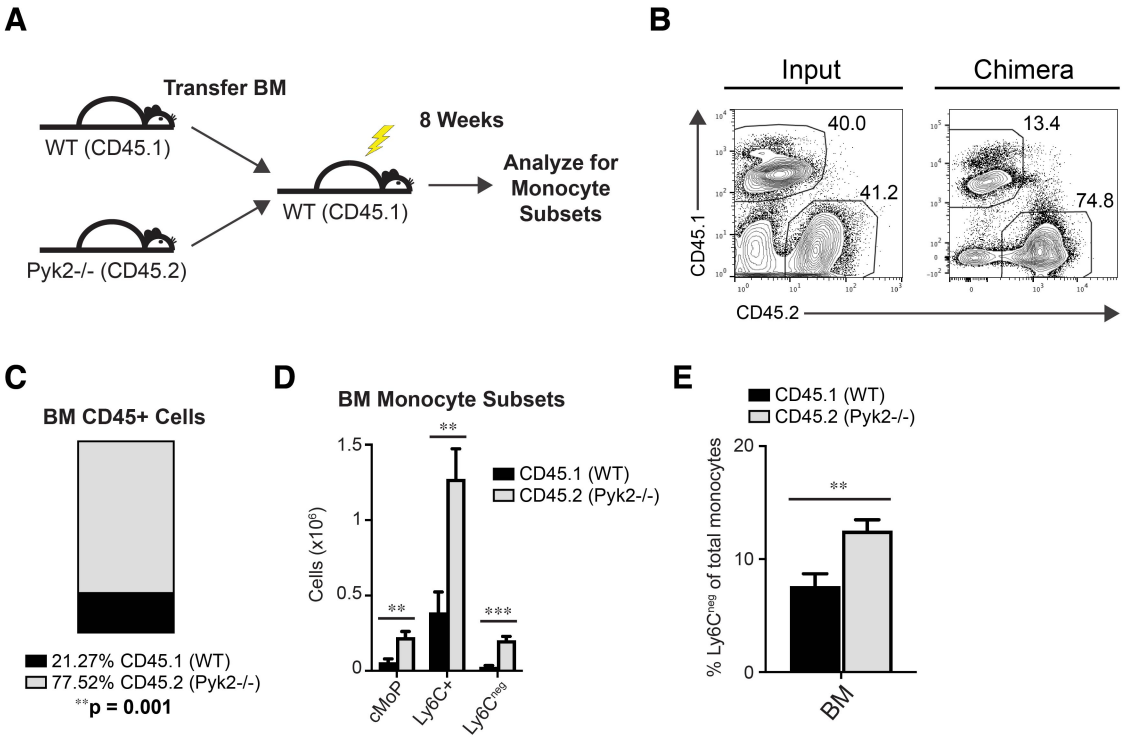

A
Transfer BM
WT (CD45.1)
8 Weeks
WT (CD45.1)
Analyze for Monocyte Subsets
Pyk2-/- (CD45.2)
B
Input
Chimera
40.0
41.2
13.4
74.8
CD45.1
CD45.2
C
BM CD45+ Cells
21.27% CD45.1 (WT)
77.52% CD45.2 (Pyk2-/-)
**p = 0.001
D
BM Monocyte Subsets
CD45.1 (WT)
CD45.2 (Pyk2-/-)
Cells (x10^6)
1.5
1
0.5
0
**
**
***
cMoP
Ly6C+
Ly6C^neg
E
CD45.1 (WT)
CD45.2 (Pyk2-/-)
% Ly6C^neg of total monocytes
20
10
0
**
BM

Figure 2.8 Loss of Pyk2 confers a competitive advantage in the establishment or maintenance of BM hematopoietic lineage cells. (A) Schematic showing experimental design for competitive BM transfers. Lethally irradiated CD45.1+ WT mice received whole bone marrow ($5x10^6$ total cells) mixed 1:1 from CD45.1+ WT and CD45.2+ Pyk2-/- donors, followed by reconstitution for 8 weeks. Single cell preparations of BM were analyzed by flow cytometry. (B) Representative flow cytometry contour plots show whole bone marrow mixed 1:1 from CD45.1+ WT and CD45.2+ Pyk2-/- donors transferred into lethally irradiated recipient mice (left), compared with a chimeric animal eight weeks after receiving the BM transfer (right). (C) Percentage of allotype-specific CD45+ cells among total CD45+ BM cells in mixed chimeric animals (n=7). (D) Total numbers of allotype-specific BM monocyte subpopulations (n=7). (E) Percentage of $Ly6C^{neg}$ monocytes among total monocytes for each CD45 allotype (n=7).

** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney test for panel C; unpaired Student's t-test for panel D and E).

(cMoP and/or Ly6C+ cells) and/or a failure of the Ly6C^{neg} monocytes to undergo cell cycle arrest. These possibilities were evaluated by measuring BrdU incorporation into monocyte subsets 2 hours following a single intraperitoneal injection of BrdU. Under these conditions, proliferating cells are marked with BrdU in the absence of any appreciable differentiation of the cells [46]. As expected, cMoPs contained the highest percentage of BrdU+ cells, followed by Ly6C+ monocytes (Figures 2.9A-B, black bars). Like their WT counterparts, Pyk2-/- Ly6C^{neg} monocytes failed to incorporate BrdU during this 2-hour pulse (Figures 2.9A-B). The fraction of Pyk2-deficient cMoP and Ly6C+ monocytes incorporating BrdU was also not elevated compared with WT controls; in fact, the opposite was the case (Figure 2.9B). Similar analyses performed in chimeric mice indicate that this is a monocyte-intrinsic effect (Figure 2.9C). Based on these data, the greater accumulation of Ly6C^{neg} monocytes observed in Pyk2-/- mice cannot be due to increased proliferation of one or more monocyte populations.

We next examined the half-life of Ly6C^{neg} monocytes. Because these cells are non-proliferating and do not incorporate BrdU, their accumulation over time can be followed by tracing precursors (cMoP and Ly6C+ monocytes) that received a single pulse of BrdU [46]. Coincident with the reduced numbers of cMoP and Ly6C+ monocytes present in the BM of Pyk2-deficient mice (Figure 2.5C), the absolute number of BrdU-labeled cells was reduced in the Pyk2-/- BM at day 0 (Figure 2.9D). The fraction of the initial pool of labeled precursors that

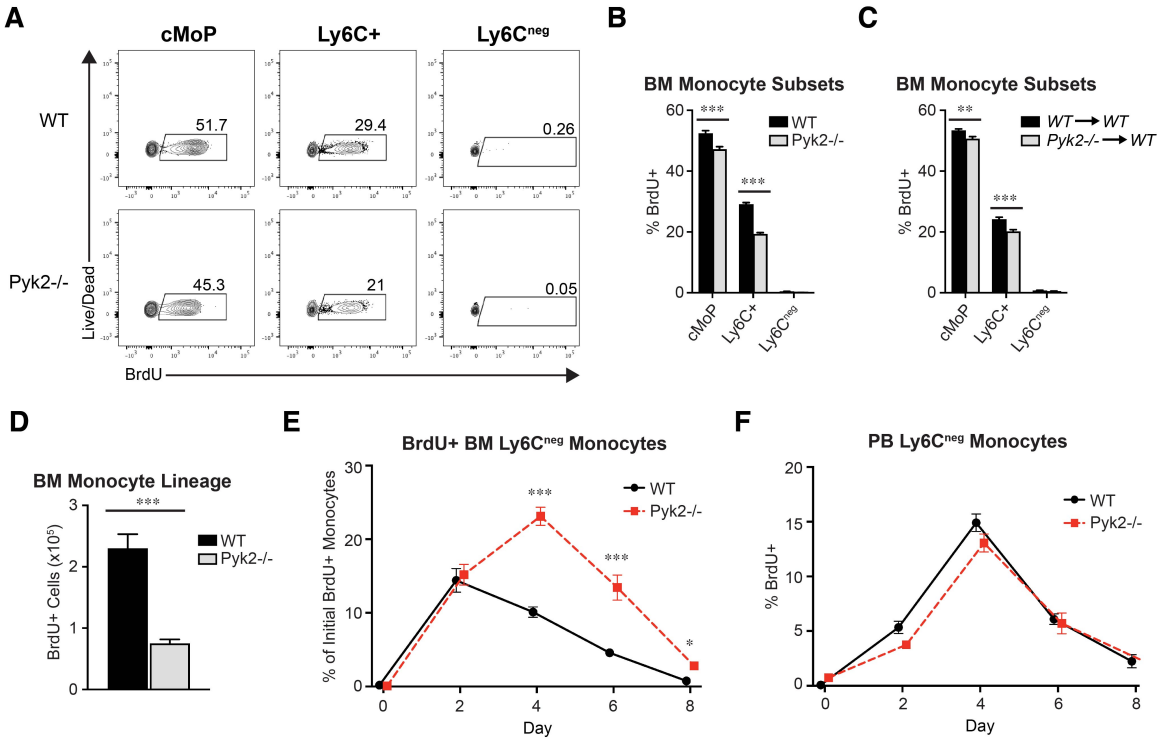

A
cMoP
Ly6C+
Ly6C^neg
WT
Pyk2-/-
51.7
29.4
0.26
45.3
21
0.05
Live/Dead
BrdU
B
BM Monocyte Subsets
WT
Pyk2-/-
% BrdU+
cMoP
Ly6C+
Ly6C^neg
C
BM Monocyte Subsets
WT → WT
Pyk2-/- → WT
% BrdU+
D
BM Monocyte Lineage
BrdU+ Cells (x10^5)
WT
Pyk2-/-
E
BrdU+ BM Ly6C^neg Monocytes
% of Initial BrdU+ Monocytes
Day
F
PB Ly6C^neg Monocytes
% BrdU+
Day

Figure 2.9 Pyk2 may control the turnover of lineage-traced Ly6C^{neg} BM monocytes. (A) The *in vivo* proliferative capacity of BM cMoPs, Ly6C+, and Ly6C^{neg} monocytes was assessed following a two-hour i.p. BrdU pulse. Representative contour plots display the percentage of monocyte subpopulations that incorporated BrdU, as assessed by flow cytometry. (B) Quantification showing the percentage of BrdU+ monocytes within each subpopulation of BM monocytes (WT n=8, Pyk2-/- n=8). Data shown are acquired from 2 independent experiments. (C) Percentage of BrdU+ monocytes within each subpopulation of BM monocytes from WT→WT and Pyk2-/-→WT chimeric mice following a two-hour i.p. BrdU pulse (WT→WT n=11, Pyk2-/-→WT n=11). (D) Absolute numbers of BrdU-labeled BM monocytes are presented (WT n=8, Pyk2-/- n=8). (E) Time course of BrdU+ Ly6C^{neg} monocytes that accumulate in the BM, represented as the percentage of total BrdU+ monocytes that were initially labeled at day 0 (WT n≥8, Pyk2-/- n≥8 per time point). (F) Time course of BrdU+ Ly6C^{neg} monocytes in the PB, represented as the proportion of Ly6C^{neg} cells that stained positive for BrdU by flow cytometry (WT n≥8, Pyk2-/- n≥8 per time point).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired Student's t-test).

differentiated into BrdU-labeled Ly6C^{neg} monocytes in the BM was then determined at 2,4, 6, and 8 days (Figure 2.9E). After 2 days, approximately 15% of the BrdU-labeled monocytes were Ly6C^{neg} in both Pyk2-/- and WT BM, indicating that the precursor cells differentiated into Ly6C^{neg} monocytes at similar rates. In accordance with the 2-3 day half-life reported for these cells [46], BrdU-labeled Ly6C^{neg} monocytes in WT BM began to decrease by day 4 and were completely lost by day 8. However, BrdU-positive Ly6C^{neg} monocytes continued to accumulate in Pyk2-/- BM through day 4, after which they began to decline. As was the case for WT cells, the BrdU-labeled Ly6C^{neg} Pyk2-/- monocytes were almost completely lost by day 8.

One possible explanation for the prolonged accumulation of Pyk2-/- Ly6C^{neg} monocytes in the BM is that they are less efficient at egressing to the periphery than WT cells. However, this is not likely to be the case since BrdU-labeled WT and Pyk2-deficient Ly6C^{neg} monocytes accumulated in the PB with similar kinetics (Figure 2.9F). Alternatively, Ly6C^{neg} monocytes could have a survival benefit in the absence of Pyk2, leading to a longer half-life in the BM. In support of this possibility, we found that the proportion of apoptotic and dead Ly6C^{neg} monocytes was decreased in Pyk2-/- BM compared to WT BM as measured by annexin V and DAPI incorporation, respectively (Figure 2.10A). Furthermore, active caspase expression, another hallmark of apoptosis, was reduced in BM Ly6C^{neg} monocytes in the absence of Pyk2 (Figure 2.10B). This reduction in apoptosis and cell death observed in Pyk2-deficient Ly6C^{neg} BM monocytes was

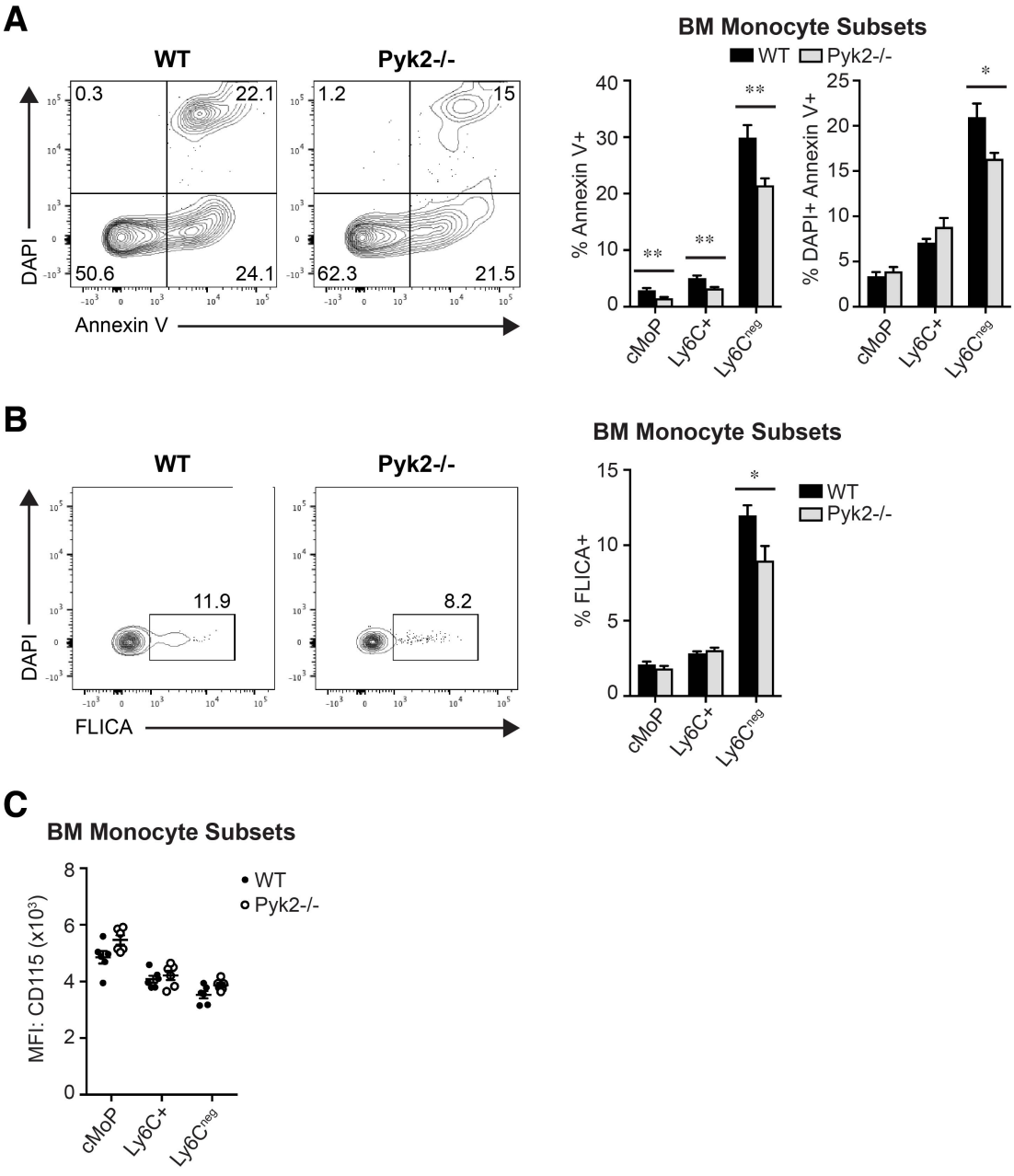

A
WT
Pyk2-/-
0.3
22.1
50.6
24.1
1.2
15
62.3
21.5
DAPI
Annexin V
BM Monocyte Subsets
WT
Pyk2-/-
% Annexin V+
% DAPI+ Annexin V+
cMoP
Ly6C+
Ly6C^neg
B
11.9
8.2
FLICA
% FLICA+
C
MFI: CD115 (x10^3)

Figure 2.10 Pyk2-deficient Ly6C^{neg} BM monocytes exhibit reduced apoptosis *in vivo*. (A) Representative contour plots display annexin V staining and DAPI incorporation in BM Ly6C^{neg} monocytes from WT and Pyk2-/- mice, assessed by flow cytometry. The adjacent graphs show the average percentage of apoptotic (DAPI^{neg} /annexin V+, left) or dead (DAPI+/annexin V+, right) monocytes in BM monocyte subpopulations (WT n=8, Pyk2-/- n=8). Data are acquired from 2 independent experiments. (B) Representative contour plots display active caspase (FLICA) staining in BM Ly6C^{neg} monocytes from WT and Pyk2-/- mice, assessed by flow cytometry. The adjacent graph shows the percentage of BM monocyte subsets expressing cleaved (active) caspases, assessed by flow cytometry (WT n=8, Pyk2-/- n=8). Data are acquired from 2 independent experiments. (C) Quantification of the mean fluorescence intensity of cell surface CD115 (M-CSFR) for WT and Pyk2-/- BM monocyte subpopulations.

* $p < 0.05$, ** $p < 0.01$ (unpaired Student's t-test).

recapitulated in chimeric WT hosts receiving Pyk2-/- BM, indicating that loss of Pyk2 confers a survival advantage for Ly6C^{neg} monocytes in a cell-autonomous manner (Figure 2.11). Notably, while M-CSF receptor (CD115) signaling is an established regulator of monocyte lineage cell survival at homeostasis [60-62], the survival advantage for BM Ly6C^{neg} monocytes deficient in Pyk2 did not correlate with changes in surface expression of CD115 (Figure 2.10C).

To further explore the role of Pyk2 in monocyte survival, BM cells from WT and Pyk2-/- mice were cultured *in vitro* for 4 hours in the presence or absence of serum. Under these conditions, monocytes have been reported to undergo a rapid onset of apoptosis [66]. As was reported by Breslin *et al.* [325], CD115 surface staining on monocytes was diminished when the cells were maintained *ex vivo* at temperatures above 4^{o}C (Figure 2.12A). Consequently, monocytes cultured under these conditions were identified as CD117^{neg}CD11b+F4/80^{low} (Figure 2.12B). Under both serum-supplemented (complete media) and serum-free conditions, the percentage of dead Ly6C+ and Ly6C^{neg} monocytes was reduced in the absence of Pyk2 (Figure 2.12C), while the absolute number of live cells was increased (Figure 2.12D). Together, these data suggest that Pyk2 controls the steady state level of monocytes by promoting the turnover of monocyte subsets under homeostatic conditions.

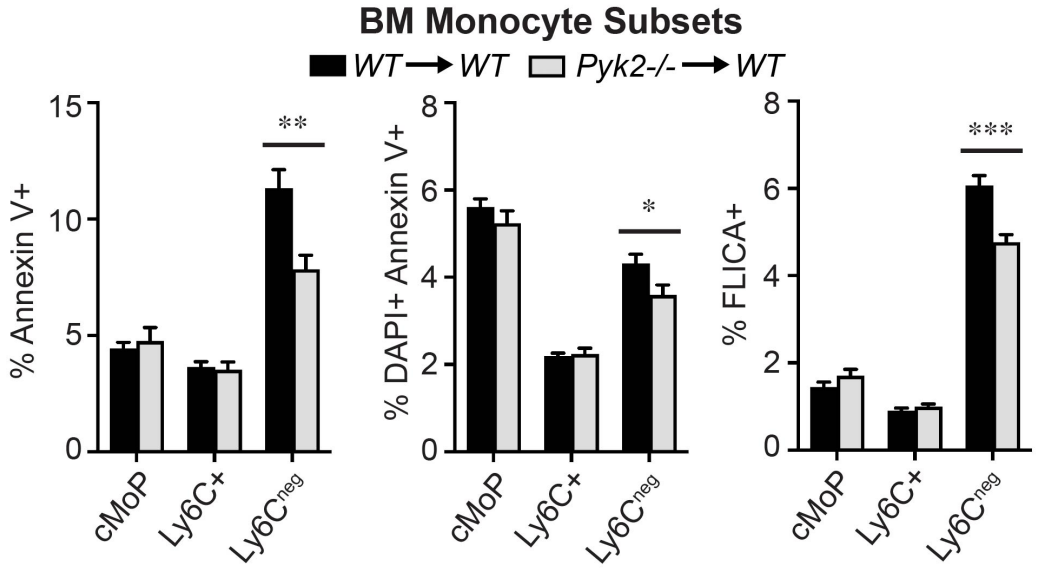

BM Monocyte Subsets
WT → WT
Pyk2-/- → WT
% Annexin V+
% DAPI+ Annexin V+
% FLICA+
**
*
***
cMoP
Ly6C+
Ly6Cneg

Figure 2.11 Pyk2 deficiency may promote Ly6C^{neg} BM monocyte survival *in vivo* via a cell-autonomous mechanism. Percentage of BM monocyte subsets staining positive for annexin V (left), DAPI (middle), or cleaved caspases (right) in WT→WT and Pyk2-/-→WT chimeric mice, assessed by flow cytometry (WT→WT n=12, Pyk2-/-→WT n=12). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired Student's t-test).

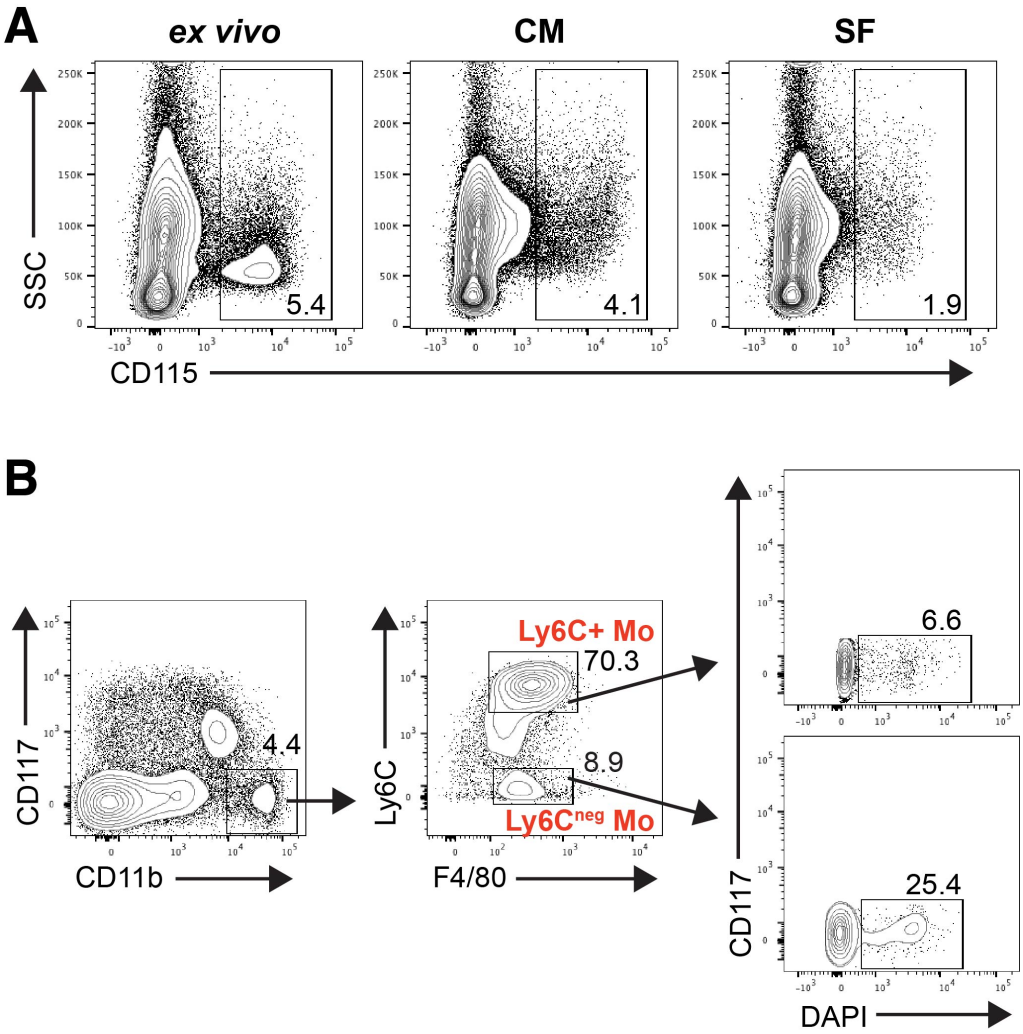

A
ex vivo
CM
SF
SSC
CD115
5.4
4.1
1.9
B
CD117
CD11b
4.4
Ly6C
F4/80
Ly6C+ Mo
70.3
8.9
Ly6C^neg Mo
6.6
25.4
CD117
DAPI
C
% DAPI+
WT
Pyk2-/-
***
***
**
***
Ly6C+
Ly6C^neg
Ly6C+
Ly6C^neg
CM
SF
D
Live Monocytes (x10^5)
WT
Pyk2-/-
***
***
***
***
Ly6C+
Ly6C^neg
Ly6C+
Ly6C^neg
CM
SF

Figure 2.12 Loss of Pyk2 promotes BM monocyte survival *in vitro*. WT or Pyk2-/- BM was cultured in RPMI media supplemented with 10% FBS (complete media; CM) or RPMI media alone (serum-free; SF). After 4 hours, non-adherent and adherent cells were collected and analyzed by flow cytometry. BM was isolated and pooled from 5 mice/genotype. Data was generated from 5 replicates/genotype. (A) Representative contour plots display CD115 expression from WT BM kept on ice (left), or cultured under CM (middle) or SF conditions (right) for 4 hours. Dead cells and doublets were excluded prior to the displayed gating. Numbers within the outlined areas indicate the percentage of gated cell populations. (B) Flow cytometry gating strategy to identify monocyte subpopulations *in vitro*. Cell death in monocyte subsets was assessed by flow cytometry using DAPI incorporation. Doublets and Ly6G+ neutrophils were excluded prior to the displayed gating. (C) The average percentage of dead monocytes among monocyte subpopulations. (D) Total numbers of live monocyte subsets identified as $DAPI^{neg}$, assessed by flow cytometry.

** $p < 0.01$, *** $p < 0.001$ (unpaired Student's t-test).

2.4 Discussion

The cellular factors that control the homeostatic maintenance of monocyte subsets remain poorly understood. In this study, we sought to characterize the expression of the tyrosine kinase Pyk2 and its function in the development and survival of lineage-committed monocyte subpopulations at steady-state. Our data indicate that Pyk2 protein expression increases as monocytes differentiate to a Ly6C^{neg} state, and that Pyk2 controls the relative proportion of monocyte populations in the BM and periphery through a monocyte-intrinsic process. Furthermore, we present both *in vivo* and *in vitro* evidence showing that Pyk2 promotes apoptosis of Ly6C^{neg} monocytes, thereby contributing to the rapid turnover of monocyte populations at steady-state.

2.4.1 Pyk2 expression is upregulated in Ly6C^{neg} monocytes.

While Pyk2 expression has been characterized in several myeloid lineage cell types [273, 259, 326, 327], the relative expression of this molecule across the continuum of monocyte differentiation had not been previously examined. Here, we report that, under homeostatic conditions, Pyk2 expression increases in Ly6C^{neg} monocytes. In support of our findings, Pyk2 mRNA transcript levels are reportedly elevated in Ly6C^{neg} monocytes isolated from BM and PB compared with Ly6C+ monocytes from the same tissues [328]. This suggests that the increase in Pyk2 protein expression observed in these cells may arise from transcriptional upregulation of the gene encoding Pyk2. While the molecular factors that govern its expression have not been established, it is tempting to

speculate that the transcription factor CEBP-β may control Pyk2 expression in monocyte populations. Although CEBP-β protein levels have not been specifically measured in monocyte subsets, this transcription factor has long been implicated in driving gene expression programs associated with macrophage maturation [329, 330]. Additionally, CEBP-β association with the Pyk2 promoter was shown to induce Pyk2 expression during monocyte differentiation to macrophages following PMA-treatment of NB4 cells [305].

2.4.2 Pyk2-deficient mice exhibit reduced BM cellularity.

While Pyk2-/- mice were initially characterized as being mildly osteopetrotic [290-292], BM hematopoiesis had not previously been fully characterized in these mice. In this report, we show that Pyk2-deficient mice exhibit a consistent reduction in their BM cellularity compared to WT controls. This may result from a defect in BM production considering that rapid BrdU-incorporation studies revealed a significant reduction in the percentage of proliferating monocytes and monocyte precursors present in Pyk2-deficient BM compared with WT mice (Figure 2.9B). Defects in BM production have been reported in other mouse models of osteopetrosis, including mice deficient for M-CSF (*op/op* mice) and the M-CSF receptor [49, 51, 331]. The paucity of BM cells in both of these mice was attributed to physical limitations restricting hematopoiesis resulting from a significantly smaller bone marrow cavity. Our data suggest a similar phenomenon in Pyk2-/- mice, since transfer of Pyk2-deficient BM into lethally irradiated WT

mice resulted in reconstitution of BM cells to levels of WT control mice (Figure 2.7C).

2.4.3 Pyk2 may promote turnover of Ly6C^{neg} monocytes through the induction of apoptotic pathways.

As described above, Pyk2-/- mice manifest reduced BM cellularity compared with WT controls (Figure 2.5A). This results in a reduction in the absolute numbers of cMoP and Ly6C+ monocytes, but surprisingly, this did not also result in reduced numbers of Ly6C^{neg} monocytes (Figure 2.5C). In contrast to the BM, total immune cellularity is similar between WT and Pyk2-/- mice in the spleen (data not shown). In this organ, Ly6C^{neg} monocyte numbers were elevated in Pyk2-/- mice compared with WT controls (Figure 2.6B). Thus, Ly6C^{neg} monocytes consistently make up a greater proportion of the total monocytes present in both the BM and periphery of Pyk2-/- animals compared with WT mice (Figure 2.5D and Figure 2.6A). We go on to demonstrate that the over-representation of Ly6C^{neg} monocytes observed in Pyk2-/- mice also occurs with transfer of Pyk2-/- BM into an irradiated WT host (Figure 2.7F), indicating that this phenomenon is intrinsic to the monocytes. Furthermore, our data show that Ly6C^{neg} monocytes have a survival benefit in the absence of Pyk2 (Figures 2.9-2.12), which likely accounts for the over-representation of these cells in the BM and periphery of Pyk2-/- animals.

Monocytes spontaneously undergo cell death *in vitro* when cultured in SF conditions, mediated at least in part by Fas death receptor signaling and caspase activation [70, 332-337]. These findings have prompted the theory that apoptosis represents the default cellular program for monocytes, though the molecular signals contributing to this remain largely undefined. Here, we demonstrate that endogenous Pyk2 functions to promote the turnover of Ly6C^{neg} monocytes during homeostatic conditions (Figure 2.10) and under conditions of experimentally-induced cell death (Figure 2.12). This could be mediated via the default apoptosis pathway since Pyk2-deficient Ly6C^{neg} monocytes in the BM exhibited reduced caspase activation compared with WT controls (Figure 2.10B). However, the Pyk2-/- Ly6C^{neg} monocytes that originated from cMoPs/Ly6C+ cells in the BM were ultimately depleted from the BM and periphery, indicating that Pyk2-independent mechanisms are also in place to control the half-life of these cells (Figure 2.9E). Notably, ectopic overexpression of Pyk2 has been shown to induce apoptosis in multiple cell lines that do not normally express this protein [294-297, 321]; however, prior to the current study, endogenous levels of Pyk2 had not been shown to trigger cell death at steady-state.

While the paucity of cMoP and Ly6C+ monocytes observed in Pyk2-/- compared to WT mice (Figure 2.5C) might at first glance suggest a role for Pyk2 in the development or survival of these populations, transfer of Pyk2-/- BM into WT hosts resulted in normal numbers of cMoP and Ly6C+ monocytes (Figure 2.7E). Thus, the accumulation of these subsets was not dependent on monocyte-

intrinsic activities of Pyk2. This is consistent with the low level of Pyk2 expression in these subsets (Figure 2.3A). Rather, we conclude that the reduced numbers of cMoP and Ly6C+ monocytes arise as a consequence of deficiencies in overall BM production in the Pyk2-/- mice (Figure 2.5A). Our data also argue against a monocyte-intrinsic role for Pyk2 in regulating cMoP and Ly6C+ monocyte survival during steady-state conditions *in vivo*, since these cells exhibited similar levels of annexin V and active caspase staining in the presence or absence of Pyk2 when present in a WT BM environment (Figure 2.11). This is in contrast to Pyk2-/- Ly6C^{neg} monocytes, which displayed less cell death under these conditions. It is worth noting, however, that *in vitro* culture of BM did reveal increased viability of Ly6C+ monocytes in the absence of Pyk2 (Figures 2.12C-D). This may be due to the fact that this experimental system triggers exceptionally rapid cell death and therefore does not reflect a typical homeostatic environment *in vivo.* It would be interesting to determine whether Pyk2 controls apoptosis of Ly6C+ monocytes in response to stress or under other physiological conditions in which the half-life of these cells is significantly altered.

Exogenous factors that promote the survival of monocytes have been shown to suppress the default apoptotic pathway [49, 51, 66, 316]. During homeostasis, this is chiefly mediated through M-CSF signaling [49, 51]. Marsh and colleagues have shown that the PI3K/AKT pathway is activated in monocytes stimulated with recombinant M-CSF, leading to repression of caspase activity and prolonged survival in culture [67-69]. Moreover, inhibition of the PI3K pathway was shown to

induce apoptosis in cultured human PB monocytes [332]. Together, these findings underscore the importance of M-CSF-stimulated PI3K signaling in impeding the *de facto* apoptotic program and prolonging monocyte lifespan. Interestingly, M-CSF administration was reported to induce tyrosine phosphorylation of Pyk2 in the THP1 human monocytic cell line cultured *in vitro* [338]. This study also showed that the M-CSFR, as well as PI3K, co-immunoprecipitated with Pyk2 in response to M-CSF stimulation [338]. However, the authors did not go on to specifically test the implications of these events on monocyte survival; instead, they speculated that M-CSF-mediated activation of Pyk2 was potentially linked with regulation of downstream signaling pathways that affect cell morphology. It is possible that M-CSF-dependent Pyk2 phosphorylation and interactions with M-CSFR/PI3K do not impact cell survival, or that the activation of Pyk2 in response to M-CSF is unique to the THP1 cell line. Alternatively, Pyk2 may have dual roles, functioning on the one hand to promote apoptosis under conditions that favor monocyte turnover and on the other hand to promote survival in the presence of high levels of M-CSF. Clearly, the mechanistic underpinnings and implications regarding the interaction between Pyk2, M-CSF survival signaling, and the canonical apoptotic pathway warrant further exploration.

In addition to M-CSF, prolonged survival of monocytes in culture also occurs in response to pro-inflammatory signals. For example, exposure of human PB monocytes to a variety of soluble inflammatory factors *in vitro* (including LPS,

TNF-α, IL-1β, GM-CSF, and IL-18) was shown to suppress caspase activation through the PI3K/AKT pathway [69, 335, 336]. It is not clear whether inflammatory cues also extend the half-life of monocytes *in vivo*. While our study implicates Pyk2 as a pro-apoptotic factor in monocytes at homeostasis, the role of Pyk2 in regulating monocyte survival during times of inflammation has not been addressed.

Given the rapid turnover of monocytes at homeostasis and the data presented above, we suggest that endogenous Pyk2 operates within the constitutive signaling program that drives apoptosis of these cells. However, in light of its increased expression and established role in adhesion signaling and migration of macrophages and other cell types [273] [259], it is interesting to speculate that Pyk2 may have additional functions in Ly6C^{neg} monocytes. For example, Ly6C^{neg} monocytes characteristically “crawl” along the resting endothelium in a manner distinct from the typical leukocyte “rolling and adhesion” process [24, 25]. This directional motility, which is essential to Ly6C^{neg} monocyte “patrolling and scavenging” functions, is dependent on integrin signaling [24]. Since Pyk2 is highly expressed in these cells and functions in signaling pathways downstream of integrin engagement (reviewed in [274, 319]), it is uniquely positioned as a potential regulator of integrin-mediated signaling and migration of Ly6C^{neg} monocytes on the endothelium. Clearly, future studies are necessary to further address the nature and regulation of the signals that control the onset of Pyk2

expression and its subsequent functions in monocytes, both at homeostasis and in response to inflammatory insults.

Chapter 3: The role of FAK in MPS-mediated control of murine breast tumor progression

3.1 Introduction

It is now a well-established paradigm that infiltrating leukocyte populations can profoundly impact disease progression in many cancers. Cells of the monocyte/macrophage lineage in particular comprise a significant portion of breast tumor stroma and influence many aspects of tumor development [339]. The majority of tumor-resident MPS cells derive from circulating Ly6C+ monocytes that are recruited from the periphery by a diverse array of tumor-secreted factors [157-159]. Their migration and function are subsequently determined by the stimuli they encounter in the tumor microenvironment, and evidence suggests that monocyte-derived cells are capable of either supporting or inhibiting primary tumor growth [340, 341]. For example, several reports have described direct and indirect anti-tumor responses by macrophages during the early stages of neoplastic development [169-172]. In contrast, a strong correlation between mononuclear phagocyte abundance and poor clinical outcome has been described in advanced breast carcinomas [339], and numerous studies in murine models indicate that these cells ultimately help to establish an environment conducive to tumor progression and metastasis [141, 173]. However, it should be noted that striking functional heterogeneity has also been described between subpopulations of TAMs present within the same tumor

[340, 342, 343]. In these studies, TAM localization to discrete microanatomical regions within the tumor conferred specific functional attributes to these cells; thus, it is thought that local cues can direct TAM activation states toward pro- or anti-inflammatory phenotypes that ultimately impact tumor growth.

In order to respond to the heterogeneity within the tumor microenvironment, MPS cells must be equipped to properly integrate the extracellular signals they encounter, which include growth factors, cytokines, and ECM components. Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that functions as a central regulator of adhesion signaling and motility in many cell types, including macrophages [259, 344]. FAK is recruited to focal complexes and mediates signal transduction downstream of integrin engagement with the ECM or growth factor receptor activation [345]. Through its close relationship with integrins, FAK has also been shown to function as a mechanosensor, transmitting physical cues from the environment to control cell survival, proliferation, and motility [120, 346-350]. While this molecule has been extensively studied in fibroblasts and transformed cell lines, FAK function in MPS populations is less understood. Data generated in our lab indicate that FAK is abundantly expressed in macrophages at steady state (Chapter 2; [259]). We therefore sought to determine whether FAK deficiency regulates mononuclear phagocyte trafficking and/or accumulation in breast cancer, and if tumor progression is influenced by loss of FAK-mediated activities in MPS cells.

In order to begin to assess these questions, we utilized genetically engineered mouse models. The mouse mammary tumor virus polyoma middle T antigen (MMTV-PyVmT) murine model of breast carcinoma targets expression of the PyVmT oncogene to mammary epithelial cells, resulting in 100% penetrance of mammary tumor formation and metastasis in a high percentage of animals [351]. Neoplasia is driven by the constitutive activation of multiple kinase pathways, including mitogen-activated protein kinase (MAPK), Src, and phophatidyl-inositol-3 kinase (PI3K) [352]. Gene expression profiling and patterns of biomarker expression suggest a high degree of similarity between PyVmT tumors and human luminal breast cancers [353, 354]. To assess the effect of loss of myeloid-expressed FAK on TAM functionality and breast tumor progression, myeloid-specific conditional FAK knock-out ($FAK^{\Delta myeloid}$) mice [259] were crossed with MMTV-PyVmT transgenic mice in the C57Bl/6 background. It is important to note that, while LysM-Cre is being used in our model to drive the conditional knockout of *FAK* in monocytes and macrophages, the LysM promoter is also active in other cells of the myeloid lineage, including neutrophils and DCs [355]. However, we and others have shown that the efficiency of *FAK* recombination is poor in DCs [355], resulting in sustained FAK expression in these cells. Additionally, previous work in our laboratory has determined that FAK is not expressed in neutrophils [259]. Therefore, we believe this system provides us with the unique opportunity to study the role of FAK in monocyte/macrophage-mediated control of breast cancer progression.

In this report, we provide evidence that FAK-deficiency in mononuclear phagocytes does not influence tumor initiation or progression in the early stages of PyVmT tumor growth. Rather, FAK expression in these cells appears to be important for constraining primary tumor growth after the transition to malignancy. We further demonstrate that the trafficking and accumulation of MPS populations at the site of developing tumors is FAK-independent. Therefore, we suggest that FAK activity may instead influence the localization and/or functional capacities of TAMs, which may ultimately help to control tumor growth and progression.

3.2 Materials and Methods

3.2.1 Mice. The $FAK^{\Delta myeloid}$ and MMTV-PyVmT mouse models have been previously described (Owen, 2007; Guy, 1992). For tumor studies, WT ($LysM^{wt/wt}$-$FAK^{fl/fl}$) and $FAK^{\Delta myeloid}$ ($LysM^{wt/cre}$-$FAK^{fl/fl}$) mice were crossed with MMTV-$PyVmT^{+/-}$ C57BL/6 mice to produce WT/$PyVmT^{+/-}$ and $FAK^{\Delta myeloid}$/$PyVmT^{+/-}$ mice, respectively. Female WT/$PyVmT^{+/-}$ and $FAK^{\Delta myeloid}$/$PyVmT^{+/-}$ mice (6-18 weeks of age) were age-matched for experiments. All studies were performed in accordance with University of Virginia Animal Care and Use Committee guidelines.

Animals were routinely genotyped from tail DNA and subjected to PCR analysis. The following primer sets were utilized: Cre primer set: #1

5’CTTGGGCTGCCAGAATTTCTC, #2 5’TTACAGTCGGCCAGGCTGAC, #3 5’CCCAGAAATGCCAGATTACG (Operon). Expected products: WT LysM allele 350bp, LysM Cre allele 700bp.

Primer set targeting PyVmT: #1 5’TGTGCACAGCGTGTATAATCC, #2 5’CAGAATAGGTCGGGTTGCTC (Operon). Expected product for PyVmT$^{+/-}$ mice: 200bp.

3.2.2 Tumor histology. Primary tumor masses from the #1 mammary gland (MG1) were surgically removed with forceps and scissors, fixed overnight (o/n) in 10% formalin, and transferred to 70% ethanol (ETOH). For analyses involving tumor progression prior to the detection of palpable masses, whole MG1s were excised and processed as described above. In the UVA histology core, specimens were embedded in paraffin and sectioned onto glass microscope slides (5μm sections). Hematoxylin and eosin staining was completed by the core staff. Images were acquired on the Olympus high magnification microscope in the UVA Advanced Microscopy core facility at 5X magnification. For whole MG1 scans, sections were imaged in the UVA CIIR with a 5X or 10X objective of a Carl Zeiss Axio Imager Z1/Apotome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and stitched together using Stereo Investigator software (MBF Bioscience, Williston, VT). For assessment of tumor progression in pre-palpable mammary tumors, H&E stained sections of MG1s were classified according to four distinct stages of PyVmT tumor progression that have been previously described [354, 356]. Investigators were unaware of the

genotype of the sample (blinded). To measure the total area of tumor growth in H&E stained MG1 sections, the perimeter of regions containing carcinoma or carcinoma and adenoma/MIN was traced and the square micron (μm^2) area within the region of interest was calculated using Image J software.

For immunofluorescence (IF) imaging, tumors were flash frozen in a slurry of dry ice + 100% ETOH, stored at -80C, and sectioned by the UVA histology core. Staining protocol was adapted from Engelhard Lab (Liz Thompson). Sections were fixed in acetone at -20C x 10 min., air dried, encircled with PAP pen, washed in PBS, blocked with α-CD16/32 (eBioscience 14-0161-85) [1/1000 PBS + 5% BSA] x 15 min., washed in PBS, incubated with avidin [1 drop/200"l PBS + 5% BSA] x 15 min., washed, incubated with biotin [1 drop/200"l PBS + 5% BSA] x 15 min., washed, incubated with α-CD31 (abcam) [1:100 PBS + 5% BSA] x 60min. at RT, washed 2 x PBS, incubated with α-rat Texas Red (Jackson Lab) [1:300 in PBS + 5% BSA] x 30min. at RT, washed 3 x PBS, and mounted with ProlongGold + DAPI (Life technologies). Images were acquired on the Olympus high magnification microscope at 10X magnification and subsequently merged using Adobe Photoshop software.

3.2.3 Measurement of tumor growth. For studies requiring palpable tumor assessment, MG1s were palpated for tumor growth twice weekly from 12 weeks of age to time of sacrifice. For studies requiring caliper measurement of primary tumor outgrowth, tumors were measured in two dimensions. Length (l) was

defined as maximum attainable tumor measurement in one dimension. Width (w) was defined as the measurement perpendicular to length. Tumor volume was derived using the formula [l x w^2]/2. To calculate the growth rate of carcinomas, the formula for specific growth rate (SGR) was used; [SGR = ln $(V_2/V_1)/(t_2-t_1)$] [357].

3.2.4 Harvest and preparation of single cell suspensions from mouse tissues. Peripheral blood (PB) was drawn through cardiac puncture (300-700µl/mouse) and placed in 1ml of 5mM EDTA/Hank's balanced saline solution (-Mg,-Ca) (HBSS). Spleens were excised, pushed through a 70µm filter, and washed in MACS buffer (0.5% BSA, 250mM EDTA in PBS). BM was flushed from both femora and tibiae with MACS buffer, washed in MACS buffer, and filtered through a 30µm filter. Lungs were excised, manually minced using scalpel followed by enzymatic digestion for 15 minutes at 37°C in Accumax (STEMCELL Technologies) under shaking conditions, and filtered through a 100µm filter. To remove erythrocytes, the tissues were incubated in ammonium/chloride/potassium (ACK) lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2 EDTA $2H_2O$ in H_2O) for 10 minutes on ice and quenched with complete media (10% FBS/DMEM).

Prior to the excision of mammary tissue, vascular perfusion was performed by injecting 10 ml of PBS directly into the left ventricle. For the processing of day 70 MG1s, excised mammary glands were manually minced using scalpel followed

by enzymatic digestion for 60 minutes at 37°C in 0.1mg/ml Liberase TL (Sigma) and 400ug/ml DNase I (Roche) dissolved in DMEM (Invitrogen) under shaking conditions. Red blood cell lysis was then performed, as described above. Samples were subsequently incubated in 0.25% trypsin in DMEM for 2 minutes at RT, followed by digestion in 5mg/ml dispase II (Roche) and 400ug/ml DNase I for 2 minutes at RT under gentle shaking conditions. For the processing of primary carcinomas (detectable by palpation), excised tumors were manually minced using scalpel followed by enzymatic digestion for 60 minutes at 37°C in Accumax under shaking conditions. Red blood cell lysis was then performed, as described above. Digested mammary tissue was resuspended in DMEM and strained through a 100μm filter to remove clumps prior to further processing.

3.2.5 Flow cytometry. Single cell suspensions containing approximately $1x10^6$ cells in 100μl MACS buffer were incubated with the F_C blocking antibody anti-CD16/32 (eBioscience, San Diego, CA, USA) for 10 minutes on ice. The cells were subsequently incubated with primary monoclonal antibodies to cell surface antigens for 25 minutes on ice. Antibodies were purchased from the following companies and used at the concentration suggested by the manufacturer: AbD Serotec, Oxford, UK: anti-F4/80 (Cl:A3-1[clone]); Biolegend, San Diego, CA, USA: anti-Ly6C (HK1.4), anti-CD11b (M1/70), anti-Ly6G (1A8), anti-CD192 (SA203G11); eBioscience, San Diego, CA, USA: anti-CD45.2 (104), anti-CD3e (145-2C11), anti-CD49b (DX5), anti-CD19 (MB19.1). Samples were stained concurrently with fluorescence minus one (FMO) antibody panels. Samples were

washed in MACS buffer, and incubated with fixable live/dead cell stain (Invitrogen, Carlsbad, CA, USA) for 30 minutes on ice. Samples were then washed in MACS buffer and fixed for 20 minutes on ice with Cytofix (BD Biosciences, San Jose, CA, USA), resuspended in MACS buffer, and data were acquired on the Cyan ADP LX (Beckman Coulter, Brea, CA, USA) in the UVA flow cytometry core. FlowJo software (Tree Star Inc., v.10, Ashland, OR, USA) was used for data analysis. Absolute numbers were calculated using Accucount beads (Spherotech, Lake Forest, IL, USA) according to the manufacturer's instructions.

3.2.6 Cell trafficking experiments. Single cells suspensions from BM of WT and $FAK^{\Delta myeloid}$ mice were prepared as described above. CD11b+ cells were then isolated using the mouse MACS CD11b Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Briefly, F_C receptors were blocked with anti-CD16/32 (eBioscience, San Diego, CA, USA) for 10 minutes on ice, and the cells were subsequently incubated with anti-CD11b magnetic beads and isolated by magnetic column. Cells were then fluorescently labeled using CellTracker (Fisher) dyes (WT: CellTracker Green; $FAK^{\Delta myeloid}$: CellTracker Orange) according to the manufacturer's instructions. Labeled cells were enumerated by hemacytometer, and $1x10^7$ cells from each genotype were resuspended into 300ul PBS/recipient mouse for adoptive transfer. Labeled cells were adoptively transferred into late carcinoma tumor-bearing WT/PyVmT mice by tail vein injection. Purity of the isolated CD11b+

populations and the ratio of fluorescently labeled cells in the preparation were assessed by flow cytometry. Thirty minutes after adoptive transfer of the fluorescently labeled cells, organs and primary MG1 tumors were harvested and processed into single cell suspensions, or flash frozen for IF analysis, as described above. Tumor samples were magnetically enriched for CD11b+ cells, and flow cytometry to detect fluorescently labeled cells in the indicated organs was subsequently performed.

3.2.7 Statistical analysis. Comparisons between groups were made using an unpaired two-tailed student t-test, Mann-Whitney test, or log-rank statistic. P values ≤ .05 (*), ≤ .01 (**), and ≤ .001 (***) were considered statistically significant.

3.3 Results

3.3.1 FAK expression in mononuclear phagocytes does not control PyVmT tumor initiation or the early stages of tumor progression

In the PyVmT murine model of breast carcinoma, a four-stage classification scheme of tumor progression has been developed based on distinct histopathological changes in ductal morphology that reflect the successive advancement to malignancy ([354]; Figure 3.1A). Tumor initiation is characterized by the development of hyperplasia, which can be identified by altered ductal architecture and a slight increase in nuclear/cytoplasmic ratio in

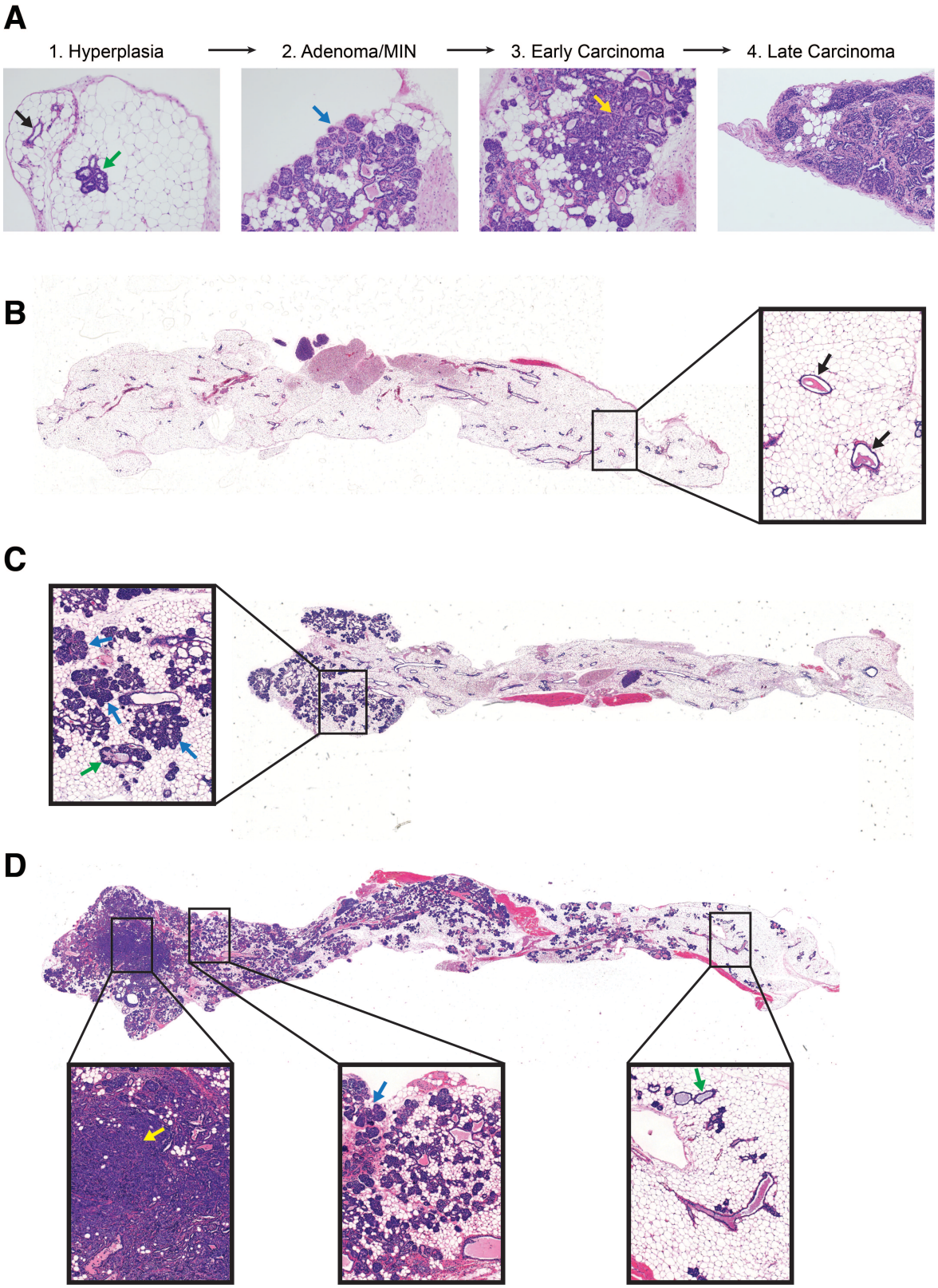

A
1. Hyperplasia
2. Adenoma/MIN
3. Early Carcinoma
4. Late Carcinoma
B
C
D

Figure 3.1 Characterization of tumor progression in the PyVmT murine model of breast cancer. (A) Representative images from hematoxylin and eosin (H&E) stained mammary sections display the histology of primary lesions during the sequential stages of tumor progression in PyVmT mice. Colored arrows point out typical morphology in normal ducts (black arrow), hyperplastic lesions (green arrow), regions of adenoma/MIN (blue arrow), and early carcinoma (yellow arrow). Images were captured at 10x magnification. (B-D) Excised #1 mammary glands (MG1s) were sectioned along their length and H&E stained. High-resolution images were captured with a 5X objective of a motorized scanning microscope and stitched together using imaging software. Images from MG1s are orientated from nipple proximal (left) to nipple distal (right). Insets display regions of interest at 10X magnification. (B) MG1 section from a PyVmT transgene^{neg} mouse. Ducts exhibiting normal morphological features are presented in the inset (black arrows). (C) MG1 section from a PyVmT mouse aged 8 weeks. Colored arrows point out regions of hyperplasia (green arrow) and adenoma/MIN (blue arrows) in the inset. (D) MG1 section from a PyVmT mouse aged 12 weeks. Insets display regions of carcinoma in the primary tumor (left panel, yellow arrow), proximal regions of adenoma/MIN (middle panel, blue arrow), and distal regions of hyperplasia (right panel, green arrow).

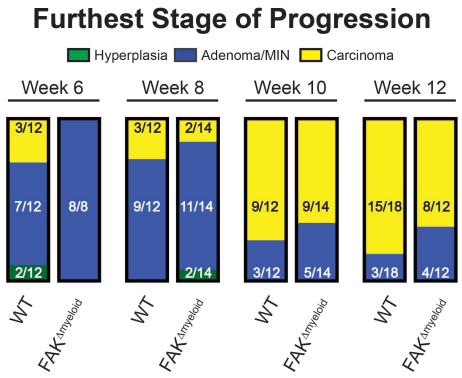
the epithelial cells (compare ductal morphology of hyperplastic lesion (green arrow) to normal ducts (black arrow) in Figure 3.1A). Hyperplastic ducts progress to adenoma/MIN lesions, consisting of closely packed acini that are enlarged by an expanding number of epithelial cells that fill the structure (Figure 3.1A, blue arrow). These cells are typically highly proliferative but exhibit minimal cytological atypia. Loss of basement membrane and distinct acinar structures, concomitant with the presence of stromal infiltrate and greater cytological atypia, mark the progression of adenoma/MIN regions to the early carcinoma stage (Figure 3.1A, yellow arrow). In the late carcinoma stage, malignancies are composed of solid sheets of transformed epithelial cells that display variable cellular size and marked nuclear pleomorphism (Figure 3.1A, far right panel).

In the PyVmT model, pre-malignant lesions are first observed proximal to the nipple around the main milk-collecting duct, followed by the appearance of multiple foci in distal ducts [354]. Compared with PyVmT transgene^{neg} mice (Figure 3.1B), ducts in PyVmT mice exhibited morphological features of adenoma/MIN and hyperplasia by 8 weeks of age (Figure 3.1C), although phenotypically normal ducts can also be observed in regions of the mammary gland more distal to the nipple. By 12 weeks of age, the primary tumor had often advanced to the carcinoma stage, while retaining proximal regions of adenoma/MIN (Figure 3.1D). We noted that the transition to early carcinoma preceded the ability to detect these tumors by palpation. Therefore, to determine if FAK expression in mononuclear phagocytes influences tumor control during the

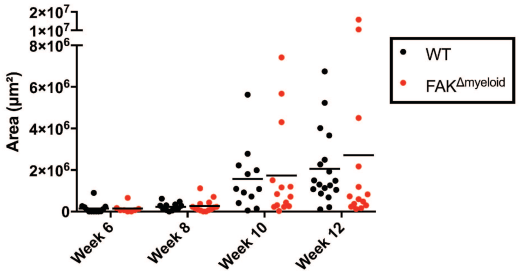
early stages of initiation/growth, we began by assessing neoplastic progression in mammary glands from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT transgenic mice prior to the advent of palpable tumors. H&E stained sections from mice aged 6, 8, 10, and 12 weeks were evaluated for the furthest observable stage of primary tumor progression. Considerable variability in tumor latency had previously been described based on anatomical location of individual mammary glands, with tumors from mammary gland 1 (MG1) appearing significantly earlier than those that derive from mammary glands 2-4 [358]. To minimize variability in our analyses, we therefore restricted our investigation to tumor progression in MG1.

Irrespective of genotype, an increasing percentage of MG1s displayed regions of carcinoma as a function of time (Figure 3.2A). Additionally, no substantial differences were observed in the proportion of WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT tumors that had progressed to the carcinoma stage at any time point, suggesting that the initial transition from adenoma/MIN to carcinoma was not likely to be regulated by FAK expression in mononuclear phagocytes. We also assessed the total area of the MG1 sections that displayed morphological hallmarks of carcinoma. Our data indicate that the size of carcinomas was similar in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT tumors in pre-palpable stages (Figure 3.2B). Similar results were attained when we extended our analysis to include proximal regions of adenoma/MIN as well (Figure 3.2C). Together, these data indicate that FAK activity in MPS cells does not regulate

A


Furthest Stage of Progression
Hyperplasia
Adenoma/MIN
Carcinoma
Week 6
Week 8
Week 10
Week 12
3/12
7/12
2/12
8/8
3/12
9/12
2/14
11/14
2/14
9/12
3/12
9/14
5/14
15/18
3/18
8/12
4/12
WT
FAKΔmyeloid

B


Carcinoma
Area (μm²)
2×10⁷
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
Week 6
Week 8
Week 10
Week 12
WT
FAKΔmyeloid

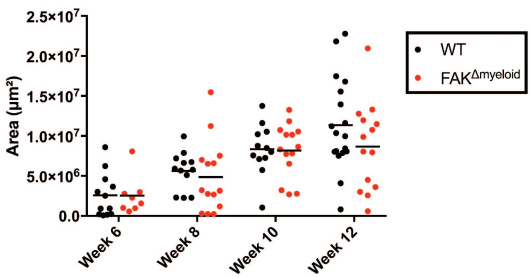

Adenoma/MIN + Carcinoma
Area (μm²)
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0.0
Week 6
Week 8
Week 10
Week 12
WT
FAKΔmyeloid

Figure 3.2 FAK expression in macrophages does not control PyVmT tumor initiation or the early stages of tumor growth. (A) Histopathological progression of primary mammary tumors in $FAK^{\Delta myeloid}$/PyVmT and control MG1s was assessed based on morphological characteristics. Data are presented as the proportional distribution of samples that had reached hyperplasia (green), adenoma/MIN (blue), or carcinoma (yellow) as the furthest observable stage of disease progression at the indicated ages. (B) Tumor growth was assessed by quantifying the total area (μm^2) of carcinoma (left), or adenoma/MIN and carcinoma (right) in $FAK^{\Delta myeloid}$/PyVmT and control MG1 samples isolated from mice at the indicated ages. Areas representative of carcinoma or adenoma/MIN and carcinoma were traced and quantified using Image J software.

tumor initiation or the early stages of progression in the PyVmT model of breast cancer.

3.3.2 Loss of FAK expression in mononuclear phagocytes accelerates the outgrowth of PyVmT breast carcinomas

To determine if FAK expression in monocytes/TAMs controls tumor growth after the transition to carcinoma, we tracked the appearance of palpable masses in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT MG1s over time. FAK$^{\Delta myeloid}$/PyVmT MG1s displayed significantly accelerated development of discernible tumor masses compared with WT/PyVmT MG1s (Figure 3.3A, left). On average, palpable tumors could be observed in FAK$^{\Delta myeloid}$/PyVmT MG1s approximately 1.5 weeks prior to tumors in WT/PyVmT MG1s (Figure 3.3A, right). Histological sections of primary tumors harvested at the palpable stage uniformly exhibited regions of advanced carcinoma (data not shown). Given that the progression of adenoma/MIN lesions to the carcinoma stage did not appear to be accelerated in FAK$^{\Delta myeloid}$/PyVmT mice (Figure 3.2), our data strongly suggest that primary tumor growth is regulated by FAK expression in TAMs after the transition to malignancy (modeled in Figure 3.3B). In accordance with the decreased tumor latency, the average tumor volume was significantly greater in FAK$^{\Delta myeloid}$/PyVmT primary tumors assessed at week 17 and 18 (Figure 3.3C). Once palpable masses were observed, however, tumor growth proceeded at similar rates in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice (Figure 3.3D), suggesting that tumor

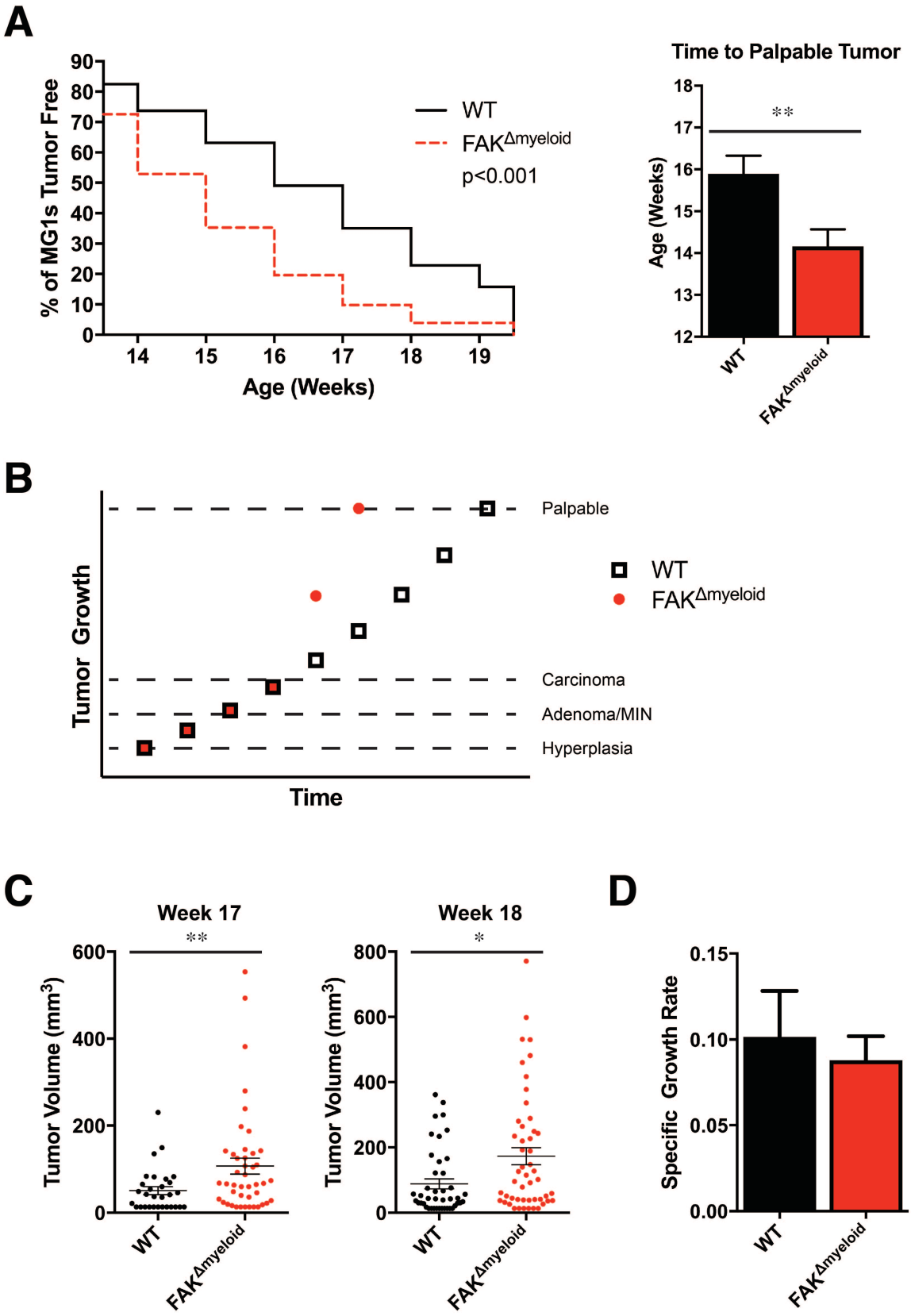

A
% of MG1s Tumor Free
Age (Weeks)
WT
FAKΔmyeloid
p<0.001
Time to Palpable Tumor
Age (Weeks)
**
WT
FAKΔmyeloid
B
Tumor Growth
Time
Palpable
Carcinoma
Adenoma/MIN
Hyperplasia
WT
FAKΔmyeloid
C
Week 17
**
Week 18
*
Tumor Volume (mm3)
WT
FAKΔmyeloid
D
Specific Growth Rate
WT
FAKΔmyeloid

Figure 3.3 Loss of FAK in myeloid cells accelerates PyVmT mammary tumor outgrowth. (A) MG1s in FAK$^{\Delta myeloid}$/PyVmT and control mice were palpated for tumor growth. Left, Kaplan-Meier plot depicts the percentage of mammary glands that remained tumor-free at each week of observation. Data were analyzed by the log rank test. Right, graph depicts the average length of time to observable tumor per mammary gland. ** $p < 0.01$ (Mann-Whitney test). Data are representative of 57 WT and 51 FAK$^{\Delta myeloid}$ MG1s, respectively. (B) Model portraying average tumor progression in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT MG1s over time. Our data indicate that tumor growth is not accelerated in FAK$^{\Delta myeloid}$/PyVmT MG1s compared with WT/PyVmT MG1s prior to the initiation of carcinoma (see Figure 3.2). Tumors harvested at the palpable stage uniformly exhibit histological hallmarks of advanced carcinoma. We therefore propose that primary tumor growth is accelerated in FAK$^{\Delta myeloid}$/PyVmT mice during the early stages of carcinoma, resulting in accelerated time to palpable tumors. (C) MG1 tumor volumes were determined by caliper measurement at the indicated time points. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney test). Week 17: n=30 WT/PyVmT; n=44 FAK$^{\Delta myeloid}$/PyVmT; Week 18: n=43 WT/PyVmT; n=50 FAK$^{\Delta myeloid}$/PyVmT. (D) Tumor growth rates were evaluated using the formula for Specific Growth Rate (SGR); [$SGR = \ln (V_2/V_1)/(t_2-t_1)$]. Time points chosen reflect first measurable tumor (t_1) and required sacrifice due to tumor burden (t_2). Tumor volumes were determined by caliper measurement. Data are representative of 10 WT and 16 FAK$^{\Delta myeloid}$ MG1s, respectively.

outgrowth during the early stages of carcinoma is regulated by FAK expression in MPS populations.

3.3.3 The recruitment and accumulation of MPS populations in PyVmT tumors is independent of FAK

FAK promotes directional migration downstream of integrin and chemotactic signaling in many cell types [268]. Given that most tumor-resident MPS cells derive from circulating Ly6C+ monocytes [157-159], we wondered whether FAK was important for the trafficking of BM-derived myeloid cells to the primary tumor. To measure the recruitment of peripheral myeloid cells to the primary tumor in an acute setting, CD11b+ cells were isolated from the BM of WT and $FAK^{\Delta myeloid}$ mice via magnetic bead enrichment, fluorescently labeled with lipophilic dye, and adoptively transferred into WT/PyVmT tumor-bearing mice. Prior to the transfer, WT and $FAK^{\Delta myeloid}$ cells were mixed at a 1:1 ratio, and could be distinguished by the color of fluorescence labeling using flow cytometry (Figure 3.4A). Thirty minutes after tail vein injection, adoptively transferred cells could be observed in the PB, spleen, lung, and primary tumor of recipient mice by flow cytometry. Our data indicate that FAK-deficient myeloid cells showed no defect in their ability to traffic to any of these sites (Figure 3.4B). While vascular perfusion was performed prior to harvesting tumors and peripheral organs, we nevertheless sought to confirm that the labeled cells had indeed extravasated from the tumor vasculature by measuring the proximity of adoptively transferred cells to endothelial cells stained with an anti-CD31 antibody. Numerous instances of

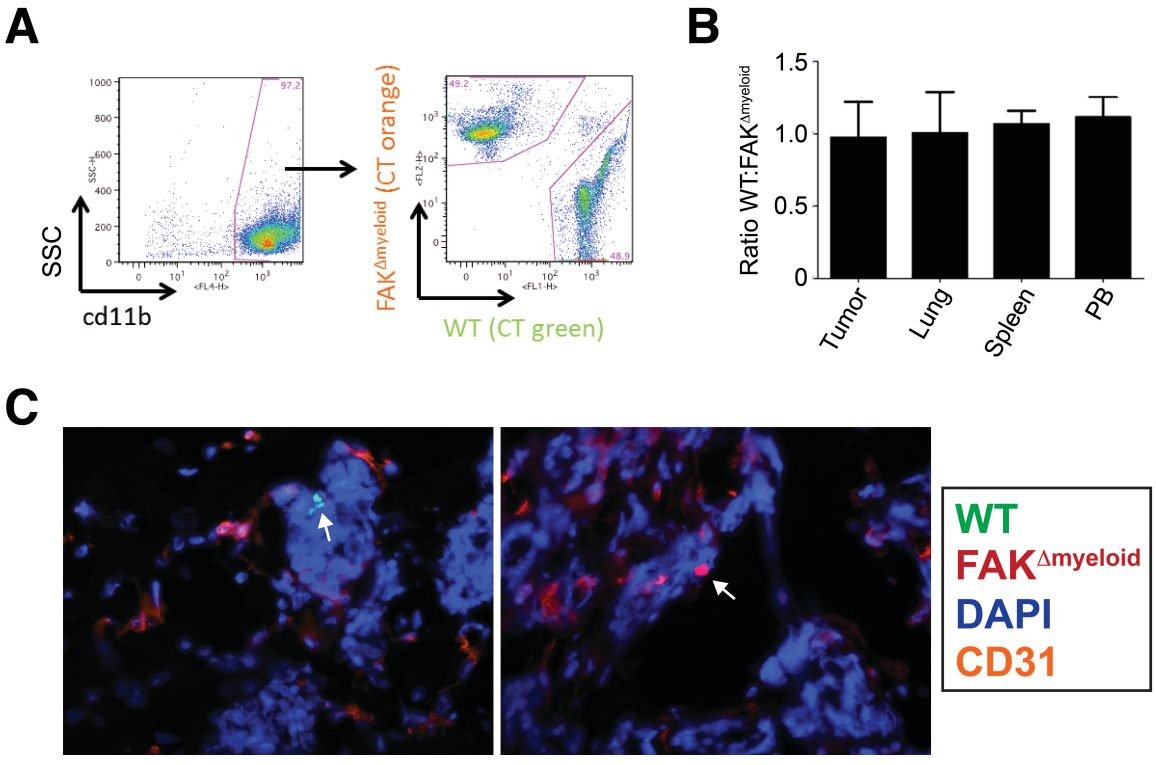

A
SSC
cd11b
FAKΔmyeloid (CT orange)
WT (CT green)
B
Ratio WT:FAKΔmyeloid
1.5
1.0
0.5
0
Tumor
Lung
Spleen
PB
C
WT
FAKΔmyeloid
DAPI
CD31

Figure 3.4 FAK is not required for the recruitment of BM-derived myeloid cells to PyVmT tumors. MACS-selected CD11b+ cells isolated from the BM of WT and $FAK^{\Delta myeloid}$ mice were labeled with CellTracker Green or CellTracker Orange fluorescent dyes, respectively. Labeled cells were then mixed at 1:1 ratio and adoptively transferred into tumor-bearing WT/PyVmT mice to monitor their trafficking. (A) Flow cytometry dot plots display the purity of CD11b+ enrichment (left), and the relative proportion of fluorescently labeled cell populations (right, WT: green channel; $FAK^{\Delta myeloid}$: red channel) in preparations for adoptive transfer. (B) Single cell suspensions generated from the tumor, lung, spleen and PB of recipient mice were analyzed by flow cytometry for the presence of labeled cells thirty minutes after adoptive transfer. Bar graph depicts the ratio of WT to $FAK^{\Delta myeloid}$ cells in the indicated organs. Data were compiled from 3 independent experiments (n=7 mice). (C) Tumor sections from recipient mice were analyzed for the proximity of adoptively transferred cells to vasculature. Immunofluorescent staining was performed to distinguish endothelial cells (anti-CD31: orange) and cell nuclei (DAPI: blue). Representative images display adoptively transferred WT (green) and $FAK^{\Delta myeloid}$ (red) cells proximal to the tumor vasculature (see arrows).

adoptively transferred cells could be visualized within the tumor bed, proximal to the tumor vasculature, suggesting that these cells had indeed extravasated (representative images displayed in Figure 3.4C). The visualization of labeled cells in tumor sections was rare overall, precluding our ability to quantify these data. Nonetheless, our findings suggest that the recruitment of BM-derived myeloid cells to primary PyVmT tumors is FAK-independent.

In support of this conclusion, we found that FAK expression in BM and peripheral Ly6C+ monocyte populations is extremely low (Chapter 2, see Figure 2.1). In contrast, macrophage populations, including TAMs, abundantly express FAK (Michael Gutknecht, personal communication). Alternatively, we hypothesized that FAK may control the differentiation, survival, or proliferation of mononuclear phagocytes in developing carcinomas such that FAK deficiency would alter the representation of these cell populations and consequently affect tumor growth. To test this hypothesis, we performed immunophenotyping of MG1s from mice aged 10 weeks, which was the time at which a significant proportion of primary tumors had advanced to the early carcinoma stage (Figure 3.2). MG1s from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice were excised, digested, and analyzed by flow cytometry for the accumulation of monocytes/M-MDSCs and TAMs (Figure 3.5A). No differences in the relative representation of tumor-resident monocytes/M-MDSCs or TAMs were observed as a function of FAK expression in these cells (Figure 3.5B). Unfortunately, technical limitations (involving considerable variability in total cell counts from our preparations)

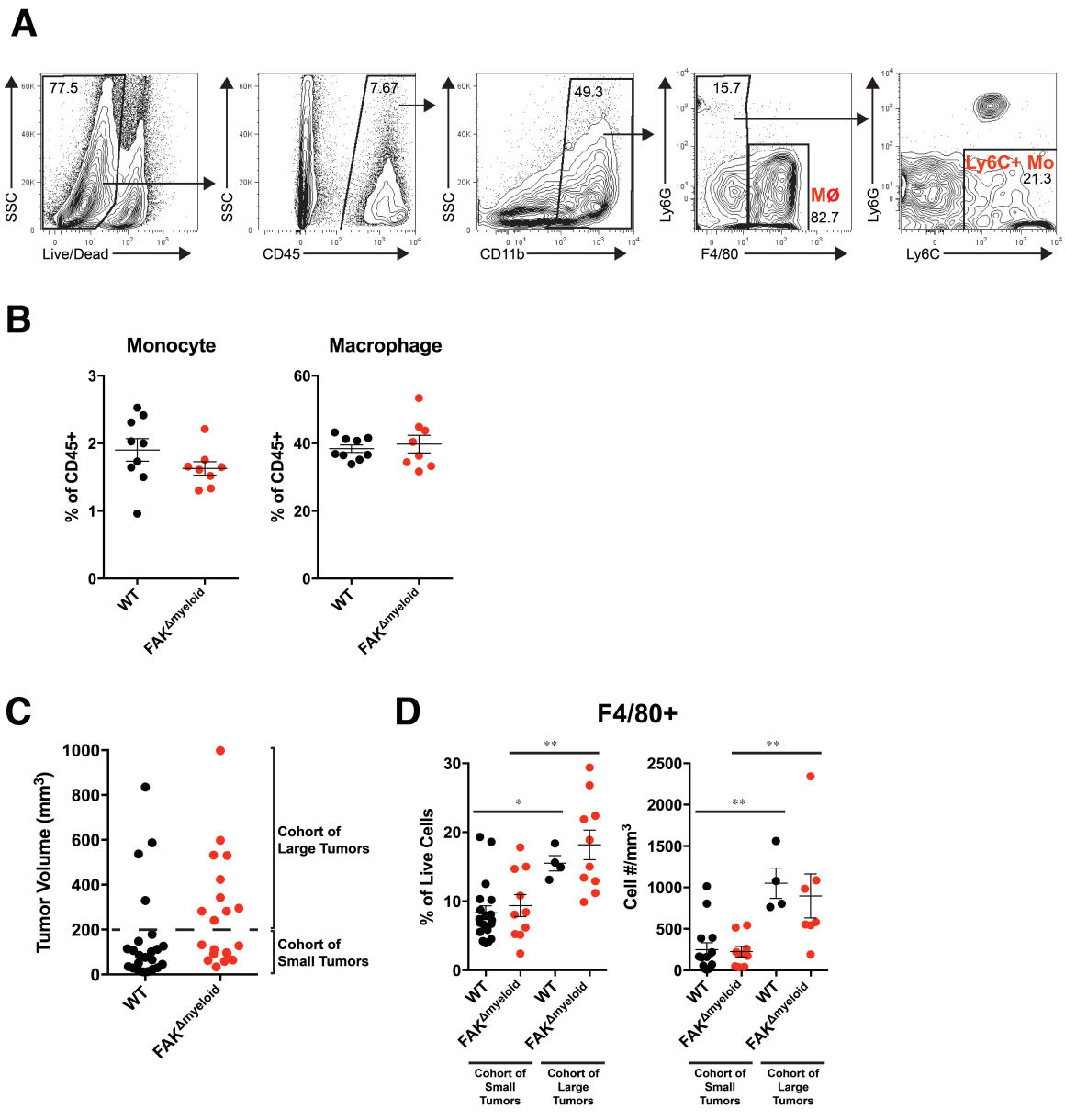

A
77.5
SSC
Live/Dead
7.67
CD45
49.3
CD11b
15.7
MØ
82.7
Ly6G
F4/80
Ly6C+ Mo
21.3
Ly6C
B
Monocyte
Macrophage
% of CD45+
WT
FAKΔmyeloid
C
Tumor Volume (mm3)
Cohort of Large Tumors
Cohort of Small Tumors
D
F4/80+
% of Live Cells
Cell #/mm3
Cohort of Small Tumors
Cohort of Large Tumors

Figure 3.5 Monocyte and macrophage accumulation in PyVmT tumors is independent of FAK. (A) Flow cytometry dot plots depict representative gating strategies to identify mononuclear phagocyte populations in cell preparations generated from week 10 PyVmT MG1s. (B) Percentage of monocytes (left) and macrophages (right) among total CD45+ cells. (C) MG1 tumor volumes from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice aged 18-19 weeks were determined by caliper measurement. Tumors were binned into cohorts according to size for further analysis. (D) Single cell suspensions generated from the tumors presented in panel C were analyzed by flow cytometry for the percentage of F4/80+ cells among live cells (left), and the absolute number of F4/80+ cells per mm^3 of tumor (right). * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney test).

precluded our ability to also determine absolute numbers of MPS subpopulations that were present in these samples.

A hallmark of the PyVmT model is the increasing accumulation of TAMs over the course of primary tumor outgrowth [354]. We next wanted to assess whether the persistence of MPS populations during the advanced stages of carcinoma was FAK-dependent, since changes in the accumulation of these populations could alter tumor growth dynamics. MG1 tumors from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice aged 18-19 weeks were measured by caliper and binned into “large” and “small” cohorts based on a predetermined tumor volume threshold of 200 mm^3 (Figure 3.5C). Our rationale for this approach was to assess TAM accumulation as a function of tumor size rather than mouse age, as the timing of tumor initiation and disease progression is inherently variable in spontaneous tumor models. The relative representation and absolute numbers of TAMs (defined as F4/80+ cells) were determined by flow cytometry (Figure 3.5D). As expected, TAM infiltration was significantly greater comparing the WT/PyVmT cohort of large tumors to smaller tumors of the same genotype. This phenotype was also observed in $FAK^{\Delta myeloid}$/PyVmT mice, indicating that the continued accumulation of TAMs in more advanced stages of the disease is independent of FAK. Furthermore, no significant differences in TAM accumulation were observed comparing size-matched tumors from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice. Altogether, our data indicate that FAK does not control the accumulation or differentiation states of MPS cells during the early or

late stages of carcinoma, suggesting that FAK may instead regulate mononuclear phagocyte localization or activities in the tumor microenvironment that may ultimately impact carcinoma outgrowth.

3.3.4 Myeloid-expressed FAK may contribute to the immune composition of PyVmT carcinomas

TAMs have been shown to regulate the recruitment and activity of other leukocyte populations, which can also profoundly impact tumor progression. We therefore examined if the loss of FAK in mononuclear phagocytes resulted in altered immune composition of developing carcinomas. Single cell preparations from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT MG1s were analyzed by flow cytometry for the presence of T cells, B cells, and NK cells (defined as CD3+, CD19+, and CD49b+, respectively) (Figure 3.6A). While no changes in the relative amounts of B cells and T cells were observed, tumors from $FAK^{\Delta myeloid}$/PyVmT mice exhibited a significant reduction in the percentage of NK cells present among total leukocytes compared with WT/PyVmT tumors (Figure 3.6B). The reduction in the representation of tumor-associated NK cells was not due to a global paucity of this cell lineage in $FAK^{\Delta myeloid}$/PyVmT mice, as the relative proportion and absolute numbers of splenic NK cells were similar in WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice (Figure 3.6C). These preliminary findings suggest that mononuclear phagocytes may rely on FAK activity for the recruitment or preservation of NK cell populations in developing breast carcinomas, which could ultimately have important implications for tumor control. However, these data

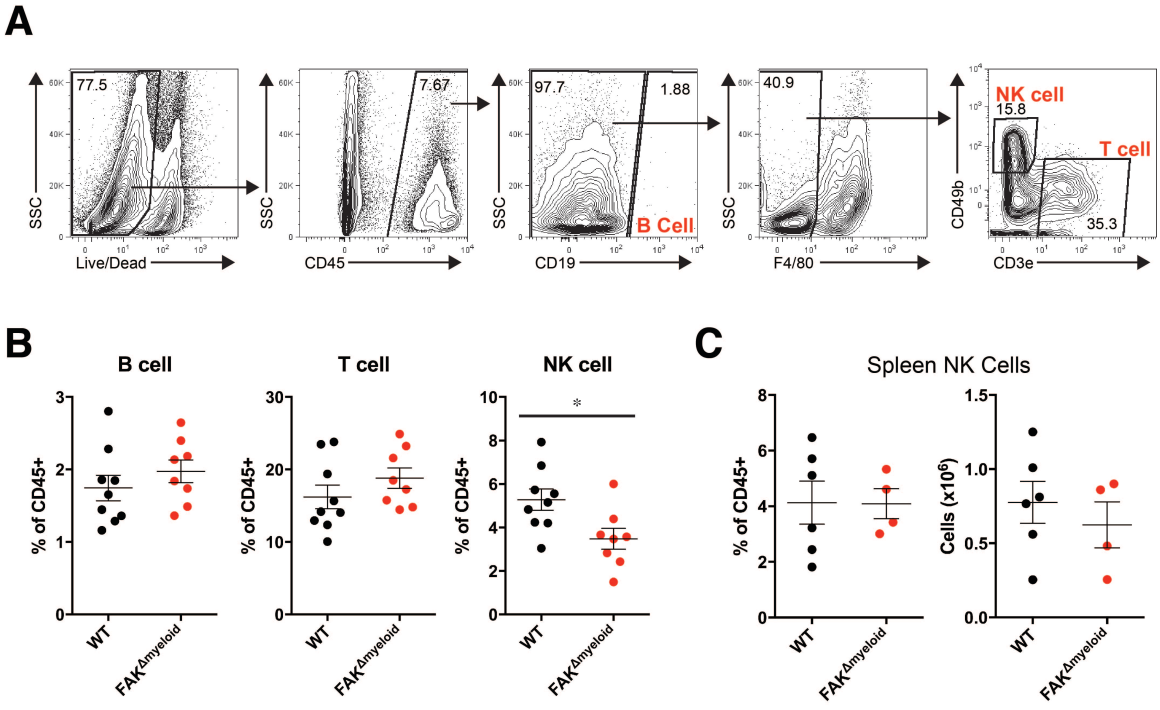

A
77.5
SSC
Live/Dead
7.67
CD45
97.7
1.88
B Cell
CD19
40.9
F4/80
NK cell
15.8
T cell
35.3
CD49b
CD3e
B
B cell
T cell
NK cell
% of CD45+
WT
FAKΔmyeloid
*
C
Spleen NK Cells
Cells (x10^6)

Figure 3.6 Myeloid-expressed FAK may regulate NK cell accumulation in PyVmT carcinomas. (A) Single cell suspensions were generated from week 10 PyVmT MG1s and analyzed by flow cytometry. Dot plots depict representative gating strategies to identify immune cell subpopulations. (B) Percentage of B cell lineage (left), T cell lineage (middle), and NK cells (right) among total CD45+ cells. * $p < 0.05$ (unpaired Student's t-test). (C) Single cell suspensions were generated from the spleen of WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice aged 10 weeks. Samples were analyzed by flow cytometry for the presence of NK cells (identified as CD45+$CD19^{neg}F4/80^{neg}$CD49b+). Graphs display the percentage of NK cells among total CD45+ cells (left), and the absolute number of NK cells/spleen (right).

should be interpreted with caution as we were unable to determine absolute cell counts in samples generated from pre-palpable tumors. It therefore remains to be clarified whether the reduced percentage of NK cells among total leukocytes reflected a reduction in total NK cell numbers within developing carcinomas from $FAK^{\Delta myeloid}$ MG1s.

3.4 Discussion

There is considerable evidence that tumor associated mononuclear phagocyte populations can profoundly impact breast cancer progression based on their ability to either support or inhibit growth and metastasis [359]. The goal of our study was to determine the fundamental contribution of FAK, a critical mediator of adhesion signaling and motility, to macrophage functions regulating breast tumor progression. Using a spontaneous murine model of breast cancer (PyVmT), we demonstrate that loss of FAK expression in MPS cells does not influence tumor initiation/progression during the early stages of tumor development (Figure 3.2), or the rate of tumor growth during the late stages of advanced carcinoma (Figure 3.3D). Rather, our data indicate that FAK facilitates MPS-mediated constraint of primary tumor outgrowth immediately after the transition from adenoma/MIN to carcinoma (see Figure 3.3B for model). In support of this model, $FAK^{\Delta myeloid}$ mice exhibited accelerated time to the appearance of palpable tumors, which contain large regions of carcinoma (Figure 3.3A), concomitant with increased tumor volume in late stage carcinomas (Figure

transfer experiments indicated that trafficking of myeloid cells to the tumor did not rely on FAK (Figure 3.4B). Furthermore, FAK did not appear to regulate the maintenance or escalating accumulation of MPS populations during the early and late carcinoma stages (Figure 3.5). Together, these data support a step-wise model (see Figure 3.7) in which inflammatory monocytes are recruited to the developing tumor and extravasate into the tumor mass via FAK-independent processes (A), at which point they upregulate FAK expression and differentiate into TAMs (or vice versa) (B) and migrate to heterogenous microdomains within the tumor (C) where they perform their effector functions (D). In this way, we propose that FAK may be important for regulating aspects of TAM activity and/or localization that ultimately result in the control of carcinoma outgrowth.

To date, much of our efforts have centered on characterizing the precise stages of PyVmT tumor development that are influenced by FAK activity in MPS populations. While our data indicate that FAK expression in MPS cells contributes to tumor constraint during the early carcinoma stage, the mechanisms that control this process are essentially unknown. As mentioned above, TAMs have been shown in some cases to adopt phenotypes that actively inhibit tumor progression [170-172], although the molecular factors that promote this process are not well characterized. It is therefore possible that FAK activity may normally facilitate these anti-tumor capabilities in TAMs, which could result in the delayed tumor outgrowth we observe in WT/PyVmT compared to $FAK^{\Delta myeloid}$/PyVmT mice. This could be achieved through directed activity against

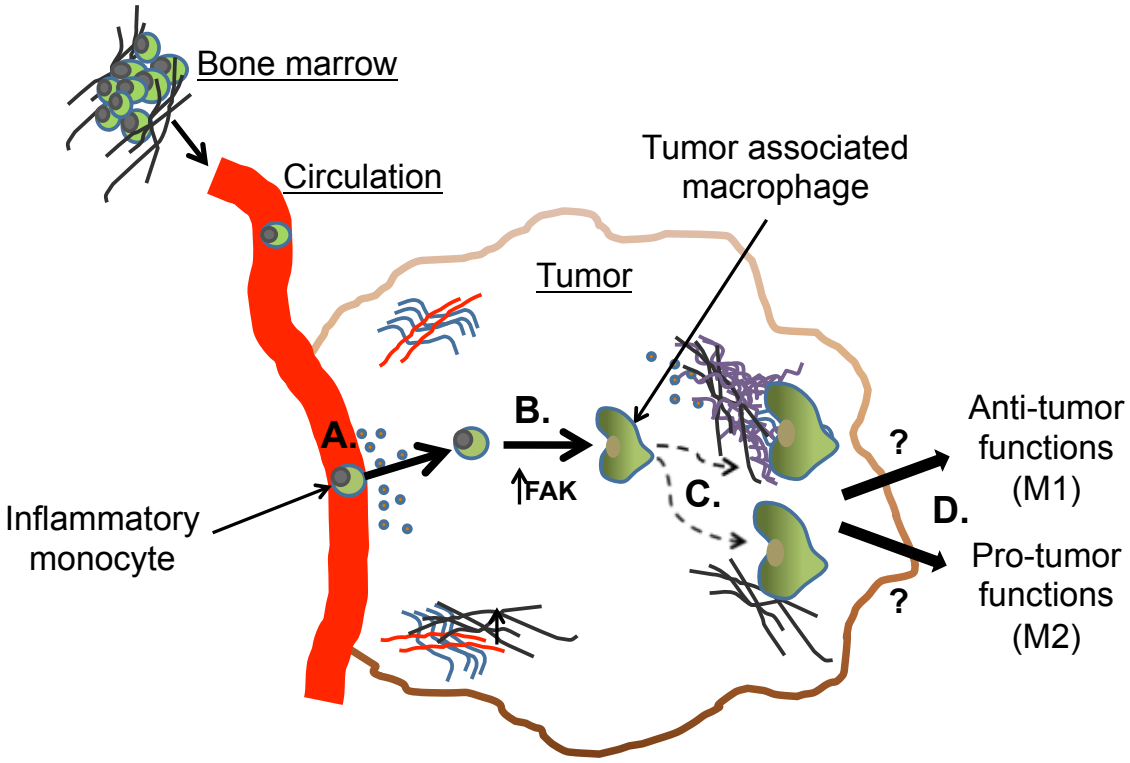

Bone marrow
Circulation
Tumor associated
macrophage
Tumor
A.
B.
FAK
C.
D.
?
?
Anti-tumor
functions
(M1)
Pro-tumor
functions
(M2)
Inflammatory
monocyte

Figure 3.7 Model for FAK-dependent regulation of PyVmT tumor growth by macrophages. The schematic illustrates potential FAK-dependent and -independent mononuclear phagocyte processes/functions in the tumor microenvironment. (A) Immature monocytes devoid of FAK expression are recruited to the tumor by soluble factors and exit the vasculature, (B) mature into TAMs and upregulate FAK expression, and (C) localize to distinct microanotomical niches within the tumor, (D) where their activities can either support or inhibit tumor growth and progression.

the tumor cells themselves, as has been previously described [170], and/or indirectly by regulating the recruitment, survival, or activation states of other leukocyte populations. Data presented above showing that $FAK^{\Delta myeloid}$ mice exhibited decreased NK cell representation in developing carcinomas are consistent with the latter possibility (Figure 3.6B) since NK cell activity has been implicated in controlling tumor growth in a number of models [360]. However, this regulation is typically exerted during the early stages of tumor initiation, rather than in more developed tumors. In addition, it is important to note that the immunophenotyping that was performed for the current study was relatively cursory and did not allow for the distinction between cell lineage subsets or activation states. Furthermore, total numbers of leukocyte subpopulations within the developing carcinomas were not assessed. Therefore, future studies that incorporate more nuanced approaches to determine the precise contributions of myeloid-expressed FAK in influencing the overall immune composition of PyVmT tumor stroma are warranted.

A second possible explanation for how FAK expression in macrophages may impact tumor outgrowth is by controlling the ability of TAMs to navigate through and respond to environmental signals within physically distinct microdomains of the tumor. This is particularly feasible given its established role in macrophage motility downstream of integrin signaling [259]. Within carcinomas, stratified microdomains containing varying extents of vasculature, hypoxia, necrosis, and extracellular matrix deposition are evident, and the landscape of these domains

constantly evolves throughout tumor progression. Macrophages are highly versatile and respond to localized cues in the tumor microenvironment that direct their activities. Intravital studies in the PyVmT model have shown that TAM directional migration and polarization are dictated by their localization within the tumor microenvironment [361]. Using immunohistological approaches, other investigators have reported differences in surface marker expression based on TAM localization patterns within the tumor [341, 362]. Thus, the reduced tumor control exhibited by $FAK^{\Delta myeloid}$/PyVmT mice may be due to impaired motility of FAK-deficient TAMs within the tumor, preventing them from reaching microanatomical locations that promote their conversion to a more pro-inflammatory (anti-tumor) phenotype. Future studies are needed to assess directional migration, localization patterns, and/or activation states of FAK-deficient TAMs during tumor progression.

TAMs also play a prominent role in stimulating angiogenesis via the production of soluble mediators, including VEGF, IL-8, CXCL8, and TNF-α [171, 204, 205, 209]. While the roles of FAK in TAM-mediated angiogenesis and metastasis were not addressed in our work, we speculate that these processes could be enhanced in $FAK^{\Delta myeloid}$/PyVmT tumors given our results indicating accelerated primary tumor growth. Indeed, angiogenesis is reportedly critical in the PyVmT model for progression of primary tumors to a metastatic phenotype [356]. M-CSF-deficient (*op/op*) mice, which have a global paucity of macrophages, exhibit impaired vascular development, a significant delay in the onset of late carcinoma,

and reduced lung metastases. Importantly, restoration of TAMs into the primary tumors of *op/op* mice reversed these phenotypes. Subsequent reports have demonstrated that TAMs also interact directly with tumor cells and promote their migration and extravasation from the site of primary growth [200, 210, 363].

It should be noted that results from the aforementioned studies are consistent with our findings showing that MPS cells with or without FAK do not regulate tumor growth in the early stages of tumor development. Interestingly, primary tumors from *op/op* mice showed a significant delay in progression to late carcinoma, while $FAK^{\Delta myeloid}$ mice exhibited enhanced carcinoma growth. Together, these data indicate to us that (1) TAMs facilitate carcinoma progression in the PyVmT model, and (2) loss of FAK in mononuclear phagocytes is functionally distinct from the depletion of TAMs. One possible explanation for this discrepancy is that TAMs lacking FAK may be more prone toward "M2-like" activation states that support the growth/progression of carcinomas. Given that FAK deficiency does not inhibit the accumulation of TAMs in PyVmT carcinomas (Figure 3.5), these "M2-polarized" cells would then be capable of accelerating carcinoma outgrowth. In support of this possibility, the vast majority of studies attribute TAM activities during later stages of cancer to a tumor-promoting function [141, 173]. DeNardo *et al*. showed that TAM M2 activation states are stimulated by soluble IL-4 secreted by tumor-infiltrating CD4+ TH2 cells in the PyVmT model [180]. The polarized TAMs subsequently promoted tumor cell invasion via the secretion of EGF. Future studies are

needed to address the potential contribution of FAK toward macrophage activation states and the molecular mechanisms that could potentially drive this process, particularly because this could have important therapeutic implications. Pharmacological inhibitors of FAK have been developed and are currently being tested for the treatment of cancers [364]. TAMs constitute a significant portion of breast tumor stroma; thus, if loss of FAK activity in TAMs indeed results in the polarization of these cells toward phenotypes that are generally growth promoting, this could potentially stunt the efficacy of FAK inhibitors as therapeutic agents.

Chapter 4: Perspectives and future directions

4.1 Introduction

Cells of the MPS can be found in nearly every tissue of the body and participate in fundamental processes that are critical for the maintenance of homeostasis and the resolution of disease. The goals of my dissertation research were to (1) characterize the relative expression levels of FAK family kinases in mononuclear phagocyte populations, (2) evaluate the role of Pyk2 in regulating monocyte population dynamics at homeostasis, and (3) define the contribution of FAK to monocyte/macrophage-mediated control of tumor growth and development in a murine model of breast cancer. We showed for the first time that FAK and Pyk2 exhibit distinct expression patterns in monocyte and macrophage subpopulations. Our results further indicated that Pyk2 expression is upregulated in Ly6C^{neg} monocytes, and in turn functions to promote apoptosis of these cells during steady-state conditions. We also showed that FAK is highly expressed in macrophages, but not monocytes, and that breast tumor growth can be regulated by loss of FAK expression in TAMs. The following discussion will provide a summary of how these findings contribute to the field of mononuclear phagocyte biology and raise compelling new directions for future research.

4.2 The regulated expression of FAK family kinases in MPS populations

Prior to this work, there were conflicting data regarding the expression of FAK family kinases in mononuclear phagocytes as well as some populations of immature progenitor cells in the BM. For example, Kume *et al.* demonstrated that FAK was not expressed in whole BM cells, but could be detected when these cells were cultured under conditions that promoted their differentiation to macrophages [310]. In a different report, FAK protein expression was observed in immature Sca1+ hematopoietic progenitor cells isolated from the BM, which are able to give rise to cells of the myeloid lineage [322]. Multiple studies using monocytes isolated from PB and cultured with growth factors *in vitro* reported that these cells lacked FAK but did not assess Pyk2 expression [303-305, 307]. Several early publications concluded that macrophages also did not express FAK [304, 306]; however, more recent studies have shown that tissue resident macrophages express abundant amounts of both FAK and Pyk2 [259, 273].

The variable and at times discrepant results generated from these reports highlighted the need for precise characterization of FAK family kinases across the continuum of monocyte/macrophage differentiation states under physiological conditions. Indeed, it should be noted that the cellular preparations that were analyzed in the aforementioned studies were not defined in a consistent manner and likely represented heterogeneous populations of monocytes/macrophages. We therefore decided to assess FAK and Pyk2 protein expression in MPS subpopulations by isolating specific subsets of mononuclear phagocytes directly

from the organs in which these cells normally reside (Chapter 2). Our results indicate that FAK is poorly expressed in monocytes and committed monocyte lineage progenitor cells, but is significantly upregulated in mature macrophages. Pyk2, in contrast, is expressed in both monocytes and macrophages; however, Pyk2 expression is markedly higher in Ly6C^{neg} monocytes and macrophages compared with Ly6C+ monocytes and cMoPs. These results demonstrate subset-specific patterns of FAK and Pyk2 expression in mononuclear phagocyte populations, which suggests a requirement for tightly regulated control over the expression of these kinases that may correlate with functions that are confined to particular differentiation states.

The molecular factors that govern the expression of FAK kinases in MPS cells were not addressed in this thesis. However, data from publicly available gene expression databases are consistent with the protein expression patterns that we observed in the monocyte and macrophage populations [328]. This suggests that protein expression of FAK kinases may be transcriptionally regulated in these cells. Transcriptional control of FAK expression has mostly been studied in the context of transformed cells, where the transcription factors nuclear factor-κB (NF-κB), NANOG, argonaute 2 (AGO2), and PEA3 have been shown to increase FAK (*PTK2*) promoter activity [365-368]. Conversely, p53 has been shown to repress *PTK2* promoter activity [369]. It is currently unknown whether these factors also regulate FAK expression during the differentiation of monocytes to macrophages. Transcriptional control of Pyk2 has not been well characterized;

however, one study reported the association of the transcription factor CEBP-β with the promoter region of the Pyk2 gene (*PTK2b*) and the subsequent induction of Pyk2 protein expression during monocyte differentiation to macrophages in the NB4 cell line [305]. Additional studies are necessary to determine how both cell-intrinsic and -extrinsic factors control FAK and Pyk2 protein expression during distinct stages of monocyte and macrophage differentiation.

Flow cytometry provides a powerful tool to study mononuclear phagocyte subpopulations during precise stages of the differentiation process. Unfortunately, commercially available antibodies to FAK and Pyk2 have been tested extensively by our lab and are not suitable for flow cytometry due to excessive non-specific antibody binding. To assess the expression of FAK family kinases in MPS subpopulations, our approach was therefore to isolate FACS sorted populations and subsequently perform western blotting on extracts prepared from these samples. One technical obstacle to this approach is the relative scarcity of certain MPS subsets, which limits the number of cells that can be isolated for protein expression analyses. Thus, while it is clear that FAK expression in monocytes is considerably lower than in macrophages, we cannot rule out the possibility that low levels of FAK may be expressed in one or more monocyte subset. Another drawback to this approach is the inherent loss of potentially relevant information regarding FAK and Pyk2 expression as a function of heterogeneity within MPS populations. For example, it is possible that Pyk2 expression may be upregulated in a small subset of Ly6C^{neg} monocytes that

could be distinguished with the use of additional markers. Similarly, FAK may only be expressed in a subset of splenic macrophages, or its expression might vary in macrophages isolated from different tissues. An alternative approach that would circumvent these limitations would be to develop FAK or Pyk2 reporter mice, similar to $CX_3CR1^{GFP/+}$ mice, which would allow for the detection of these proteins using fluorescence as a surrogate marker. Notably, a similar method has been employed by the Hettinger group in characterizing expression of the transcription factor NR4A1 in monocyte subsets [44].

4.3 Perspective on the role of Pyk2 as a promoter of cell death in Ly6C^{neg} monocytes

One question that arises from the observation that FAK and Pyk2 protein expression is not equivalent across MPS cell populations is whether these proteins regulate distinct functions in these subsets. The relative absence of FAK expression in monocytes, together with the pronounced upregulation of Pyk2 expression in the Ly6C^{neg} fraction of monocytes, led us to investigate the role of Pyk2 in the differentiation and accumulation of monocyte subpopulations. We found that Pyk2-deficiency led to an over-representation of Ly6C^{neg} monocytes, concomitant with reduced indicators of apoptosis in these cells, suggesting that Pyk2 functions to promote the turnover of Ly6C^{neg} monocytes at homeostasis. While exogenous overexpression of Pyk2 has previously been shown to induce apoptosis in several cell types [294-297, 321], this is the first time to our

knowledge that endogenous levels of Pyk2 have been shown to promote cell death. Additionally, our study is the first to demonstrate a role for Pyk2 in promoting apoptosis of monocytes.

Further experimentation is needed to elucidate the signaling mechanisms that control Pyk2-mediated cell death in Ly6C^{neg} monocytes. To date, several factors have been identified that promote the survival of Ly6C^{neg} monocytes, including M-CSF, CX_3CL1, and the transcription factor NR4A1 [44, 49, 51, 66, 316]. However, very little is known about the molecular factors that actively promote apoptosis in these characteristically short-lived cells. Monocytes spontaneously undergo apoptosis when cultured *ex vivo*, and Fas-FasL signaling has been implicated in driving this process via the repression of Bcl-2 signaling and the activation of capsases [332, 333]. However, there is currently no evidence linking canonical Fas signaling with Pyk2-mediated apoptosis; in fact, one report showed that Pyk2 was activated downstream of dexamethasone (Dex)-induced apoptosis but not Fas mAb-induced apoptosis in multiple myeloma cells [295]. The authors of this study went on to show that overexpression of kinase-inactive Pyk2 prevented Dex-induced apoptosis, but did not affect Fas mAb-induced cell death, suggesting that Pyk2 may promote apoptosis in a Fas-independent manner.

Our work in reciprocal chimeric mice indicate that the reduced apoptosis observed in Ly6C^{neg} monocytes from Pyk2-/- mice cannot be entirely attributed to

an altered stromal environment present in the BM of these animals; rather, this phenotype is at least in part driven by loss of Pyk2 in hematopoietic cells. To further illustrate a survival advantage for Ly6C^{neg} monocytes, Pyk2 conditional knockout mice could be developed using the Cre-LoxP system. In this model, constitutive Cre expression driven by the LysM-promoter would induce myeloid-specific deletion of Pyk2, similar to the FAK$^{\Delta myeloid}$ mice described in Chapter 3 of this thesis. These mice could potentially provide supportive evidence demonstrating myeloid cell-autonomous effects of Pyk2 deficiency on the accumulation of Ly6C^{neg} monocytes. Another approach would be to transfer FACs-isolated MDPs or cMoPs from WT and Pyk2-/- mice into lethally irradiated recipient mice in a mixed chimera experiment, and subsequently track the differentiation and survival of Ly6C^{neg} monocytes over a short period of time. This experiment would allow us to directly assess the contribution of Pyk2 to non-classical monocyte lifespan. Given our current understanding of Pyk2 function in these cells, our expectation would be that Pyk2-deficient progeny would outlive WT cells. Similar experiments could also be performed by transferring fluorescently labeled monocytes into non-irradiated recipient mice. Under these conditions, the genotype of the adoptively transferred cells would be distinguished by either the color of fluorescence staining used to label the donor cells, or the CD45 allotype of the donor cells.

Our data indicate that Pyk2-deficient Ly6C^{neg} monocytes accumulate in the BM and spleen of chimeric mice to a greater extent than WT Ly6C^{neg} monocytes, and

this is concomitant with decreased levels of apoptosis in BM Ly6C^{neg} monocytes. However, this phenotype was not observed in the PB, where similar numbers of WT and Pyk2-/- Ly6C^{neg} monocytes were observed. One possible explanation for this phenomenon is that the BM and spleen, but not the PB, provide a specialized environment that allows for the turnover of some Ly6C^{neg} monocytes via a mechanism that is at least partially dependent on Pyk2. Interestingly, increased expression of CXCR4 has been shown to direct aged neutrophils from the circulation to the liver, spleen, and BM for elimination [370, 371]. Although a similar process has not been demonstrated in monocytes, it is interesting to speculate that Pyk2 may facilitate the localization or signaling necessary for the proper removal of aged Ly6C^{neg} monocytes in the BM and spleen.

Another probing question that arises from these studies is why Ly6C^{neg} monocytes utilize Pyk2 for their turnover, but macrophages (which also abundantly express Pyk2) are much longer lived. One potential explanation is that the macrophages are exposed to distinct microenvironmental stimuli, including survival factors, that prolong their lifespan. It should also be noted that, unlike monocytes, macrophages express FAK in addition to Pyk2. Notably, FAK has been shown to promote survival signaling in several cell types [250, 264]. Hence, it is possible that the upregulation of FAK in these cells is needed in part to oppose the function of Pyk2 as a cell death promoter. In this model, FAK and Pyk2 would fulfill opposing roles; of note, this functional relationship has previously been demonstrated in other contexts [249].

4.4 Functional implications for Pyk2 activity in Ly6C^{neg} monocytes

What then is the practical importance of restricting the compartment of Ly6C^{neg} monocytes? It is unknown at this point why short-lived innate immune cells are produced and die at such rapid rates. Some have speculated that this may ensure the timely turnover of these cells during inflammatory reactions, potentially providing a protective measure against excessive tissue damage during instances of chronic inflammation [372]. At homeostasis, however, it is unclear whether an expanded Ly6C^{neg} monocyte compartment would have positive or deleterious consequences. Functionally, these cells have been linked to scanning of the vasculature and uptake of microparticles along the endothelium [24]. Thus, these cells purportedly act as intravascular caretakers that remove debris from the vasculature and are poised to respond to danger signals. Ly6C^{neg} monocytes have also been shown to cross-present antigens from phagocytosed cells to T cells in the spleen, thereby contributing to tolerogenic responses [373]. Given that Pyk2-/- mice do not display overt phenotypic abnormalities, the modest overabundance of Ly6C^{neg} monocytes we have observed likely does not contribute to pathologies in the absence of stimuli. However, it would be interesting to assess responses related to the maintenance of vascular integrity in the Pyk2-deficient mice.

Although Ly6C^{neg} monocytes rarely extravasate from the vasculature during physiological conditions, these nonclassical monocytes have been reported to enter peripheral tissues and differentiate into resident macrophages in response

to some inflammatory cues [24]. As opposed to Ly6C+ inflammatory monocytes, the Ly6C^{neg} monocytes produce low levels of proinflammatory cytokines and are thought to generally contribute to wound healing processes and the resolution of inflammation [18]. After myocardial infarction (MI), Ly6C^{neg} monocytes were shown to traffic to the infarcted heart during the reparative phase of the injury response, and mice deficient in these nonclassical monocytes displayed adverse cardiac remodeling following MI [98, 374]. Other protective roles for Ly6C^{neg} monocytes have been demonstrated in mouse models of arthritis and Alzheimer's disease [375, 376]. In the context of our findings, these results suggest that Pyk2-deficient mice might exhibit enhanced wound healing due to the increased numbers of Ly6C^{neg} monocytes that they harbor. However, others have reported aggravation of pathologies following Ly6C^{neg} monocyte recruitment, including those associated with atherosclerosis and kidney disease [377]. Thus, the contribution of nonclassical monocytes to either the promotion or resolution of inflammatory reactions may be context-dependent. Of note, any disease paradigms applied to our model would need to account for the global nature of the Pyk2-deficiency in our mice. Alternatively, Pyk2 conditional knockout models could be developed to explore monocyte-specific contributions to disease resolution.

While our work demonstrates that Pyk2 promotes the turnover of Ly6C^{neg} monocytes, this does not exclude the possibility that Pyk2 has other functional activities in these cells. The majority of our current understanding of Ly6C^{neg}

function comes from sophisticated intravital imaging experiments tracking the patrolling and scavenging activities of these cells along the luminal side of the vasculature. This activity is dependent on the β2 integrin complex (lymphocyte function-associated antigen-1; LFA-1), as antibody blockade to either subunit of the LFA-1 complex (CD11a or CD18) leads to rapid disassociation of Ly6C^{neg} monocytes from the vasculature [24, 25]. Furthermore, genetic ablation of the LFA-1 ligands, intercellular adhesion molecule 1 (ICAM1) and ICAM2, also results in dissociation of Ly6C^{neg} monocytes from the endothelial wall [25]. The contribution of signaling molecules like Pyk2 that function downstream of integrin activation to these patrolling activities is unknown. However, in several other immune cell types, Pyk2 is known to be activated in response to LFA-1 stimulation [378-382]. Given this information, it would be interesting to assess the contribution of Pyk2 toward Ly6C^{neg} monocyte patrolling capabilities. For intravital imaging studies, this would require isolation and fluorescence labeling of Pyk2-deficient Ly6C^{neg} monocytes prior to adoptive transfer into a recipient mouse. Alternatively, a Pyk2-/- fluorescent reporter mouse could be developed and utilized, as previously described. Given the established role of Pyk2 in promoting the directional movement of macrophages, we might expect Pyk2-deficient monocytes to show impaired patrolling or scavenging capabilities. If this were the case, follow-up experiments testing the role of Pyk2 in the response of Ly6C^{neg} monocytes to vascular damage would be warranted.

4.5 Perspective on FAK in macrophage control of breast tumor growth

Our data indicate that FAK is highly expressed in macrophages (Chapter 2), and that MPS cells may utilize FAK to restrict the growth of primary tumors in the PyVmT murine model of breast cancer (Chapter 3). While our results show that the accumulation of TAMs in the developing tumor is likely governed by FAK-independent processes, the accelerated tumor progression observed in $FAK^{\Delta myeloid}$/PyVmT mice may still be the result of aberrant localization of TAMs within the tumor. Future research should therefore establish the contribution of FAK to TAM localization within the architecture of tumors transitioning from early carcinoma to late carcinoma by determining macrophage localization to subdomains within the tumor. This could be accomplished through histological examination of WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT MG1s to quantify the number of TAMs within the tumor nest and the surrounding stroma, as well as the proximity of TAMs to the vasculature using techniques that have been previously described [343, 383]. If FAK is important for migration into the nest of the tumor, we would expect there to be greater infiltration of TAMs away from the vasculature and into the tumor nest in samples isolated from WT/PyVmT compared with $FAK^{\Delta myeloid}$ /PyVmT mice. A similar disparity in TAM infiltration would be expected in angiogenic or fibrotic areas if FAK is required for the TAMs to respond to chemotactic signals emanating from those regions of the tumor. These histological analyses would rely heavily upon qualitative assessment of microdomains within the tumor, including areas of neoangiogenesis and the subdivision of tumor stroma from tumor nest; it would therefore be useful to

consult with a pathologist to discuss experimental design, data analysis, and quantification of IHC staining during the course of experimentation. Of note, it seems unlikely to us that regions of necrosis and/or hypoxia drive the accelerated tumor growth observed in $FAK^{\Delta myeloid}$ mice, given that these features are generally associated with tumors that have already progressed to late carcinoma.

Alternatively, tumor outgrowth may be accelerated in $FAK^{\Delta myeloid}$/PyVmT mice because of altered TAM functionality. Indeed, even if FAK is important for macrophage migration within the tumor, this would not discount the possibility that there may also be fundamental changes to the activation state of these TAMs, given the importance of cellular crosstalk within microdomains of a developing tumor. For this reason, it would be useful to also assess the functional characteristics of macrophages populating tumors that are undergoing the transition from early to late carcinoma. We could begin to characterize TAM activation states by sorting TAMs from WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT tumors and performing quantitative RT-PCR to compare gene expression of known functional markers such as IL-12, NOS2, IL-1, IL-6, IL-23 ("M1-like"), and IL-10, ARG1, TGF-β, and IL-4Rα ("M2-like"). These results would provide a picture of the overall gene expression of TAM populations in MG1s; parallel flow cytometric analyses could also performed to identify functional subpopulations of polarized macrophages in MG1s. Since soluble factors produced by TAMs can influence tumor growth and progression, *ex vivo* culture methods of

macrophages isolated from developing tumors could be employed to measure a broad spectrum of soluble factors in the supernatant that may be produced by these TAMs. Using these results as a guide, we could further test for differences in growth factor and cytokine production using ELISAs to measure protein levels of specific factors present in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT MG1s.

Additionally, we could determine whether macrophage activation state is intrinsically regulated by FAK outside of the context of the tumor microenvironment. Using pharmacological inhibitors to FAK activation, as well as our genetic models of conditional FAK knockout, macrophages could be cultured from bone marrow *in vitro* and the soluble factors present in the supernatants from these cultured cells could be measured using multiplex analysis. Moreover, the polarization state of these macrophages could again be determined using the approaches outlined above. These experiments may help to clarify whether FAK signaling in macrophages fundamentally controls their polarization to a given state, irrespective of its role in governing the capacity of these cells to migrate.

The contribution of Pyk2 to MPS-mediated control of breast tumor growth was not assessed in this study. Given that Pyk2 is also highly expressed in macrophages, and the fact that FAK and Pyk2 have been shown to exhibit some overlapping functions [298, 384, 385], it will be important to assess whether Pyk2 expression in MPS cells can also regulate tumor growth. However, several elements of the global Pyk2 knockout model would require careful consideration.

First, while Pyk2 expression is restricted mainly to cells of the CNS and hematopoietic cells, upregulation of Pyk2 expression has also been reported in some breast cancers [386]. Hence, a conditional knockout mouse would be required to assess tumor control by Pyk2 specifically in myeloid cells. Secondly, unlike FAK, Pyk2 is also highly expressed in some subsets of monocytes and neutrophils. Therefore, any functional consequences of Pyk2 deficiency on tumor growth could not be attributed solely to TAMs in this model. Nevertheless, studying the contribution of Pyk2 in myeloid-mediated control of tumor growth represents an attractive area of study that may have important therapeutic implications.

4.6 Concluding remarks on FAK and Pyk2 in mononuclear phagocytes

In conclusion, the data and perspectives presented in this thesis provide novel insights into previously unappreciated roles for FAK and Pyk2 in regulating MPS populations. Together, our findings indicate that Pyk2 is important for the homeostatic maintenance of monocyte subpopulations, and that FAK contributes to the control of breast tumor growth by TAMs. While we remain in the early stages of understanding the mechanistic underpinnings that drive these processes, our findings provide a stepping-stone for future studies that may uncover additional functions for FAK and Pyk2 in controlling important biological processes during homeostasis and disease.

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