Type I IFN, Toll-like Receptor Signaling and Myeloid Homeostasis

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Abstract

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The roles of toll-like receptor and type I interferon signaling in modulating myeloid homeostasis are incompletely defined. Here, we investigate how these factors interact to influence myeloid development in the bone marrow and myeloid cell egress from the bone marrow. The findings described here advance our knowledge of basic immunology and may have clinical applications in the contexts of autoimmunity and infection.

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Dedication

I would principally like to thank my wife Amy, my parents Jeffrey and Jeanie and my sister Amy for their unwavering support and love. I would also like to thank Amy's parents for their gracious support.

Commonly used abbreviations

BM, bone marrow

CAR, CXCL12-abundant reticular

CCL, CC chemokine ligand

CDP, common dendritic cell progenitor

CLP, common lymphoid progenitor

CMP, common myeloid progenitor

DC, dendritic cell

eGMP, emergency granulocyte/macrophage progenitor

GMP, granulocyte/macrophage progenitor

IFN, interferon

$IFNAR^{-/-}$, interferon receptor alpha deficient

i.p., intraperitoneal

ISG, IFN stimulated gene

LCMV, Lymphocytic Choriomeningitis Virus

Lin, Lineage

LT-HSC, long-term hematopoietic stem cell

LSK, $Lineage^{-}Sca1^{+}c\text{-}Kit^{+}$ surface phenotype

MPP, multipotent progenitor

MSC, mesenchymal stem cells

mTOR, mammalian target of rapamycin

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

PI3K, phosphatidylinositide 3-kinase

TLR, Toll-like receptor

qPCR, quantitative real time PCR

pDC, plasmacytoid dendritic cell

SLE, systemic lupus erythematous

ST-HSC, short-term hematopoietic stem cell

TLR, Toll-like receptor

$TLR7^{-/-}$, TLR7 deficient

WT, wild type.

Chapter 1: Introduction

The immune system is the network of tissues, cells and vasculature that operates to keep the host free from infection and cancer, while avoiding immunopathology and autoimmunity[1]. These processes are carried out in large part by the cells of the immune system, which are able to traverse the body, modify their population dynamics and function to fight pathogens and then, save for pathogen-specific memory cells, reset after infection to approximate steady state numbers and behavior. Most cells of the immune system originate from hematopoietic stem cells in the bone marrow and terminally mature immune cells are broadly divided into two groups: adaptive cells and innate cells. Adaptive immune cells include B cells that generate antibodies and T cells which perform helper and cytotoxic functions. Adaptive cells are notable for displaying antigen specificity and exhibiting long-lived memory, whereas innate cells lack these qualities. Innate immune cells include cells of the myeloid lineage, such as macrophages, dendritic cells, monocytes and neutrophils, as well as innate lymphocytes, such as natural killer cells and innate lymphoid cells. Myeloid cells are capable of phagocytosis, antigen presentation, pathogen recognition and eradication, as well as promotion of tissue homeostasis. The work presented here focuses on the dynamics of myeloid cell development in the bone marrow and egress from this tissue under conditions of inflammation.

Research into the cells that would comprise the innate immune system began over a century ago when Ilya Metchnikoff observed the process of phagocytosis and described “pathological inflammation” and “physiological inflammation,” in which he noted motile, phagocytic cells engulfing and eating damaged tissue or pathogens, respectively[2]. The discovery of phagocytosis and its nascent implications garnered Metchnikoff a share of the 1908 Nobel Prize in Physiology and Medicine and his work is considered the first to focus on cells that are now broadly considered to be members of the innate immune system. Our understanding of the constituents and coordinated functions of the immune system as a whole have progressed substantially since Metchnikoff’s time. An important conceptual step forward occurred when Charles Janeway predicted that “nonclonal recognition of nonself patterns plays a major role in immune system function and in host defense”[3]. Janeway contended that certain cells of the immune system have the ability to discriminate self from non-self through the expression of pattern recognition receptors (PRR) and that engagement of these receptors initiates an inflammatory response.

We now know that several families of germ-line encoded PRR exist and are broadly expressed on cells of the innate immune system. Moreover, we have learned that engagement of PRR is important to promote maturation, antigen presentation and inflammatory cytokine secretion by innate cells, all of which are important components of the cross-talk required between the innate and adaptive arms of the immune system to foment a complete immune response.

Toll-like receptors (TLR) are a family of transmembrane PRR. There are 10 TLR genes in humans and 12 in mice. These receptors possess leucine-rich repeats, TIR domains, and signal through the adapter proteins MYD88 and/or TRIF. Conserved mammalian TLR1, 2, 4, 5 and 6 are expressed on the surface of cells and recognize bacterial cell wall components or flagellin. TLR3, 7, 8, 9 and 13 are expressed intracellularly in endosomal compartments and are specific for nucleic acids. TLR10 function is still being elucidated and is only expressed in humans. TLR11, 12 and 13 are specifically expressed in mice, not humans. Upon ligation of their cognate receptors, TLR initiate inflammatory signaling via mitogen associated protein kinase (MAPK), NF-kB and interferon regulatory factory (IRF) pathways[4]. TLR7 is specific for single-stranded ribonucleic acid (ssRNA) and is highly expressed by plasmacytoid dendritic cells[5,6].

TLR are now intimately associated with the immune system but the *Toll* gene was first implicated as a key gene in Drosophila embryonic development[7]. In addition to expression on mature myeloid cells, where they directly participate in innate immunity to infection, TLR are expressed by human and mouse hematopoietic stem cells (HSC) and myeloid-committed progenitor cells[8-10]. Moreover, TLR signaling in stem and progenitor cells biases their commitment toward the myeloid lineage[9]. This observation was intriguing to us and formed the basis for my thesis project, which focused on what inflammatory signaling pathways contribute to perturbations in myeloid peripheral homeostasis. The homeostasis of immune cells can be altered in a number of ways including changes in development in the bone marrow, egress of mature myeloid cells from the bone marrow or modifications in peripheral survival or proliferation, among other possibilities. Our studies focused on the bone marrow and the changes inflammation can exact on hematopoietic development into myeloid cells and bone marrow egress of these populations.

In the steady state, mature myeloid cells develop in the bone marrow from a pluripotent, heterogeneous group of HSC**.** Self-renewing long-term hematopoietic stem cells (LT-HSC) develop into

short-term HSC (ST-HSC) and then into multipotent progenitor cells (MPP). These cells together compose what is referred to as the "LSK" (Lineage$^-$Sca1$^+$c-Kit$^+$) fraction of the bone marrow. MPP mature into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) that are poised to preferentially generate the cells that compose the mature lymphoid and myeloid compartments, respectively[11]. The specifics of myeloid development are currently under intensive study but a general understanding has emerged. Nearly all mature myeloid cells and dendritic cells develop from the CMP, including monocytes, neutrophils, F4/80^{low} macrophages, conventional dendritic cells and plasmacytoid dendritic cells[12,13]. These mature cells pass through at least one more multipotent progenitor stage before complete differentiation. The granulocyte macrophage progenitor (GMP) and common dendritic cell precursor (CDP) derive directly from the CMP. GMP produce neutrophils and monocytes[13]. Monocytes are able to further develop into F4/80^{low} macrophages and inflammatory dendritic cells in the periphery. F4/80^{high} tissue-resident macrophages develop from a yolk sac progenitor, independent of CMP[14]. CDP produce all conventional DC subsets and pDC, but not monocytes, macrophages or neutrophils *in vivo*[15,16] (Fig. 1.1). More committed, clonogenic monocyte/macrophage and pDC progenitor cells have been recently described[17,18]. The macrophage DC precursor (MDP), another myeloid progenitor cell, phenotypically overlaps with CMP, GMP and CDP populations[19-21] but was recently shown to lack the ability to give rise to macrophages and dendritic cells at a clonal level[22]. Table 1 lists hematopoietic stem, lymphoid- or myeloid-committed progenitor cells and their progeny with the phenotypic markers used to identify them by flow cytometry in our studies.

The hematopoietic system is capable of rapidly increasing myeloid cell output to combat pathogens and to restore peripheral homeostasis after infection. The precise hematopoietic changes depend on the particular infectious agent, but infection-induced hematopoietic alterations generally include an increase in the number of cells identified as primitive HSC, upregulation of the interferon-inducible marker Sca-1 by intermediate hematopoietic precursor cells and a diminishment of lymphoid cell generation in favor of myeloid cell development[23-27]. This demand-driven hematopoietic state is a component of "emergency myelopoiesis," a process in which myeloid effector cells are rapidly generated in the bone marrow and shunted to the periphery[27,28] (Fig. 1.2). Indeed, Lymphocytic Choriomeningitis virus, Vaccinia virus, *Ehrlichia muris, Plasmodium chabaudi*, *Mycobacterium avium, Listeria*

monocytogenes, Escherichia coli or *Staphylococcus aureus* infection results in significant deviations in steady state BM progenitor population dynamics, such as diminishment of CMP, increases in LSK and changes in the number of monocyte progenitors[26,29-35].

The factors that promote emergency myelopoiesis have not been completely elucidated. Some studies have implicated direct pathogen sensing by bone marrow hematopoietic stem and progenitor cells via TLR that initiates a cell-intrinsic myeloid differentiation program[9]. TLR signaling may also indirectly influence hematopoiesis by inducing production of inflammatory cytokines by mature cells or progenitors[36,37]. Indeed, inflammatory cytokines have been shown to modulate hematopoiesis[26,29,31,38,39]. For example, IFNγ can act upon GMP to upregulate myeloid-associated transcription factors[29] or can act upon non-hematopoietic BM stromal cells to induce IL-6 signaling, thereby downregulating transcription factors that inhibit myeloid differentiation in stem and progenitor cells[38]. Other work has shown that type I IFN, an antiviral cytokine family, can act directly on quiescent long-term HSC (LT-HSC) and cause them to enter the cell cycle[40-42]. The role of type I IFN in influencing lineage commitment and developmental kinetics during hematopoiesis is unknown. Further, no studies have focused on understanding how hematopoiesis and peripheral myeloid homeostasis can be altered in vivo in the context of increased TLR signals and type I IFN.

Another mechanism that can affect the peripheral homeostasis of myeloid cells is the egress of mature myeloid cells from the bone marrow into the periphery. The bone marrow cavity, in addition to being the site of hematopoiesis, is a reservoir of mature inflammatory monocytes and neutrophils. These cells leave the bone marrow and enter blood circulation in response to chemokine signaling. We are most interested in the behavior of inflammatory monocytes, which express high levels of the chemokine receptor CCR2. Ligation of this receptor by ligands CCL2, -7, and -12 leads to monocyte egress from the bone marrow[43]. Infection with bacterial, viral or fungal pathogens drives monocyte egress from the bone marrow to the periphery[44-47]. However, the mechanisms operating in the bone marrow cavity underpinning these observations during viral infection have not been investigated.

It is important to understand how myeloid cells develop and how their population dynamics are regulated in order to design therapeutics tailored to retard or accelerate the development of specific myeloid populations, depending on the disease- or infection-state[48]. Here, we have elucidated novel roles

for TLR7 and type I IFN in both directly and indirectly changing myeloid cell development. Furthermore, we have illustrated the roles of these signaling pathways in hematopoietic and non-hematopoietic cells as being key to driving the egress of inflammatory monocytes from the bone marrow into the bloodstream early after viral infection. Recombinant type I IFN is used as a treatment for some cancers[49], Multiple Sclerosis[50] and Hepatitis C Virus infection[51] but has shown limited efficacy in these situations. Additionally, type I IFN and TLR7 signaling are important in the pathogenesis of the autoimmune disease systemic lupus (SLE)[52]. Therefore, in addition to enhancing our knowledge of basic immunology, our work elucidating how TLR7 and the type I IFN cytokine family act to influence myeloid cell homeostasis may have clinical implications for cancer, viral infections and autoimmune disorders.

	Gating strategy
Progenitors	
LT-HSC	$Lin^-CD117^+Sca1^+CD135^-CD34^-$
ST-HSC	$Lin^-CD117^+Sca1^+CD135^-CD34^+$
MPP	$Lin^-CD117^+Sca1^+CD135^+CD34^+$
CMP	$Lin^-CD117^+Sca1^-CD16/32^{low/-}CD34^+$
GMP	$Lin^-CD117^+Sca1^-CD16/32^+CD34^+$
CDP	$Lin^-Sca1^-CD135^+IL7R\alpha^-$
eGMP	$Lin^-CD117^+Sca1^+CD16/32^+CD34^+$
CLP	$Lin^-CD117^{int}Sca1^{int}IL7R\alpha^+$
Data not shown	
LT-HSC	$Lin^-CD117^+Sca1^+CD135^-CD150^+CD34^-$
ST-HSC	$Lin^-CD117^+Sca1^+CD135^-CD150^+CD34^+$
MPP	$Lin^-CD117^+Sca1^+CD135^+CD150^-CD34^+$
Mature Bone Marrow	
pDC	$CD11b^-$Siglec H^+BST2^+
Inflammatory Monocyte	$CD11b^+Ly6C^+Ly6G^-$
Neutrophil	$CD11b^+Ly6C^+Ly6G^+$
Spleen	
pDC	Siglec H^+BST2^+
$CD8\alpha^+$DC	$BST2^-$/Siglec $H^-B220^-/CD19^-CD11c^+MHCII^+CD8\alpha^+$
$33D1^+$DC	$BST2^-$/Siglec $H^-B220^-/CD19^-CD11c^+MHCII^+CD8\alpha^-33D1^+$
$CD8\alpha^-33D1^-$DC	$BST2^-$/Siglec $H^-B220^-/CD19^-CD11c^+MHCII^+CD8\alpha^-33D1^-$
Inflammatory monocyte	$CD11b^+Ly6C^+Ly6G^-$
Neutrophil	$CD11b^+Ly6C^+Ly6G^+$

Table 1. Hematopoietic stem, lymphoid- or myeloid-committed progenitor cells, their mature progeny and the phenotypic markers used to identify them in our studies.

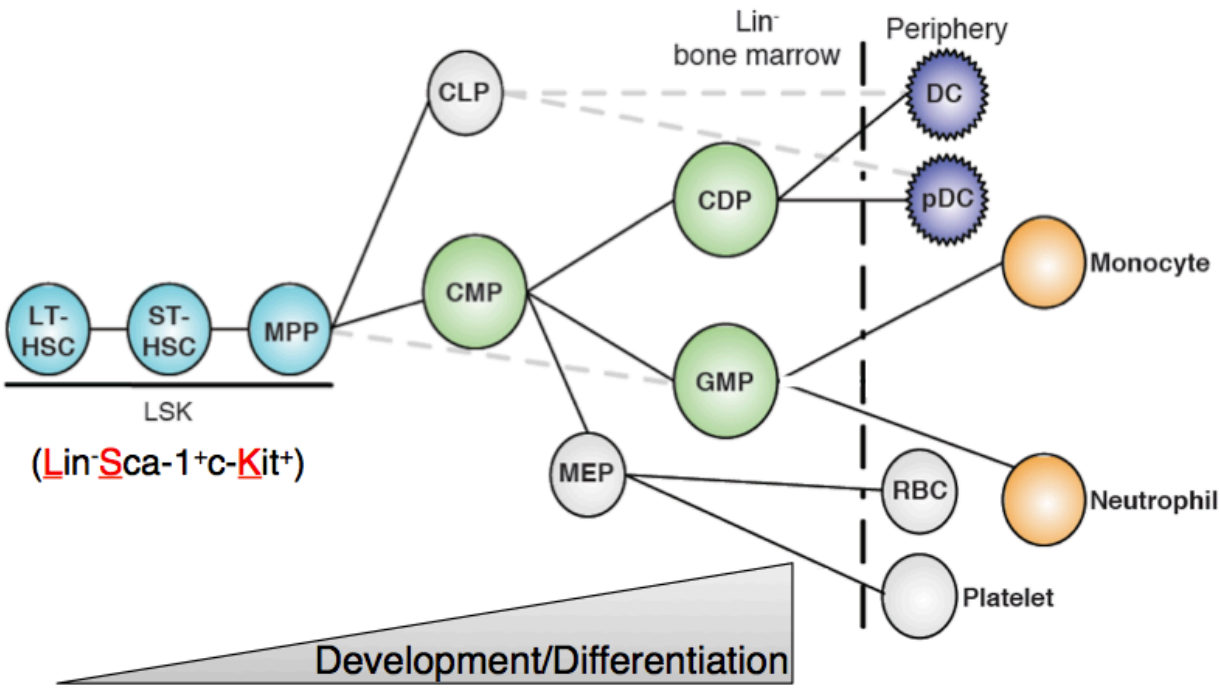

Figure 1.1. Myeloid development.

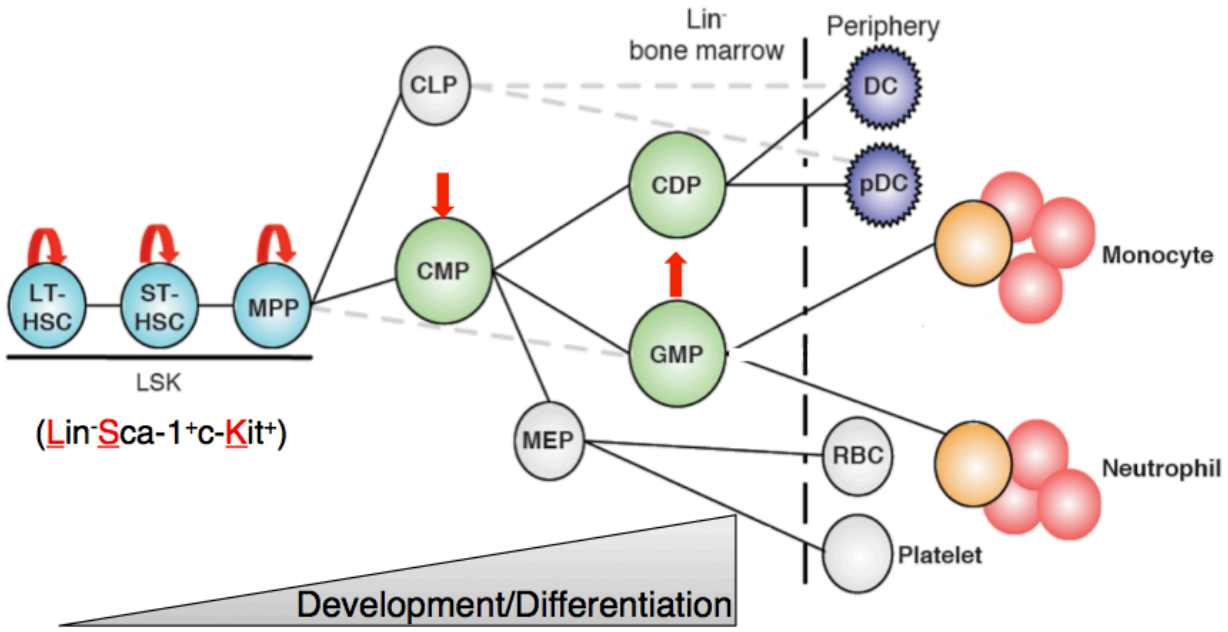

Figure 1.2. Emergency myelopoiesis.

Hallmarks of emergency myelopoiesis in the bone marrow include an increase in LSK, a decrease in CMP, a decrease in Megakaryocyte erythrocyte precursors (MEP) and an increase in GMP. In the periphery, these hematopoietic changes result in increased monocytes and neutrophils.

Chapter 2: Type I IFN Drives Emergency Myelopoiesis and Peripheral Myeloid Expansion during Chronic TLR7 Signaling

Abstract

Mice overexpressing TLR7 (TLR7.1 mice) are a model of SLE pathogenesis and exhibit peripheral myeloid expansion. We show that TLR7.1 mice have a dramatic expansion of splenic cells that derive from granulocyte/macrophage progenitors (GMP) compared to WT mice. In the bone marrow, TLR7.1 mice exhibited hallmarks of emergency myelopoiesis and contained a discrete population of Sca-1^+ GMP, termed emergency GMP (eGMP), that are more proliferative and superior myeloid precursors than classical Sca-1^- GMP. The emergency myelopoiesis and peripheral myeloid expansion in TLR7.1 mice was dependent on type I IFN signaling. TLR7 agonist administration to non-transgenic mice also drove type I IFN-dependent emergency myelopoiesis. TLR7.1 plasmacytoid DC were cell-intrinsically activated by TLR7 overexpression and constitutively produced type I IFN directly ex vivo. This work shows that type I IFN can act upon myeloid progenitors to promote the development of eGMP, which leads to an expansion of their progeny in the periphery.

Introduction

The hematopoietic system is capable of rapidly increasing myeloid cell output to combat pathogens. Accordingly, viral, bacterial and parasitic infection results in significant reprogramming of steady state hematopoiesis[39]. This demand-driven hematopoietic state is a component of "emergency myelopoiesis," a process in which myeloid effector cells are rapidly generated in the bone marrow (BM) and shunted to the periphery[27,39].

The mechanisms governing the shift from steady state hematopoiesis to emergency myelopoiesis are not completely understood. In vitro studies indicate that TLR signaling in hematopoietic progenitor cells can intrinsically alter their development into different hematopoietic lineages[9]. TLR signaling may also indirectly influence hematopoiesis by inducing production of cytokines, including the type I IFN family. Type I IFN can act directly on quiescent Long-Term Hematopoietic Stem Cells (LT-HSC) and cause them to enter the cell cycle[40], but the role of type I IFN in influencing myeloid development is unknown. Here, we use mice with myeloid expansion due to overexpression of TLR7 through a bacterial artificial chromosome transgene (TLR7.1 mice), TLR7 agonist administration and in vitro culture of myeloid progenitor cells[53] to show that type I IFN driven by chronic TLR7 signaling can drive emergency myelopoiesis.

Results and Discussion

TLR7.1 mice have differential expansion of splenic myeloid populations

TLR7.1 mice were previously shown to develop severe splenomegaly characterized by the expansion of cells expressing the surface markers CD11c and CD11b[53], which mark a variety of myeloid populations. We more precisely defined the myeloid expansion in TLR7.1 mice and observed that TLR7.1 mice exhibited an increased frequency and absolute number of inflammatory monocytes and neutrophils in their spleens compared to WT mice (Fig. 2.1A). Unexpectedly, we found that TLR7.1 mice exhibited a diminished percentage of splenic plasmacytoid dendritic cells (pDC), CD8α$^+$ DC and CD8α$^-$33D1$^+$ DC, but an increased absolute number compared to WT mice (Fig. 2.1B). We also identified a CD11c$^+$MHCII$^+$ DC population lacking expression of CD8α and 33D1 that was increased by percentage and absolute number in TLR7.1 mice compared to WT mice (Fig. 2.1B). CD8α$^-$33D1$^-$ DC expressed high levels of CD11b, Sirpα, and CX3CR1, but low levels of the endothelial marker ESAM (Fig. 2.1C), indicating they belong to the granulocyte/macrophage progenitor (GMP)-derived ESAM^{lo} subset of CD11b$^+$ DC[54].

The paradoxical decrease in percentage and increase in absolute number of pDC, CD8α$^+$ DC and CD8α$^-$33D1$^+$ DC was due to the significant increase in spleen size and cellularity in TLR7.1 mice compared to WT mice (Fig. 2.1D). To resolve a clearer picture of splenic myeloid homeostasis in TLR7.1 mice, we calculated the fold change in cell number of each myeloid population between TLR7.1 and WT mice. This showed that GMP-derived inflammatory monocytes, neutrophils and CD8α$^-$33D1$^-$ DC were dramatically expanded (20-25x increase), whereas common dendritic cell progenitor (CDP)-derived CD8α$^-$33D1$^+$ DC, CD8α$^+$ DC and pDC were modestly increased (2-3x increase) in TLR7.1 mice compared to WT mice (Fig. 2.1E).

TLR7.1 mice exhibit emergency myelopoiesis

In the steady state, myeloid cells develop in a step-wise fashion from LT-HSC to short term-HSC (ST-HSC) to multipotent progenitor cells (MPP), which together compose the "LSK" (Lineage$^-$Sca1$^+$c-Kit$^+$) fraction of the BM. MPP mature into common myeloid progenitors (CMP) or lymphoid-primed MPP, that further develop through a series of increasingly committed progenitor cells, such as the GMP and CDP, to give rise to mature myeloid cells[55]. During inflammation, hematopoiesis shifts toward emergency myelopoiesis to rapidly generate more effector cells. Due to the preferential expansion of GMP progeny in

TLR7.1 mice, we hypothesized that TLR7 overexpression caused a permanent state of emergency myelopoiesis leading to peripheral myeloid expansion.

TLR7.1 mice had an increased absolute number of LSK cells compared to WT mice, with significant increases in each of the three progenitors that comprise this compartment, LT-HSC, ST-HSC and MPP, identified using either *Flk2*/CD135 (Fig. 2.2A, B) or the SLAM family member CD150 (data not shown). TLR7.1 mice had fewer CMP and more GMP compared to WT mice using steady state gating strategies (Fig. 2.2A, 2.2C). Strikingly, TLR7.1 mice exhibited a significant increase in Sca-1$^+$ GMP, identified as CD34$^+$CD16/32$^+$ cells within the LSK gate, compared to WT mice (Fig. 2.2A, 2.2D). We termed these Sca-1$^+$ GMP "emergency GMP" (eGMP) as they appear during emergency myelopoiesis in this system, as well as during *Ehrlichia muris* and *Plasmodium chabaudi* infection[26,31]. Therefore, TLR7.1 mice are undergoing emergency myelopoiesis in the BM, characterized by an increase in LSK cells, a decrease in CMP, an expansion of GMP and the appearance of eGMP. The number of CDP and common lymphoid precursor cells (CLP), which have been shown to have DC potential[9], were similar in WT and TLR7.1 mice (Fig. 2.1E).

Cell-extrinsic changes in myeloid development in TLR7.1 mice.

The alterations in myelopoiesis and peripheral myeloid homeostasis in TLR7.1 mice suggested that TLR7 overexpression might drive a cell-intrinsic expansion of hematopoietic progenitor cells. We first examined TLR7 expression in hematopoietic progenitors by qPCR. *Tlr7* mRNA was expressed in LSK, CMP and GMP in WT mice and overexpressed 4-8 fold in TLR7.1 mice (Fig. 2.3A). Additionally, *Tlr7* mRNA was expressed in mature WT myeloid cells in the spleen and BM and overexpressed 3-12 fold in TLR7.1 mice.

To assess whether TLR7 overexpression drives cell-intrinsic myeloid expansion in TLR7.1 mice, we generated mixed BM chimeric mice in which lethally irradiated hosts were reconstituted with congenically marked WT and TLR7.1 BM at a 1:1 ratio. Both TLR7.1:WT mixed BM chimeric mice and WT mice reconstituted with 100% TLR7.1 BM exhibited splenomegaly, showing that splenic expansion occurs when TLR7 is overexpressed only in hematopoietic cells (Fig. 2.3B). TLR7.1:WT mixed BM chimeric mice exhibited a phenotype similar to TLR7.1 mice including increases in GMP and eGMP in the BM and expansion of inflammatory monocytes and neutrophils in the spleen compared to control

chimeras (data not shown). However, we saw no advantage of TLR7.1 over WT BM hematopoietic progenitors (Fig. 2.3C). This was surprising as LSK, CMP and GMP have been shown to express TLR, and in vitro TLR ligation can cause the myeloid differentiation of these progenitors[9]. We also saw no advantage of TLR7.1 cells in producing neutrophils or monocytes (data not shown). Our observation that TLR7 signals in BM progenitor cells do not drive myeloid differentiation in vivo leaves open the question of why LSK, CMP and GMP express TLR and if there particular situations where TLR signaling can intrinsically drive myeloid differentiation in vivo.

TLR7.1 mice exhibit a type I IFN signature in BM myeloid progenitors.

Because emergency myelopoiesis in TLR7.1 mice was cell-extrinsic, we looked for alterations in serum cytokines in TLR7.1 mice. We found no differences in cytokines with known roles in myelopoiesis (Fig. 2.3D and data not shown). Next, we asked whether type I IFN, a family of cytokines implicated in early hematopoiesis, inflammation and SLE, but not included in our cytokine panel, might be increased in the BM of TLR7.1 mice. TLR7.1 mice have an IFN signature in the spleen[53], though we were unable to detect IFNα or IFNβ protein in the serum of TLR7.1 mice by ELISA or bioassay (data not shown). Therefore, we focused on whether type I IFN was acting directly on hematopoietic progenitor cells in TLR7.1 mice. We sorted total LSK, CMP and GMP from WT and TLR7.1 BM and assayed these cells by qPCR for expression of interferon-stimulated genes (ISG). TLR7.1 progenitors exhibited higher expression of ISG than WT cells (Fig. 2.4A), showing that LSK and myeloid progenitor cells in TLR7.1 mice have been exposed to and responded to type I IFN in vivo.

Bone marrow pDC are cell-intrinsically activated by TLR7 overexpression and constitutively produce type I IFN.

The ISG expression in TLR7.1 myeloid progenitors suggested that increases in type I IFN in the BM environment may cause the myeloid expansion seen in TLR7.1 mice. We hypothesized that pDC in the BM, which express the highest amounts of *Tlr7* mRNA in both WT and TLR7.1 mice (Fig. 2.3A), were generating type I IFN constitutively due to TLR7 overexpression. TLR7.1 BM pDC had increased expression of MHCII compared to WT BM pDC (Fig. 2.4B, Fig. 2.3E), supporting the hypothesis that TLR7 overexpression leads to pDC activation. Analysis of mixed BM chimeras showed that within the

same mouse, TLR7.1 BM pDC had higher surface MHCII than WT BM pDC (Fig. 2.4C, Fig. 2.3F). Therefore, BM pDC were cell-intrinsically activated due to TLR7 overexpression.

Next, we examined whether TLR7.1 BM pDC were generating type I IFN. pDC, inflammatory monocytes and neutrophils were sorted from the BM of WT and TLR7.1 mice and prepared for qPCR directly ex vivo. TLR7.1 BM pDC were generating higher amounts of *Ifna1* and *Ifnb* mRNA in vivo compared to WT BM pDC (Fig. 2.4D). Neither BM inflammatory monocytes nor neutrophils were constitutively producing type I IFN mRNA (Fig. 2.4D). These data indicate that TLR7 overexpression drives cell-intrinsic activation of BM pDC and the generation of type I IFN that may then act upon BM hematopoietic progenitor cells. It is interesting to speculate that these professional type I IFN-producing cells are found in the BM to mediate type I IFN-dependent changes in hematopoiesis.

Lack of type I IFN signaling limits emergency myelopoiesis in TLR7.1 mice

To directly test whether type I IFN is responsible for the peripheral myeloid expansion and emergency myelopoiesis observed in TLR7.1 mice, we bred TLR7.1 mice to mice lacking the type I IFN receptor (IFNARKO). The weight and overall cellularity of the spleens of TLR7.1xIFNARKO mice were significantly reduced compared to TLR7.1 mice, though they were still increased compared to WT mice (Fig. 2.5A and data not shown). The increase in absolute number of TLR7.1 inflammatory monocytes and neutrophils compared to WT was largely dependent on type I IFN signaling, whereas the number of $CD8\alpha^{-}33D1^{-}$ DC, though significantly lower in TLR7.1xIFNARKO mice than TLR7.1 mice, was more modestly reduced (Fig. 2.5B).

As we observed mitigation of the TLR7.1 peripheral myeloid expansion in TLR7.1xIFNARKO mice, we postulated that the emergency myelopoiesis observed in TLR7.1 mice would be diminished. Consistent with this hypothesis, the increased number of LSK cells in TLR7.1 mice was almost completely reduced to WT numbers in TLR7xIFNARKO mice (Fig. 2.3G). The reduction in CMP numbers in TLR7.1 mice was reversed in the TLR7.1xIFNARKO mice, but the increase in GMP numbers was not affected by the lack of type I IFN signaling (Fig. 2.5C). Strikingly, eGMP were nearly absent in TLR7.1xIFNARKO mice (Fig. 2.5D). Therefore, as emergency myelopoiesis and peripheral myeloid expansion seen in the TLR7.1 mice largely depends upon type I IFN signaling, we believe that TLR7.1 mice represent a model of chronic type I IFN-driven myeloid expansion.

TLR7 agonist administration drives emergency myelopoiesis in WT, but not IFNARKO mice

To assess whether chronic TLR7 signaling can drive type I IFN-dependent emergency myelopoiesis in a non-transgenic system, the TLR7 agonist gardiquimod was serially administered to WT and IFNARKO mice for 6 days. A significant increase in eGMP was observed in WT, but not IFNARKO, mice treated with TLR7 agonist (Fig. 2.6A, 2.6B). Further, other hematopoietic alterations consistent with the process of emergency myelopoiesis, such as increases in LSK, decreases in CMP and increases in GMP were observed in WT mice treated with TLR7 agonist (Fig. 2.3H). This approach validated our observations made in the TLR7.1 system that TLR7-driven type I IFN can act upon bone marrow progenitor cells to drive emergency myelopoiesis.

eGMP produce more myeloid cells in vitro and are more proliferative than GMP in vivo

We hypothesized that type I IFN-dependent eGMP are better myeloid progenitors than GMP. To test this prediction, equivalent numbers of WT GMP, TLR7.1 GMP and TLR7.1 eGMP were sorted and plated in methylcellulose media containing IL-3, IL-6 and SCF, which support differentiation of granulocyte and macrophage colonies. eGMP generated more total colonies, and a greater proportion of these colonies were mixed-lineage granulocyte/macrophage CFU (CFU-GM), than GMP (Fig. 2.7A and Fig. 2.3I). eGMP-derived colonies were also much larger than those generated by GMP (data not shown). Strikingly, TLR7.1 eGMP produced approximately 4-fold more cells than WT or TLR7.1 GMP (Fig. 2.7B, 2.7C). GMP yielded similar colony numbers, proportions of CFU-GM and cell numbers whether they derived from WT or TLR7.1 mice, suggesting that GMP exposed to type 1 IFN, but that do not express Sca-1, do not increase their differentiative capacity. These data demonstrate that eGMP are superior progenitors of myeloid cells compared to GMP.

Our in vivo observations and our in vitro CFU data suggest that eGMP possess different functional properties than GMP that allow them to more efficiently generate mature myeloid cells. GMP were identified by flow cytometry as being Sca-1^{-}, whereas eGMP were Sca-1^{+}. Interestingly, Sca-1 expression has been shown to correlate with cell cycle activity in hematopoietic progenitor cells during some settings of emergency hematopoiesis[40,56]. Significantly more TLR7.1 eGMP had progressed through S phase of the cell cycle during a one hour BrdU pulse than GMP from either WT or TLR7.1 mice (Fig. 2.7D). This increased proliferation defines a functional difference between eGMP and GMP that may

allow eGMP to more efficiently generate myeloid progeny. Sca-1, a gpi-anchored cell surface protein, has been shown to drive ERK phosphorylation and the MEK/ERK axis promotes both myeloid differentiation and proliferation[56-58]. Therefore, Sca-1 may signal though ERK to drive both proliferation and differentiation of eGMP. Interestingly, cells sharing the phenotype of eGMP have been observed in *Ehrlichia muris* and *Plasmodium chabaudi* infection and have been shown to be type II IFN dependent[26,31]. Thus, our work is the first to define the role of type I IFN in driving eGMP generation.

Here we link chronic TLR7 signaling to type I IFN production, which can then act at the level of hematopoiesis to drive emergency myelopoiesis and peripheral expansion of inflammatory monocytes and neutrophils. This may be the mechanism underlying a number of previously published models of TLR7-driven inflammation. For example, SLE-prone BXSB mice and mice serially treated with TLR7/8 agonist display selective increases in inflammatory monocytes and neutrophils[59,60]. *Unc93b1*^{D34A} mutant mice, in which TLR7 signaling is increased, exhibit increases in spleen size and expansion of CD11b^{+}CD11c^{+} cells, inflammatory monocytes and neutrophils, similar to TLR7.1 mice[61]. The etiology of these observations was unclear, and our results would suggest that type I IFN-driven emergency myelopoiesis is occurring.

Our results are particularly interesting given that TLR7.1 mice are a model of SLE. SLE patients have gene signatures in their blood reflective of both type I IFN signaling and granulopoiesis[62,63]. SLE pathogenesis is driven in part by immune complexes, formed by anti-nucleic acid antibodies and their antigens provided by dying neutrophils undergoing type I IFN-driven NETosis. These immune complexes are recognized by pDC and elicit TLR-dependent type I IFN secretion, generating an autoimmune circuit[64-66]. Our data suggest that type I IFN produced by pDC due to chronic TLR7 signaling promotes development of neutrophils in the BM, which may then go on to participate in the chronic feed-forward loop between neutrophils and pDC; thereby, providing a mechanistic link between the granulopoiesis and type I IFN signatures in SLE. In support of this model, TLR7.1 mice require B cells for myeloid expansion[66], suggesting that RNA/anti-RNA immune complexes drive pDC type I IFN production in these mice. Our observations linking type I IFN to myelopoiesis may also have implications for other situations of increased type I IFN, including acute and chronic viral infections as well as type I IFN treatment of infections, such as hepatitis C virus.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River or bred in our vivarium. TLR7.1 mice [53] and IFNARKO mice were provided by S. Bolland (NIH) and D.B. Stetson (University of Washington), respectively. Mixed BM chimeric mice were generated by lethally irradiating C57BL/6 x B6.SJL F1 mice and reconstituting them with a 1:1 ratio of C57BL/6 (CD45.2) and B6.SJL (CD45.1) or TLR7.1 (CD45.2) and B6.SJL (CD45.1) BM cells. Mice were housed at the University of Washington or Benaroya Research Institute. Experiments were performed under IACUC-approved protocols.

Cell isolation

Lineage-negative progenitor cells from 2 femurs and tibias were enriched from RBC-lysed BM using a Lineage Cell Depletion Kit (Miltenyi Biotec). Biotin-conjugated mAbs to CD11b, Gr1, F4/80, CD11c, CD3 and NK1.1 were added to the lineage-negative cell suspensions for negative gating during flow cytometry. Splenocytes were isolated by collagenase (type IV, Worthington)/DNase (Sigma Aldrich) digestion followed by calcium chelation and RBC lysis.

Flow cytometry, cell sorting, BrdU and TLR7 agonist administration

For surface staining, 2-4 x 10^6 cells were blocked with fluorescently labeled (lineage negative progenitors) or unlabeled (spleen) anti-CD16/32 mAb followed by the addition of mAbs as indicated. Samples were incubated at 4C for 30 minutes, except for anti-CD34 stains for 60-90 min. All mAbs were from eBioscience, Biolegend or Invitrogen. Data were acquired using a LSRII (BD Biosciences) and analyzed using FlowJo (TreeStar). Doublets were excluded before gating on live cells using FSC and SSC. For BrdU incorporation, mice were injected i.p. with 1 mg of BrdU (Sigma Aldrich) 1 hour before sacrifice. BrdU incorporation was assayed by using the BD BrdU Flow Kit procedure (BD Biosciences). Where indicated, 10 μg of the TLR7 agonist gardiquimod (Invivogen) was administered i.v. every other day over the course of 6 days.

Quantitative real-time PCR (qPCR) and primer sequences

Samples were prepared as previously described[67]. Primer sequences are listed below.

Tlr7	F	5' GTT CTT GAC CTT GGC ACT A 3'
219 bp	R	5' CCG TGC ATA TTC ATC GTA 3'
Ifit1	F	5' TGC TGA GAT GGA CTG TGA GG 3'
100 bp	R	5' CTC CAC TTT CAG AGC CTT CG 3'
Isg15	F	5' AAG CAG CCA GAA GCA GAC TC 3'
167 bp	R	5' CAC CAA TCT TCT GGG CAA TC 3'
Mcp1	F	5' TAG TTT TTG TCA CCA AGC TC 3'
100 bp	R	5' GAT CTC ATT TGG TTC CGA TCC 3'
Mx1	F	5' GGC AGA CAC CAC ATA CAA CC 3'
115 bp	R	5' CCT CAG GCT AGA TGG CAA G 3'
Oas1	F	5' CTT AGC ATG GAG CAC GGA CT 3'
156 bp	R	5' AGC ACC TTG GAA GCA TCT CTC 3'
Ifna1	F	5' AGT GAG CTG ACC CAG CAG AT 3'
166 bp	R	5' GGT GGA GGT CAT TGC AGA AT 3'
Ifnb	F	5' CTC CAC CAC AGC CCT CTC 3'
157 bp	R	5' CAT CTT CTC CGT CAT CTC CAT AG 3'
18s	F	5' GAG GGA GCC TGA GAA ACG G 3'
68 bp	R	5' GTC GGG AGT GGG TAA TTT GC 3'

Bio-plex assay

Serum samples were analyzed using Bio-Plex Pro Mouse Cytokine 23-plex Assay with a Bio-Plex 200 system (Bio-rad).

Colony forming unit assays

250 GMP or eGMP from WT or TLR7.1 mice were sorted and plated in duplicate for 6 days in Methocult GF M3534 (Stemcell Technologies). Colonies were scored on day 5. On day 6, cells were extracted and quantified by flow cytometry using polystyrene counting beads (Polysciences, Inc.).

Data presentation and statistical analysis

Graphs and histograms were generated using Prism (GraphPad) using a two-tailed Student's t-test or one-way ANOVA with a tukey post-test, as noted. Error bars correspond to SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Chapter 2 Figures

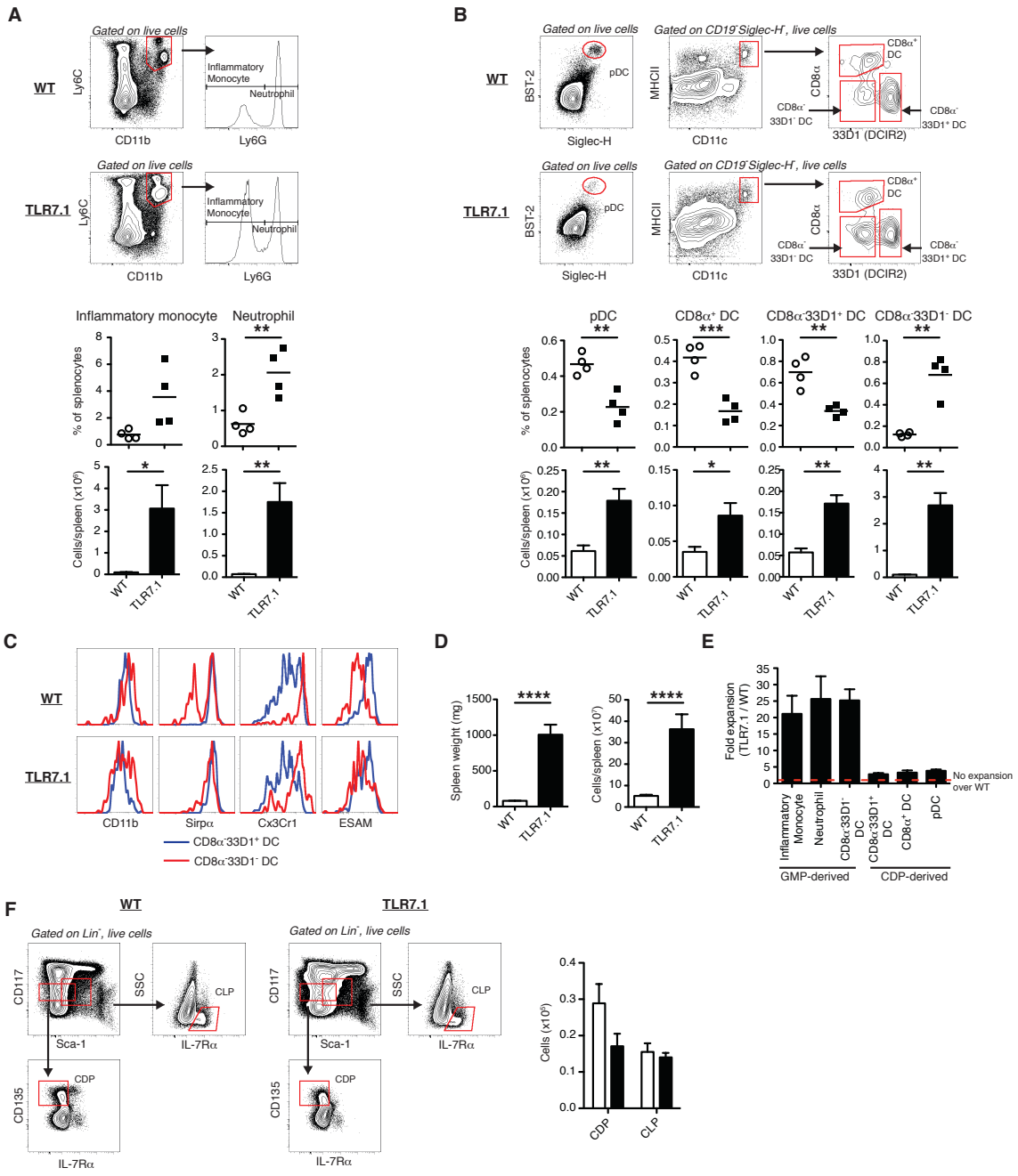

Figure 2.1. TLR7.1 mice have differential expansion of splenic myeloid populations compared to WT mice and no change in BM CDP or CLP.

(**A**) Gating strategy and representative flow cytometry plots for splenic inflammatory monocytes and neutrophils. Percentage and number of splenic inflammatory monocytes and neutrophils. Data are representative of 5 experiments. (**C**) Gating strategy for splenic pDC and DC subsets. Percentage and number of splenic pDC and DC subsets. Data are representative of 3 experiments (pDC), 10 experiments

(CD8α$^+$ DC), 6 experiments (CD8α$^-$33D1$^+$ DC and CD8α$^-$33D1$^-$ DC). (**C**) CD8α$^-$33D1$^-$ DC were gated on as in Supplemental Fig. 1C. Data are representative of three experiments. (**D**) Spleen weight and cellularity are from 6 experiments. (**E**) Fold change in absolute number of cells between TLR7.1 and WT mice was determined by averaging the number of WT and TLR7.1 cells and then dividing the TLR7.1 value by the WT value. Dotted line represents no expansion (value of 1). Data are from 4 experiments (CD8α^-33D1$^+$ DC, CD8α^-33D1$^+$ DC, inflammatory monocytes and neutrophils) or 6 experiments (pDC and CD8α^+ DC). (**F**) Gating strategy and representative flow cytometry plots for CDP and CLP in WT and TLR7.1 mice and number of CDP and CLP in WT and TLR7.1 mice. Data are from 3 experiments. (**A-B, D-F**) Data represent at least 12 mice per genotype. Significance determined by two-tailed, unpaired student's t-test.

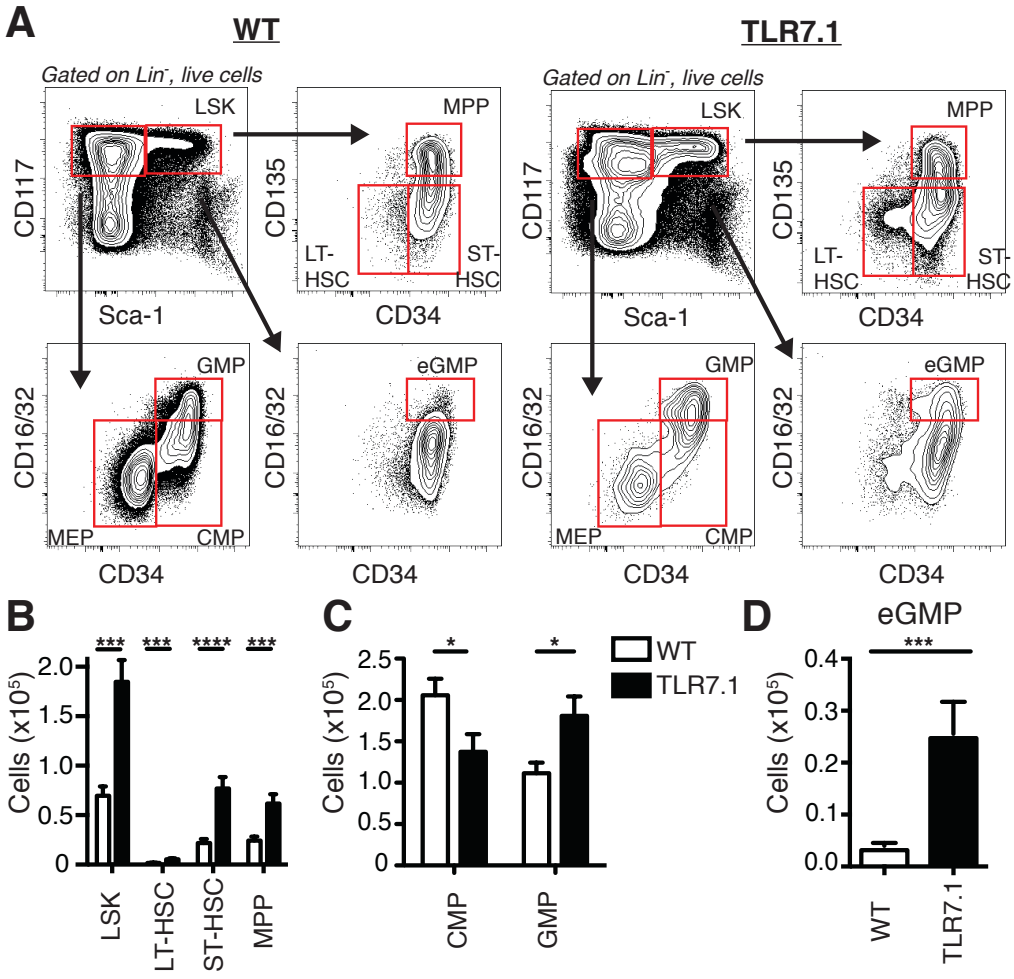

Figure 2.2. TLR7.1 mice exhibit emergency myelopoiesis in the bone marrow.

(**A**) Gating strategy and representative flow cytometry plots for BM LSK, CMP, GMP and eGMP. (**B**) Number of LT-HSC, ST-HSC and MPP from 3-5 experiments. (**C, D**) Number of CMP, GMP (C) and eGMP (D) from 5 independent experiments. Significance determined by two-tailed, unpaired student's t-test.

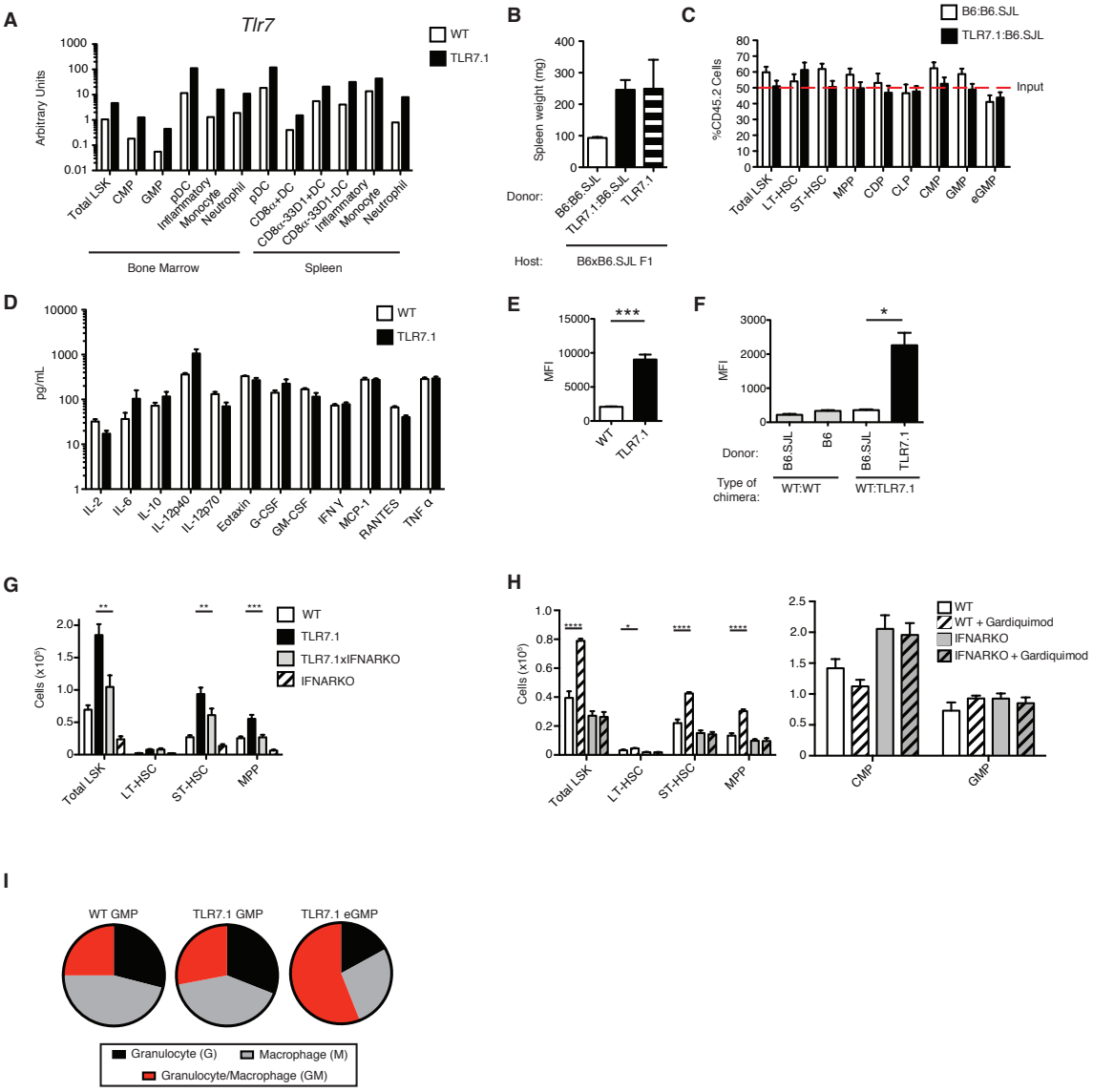

Figure 2.3 TLR7.1 phenotype is cell-extrinsic, not due to circulating inflammatory cytokines, TLR7.1xIFNARKO mice exhibit rescue of LSK expansion compared to TLR7.1 mice, TLR7 agonist administration drives type I IFN-dependent emergency myelopoiesis and TLR7.1 eGMP preferentially generate GM CFU compared to GMP.

(**A**) TLR7 expression in bone marrow progenitors, mature bone marrow cells and mature splenic cells as assessed by qPCR (>2 mice from each genotype were pooled and cells were sorted as in Fig. 2.2A and Fig. 2.1A, 2.1B; data are from two independent experiments). (**B**) Spleen weight in BM chimeras generated with mixed B6:B6.SJL, TLR7.1:B6.SJL or TLR7.1 bone marrow only transferred into irradiated B6 x B6.SJL F1 mice. B6:B6.SJL and TLR7.1:B6.SJL data are from four experiments with a total of 14 B6:B6.SJL and 15 TLR7.1:B6.SJL mixed BM chimeras; TLR7.1 bone marrow only transferred into irradiated B6 x B6.SJL F1 mice data are from 2 mice. (**C**) Percentage of $CD45.2^+$ lineage negative bone marrow progenitor cells from B6:B6.SJL (B6; white) and TLR7.1:B6.SJL (TLR7.1; black) mixed bone marrow chimeras. Data are from four experiments. B6 x B6.SJL F1 mice were used as controls for gating on $CD45.1^+$ and $CD45.2^+$ cells. (**D**) Serum from 11 WT and 8 TLR7.1 mice were analyzed for cytokines by 23-cytokine Bio-plex assay. Additional cytokines surveyed included IL-1α, -1b, -3, -4, -5, -9, -13, -17, KC, MIP1α, and MIP1β (data not shown). Data are represented as mean + SEM. (**E**) Bar graph shows the mean fluorescence intensity of MHCII staining on BM pDC. Data show 6 mice per group and are representative of 5 experiments. (**F**) MHCII expression on $CD45.2^+$ and $CD45.1^+$ BM pDC from the same mixed BM chimeric mice is shown. Bar graph shows the mean fluorescence intensity of MHCII staining for 4 mice per group and is representative of 3 experiments. (**G**) LSK WT, TLR7.1, TLR7.1xIFNARKO and IFNARKO mice were gated on as in Fig. 2.1A. Data were from 3-8 independent experiments representing at least 12 mice per genotype. (**H**) Mice were serially treated with gardiquimod as in Fig. 4. Cells were identified as in Figure 1A. Data are from two experiments with 6 mice per group. (**I**) Cells were plated as in Figure 2.7A-C. Colonies were scored on day 5 at 5-10x magnification based on manufacturer's directions. Data are from three mice of each genotype and are from 2 experiments. In bar graphs, data are represented as mean + SEM (**B-H**) or mean (**A**); Significance determined by two-tailed, unpaired student's t-test (**C-F**) or one-way ANOVA with a tukey post-test (**F-H**).

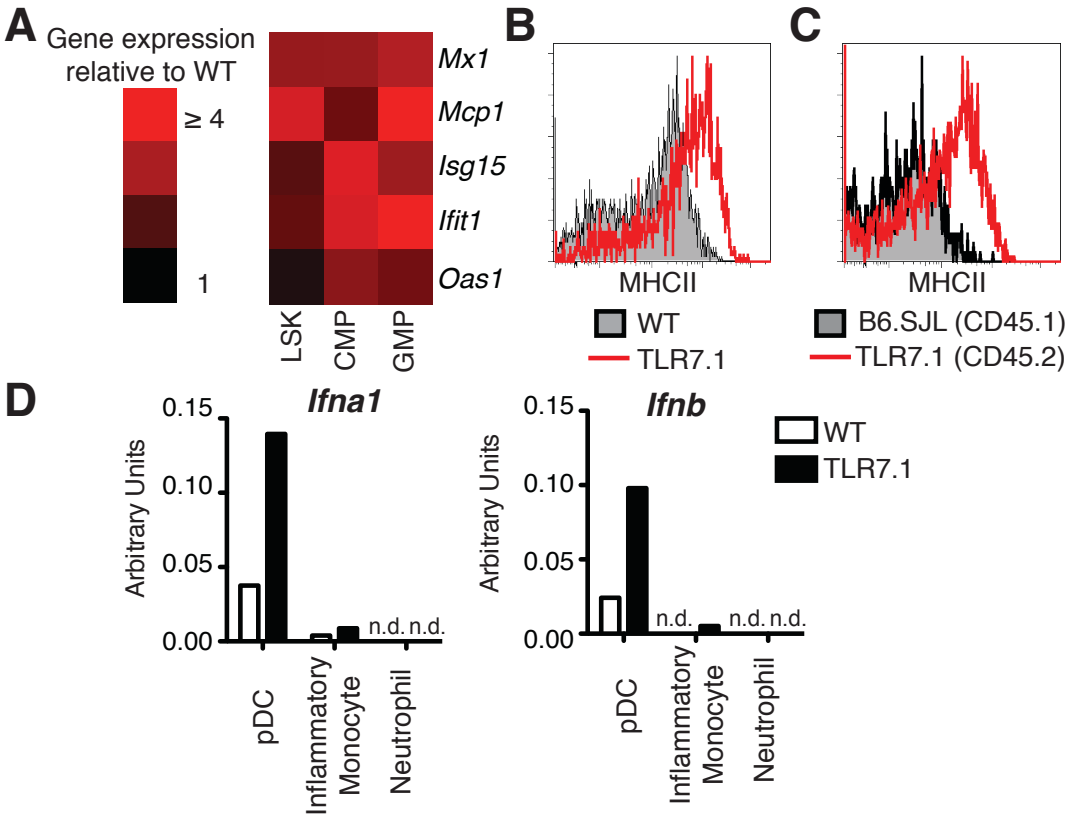

Figure 2.4 TLR7.1 myeloid progenitors exhibit a type I IFN signature in the bone marrow where pDC are constitutively activated and generating type I IFN mRNA.

(**A**) ISG mRNA from BM progenitors, sorted as in Fig. 1A and assayed by qPCR. Data are from 2 independent experiments. (**B**) BM pDC from WT and TLR7.1 mice were gated on as $CD11b^{-}Siglec\text{-}H^{+}BST\text{-}2^{+}$ and a histogram of MHCII expression is shown, representative of 5 independent experiments. (**C**) BM pDC from mixed BM chimeras generated with a 1:1 ratio of TLR7.1 and B6.SJL BM were gated as $CD45.2^{+}$ and $CD45.1^{+}$, respectively. MHCII expression on $CD45.2^{+}$ and $CD45.1^{+}$ BM pDC from the same mouse are shown, representative of 3 independent experiments. (**D**) BM cells were sorted (pDC as in Fig. 2B; inflammatory monocytes $CD11b^{+}Ly6C^{+}Ly6G^{-}$; neutrophils $CD11b^{+}Ly6C^{+}Ly6G^{+}$) and used for qPCR. Data are representative of two experiments. n.d. not detected.

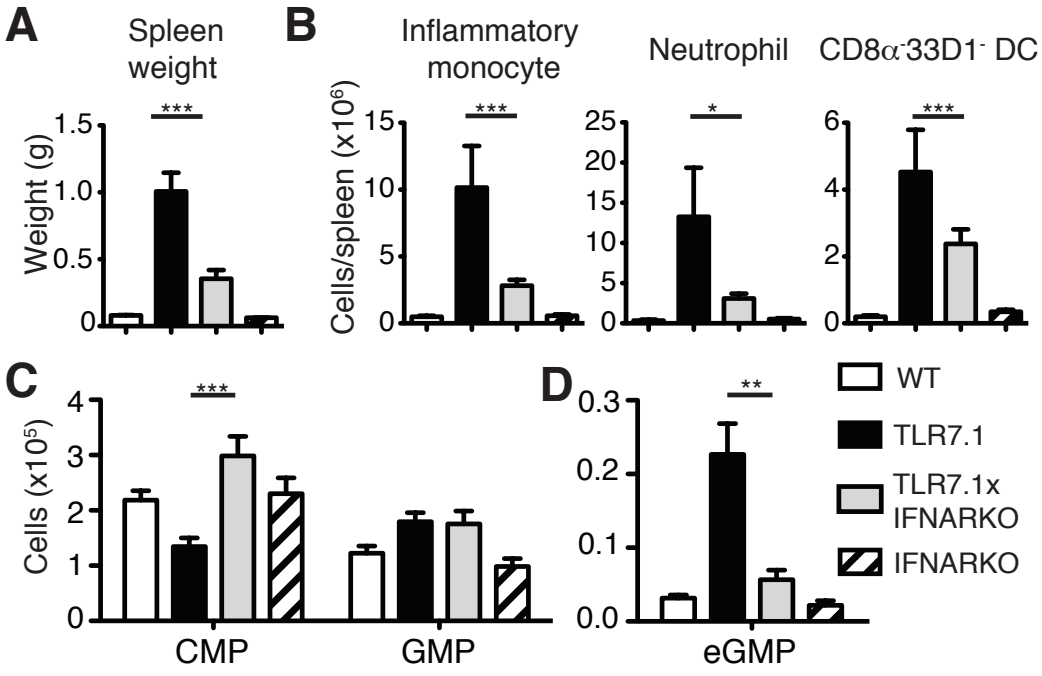

Figure 2.5. TLR7.1 emergency myelopoiesis depends on type I IFN signaling.

(**A**) Spleen weight. (**B**) Number of splenic myeloid cells were gated on as in Fig. S1A and B. (**C-D**) BM progenitor cells were gated on as in Fig. 1A. All data represent the mean of 12-15 mice assessed in at least 3 independent experiments. Significance determined by one-way ANOVA with a tukey post-test.

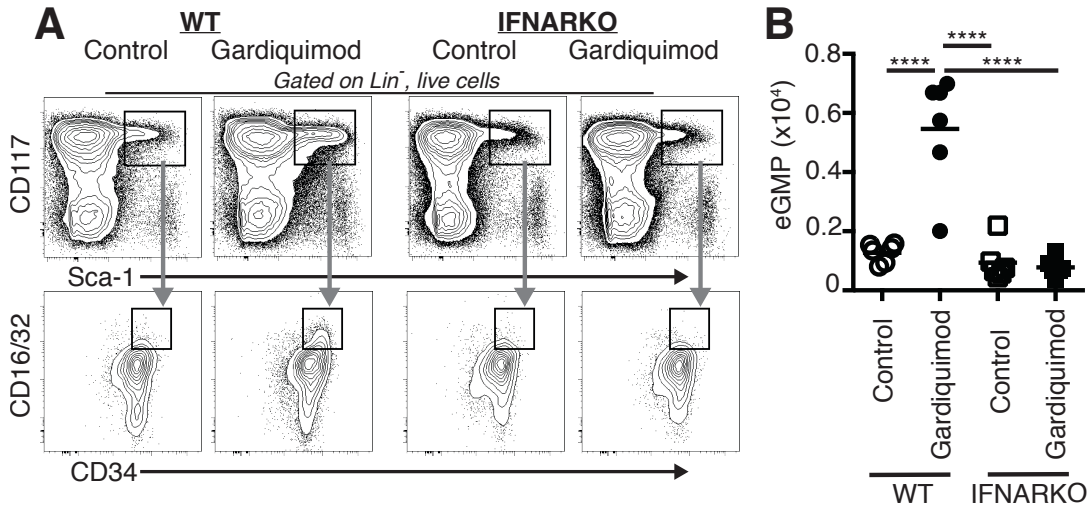

Figure 2.6 Serial TLR7 agonist administration drives type I IFN-dependent emergency myelopoiesis.

(**A**) Identification of eGMP in WT and IFNARKO mice treated with or without 10 μg gardiquimod i.v. every other day for 6 days. (**B**) Number of eGMP. Data show 6 mice per group from 2 independent experiments with each dot representing an individual mouse. Significance determined by one-way ANOVA with a tukey post-test.

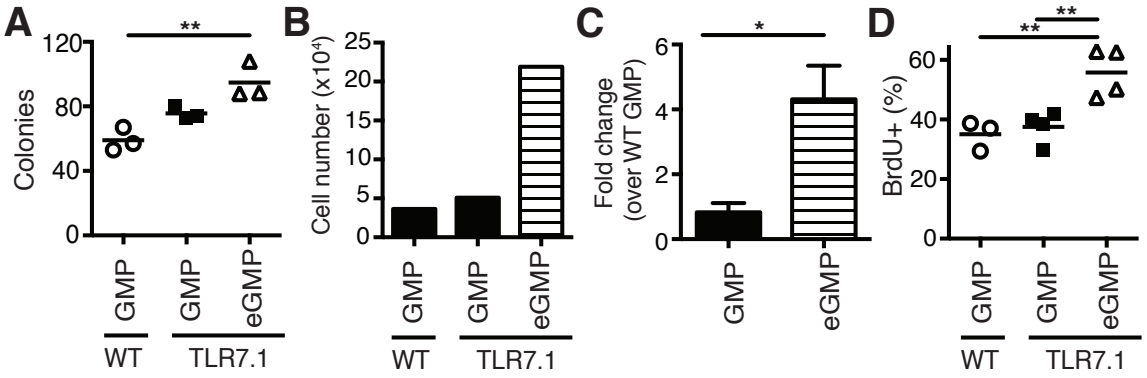

Figure 2.7. eGMP are superior myeloid precursors and more proliferative than GMP.

(**A-C**) 250 WT GMP, TLR7.1 GMP or TLR7.1 eGMP were plated in methylcellulose media with SCF, IL-3 and IL-6. (**A**) Number of colony forming units (CFU) after 5 days. (**B**) Absolute cell number generated after 6 days in culture from 1 experiment. (**C**) Fold change in cell yield over WT GMP. (A, C) Data are representative of cells sorted from 3 WT or TLR7.1 mice in 2 independent experiments. (**D**) Percent $BrdU^+$ GMP and eGMP in WT and TLR7.1 mice 1 h after injection of BrdU. Data are representative of 2 experiments. Each dot represents an individual mouse with bar representing the mean. (**A-D**) Significance determined by one-way ANOVA with a tukey post-test, two-tailed, paired or unpaired student's t-test, where appropriate.

Chapter 3: Type I IFN is an essential cofactor in TLR-driven PI3K/mTOR-dependent myeloid differentiation

Abstract

Myeloid cells develop in the bone marrow via a hematopoietic program that is adaptable to the needs of the host[27]. Type I interferon (IFN) and Toll-like receptor (TLR) signaling can modulate hematopoiesis to drive accelerated development of myeloid cells, which may be particularly important in promoting myelopoiesis during infection[9,68]. How these inflammatory mediators act on myeloid-committed progenitors to orchestrate functional changes and the consequences of this are important unresolved questions. Here we show in mice that TLR7-driven myeloid development from the common myeloid progenitor (CMP) is a cell-intrinsic, PI3K- and mTOR-dependent process that is enhanced by and requires type I IFN signaling. TLR7 signaling induced rapid PI3K-dependent phosphorylation of the mTOR target S6K in CMP and this signal was significantly increased by the addition of type I IFN, though type I IFN alone did not engage this signaling cascade. CMP integrated these inflammatory signals and responded by rapidly proliferating. CMP from $IFNAR^{-/-}$ mice were unable to effectively develop myeloid cells in response to TLR stimulation, indicating type I IFN is also required for this process. TLR-driven myeloid development from the more-committed granulocyte macrophage progenitor was not enhanced by nor required type I IFN. The results presented here redefine the roles of type I IFN and TLR signaling in the BM microenvironment, suggesting that IFN is constitutively required by some myeloid progenitors as a required accessory signal that enables efficient responses to TLR.

Introduction

Myeloid cells exert innate anti-microbial responses and contribute to tissue and metabolic homeostasis but can aberrantly suppress immune responses to infection and tumors[14,69]. It is important to understand how these cells develop in order to design therapeutics tailored to retard or accelerate the development of specific myeloid populations, depending on the disease- or infection-state[48]. Myeloid cells, such as monocytes and neutrophils, are generated in the bone marrow from self-renewing stem cells through a series of discrete myeloid-committed progenitor cells during the process of hematopoiesis. In the context of infection, the dynamics of hematopoiesis shift leading to a state in which the output of myeloid cells is increased at the expense of lymphopoiesis, called emergency myelopoiesis[27]. Human and mouse stem and progenitor cells express TLR[8,27] and *in vitro* evidence suggests that pathogen recognition via TLR is a mechanism for stem and progenitor cells to engage pathogen-associated patterns and in turn rapidly generate myeloid cells to assist in pathogen clearance[9]. It is unclear whether this is a cell-intrinsic process or an indirect phenomenon driven by autocrine or paracrine cytokines[36,39]. Type I IFN, a family of anti-viral cytokines associated with inducing an anti-proliferative state in responding cells, have paradoxically been shown to drive primitive hematopoietic stem cells to enter the cell cycle[40-42] and to promote myeloid development[68], though how the latter may occur is not known.

Results

To gain insight into how TLR signaling and type I IFN can alter myeloid development, we administered the TLR7 agonist R848, which drives robust induction of type I IFN *in vivo*[70], to WT and IFNAR$^{-/-}$ mice that are unable to respond to type I IFN[71], three days per week for two weeks. This regimen caused significant splenomegaly characterized by an increase in the number of splenic inflammatory monocytes and neutrophils in WT but not IFNAR$^{-/-}$ mice (Fig. 3.1a, b). To ascertain whether this splenic myeloid expansion was due to alterations in hematopoiesis, we analyzed the number of stem and myeloid-committed progenitor cells in bone marrow of WT and IFNAR$^{-/-}$ mice. Inflammatory monocytes and neutrophils derive from multipotent stem cells, which mature into the myeloid-committed common myeloid progenitor (CMP) that then gives rise to the granulocyte macrophage progenitor (GMP) before terminal maturation. In the bone marrow, we observed that TLR7 agonist administration promoted an increase in the primitive stem cell compartment (LSK), a decrease in CMP, and an increase in GMP in WT but not IFNAR$^{-/-}$ mice, demonstrating that emergency myelopoiesis occurs in response to TLR7 signaling in a type I IFN-dependent manner (Fig. 3.1c). These data confirm previous observations made about how TLR7 agonist administration alters hematopoiesis[68] (Fig. 2.6) and peripheral myeloid homeostasis[60] and extends them to show a requirement for type I IFN. This *in vivo* system demonstrated two important points: 1) TLR7 and type I IFN signaling promote peripheral myeloid expansion and emergency myelopoiesis, and 2) TLR7 signaling drives type I IFN-dependent alterations in hematopoiesis and myeloid development.

Evaluating changes in behavior and function of stem and myeloid-committed progenitor cells in the bone marrow during infection is problematic as Sca-1, a surface marker that delineates stem cells from myeloid-committed progenitors, is upregulated in response to type I and II IFN[25]. This obstacle has rendered our group and others unable to precisely define how CMP and GMP respond to inflammatory signaling *in vivo*[29,38,68]. To unambiguously address how these signals influence myeloid-committed progenitor behavior, we adopted an *in vitro* approach[9] in which we purified CMP and GMP by cell sorting and cultured them with IFNβ, R848, or both IFNβ and R848, and quantitated the output of CD11b^{+}F4/80^{+} myeloid cells. We observed that IFNβ alone does not drive myeloid development from CMP or GMP, but that R848 was sufficient to cause the development of CD11b^{+}F4/80^{+} cells from both cell types (Fig. 3.2a

and 3.3a). Surprisingly, IFNβ and R848 synergized in CMP to induce the highest frequency and number of $CD11b^+F4/80^+$ cells per input cell (Fig. 3.2a). GMP were more efficient producers of $CD11b^+F4/80^+$ cells than CMP in response to R848, but IFNβ did not enhance TLR7-driven myeloid development in GMP, distinct from the effect of IFNβ seen in CMP (Fig. 3.3a).

We were intrigued by the cooperative effects of type I IFN and TLR7 in CMP and investigated intracellular signaling mechanisms underpinning this observation. CMP and GMP both expressed the IFNAR1 subunit of the IFNAR receptor and could efficiently signal downstream of this receptor by phosphorylating STAT1 in response to IFNβ (Fig. 3.3b, c), therefore differences in the ability to immediately signal downstream of IFNβ to STAT1 do not explain the distinct responses of CMP and GMP to IFNβ in our myeloid cell differentiation cultures. TLR activate several signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) signaling[72]. CMP and GMP engaged the NF-κB pathway downstream of TLR7, and IFNβ did not augment this ability (Fig. 3.3d). However, R848 promoted rapid phosphorylation of the mTOR target S6K1 (S6) in CMP, and IFNβ significantly enhanced this signal in a PI3K-dependent manner (Fig. 3.2b, c). IFNβ and TLR7 signals did not synergize in GMP to augment pS6 signaling, except at one hour post treatment, but GMP phosphorylated S6 more robustly than CMP in response to R848 alone (Fig. 3.3e). IFNβ alone did not promote S6 phosphorylation in either CMP or GMP (Fig. 3.3f). Intact PI3K and mTOR signaling were crucial to myeloid development from CMP in response to R848 alone and R848 and IFNβ together, as inhibiting these signals for the entire duration of the cultures significantly reduced the output of myeloid cells (Fig. 3.2d, e and data not shown). TLR signaling, type I IFN signaling and the PI3K/mTOR pathway can drive cellular proliferation, therefore we hypothesized that these signals provided proliferative cues to CMP. Indeed, CMP cultured with R848 alone and R848 and IFNβ together, but not IFNβ alone, for 24 h proliferated significantly more than CMP cultured in media alone (Fig. 3.2f).

Consistent with the observation that R848 stimulation prompted early changes in CMP behavior, we found that removing R848 stimulation after 48 hours of the 96-hour culture did not abrogate the production of mature myeloid cells in comparison with those containing R848 for the entire culture (Fig.

3.3g). Moreover, the inhibition of PI3K signaling after 48 hours of culture with R848 alone or IFNβ and R848 did not significantly inhibit myeloid differentiation, whereas if PI3K is inhibited for the entire culture myeloid differentiation is blocked (Fig. 3.3h, 3.2d). Collectively, these *in vitro* data demonstrate that TLR7 promotes early PI3K and mTOR activation in CMP that drives myeloid differentiation through the promotion of early proliferation and that these processes are synergistically enhanced by type I IFN.

Our *in vivo* data indicated that type I IFN may be required by myeloid-committed progenitors to efficiently develop into mature myeloid cells in response to TLR signaling. To evaluate this possibility, we assessed IFNAR$^{-/-}$ CMP and GMP in our *in vitro* culture system. CMP derived from WT mice generated CD11b^{+}F4/80^{+} cells in a dose-dependent manner in response to R848, but CMP from IFNAR$^{-/-}$ mice were strikingly deficient in TLR7-driven myeloid differentiation (Fig. 3.4a). This observation held true for IFNAR$^{-/-}$ CMP treated with the TLR4 agonist lipopolysaccharide and the TLR9 agonist CpG oligonucleotide C (Fig. 3.5a). GMP from WT and IFNAR$^{-/-}$ mice generated CD11b^{+}F4/80^{+} myeloid cells in response to TLR4, 7 and 9 equally in most cases, reinforcing our observations that GMP differ substantially from CMP (Fig. 3.5b). WT and IFNAR$^{-/-}$ CMP produced CD11b^{+}F4/80^{+} cells at similar numbers in response to the homeostatic macrophage differentiation factor MCSF, demonstrating that type I IFN is specifically required to efficiently generate myeloid cells from CMP during TLR-driven differentiation (Fig. 3.4b). IFNAR$^{-/-}$ CMP and GMP signaled downstream of TLR7 to engage the NF-κB pathway at equivalent levels to WT cells (Fig. 3.5c). However, unlike WT CMP, IFNAR$^{-/-}$ CMP had reduced S6 phosphorylation and were less able to proliferate early in response to R848 compared to WT CMP (Fig. 3.4c, d). These data show that type I IFN is required to drive efficient TLR7-driven activation of the PI3K/mTOR pathways, and proliferation, in CMP.

Our finding that CMP required IFNAR signaling to efficiently produce macrophages in response to TLR agonists raised the possibility that these cells have the capacity to produce type I IFN that acts in an autocrine fashion, similar to mature macrophages[73]. We observed after 1 hour in culture with R848 that CMP generated *Ifnb* mRNA, though not *Ifna1 or Ifna4*, and subsequently upregulated the IFN-inducible genes *Isg15* and *Ifit1* consistent with early IFNβ production that then acts in an autocrine and/or paracrine manner (Fig 3.4e and data not shown). Hematopoietic stem and progenitor cells can produce inflammatory cytokines in response to TLR agonists[36], therefore we evaluated whether TLR7 signaling in

CMP produced other soluble factors that promoted myeloid development from these cells. We mixed WT and TLR7$^{-/-}$ CMP that could be distinguished by expression of different alleles of CD45 in equal numbers and cultured with R848 for 96 hours. In mixed cultures of WT CMP only, the CD11b^{+}F4/80^{+} cells generated were roughly 50% from each input CMP population (Fig. 3.4f). However, in mixed cultures of WT and TLR7$^{-/-}$ CMP, greater than 90% of the resulting CD11b^{+}F4/80^{+} cells were derived from WT CMP in response to R848, though TLR7$^{-/-}$ CMP and WT CMP retained a 50%:50% ratio of CD11b^{-}F4/80^{-} cells throughout the culture and TLR7$^{-/-}$ CMP produced CD11b^{+}F4/80^{+} cells equivalently to WT CMP in response to MCSF (Fig. 3.4f and 3.5d-e). Therefore, TLR7 signaling in CMP does not cause the production of soluble mediators of myeloid differentiation, such as MCSF or IL-6, which can act in the absence of a concurrent TLR signal. Additionally, these data show that TLR7 signaling programs cell-intrinsic myeloid differentiation from CMP that requires autocrine type I IFN signaling.

Discussion

The work presented here illuminates several novel aspects of myeloid differentiation and how inflammatory signaling modifies hematopoietic stem and progenitor cell behavior. The most novel findings of this study focus on the unique and critical roles of type I IFN in TLR-driven CMP differentiation. We show that this cytokine family acts as an accessory signal, but not on its own, to synergistically enhance TLR7 signaling and yet is also absolutely required for efficient TLR-driven myeloid maturation from CMP. These findings lend insight into paradoxes in the literature in which type I IFN has been shown to drive LSK into the cell cycle *in vivo*[40] but not *in vitro*[42]. Our data suggest that this may be through enabling efficient responses to TLR signals from dying cells that are abundant after TLR or type I IFN injection *in vivo* but not present *in vitro* upon stimulation with type I IFN. Indeed, we have seen that our findings in CMP extend to less differentiated LSK cells (unpublished observations) but not to more mature GMP, suggesting the dependence on type I IFN wanes with differentiation status.

The bone marrow microenvironment exhibits constitutive low levels of type I IFN though the biological significance of these tonic signals have remained elusive[74]. Our observation that CMP require type I IFN for TLR-driven myeloid development suggests that this chronic, low-level stimulus provides a priming signal for these myeloid progenitors that permits them to efficiently mature in response to TLR signals. This notion is validated by our *in vivo* system in which TLR7 agonist administration boosts myeloid development in WT but not IFNAR$^{-/-}$ mice, bolsters our previous observations made in a transgenic model of TLR7 overexpression in which type I IFN signaling is required for efficient myeloid development[68] and complements work indicating that germ free mice have impaired myeloid development during bacterial infection[75]. The source of this constitutive type I IFN is unknown but evidence suggests that it may derive from commensal microbiota[75,76].

TLR signals have been shown to modify the behavior of stem cells and myeloid-committed progenitors[9,36,77]. However, it is unclear from these previous studies whether TLR signals act in a cell-intrinsic manner or through the generation of inflammatory cytokines, like IFNγ[29] or IL-6[38], which promote myeloid development[39] and can be released by progenitors or mature cells[36]. We present data here that CMP can generate the type I IFN IFNβ, which acts in an autocrine fashion on these cells. However, our mixed culture experiments indicate that this CMP-derived type I IFN, and any other inflammatory

cytokines that may be generated in these assays, does not enable myeloid development in the absence of a TLR signal. These are the first results to demonstrate a definitive cell-intrinsic role for TLR signaling in myeloid development from a myeloid-committed progenitor.

Overall, this work suggests a model in which CMP receive chronic, low type I IFN signals that do not modulate their function in the steady state but allow for maximal responses to circulating TLR ligands during infection by enhancing the TLR-PI3K-mTOR pathway. In CMP, this pathway drives proliferation that allows for rapid generation of mature myeloid cells. Interestingly, the more mature GMP can respond robustly to TLR7 signaling to engage the PI3K-mTOR pathway but this signaling is not enhanced by IFNβ. This differential responsiveness to type I IFN and TLR signaling between different myeloid progenitors is interesting and raises questions about whether myeloid cells differentiated from CMP progress through the GMP stage as believed[13], and if inflammatory signals on progenitor cells may condition progeny that exhibit inflammatory or suppressive signatures. Moreover, the observation of heterogeneity in myeloid-committed progenitors may have implications in cancers as some leukemic stem cells may derive from distinct progenitor populations[78].

Materials and Methods

Mice and in vivo experiments.

Mice were 6-23 weeks of age, including wild-type C57BL/6 (CD45.2) and B6.SJL (CD45.1) from Charles River and Jackson Laboratories, C57BL/6 *TLR7*$^{-/-}$ from Jackson Laboratories and C57BL/6 *IFNAR*$^{-/-}$ mice from D. Stetson (University of Washington). Age and sex matched mice were injected three days per week for two weeks with 25 μg R848 (Invivogen) i.p. and sacrificed two days after the last injection. Cells were isolated, stained and analyzed as described[68].

In vitro experiments.

CMP and GMP were isolated as described[68]. 2500 to 20000 were plated per well of 96-well plates in complete serum-free StemPro 34 (Gibco) media with 20 ng mL^{-1} Stem Cell Factor (Peprotech), unless otherwise noted. For signaling assays and BrdU experiments, cells were rested at least 2 h before stimulation. For phosphorylation assays, cells were fixed and permeabilized followed by methanol fixation before staining for intracellular proteins. For BrdU incorporation, 10 mg mL^{-1} BrdU (Sigma-Aldrich) was added 4 hours before fixation. BrdU incorporation was assayed by using the BD BrdU Flow Kit procedure (BD Biosciences) or by methanol fixation and DNase treatment (Sigma Aldrich). For *in vitro* assays, 1 μg mL^{-1} R848, 50 units mL^{-1} IFNβ (PBL Assay Science, mammalian expressed) and 20 ng mL^{-1} MCSF (Peprotech), 100 ng mL^{-1} TNFα (Peprotech) were used, unless otherwise specified. CpG-C (Invivogen) and *Salmonella minnesota* R595 LPS (List Biological Laboratories) were used as noted. ZSTK474 and Rapamycin (Selleck) were used at 1 μM, unless otherwise noted. Cell yield was quantified using polystyrene microspheres and flow cytometry (Polysciences) as the number of CD11b^{+}F4/80^{+} events per well divided by the number of polystyrene bead events per well multiplied by the total number of polystyrene beads per well.

Quantitative real time PCR.

RNA was generated using RNeasy Plus Mini Kit and RNA cleanup kit (Qiagen). cDNA was synthesized using Qiagen reagents with random hexamers and OligoDT primers. qPCR was performed using SYBR green reagents (Takara) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression was calculated as previously described[68].

Chapter 3 Figures

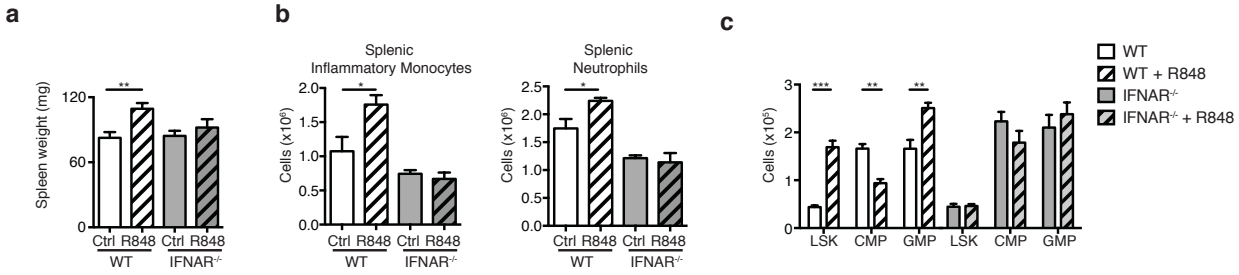

Figure 3.1. TLR7 agonist administration promotes type I IFN-dependent peripheral myeloid expansion and emergency myelopoiesis.

a-c, Mice were injected 3 times per week with 25 μg R848 i.p. for two weeks. **a**, spleen weight. **b**, numbers of splenic inflammatory monocytes and neutrophils. Cells were identified by flow cytometry as $BST2^{-}CD11b^{+}Ly6C^{+}Ly6G^{-}$ (inflammatory monocytes) or $BST2^{-}CD11b^{+}Ly6C^{-}Ly6G^{+}$ (neutrophils). **c**, numbers of pluripotent stem cells (LSK), common myeloid progenitors (CMP) and granulocyte-macrophage progenitor cells (GMP). Cells were identified by flow cytometry as $Lineage^{-}Sca1^{+}ckit^{+}$ (LSK), $Lineage^{-}Sca1^{-}ckit^{+}CD34^{+}CD16/32^{int/low}$ (CMP) or $Lineage^{-}Sca1^{-}ckit^{+}CD34^{+}CD16/32^{+}$ (GMP). n = 2 with a total of 8 WT control, 9 WT R848-injected, 7 $IFNAR^{-/-}$ control and 8 $IFNAR^{-/-}$ R848-injected mice (a) or representative of two experiments (b, c). Mean values + SEM are shown. *p < 0.05, ** p < 0.01, ***p < 0.001, two- tailed, unpaired student's t-test.

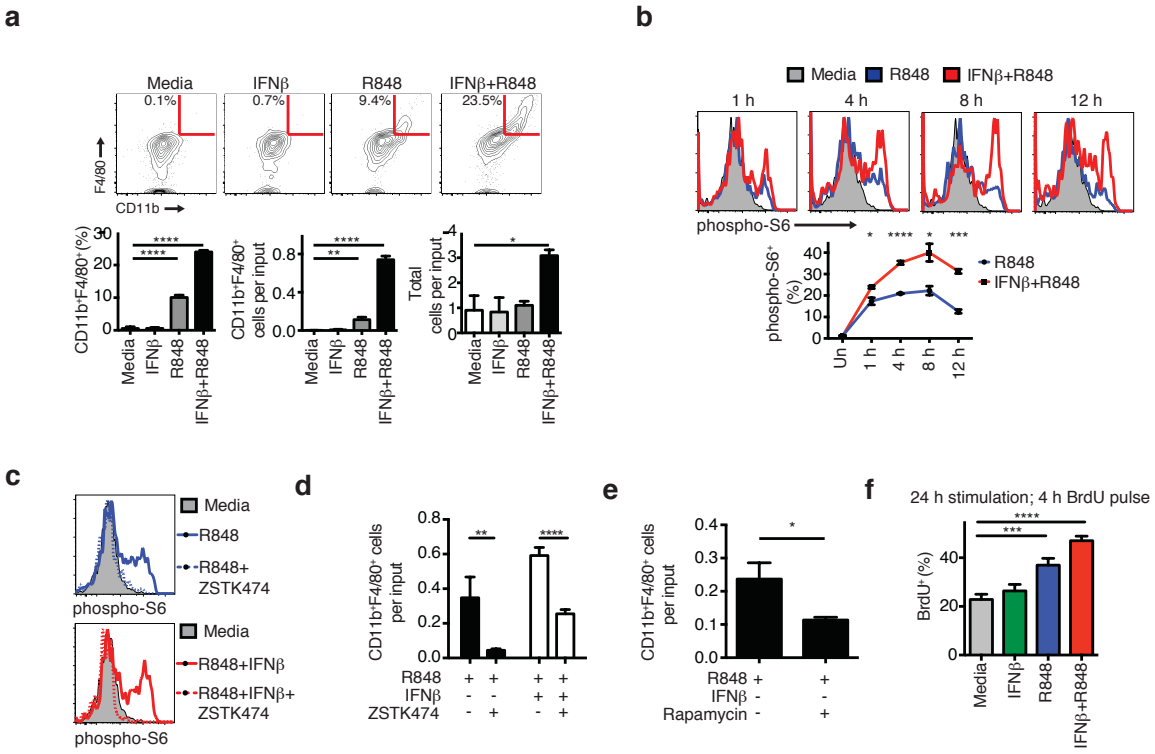

Figure 3.2. Type I IFN and TLR7 signaling synergize in CMP to promote optimal development of myeloid cells in a PI3K- and mTOR-dependent manner via increased proliferation.

a, CMP were stimulated with 50 units IFNβ mL^{-1}, 100ng mL^{-1} R848 or both as noted for 96 h. *n* = 3 independent experiments, 2-3 technical replicates per experiment. **b,** CMP were stimulated as noted for indicated times. *n* = 3 biological replicates, representative of 6. **c,** CMP were stimulated as noted for 4 h with or without 30 minute pretreatment with 10 μM ZSTK474. *n* = 3 independent experiments. **d,** CMP were stimulated as noted with or without ZSTK474 for 96 h. *n* = 3 independent experiments with 3-4 technical replicates per treatment. **e,** CMP were stimulated as noted with or without rapamycin for 96 h. *n* = 2 independent experiments, 3 technical replicates per treatment. **f,** CMP were stimulated as noted for 24 h, BrdU was added during last 4 h. *n* = 6 (IFNβ), 10 (R848) and 10 (R848+IFNβ) combined independent experiments. Mean values + s.d. (a, d, e), ± SEM (b) or + SEM (f) are shown. *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001, one way analysis of variance (ANOVA) with dunnett's post test (a, f), two-tailed, unpaired student's t-test (b, d, e).

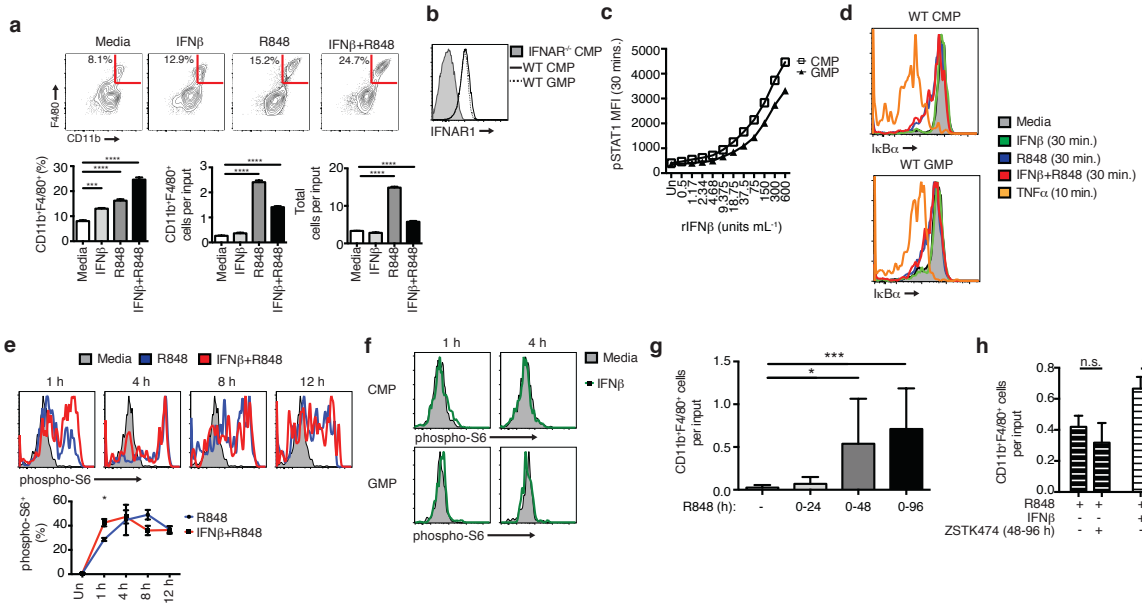

Figure 3.3. Type I IFN and TLR7 signaling do not synergize in GMP, myeloid-committed progenitors express IFNAR1 and phosphorylate STAT1 in response to IFNβ, myeloid-committed progenitors respond to TLR7 by degrading IκBα, GMP phosphorylate S6 in response to R848, myeloid-committed progenitors do not phosphorylate S6 in response to IFNβ and early TLR7/PI3K signals are important for myeloid differentiation from CMP.

a, GMP were stimulated as noted for 96 h. *n* = 2 independent experiments with 3 technical replicates per experiment. **b,** *Ex vivo* IFNAR1 expression on CMP and GMP. *n* = 3 independent experiments. IFNAR$^{-/-}$ CMP used as negative control. **c,** Cells were stimulated as noted for 30 min. *n* = 9 independent experiments. **d,** Cells were stimulated as noted. *n* = 8 independent experiments. **e,** GMP were stimulated as noted for indicated time points. *n* = 3 biological replicates, representative of 6 biological replicates. **f,** Cells were stimulated as indicated. *n* = 3 independent experiments. **g,** CMP were stimulated with R848 and stimulation was washed out as noted. *n* = 4 combined independent experiments. **h,** CMP were stimulated as noted. *n* = 3 independent experiments with 3-4 technical replicates. Mean values + s.d. (a, g, h) or ± SEM are shown. *p < 0.05, ***p < 0.001, ****p < 0.0001, ANOVA with dunnett's post test (a, g), two-tailed, unpaired student's t-test (e, h).

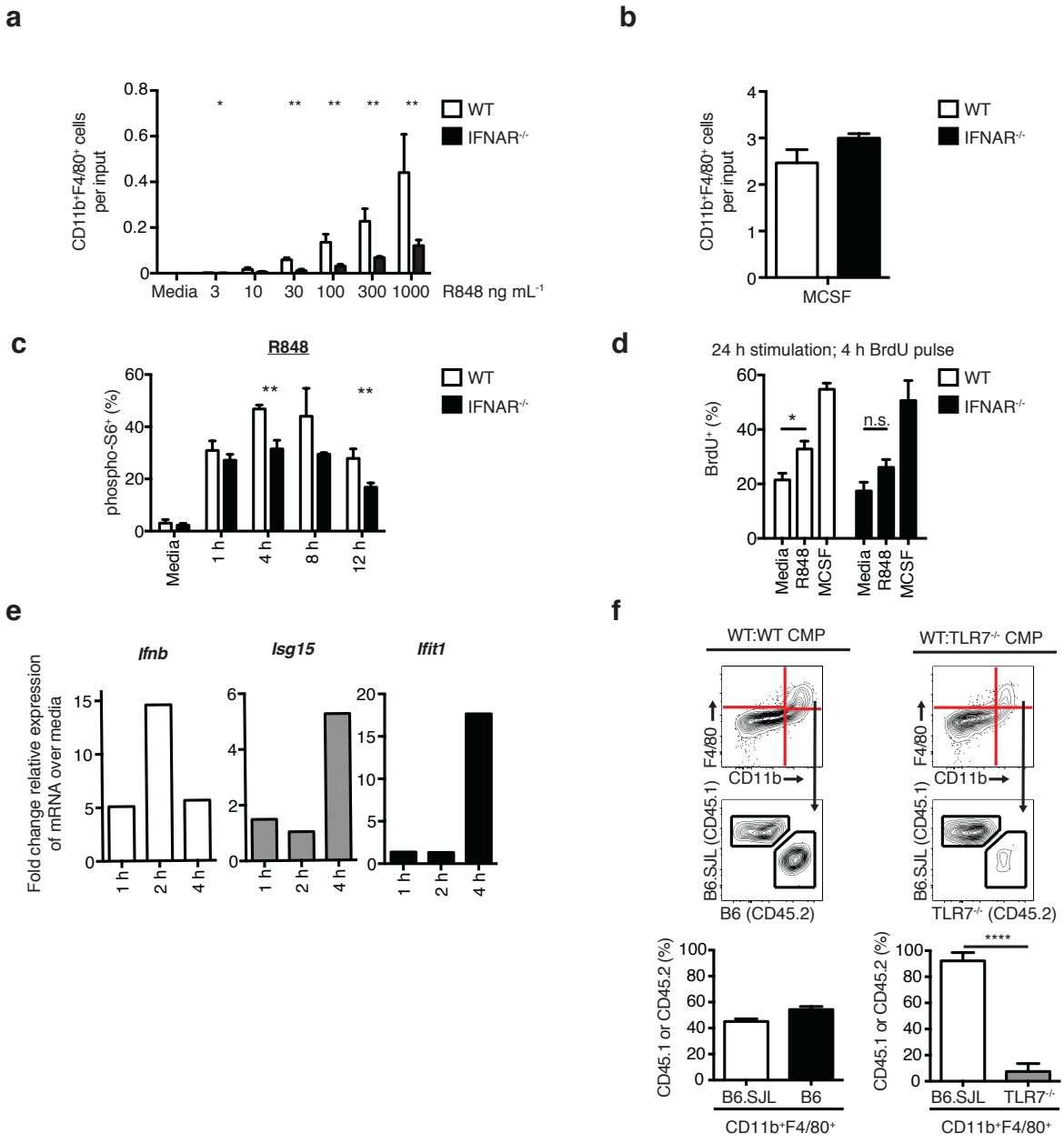

Figure 3.4. TLR7 drives cell-intrinsic, type I IFN-dependent myeloid development from CMP.

a-c, WT and IFNAR$^{-/-}$ CMP were stimulated as noted. *n* = 3 independent experiments with 3 replicates per experiment (a), *n* = 3 independent experiments with 2-3 technical replicates per experiment (b), *n* = 3 independent experiments (c). **d,** CMP were stimulated as noted for 24 h, BrdU was added during last 4 h. *n* = 7 (WT), *n* = 6 (IFNAR$^{-/-}$) combined independent experiments. **e,** CMP were cultured in 100 ng mL^{-1} SCF and stimulated with R848 for indicated times. Fold change in mRNA expression. *n* = 3 independent experiments. **f,** WT:WT and WT:TLR7$^{-/-}$ CMP were cultured at a 1:1 ratio and stimulated with R848 for 96 h. *n* = 2 independent experiments. Mean values + s.d. (a, b, d), or + SEM (d) are shown. *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001, two-tailed, unpaired student's t-test.

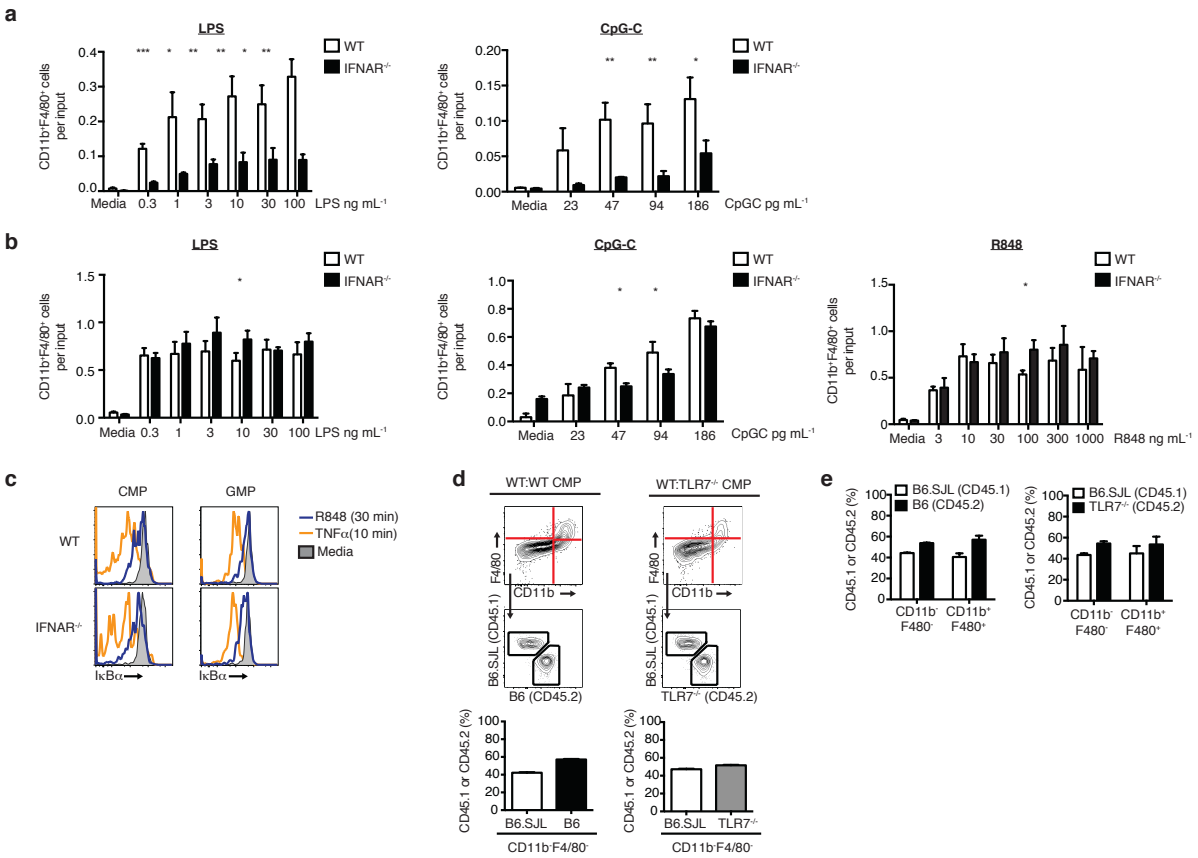

Figure 3.5. CMP, not GMP, require type I IFN for myeloid development in response to TLR signaling, IFNAR$^{-/-}$ are not deficient in acute TLR7 signaling and TLR7$^{-/-}$ CMP are able to efficiently generate myeloid cells in response to MCSF

a, CMP were cultured with LPS or CpG-C as noted for 96 h. *n* = 2 independent experiments with 3 technical replicates per experiment. **b,** GMP were with cultured LPS or CpG-C as noted. *n* = 2 independent experiments with 3 technical replicates per experiment. **c,** CMP and GMP were cultured as noted. *n* = 4 independent experiments. **d,** WT:WT and WT:TLR7$^{-/-}$ CMP were cultured at a 1:1 ratio and stimulated with R848 for 96 h. *n* = 2 independent experiments. **e,** WT:WT and WT:TLR7$^{-/-}$ CMP were cultured at a 1:1 ratio and stimulated with MCSF for 96 h. *n* = 2 independent experiments. Mean values + s.d. (a, b, d, e) are shown. *p < 0.05, ** p < 0.01, ***p < 0.001, two-tailed, unpaired student's t-test.

Chapter 4: Hematopoietic and Non-Hematopoietic Cells Promote Type I Interferon- and Toll-like Receptor 7-dependent Monocytosis During Low-dose Lymphocytic Choriomeningitis Virus Infection

Abstract

Release of inflammatory monocytes from the bone marrow (BM) into the blood is an important physiological response to infection, but the mechanisms regulating this phenomenon during viral infection are not completely defined. Here, we show that low-dose infection with Lymphocytic Choriomeningitis Virus (LCMV) caused rapid, transient inflammatory monocytosis that required type I IFN and TLR7 signaling. Both signals were critical for induction of IFN stimulated gene expression and CCR2 ligand upregulation in the BM microenvironment in response to LCMV infection. Experiments utilizing bone marrow chimeric mice demonstrated that type I IFN- and TLR7-signaling on either hematopoietic or non-hematopoietic cells was sufficient to initiate monocytosis in response to LCMV infection. BM plasmacytoid dendritic cells (pDC) generated type I IFN directly ex vivo, suggesting that pDC are a hematopoietic contributor of type I IFN in the BM early during LCMV infection. Overall, we describe novel roles for type I IFN and TLR7 signaling in non-hematopoietic cells and BM pDC in directing interferon stimulated gene and CCR2 ligand expression in the BM to initiate a burst of inflammatory monocyte egress from BM to the blood during viral infection.

Introduction

The bone marrow (BM) acts as a reservoir of inflammatory monocytes, which can be released into peripheral circulation in response to microbial challenge[43]. Inflammatory monocytes express high levels of the chemokine receptor CCR2, which binds to the chemokines CCL2, 7 and 12. Inflammatory monocyte egress from the BM into the blood in the steady state and in response to inflammation requires interactions between CCR2 and CCL2 or 7, whereas the role of CCL12 in this process is still unclear[44,45,79]. CCR2 ligands can be induced in response to either TLR stimulation or type I IFN signaling or both during infection with bacterial, fungal and viral pathogens, but how these signals are integrated in the BM niche and what effect this has in shaping the innate response to virus infection is still not completely defined[44-47,80]. Recent work has suggested that viral infection can drive monocytosis that is critical for viral clearance and survival[81,82]. However, the mechanisms by which viral infection drives BM egress of inflammatory monocytes have not been fully elucidated.

Cells of hematopoietic and non-hematopoietic origin populate the BM microenvironment. The contribution of cells from both lineages in mounting an effective inflammatory response is an area of active study. Recent work has indicated that both non-hematopoietic mesenchymal stem cells (MSC) and CXCL12-abundant reticular (CAR) cells and hematopoietic cells in the BM are capable of producing of *Ccl2* and inducing monocytosis in response to low, systemic doses of LPS and infection with the bacterium *Listeria monocytogenes*[45]. Other work has shown that infection with murine CMV, a virus with a dsDNA genome, induces CCR2 ligands in the BM primarily from F4/80$^+$ hematopoietic cells in a manner that depends largely on type I IFN[46]. What inflammatory factors, innate receptors and specific cell types determine the response of the BM microenvironment to infection with a ssRNA virus are unknown.

Plasmacytoid dendritic cells (pDC) are sentinel cells of the innate immune system that express high levels of TLR7 and 9 and are capable of rapidly secreting IFNα. pDC are unique among other dendritic cell subsets in that they fully mature and reside in appreciable numbers in the BM[83]. BM pDC have been found in mice, primates and humans, suggesting an evolutionary conserved role for these cells in the BM microenvironment[84-86]. BM pDC may represent a source of rapid, local type I IFN that uniquely influences early innate immune responses originating in the BM, such as the release of inflammatory monocytes into the circulation. Here, we investigated the mechanisms and cell types

involved in modulating BM gene expression changes that promote monocytosis during infection with the arenavirus Lymphocytic Choriomeningitis Virus (LCMV) strain Armstrong and revealed novel roles for type I IFN, TLR7, non-hematopoietic cells and BM pDC in this process.

Results

Low-dose LCMV infection promotes monocytosis at 48 hours post-infection.

To interrogate whether RNA virus infection drives inflammatory monocytosis as seen in DNA virus and bacterial infections[44,45,81,82], we first infected mice with $1x10^2$, $1x10^4$ and $2x10^5$ PFU of LCMV strain Armstrong and examined the number of leukocytes in the blood early after infection. Surprisingly, we found that doses higher than $1x10^2$ PFU promoted dose-dependent pan-leukopenia beginning as early as 24 hours after infection, with significant decreases in CD45.2$^+$ leukocytes, neutrophils, resident monocytes, B cells and T cells (Fig. 4.1A and 4.2A).

As infection with $1x10^2$ PFU did not promote pan-leukopenia and because a low-dose inoculum more accurately reflects a physiological infection, we focused on this dose to investigate whether LCMV promotes monocytosis (Fig. 4.1A). Indeed, we observed a significant increase in the frequency and absolute number of inflammatory monocytes in the blood by 48 hours post-infection at this dose but not others at the time points investigated (Fig. 4.1B-D and 4.2B). The number of blood inflammatory monocytes peaked at 48 hours post-infection and then began to wane by 72 hours post-infection (Fig. 4.1D). This increase was specific to inflammatory monocytes as the fold change in absolute cell number of these cells increased ~3-fold by 48 hours post-infection, whereas there was little change in the number of neutrophils, resident monocytes and total CD45.2$^+$ leukocytes at this or other time points (Fig. 4.1E).

Low-dose LCMV monocytosis requires type I interferon receptor- and toll-like receptor 7-signaling.

LCMV infection is characterized by a rapid burst of type I IFN followed by a later surge in IFNγ[87]. IFNγ has been shown to cause *de novo* inflammatory monocyte production from myeloid precursor cells, which results in inflammatory monocytosis at day 8 post-infection with a high dose ($1x10^5$ PFU) of LCMV[29]. However, whether type I IFN can modulate blood inflammatory monocyte homeostasis early in LCMV infection and with a more physiological dose of virus has not been investigated. To address this possibility, we inoculated WT and IFNAR$^{-/-}$ mice, which lack cell surface expression of a functional type I IFN receptor, with $1x10^2$ PFU LCMV and examined the number and frequency of inflammatory monocytes in the blood. At 48 hours post-infection, there was a significant increase in the frequency and absolute number of inflammatory monocytes in the blood in WT mice, whereas there was no significant change in blood inflammatory monocyte frequency or absolute numbers in IFNAR$^{-/-}$ mice (Fig. 4.3A).

TLR7 is an endosomal pattern recognition receptor that is specific for ssRNA and LCMV possesses a genome composed of ssRNA. Therefore, during LCMV infection TLR7-expressing cells may recognize ssRNA viral nucleic acids and then generate type I IFN. To test whether TLR7-mediated viral recognition was important in monocytosis during LCMV infection, we infected WT and TLR7-deficient ($TLR7^{-/-}$) mice with $1x10^2$ PFU LCMV. $TLR7^{-/-}$ mice did not display any signs of monocytosis at 48 hours post-infection whereas WT mice exhibited an increase in frequency and absolute number of blood inflammatory monocytes (Fig. 4.3B). Collectively, both IFNAR and TLR7 are required for an increase in the number of inflammatory monocytes in the blood at 48 hours post infection with $1x10^2$ PFU LCMV (Fig. 4.3C).

Low-dose LCMV infection induces IFNAR- and TLR7-dependent Interferon stimulated gene expression and CCR2 ligand upregulation.

We hypothesized that the type I IFN- and TLR7-dependent inflammatory monocytosis we observed in response to low-dose LCMV infection was due to inflammatory monocyte egress from the BM and that BM gene expression changes regulated this process. To evaluate this possibility, we examined gene expression changes at 36 hours post-infection in whole BM, which includes both hematopoietic and non-hematopoietic cells, as this time point preceded the spike in blood inflammatory monocytes seen at 48 hours post-infection. We observed that the interferon-stimulated genes (ISG) *Isg15* and *Ifit1* were upregulated ~6x and ~18x, respectively, in total BM of WT mice by 36 hours post-infection compared to uninfected control mice (Fig. 4.4A). The upregulation of ISG was completely dependent on IFNAR signaling, as infection of $IFNAR^{-/-}$ mice did not drive increased BM ISG expression over uninfected control mice (Fig. 4.4A). Optimal induction of BM ISG at 36 hours post-infection required TLR7 signaling. We found no significant induction of *Ifit1* by LCMV infection in $TLR7^{-/-}$ mice, whereas *Isg15* was upregulated only ~2x over uninfected mice in $TLR7^{-/-}$ mice, much lower than the ~6x induction in WT mice (Fig. 4.4A). Therefore, ISG expression during low-dose LCMV infection is partially dependent upon TLR7.

CCL2 is the principal ligand of CCR2 and the primary driver of inflammatory monocyte egress from the BM[43]. *Ccl2* can be induced by type I IFN and TLR signaling during infection with the gram positive bacteria *Listeria monocytogenes* or after injection of pure TLR agonists[44,45,80]. It is unknown how CCR2 ligands in the BM are regulated in response to LCMV. We hypothesized that type I IFN and TLR7

signaling in the BM were driving *Ccl2* upregulation during low-dose LCMV infection. To test this hypothesis, we infected WT mice with $1x10^2$ PFU LCMV and measured induction of *Ccl2* at 36 hours post-infection. *Ccl2* was upregulated ~17x in the BM of infected WT mice compared to uninfected control mice (Fig. 4.4B). Strikingly, this increase in *Ccl2* was abrogated in both $IFNAR^{-/-}$ and $TLR7^{-/-}$ mice, paralleling the lack of monocytosis we observed in these animals after infection (Fig. 4.3). We found that *Ccl7* and *Ccl12*, also ligands for CCR2, were induced in a IFNAR- and TLR7-dependent manner similar to *Ccl2* at 36 hours post-infection with $1x10^2$ PFU LCMV (Fig. 4.4B).

At 48 hours post-infection, ISG expression in the BM microenvironment still depended entirely on type I IFN signaling and optimal induction of BM ISG also required TLR7 signaling (Fig. 4.5A). Similar to 36 hours post-infection, upregulation of CCR2 ligands in the BM at 48 hours post-infection required both IFNAR and TLR7 signaling (Fig. 4.5B). LCMV is characterized by IFNγ production later than 48 hours post-infection[87]. Consistent with this, in our infection system *Ifng* mRNA there was not significantly upregulated in the BM at 36 or 48 hours post-infection with $1x10^2$ PFU LCMV (Fig. 4.4C-D). Overall, these data indicate that both type I IFN and TLR7 are critical for driving peak ISG and CCR2 ligand induction in the BM in response to low-dose LCMV infection at 36 hours and 48 hours post-infection.

Hematopoietic and non-hematopoietic cells contribute to monocytosis during low-dose LCMV infection.

Non-hematopoietic BM cells have been shown to respond to TLR agonist administration and *Listeria monocytogenes* infection by generating *Ccr2* ligands that promote inflammatory monocyte egress from the BM to the blood[45]. During murine CMV virus infection, $F4/80^+$ hematopoietic cells have been shown to produce CCR2 ligands[46]. However, viruses like LCMV provide a different array of signals to the immune system and provoke a qualitatively different immune response compared to bacteria or dsDNA viruses. Therefore, it is possible that the cellular mediators of BM gene expression differ depending on the pathogen, as they do between *Listeria monocytogenes* and MCMV infection.

Our previous experiments showed that significant changes in BM gene expression after LCMV infection depended on TLR7 and type I IFN signaling but they did not discriminate whether hematopoietic or non-hematopoietic cells were responsible for these gene changes, as both of these cell types are flushed from the bone marrow using standard procedures[45,88]. To evaluate whether hematopoietic or non-hematopoietic cells were important for responding to type I IFN and TLR7 to cause monocytosis during

low-dose LCMV infection, we generated BM chimeric mice in which radiation-sensitive hematopoietic or radiation-resistant non-hematopoietic cells could selectively respond to type I IFN or TLR7 signals. Therefore, the BM chimeric mice generated (WT→WT, TLR7$^{-/-}$→WT, IFNAR$^{-/-}$→WT, WT→TLR7$^{-/-}$ and WT→IFNAR$^{-/-}$) allowed us to dissect specifically whether type I IFN and TLR7 signaling were important on hematopoietic or non-hematopoietic cells in our infection system (Fig. 4.6A). In all cases we used congenic alleles of CD45 to assess reconstitution (Fig. 4.6B).

After infecting these chimeric mice with 1x10^2 PFU LCMV, we assessed whether monocytosis was observed at 48 hours post-infection. Surprisingly, we observed an increase in blood inflammatory monocytes after infection in all chimeric mice (Fig. 4.7A). This increase was specific to inflammatory monocytes, as we observed no change in overall leukocytes at 48 hours post-infection (Fig. 4.7B). These data demonstrate that functional redundancy exists between hematopoietic and non-hematopoietic cells in driving monocytosis in response to low-dose LCMV infection – intact type I IFN and TLR7 signaling from either compartment is sufficient to promote this response.

Next, we asked whether BM gene expression changes reflected the monocytosis observed across all chimeric mice. Indeed, WT→IFNAR$^{-/-}$, TLR7$^{-/-}$→WT and WT→TLR7$^{-/-}$ chimeric mice expressed nearly equivalent levels of BM *Isg15* and *Ifit1* compared to WT→WT chimeras, all of which were increased at least 10x over uninfected WT mice at 48 hours post-infection (Fig. 4.7C). The data from WT→IFNAR$^{-/-}$ chimeric mice demonstrate that WT hematopoietic cells in the BM are sufficient to generate ISG when non-hematopoietic cells cannot respond to IFNAR signaling. The data from TLR7$^{-/-}$→WT and WT→TLR7$^{-/-}$ chimeric mice shows that TLR7 expression on either hematopoietic or non-hematopoietic cells is sufficient to generate robust ISG in response to LCMV. Interestingly, compared to uninfected control mice, infected IFNAR$^{-/-}$→WT chimeras had no induction of BM *Isg15* and reduced induction of BM *Ifit1* compared to infected WT→WT chimeras or WT→IFNAR$^{-/-}$ chimeras (Fig. 4.7C). The fact that BM from IFNAR$^{-/-}$→WT chimeric mice, which contains IFNAR$^{-/-}$ hematopoietic cells and WT non-hematopoietic cells, had minimal induction of ISG indicated that IFNAR signaling on hematopoietic cells is important for early BM ISG induction in response to low-dose LCMV. However, that IFNAR$^{-/-}$→WT chimeric mice were still able to mount a robust increase in the numbers of blood monocytes by 48 hours

post-infection suggests that type I IFN responsiveness on hematopoietic cells is not required to elicit egress of inflammatory monocytes into the blood.

We then examined the induction of CCR2 ligands in the chimeric mice at 48 hours post-infection. WT→IFNAR$^{-/-}$, TLR7$^{-/-}$→WT and WT→TLR7$^{-/-}$ chimeras all produced similar amounts of *Ccl2*, *Ccl7* and *Ccl12* as control WT→WT chimeras after LCMV infection. These data show that TLR7 expression on either hematopoietic or non-hematopoietic cells is sufficient to generate robust CCR2 ligand expression in response to LCMV. Similar to what was found for *Ifit1* induction, IFNAR$^{-/-}$→WT chimeras had reduced induction of *Ccl2*, *Ccl7* and *Ccl12* compared to WT→WT chimeras (Fig. 4.7D). These results demonstrate that IFNAR expression on hematopoietic cells, but not non-hematopoietic cells, in the BM contribute to ISG and CCR2 ligand induction after low-dose LCMV infection. The reduced CCR2 ligand induction observed in IFNAR$^{-/-}$→WT did not affect inflammatory monocytosis in these chimeras, corroborating evidence that very low amounts of CCR2 ligands are capable of promoting monocytosis[45].

Plasmacytoid dendritic cells generate type I IFN in the bone marrow 36 hours post-infection with low-dose LCMV.

We posited that pDC, cells that express high levels of TLR7, are capable of producing prodigious amounts of type I IFN and reside in the BM, might be contributing to the TLR7 and IFNAR-dependent inflammatory monocytosis after low-dose LCMV infection. To investigate this, we sorted BM pDC from uninfected mice and mice infected with 1x10^2 PFU LCMV at 36 and 48 hours post-infection. We found that BM pDC were producing increased amounts of *Ifna4* and *Ifnb* mRNA after infection in comparison with the undetectable or low amounts seen in pDC from uninfected mice (Fig. 4-8A). These data demonstrate that pDC are hematopoietic contributors of type I IFN in the BM early in infection with low-dose LCMV.

Discussion

This study puts forth several novel findings in addition to building upon previous studies that have investigated how monocytes egress from the BM during infection. Other work has shown that monocytosis and CCR2 ligand production during infection is important for BM monocyte egress but a specific sensor that initiates this response has not been identified. Here, we show that a single TLR, TLR7, is critical in early ISG and CCR2 ligand induction during LCMV infection. Our work is also the first to show that during LCMV infection, BM monocyte egress and CCL2/7/12 production requires IFNAR signaling. Lastly, we found that both hematopoietic and non-hematopoietic cells contributed to TLR7- and IFNAR-dependent CCR2 ligand production and monocyte egress during LCMV infection. Other studies have implicated either hematopoietic or non-hematopoietic cells in promoting BM monocyte egress in response to LPS, *Listeria monocytogenes* infection and murine CMV infection. Our work indicates that both hematopoietic and non-hematopoietic cells are capable of responding to LCMV through TLR7 and driving this response to infection.

It is clear that bacterial infection, such as with *Listeria monocytogenes*, can elicit monocytosis but the literature is scant and conflicting on whether viral infection promotes this response early in infection. A study using a high dose of $1x10^5$ PFU LCMV Strain Armstrong, delivered i.p., observed no monocytosis early in infection but rather an increase in blood inflammatory monocytes by day 8 post infection due to *de novo* production of inflammatory monocytes from myeloid progenitors[29]. In contrast, work in which $1x10^2$ focus forming units of West Nile Virus was delivered s.c., described monocytosis, likely due to BM egress[82]. We found that high doses of LCMV strain Armstrong that are typically used in the literature, from $1x10^4$ - $2x10^5$ PFU delivered i.p., promoted minor increases in the number of blood inflammatory monocytes with the concomitant development of pan-leukopenia. These observations highlight the sensitivity of the innate immune system in response to pathogens and underscore the importance of the appropriate viral inoculum to study immune system function and behavior, particularly that of inflammatory monocytes.

CCR2-CCL2 and -CCL7 interactions are important in mediating egress of inflammatory monocytes from the BM to the blood[43]. The mechanistic underpinning of how this egress event occurs is unclear. It has been suggested that CCR2 ligands can be produced by cells that appose BM blood

vessels and that this event initiates the migration of inflammatory monocytes from BM cavities to vascular sinuses and into circulation[45]. MSC and CAR cells of non-hematopoietic origin and hematopoietic cells have been shown to mediate the reorganization and egress of BM monocytes during LPS administration and infection with *Listeria monocytogenes*, but only non-hematopoietic cells were shown to directly appose BM blood vessels and generate *Ccl2*, suggesting that they are crucial mediators of inflammatory monocyte egress in these models[45]. Other work has suggested that BM-resident F4/80^{+} macrophages upregulate CCR2 ligands during murine CMV infection in a manner that requires type I IFN[46]. These seemingly contradictory findings with two distinct pathogens indicate that the BM is a dynamic microenvironment that has redundant mechanisms to ensure that inflammatory monocytes can be efficiently released into circulation in the event of bacterial, viral or other microbial challenge. They also show that distinct pathogens may engage different mechanisms to elicit similar functional outcomes, such as monocyte egress from the BM, highlighting the importance of investigating monocyte egress during infections with a variety of pathogens. Our study adds to this literature by definitively showing that type I IFN- and TLR7-signaling can be important in initiating BM egress of inflammatory monocytes during viral infection, and that these signaling events can occur on either hematopoietic cells or non-hematopoietic cells.

The threshold to induce inflammatory monocyte egress from the BM is low, as LPS doses in the picogram range can effectively mobilize inflammatory monocytes into the blood[45]. Consistent with this, we observed that less BM ISG and CCR2 ligands were produced in IFNAR$^{-/-}$→WT compared to WT→WT, WT→IFNAR$^{-/-}$, TLR7$^{-/-}$→WT and WT→TLR7$^{-/-}$ chimeric mice after LCMV infection, but that all chimeric mice were able to efficiently elicit rapid monocytosis. These observations speak to the sensitivity of this system and the importance of efficient mobilization of inflammatory monocytes from the BM to the blood during infection.

The relative contributions of TLR and RIG-I-like receptor (RLR) signaling during LCMV infection are unclear. For example, TLR7$^{-/-}$ mice have been shown to have diminished levels of type I IFN one day after infection compared to WT mice[89]. On the contrary, it has been shown that mice lacking components of RLR signaling have significantly less type I IFN early in infection, whereas endosomal- or pan-TLR deficient mice are not deficient in this respect[90,91]. Here, though we do not investigate a role for RLR

signaling, we present a clear function for TLR7 signaling in response to low-dose LCMV strain Armstrong infection as mediating egress of inflammatory monocytes from the BM to the blood early during infection.

The precise role of pDC in determining the host's ability to clear virus and shape the innate immune response later in infection is unclear[92-94]. However, it has been demonstrated during LCMV infections of between $2x10^6$-$3x10^6$ PFU that pDC are activated in a TLR-dependent manner[90,95] and that these cells are ephemeral secretors of type I IFN early after infection[91]. In line with these reports, our data show that in response to $1x10^2$ PFU LCMV strain Armstrong, BM-resident pDC produce type I IFN mRNA by 36 hours post-infection. pDC are predominantly found in association with non-hematopoietic CAR cells in the BM[86]. Therefore, we propose that BM pDC cells act to secrete type I IFN that can then generate CCR2 ligands from other cells, such as CAR cells, but possibly also from MSC or hematopoietic cells such as $F4/80^+$ macrophages[45,46]. We attempted to definitively assess the role of pDC in this system by depleting these cells using BDCA2-DTR mice[93], but we found that simple delivery of diphtheria toxin to wild-type mice can cause monocyte egress making it impossible to use this model to determine the role of pDC in LCMV-dependent monocytosis (data not shown).

Monocytosis is an important component of an effective response to viral infection[81,82]. Inflammatory monocytes, once released from the BM and into the blood may go on to mature into APC, which then influence later T cell responses. Corroborating this idea, monocyte-derived APC have been shown to be important for restimulating tissue-resident effector T cell IFNγ in situ in the vaginal mucosa after Herpes Simplex Virus-2 infection[81]. We show here that cooperative signaling of type I IFN and TLR7, in both hematopoietic and non-hematopoietic cells, is crucial for modulating BM gene expression to influence early innate immune cell population dynamics in the periphery during low-dose LCMV infection. These data highlight that the BM is an important lymphoid organ in early anti-viral defense in response to LCMV and possibly other RNA virus infection.

Materials and Methods

Mice, bone marrow chimeric mice and LCMV infection

C57BL/6 and B6.SJL mice were purchased from Charles River or bred in our vivarium. IFNAR$^{-/-}$ mice were provided by D.B. Stetson (University of Washington) and TLR7$^{-/-}$ mice were purchased from Jackson Laboratories and bred in our vivarium. Mice were housed at Benaroya Research Institute and all experiments were performed under IACUC-approved protocols. BM chimeric mice were generated by lethally irradiating (1000 rad) recipient mice and reconstituting them with 4x10^6 donor BM cells. In all cases, recipients and donors differed in CD45 allele expression for assessment of chimerism. For infections, 1x10^2, 1x10^4 or 2x10^5 PFU of LCMV strain Armstrong were administered to mice i.p.

Cell isolation, cell surface staining, flow cytometry and cell sorting

To obtain samples from the blood, mice were bled via saphenous vein into 10 μL of heparin (25 mg/mL; Sigma-Aldrich, Cat. H3149). 30 μL of heparinized blood was depleted of red blood cells with Ack (Lonza, Cat. 10-548E). Blood cells were blocked with unlabeled anti-CD16/32 mAb followed by the addition of mAbs as indicated in a final volume of 50 uL. Cells were typically labeled with the following panel of monoclonal antibodies: CD19 AF647 (clone: eBio1D3; 1:200; eBioscience), TCRβ APC (clone: H57-597; 1:200; eBioscience), Ly6G Pacific Blue (clone: 1A8; 1:400; Biolegend) CD115 PE (clone: AFS98; 1:100; eBioscience), CD11b PerCp-Cy5.5 (clone: M1/70; 1:600; eBioscience), Ly6C FITC (clone: HK1.4; 1:400; Biolegend) and CD45.2 Alexa Flour 700 (clone: 104; 1:200; eBioscience). Samples were incubated at 4C for 30 minutes for surface stains, then washed and fixed in 4% paraformalydehyde. Blood cell numbers were quantified using polystyrene microspheres (Polysciences, Cat. 18328). The same animal was tracked longitudinally across all time points for determination of cell numbers in the blood. Data for all time points was acquired on the same day using the same bead lot on an LSRII (BD Biosciences) and analyzed using FlowJo (TreeStar). Live cell were analyzed by using FSC and SSC. BM pDC were isolated and sorted as described previously[68].

Quantitative Real Time Polymerase Chain Reaction

BM was eluted directly into RLT Plus buffer and RNA was generated using RNeasy Plus Mini Kit (Qiagen, Cat. 74136). cDNA was synthesized using Applied Biosystems (Cat. 4368814) or Qiagen

reagents with random hexamers and OligoDT primers. qPCR was performed using SYBR green reagents (Takara) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression was calculated as previously described[68]. Primer sequences are as follows: *Hprt*: **F**, TGA AGA GCT ACT GTA ATG ATC AGT CAA C; **R**, AGC AAG CTT GCA ACC TTA ACC A, *Isg15*: **F**, AAG CAG CCA GAA GCA GAC TC; **R**, CAC CAA TCT TCT GGG CAA TC, *Ifit1*: **F**, GCC ATT CAA CTG TCT CCT G; **R**, GCT CTG TCT GTG TCA TAT ACC, *Ccl2*: **F**, AAC TGC ATC TGC CCT AAG GTC; **R**, AGT GCT TGA GGT GGT TGT GGA, *Ccl7*: **F**, GAT CTC TGC CAC GCT TCT GT; **R**, ATA GCC TCC TCG ACC CAC TT, *Ccl12*: **F**, ATT TCC ACA CTT CTA TGC CTC CT; **R**, ATC CAG TAT GGT CCT GAA GAT CA, *Ifng*: **F,** GGC TGT TTC TGG CTG TTA CTG C; **R** ACT CCT TTT CCG CTT CCT GAG G.

Data presentation and statistical analysis

Graphs were generated and statistical analyses were performed as noted in figure legends using Prism (GraphPad).

Chapter 4 Figures

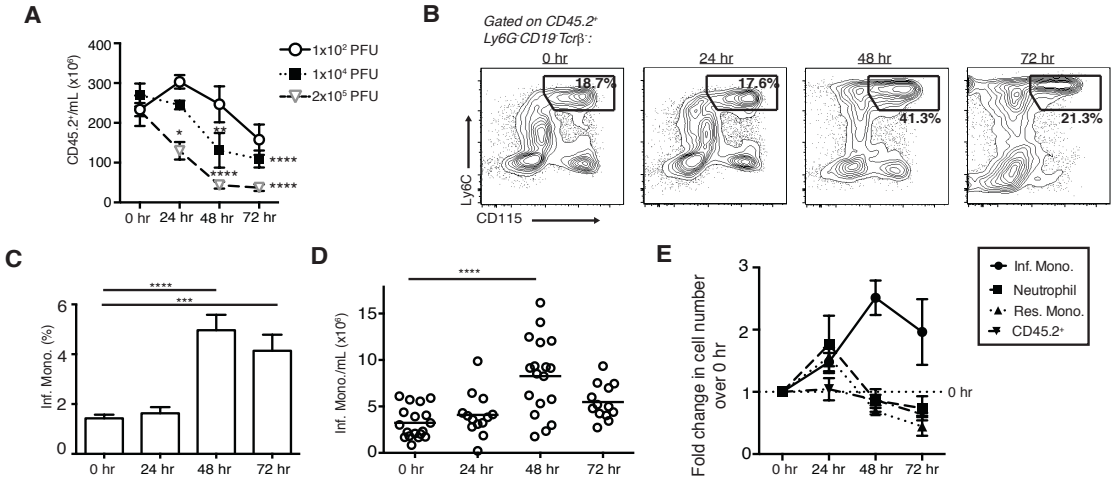

Figure 4.1. Low-dose LCMV promotes monocytosis.

(**A**) Absolute number of blood CD45.2+ cells following infection with $1x10^2$ PFU, $1x10^4$ PFU or $2x10^5$ PFU LCMV strain Armstrong. *n* = 5 mice per group. Statistical differences reflect changes from 0 hr. (**B**) Gating strategy and representative flow cytometry plots for identifying inflammatory monocytes ($CD45.2^+Ly6G^-Tcr\beta^-CD19^-CD115^+Ly6C^+$) in the blood. Percentage (**C**) and absolute number (**C**) of blood inflammatory monocytes at indicated time points post-infection with $1x10^2$ PFU LCMV strain Armstrong. Data are from seven (**C**) and five (**D**) experiments. (**E**) Fold change in number of blood cells over 0 hour. Inflammatory monocytes were identified as in Figure 1B. Neutrophils were identified as $CD45.2^+Ly6G^+$. Resident monocytes were identified as $CD45.2^+Ly6G^-Tcr\beta^-CD19^-CD115^+Ly6C^-$. $CD45.2^+$ cells were identified as $CD45.2^+$. Data from 24 hours, 48 hours and 72 hours post-infection are from three, five and three experiments, respectively. Data are presented as mean ± SEM (A, E), + SEM (C) or mean (D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, as determined by one way ANOVA with dunnett's multiple comparison post test.

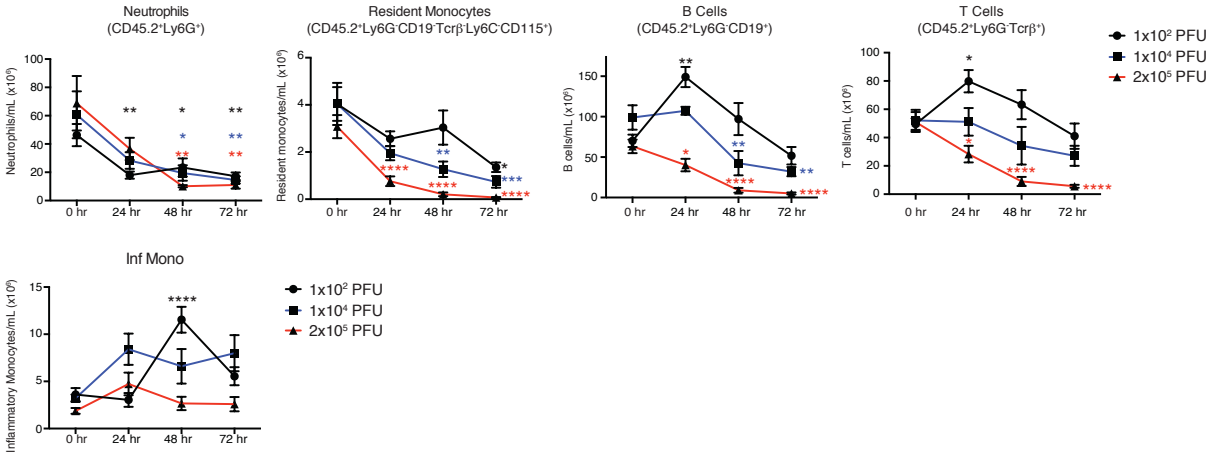

Figure 4.2. High doses of LCMV promote blood leukopenia.

(**A**) Absolute number of blood neutrophils, resident monocytes, B cells and T cells following infection with $1x10^2$ PFU (black), $1x10^4$ PFU (blue) or $2x10^5$ PFU (red) LCMV strain Armstrong. (**B**) Absolute number of blood inflammatory monocytes following infection with $1x10^2$ PFU (black), $1x10^4$ PFU (blue) or $2x10^5$ PFU (red) LCMV strain Armstrong. (**A-B**) *n* = 5 mice per group. Statistical differences reflect changes from 0 hr. Data are presented as ±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, as determined by one way ANOVA with dunnett's multiple comparisons post test.

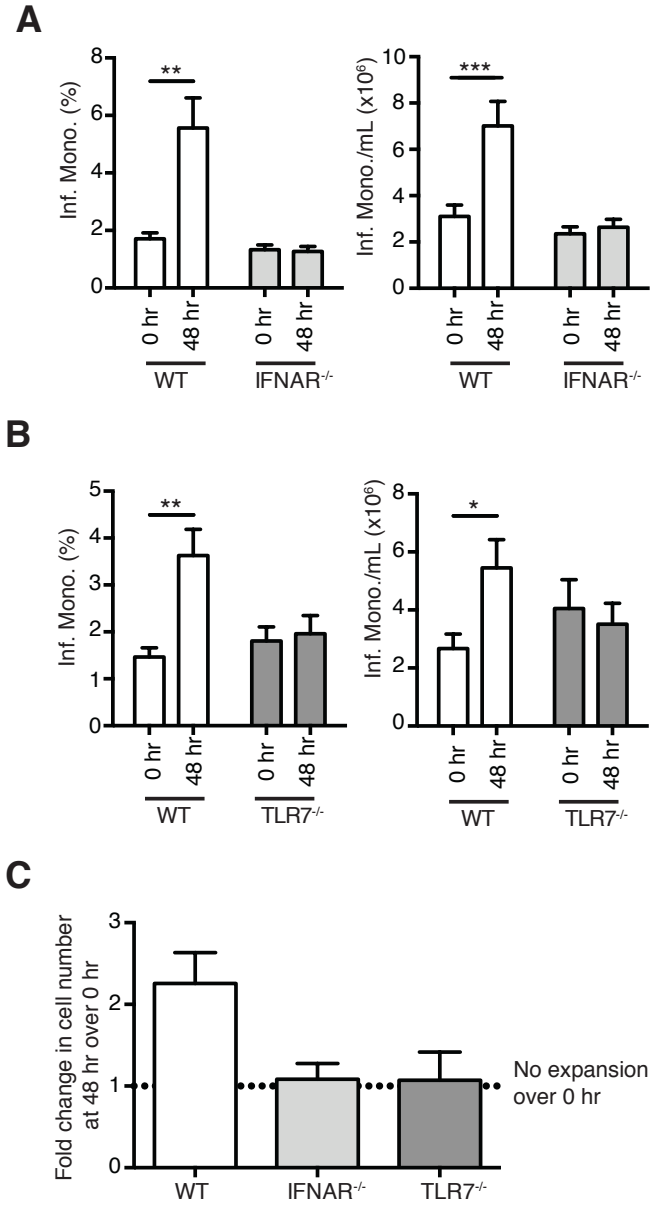

Figure 4.3 Low-dose LCMV-driven monocytosis requires type I IFN and TLR7 signaling.

(**A**) Frequency and absolute number of blood inflammatory monocytes in WT and IFNAR$^{-/-}$ mice at 0 and 48 hours post-infection with 1x10^2 PFU LCMV strain Armstrong. Data are from 4 experiments with 13 WT and 16 IFNAR$^{-/-}$ mice. (**B**) Frequency and absolute number of blood inflammatory monocytes in WT and TLR7$^{-/-}$ mice at 0 and 48 hours post-infection. Data are from 3 experiments with 9 WT and 9 TLR7$^{-/-}$ mice. (**C**) Fold change in the absolute number of blood inflammatory monocytes at 48 hours post-infection over 0 hours. Data are from three experiments. (**A-C**) Data are presented as mean + SEM. (**A-B**) *p<0.05, **p<0.01, ***p<0.001, as determined by paired student's t-test.

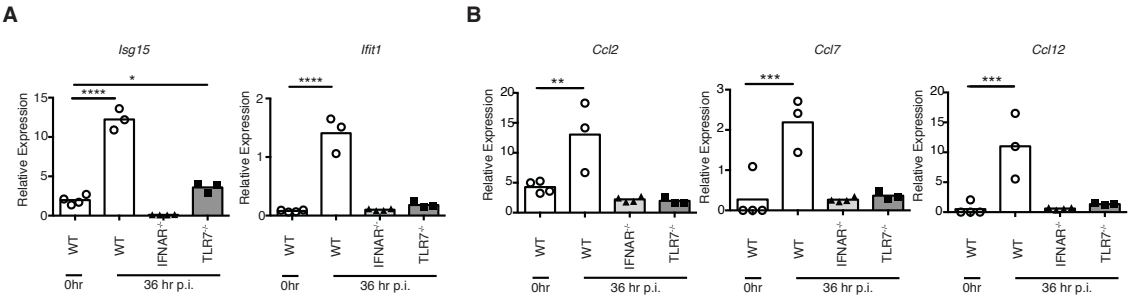

Figure 4.4. Low-dose LCMV infection promotes ISG and CCR2-ligand mRNA in the BM in a type I IFN- and TLR7-dependent manner.

(**A-B**) Whole BM was assayed by qPCR for gene expression in uninfected WT mice and infected WT, IFNAR$^{-/-}$ and TLR7$^{-/-}$ mice at 36 hr. post-infection with 1x10^{2} PFU LCMV strain Armstrong. Data are representative of 13 (WT), 10 (IFNAR$^{-/-}$) and 11 (TLR7$^{-/-}$) infected mice from three or more experiments. In bar graphs, data are presented as mean. Each dot represents one mouse. *p<0.05, **p<0.01, ***p<0.001,****p<0.0001, as determined by one way ANOVA with dunnett's multiple comparisons post test. In samples where transcripts were not detectable (*Ccl7* and *Ccl12* in 3 out of 4 uninfected mice) values were quantified as zero.

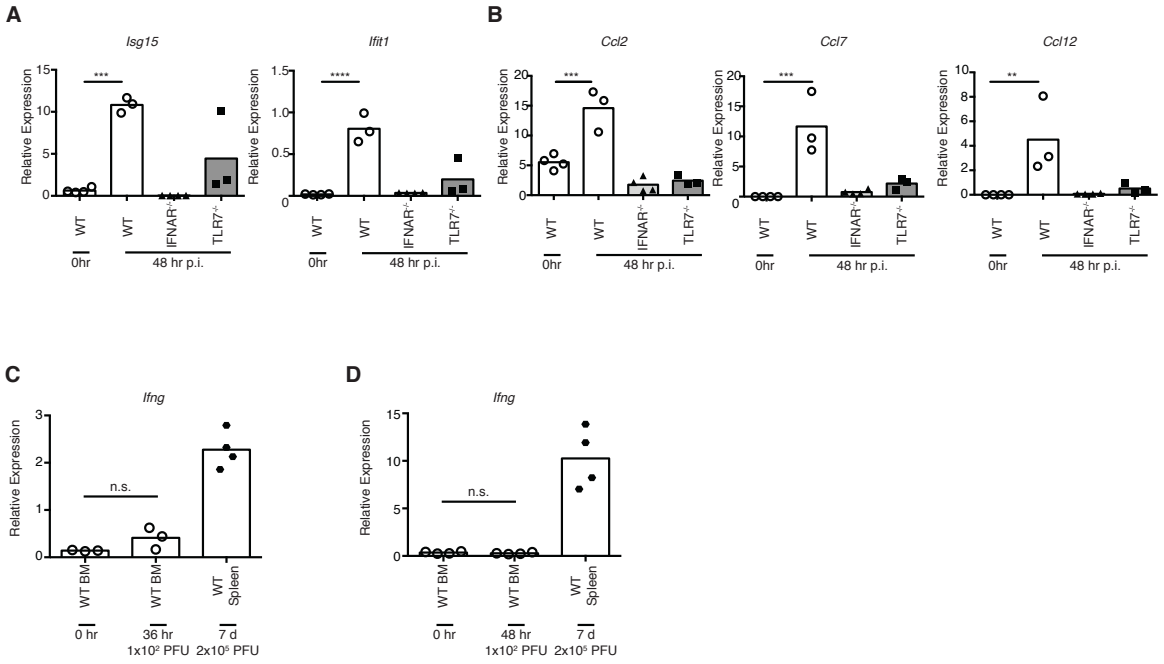

Figure 4.5. Low-dose LCMV infection promotes ISG and CCR2-ligand mRNA in the BM in a type I IFN- and TLR7-dependent manner at 48 hours post-infection and does not promote BM Ifng.

(**A-B**) Mice sacrificed 48 hours post-infection. Data are representative of 6 (WT), 7 (IFNAR$^{-/-}$) and 6 (TLR7$^{-/-}$) infected mice from two experiments. (**C**) Mice sacrificed 36 hours post-infection. BM data are representative of 6 uninfected and 7 infected mice. (**D**) Mice sacrificed 48 hours post-infection. BM data are representative of 6 uninfected and 7 infected mice. (**C-D**) Whole spleen samples from 4 mice infected with 2x10^5 PFU LCMV were used as a positive control and not included in statistical analysis. In bar graphs, data are presented as mean. Each dot represents one mouse. **p<0.01, ***p<0.001,****p<0.0001, as determined by one way ANOVA with dunnett's multiple comparisons post test (A-B) or two tailed, unpaired student's t test (**C-D**). In samples where transcripts were not detectable (*Ccl7* and *Ccl12* in 4 out of 4 uninfected mice at 48 hours post-infection) values were quantified as zero.

A

Chimera	Hematpoietic compartment	Non-hemaotpoietic compartment	Comparison determines
WT → WT	WT (B6)	WT (B6.SJL)	Control
IFNARKO → WT	IFNARKO	WT (B6.SJL)	What hematopoietic compartments IFNAR signaling is required in for optimal response to LCMV
WT → IFNARKO	WT (B6.SJL)	IFNARKO	
TLR7KO → WT	TLR7KO	WT (B6.SJL)	What hematopoietic compartments TLR7 signaling is required in for optimal response to LCMV
WT → TLR7KO	WT (B6.SJL)	TLR7KO	

B

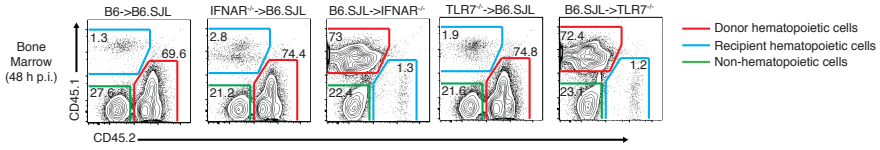

Figure 4.6. BM chimera analysis.

(**A**) Table of BM chimeras generation and interpretation. (**B**) BM (48 hours post-infection) reconstitution from representative chimeras showing donor and recipient hematopoietic cells ($CD45.1^+$ and $CD45.2^+$) and non-hematopoietic cells ($CD45.1^-CD45.2^-$).

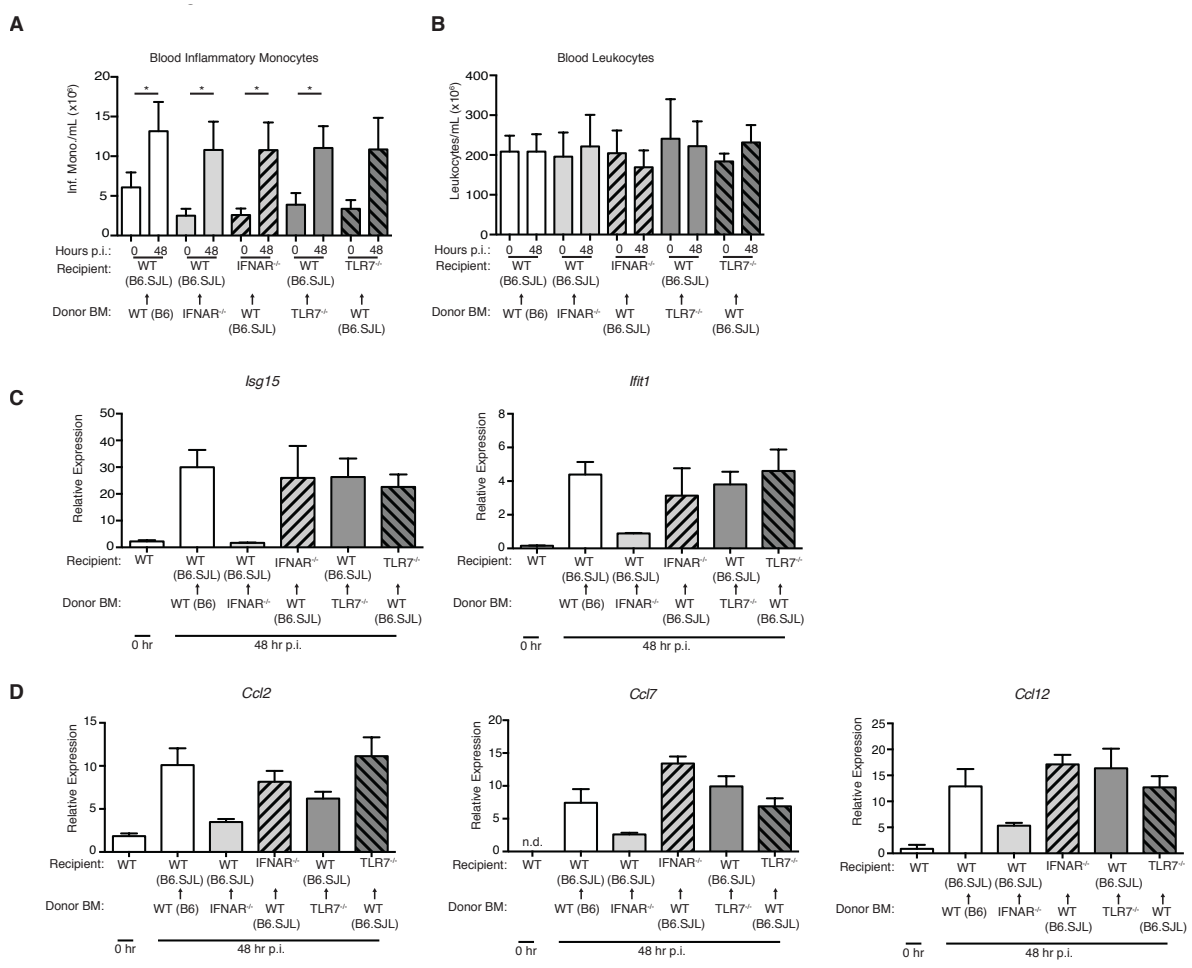

Figure 4.7. Hematopoietic and non-hematopoietic cells contribute to monocytosis in response to low-dose LCMV.

(**A-D**). Chimeric mice were generated by lethally irradiating recipient mice and transferring $4x10^6$ donor cells as noted. Mice were reconstituted for 6 weeks before infection with $1x10^2$ PFU LCMV strain Armstrong. (**A**) Absolute number of donor inflammatory monocytes at 0 and 48 hours post infection. Inflammatory monocytes were identified as $CD45.1^+$ or $CD45.2^+Ly6G^-Tcr\beta^-CD19^-CD11b^+Ly6C^+$. (**B**) Absolute number of donor leukocytes at 0 and 48 hours post infection. Donor leukocytes were identified as $CD45.1^+$ or $CD45.2^+$. (A-B) Data are from 6 B6→B6.SJL, 5 IFNAR$^{-/-}$→B6.SJL, 6 B6.SJL→IFNARKO$^{-/-}$, 4 TLR7$^{-/-}$→B6.SJL, and 3 B6.SJL→TLR7$^{-/-}$ chimeric mice. Data are presented as mean + SEM. *$p<0.05$, as determined by paired student's t-test. (**C-D**) Whole BM was assayed by qPCR for gene expression as in Fig. 3. Data are from 3 uninfected mice, 6 B6→B6.SJL, 5 IFNAR$^{-/-}$→B6.SJL, 3 B6.SJL→IFNAR$^{-/-}$, 4 TLR7$^{-/-}$→B6.SJL, and 3 B6.SJL→TLR7$^{-/-}$ chimeric mice at 48 hours post infection. Data are presented as mean + SEM.

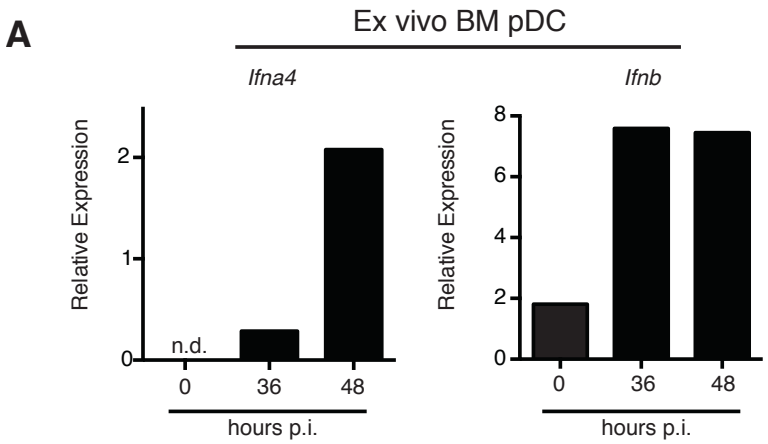

Figure 4.8. Bone marrow plasmacytoid dendritic cells generate type I IFN by 36 hours post-infection with low-dose LCMV.

(A) BM pDC (CD11b-BST2+Siglec-H+) were sorted from uninfected and mice infected with $1x10^2$ PFU LCMV strain Armstrong at indicated time points. Data are representative of 2 experiments in which BM pDC were sorted and pooled from 2 mice per time point. In bar graphs, data are presented as mean.

Chapter 5: Concluding Remarks

Myeloid cells are critical components of the immune system. The work presented here increases our understanding of how the homeostasis of these cells is modulated during inflammation using models of autoimmunity and infection. Inflammatory monocyte egress from the bone marrow during infection is a critical first step in pathogen eradication. Our work is the first to show that type I IFN and TLR7 on either hematopoietic and non-hematopoietic cells is sufficient to mediate gene changes that allow for monocytes to leave the bone marrow and enter the blood stream. Future work in this area will reveal whether other TLR are important for inflammatory monocyte egress in response to different pathogens and the specific consequences suffered during infection if the host is unable to mediate effective bone marrow egress of inflammatory monocytes.

Myeloid development is an area of active research and the results presented here will advance the field. Our findings that myeloid-committed progenitors from the BM utilize TLR signaling directly and CMP, but not GMP, require type I IFN as a cofactor for efficient TLR-induced differentiation are very intriguing. However, it remains an open question as to why myeloid-committed progenitor cells are able to respond directly to TLR signals and whether this provides some advantage over integrating inflammatory cytokine signals produced by other cells. The BM cavity is highly vascularized and pathogens such as LCMV and HIV have been detected in this milieu[96,97], suggesting that BM progenitor cells may engage directly with microbes during infection. Yet, while the BM environment is permissive to infectious agents, stem cells are thought to reside in specialized immune-privileged and immunosuppressive niches[98] that may impede pathogen encounter and an ensuing inflammatory response. The precise location of myeloid-committed progenitors is unknown, but interestingly, CXCR4, a chemokine receptor that retains stem and progenitor cells in the BM, can inhibit TLR signaling in macrophages[99]. Therefore, our in vitro results in which myeloid-committed progenitors are cultured without the signals provided by their resident niche may better approximate how CMP and GMP behave in peripheral sites after mobilization. Indeed, small numbers of stem cells and myeloid-committed progenitors circulate throughout the blood, lymphatics and tissues continually[100]. Therefore, the ability of myeloid-committed progenitor cells to sense TLR ligands, as opposed to requiring inflammatory cytokine signals from another sentinel cell, may be the

most rapid means for these cells to replenish innate immune effector cells in the periphery during infection. Additionally, stem and progenitor cell mobilization is a frequent response to infection, which may increase the pool of myeloid progenitors that respond to pathogens through TLR signaling.

The results presented here show how myeloid progenitor cells respond to TLR and describe that this interaction drives increased progenitor proliferation that results in a quantitative increase in myeloid cell number. Our work does not dissect whether this qualitatively impacts the cells that derive from CMP or GMP under TLR7-stimulating conditions versus steady-state circumstances. It is attractive to hypothesize that mature myeloid cells developed from CMP or GMP cultured with TLR agonists will be "conditioned" to exhibit different gene expression and function than cells derived from CMP or GMP cultured with homeostatic myeloid growth factors such as MCSF. For example, it would be advantageous to the host if the myeloid cells produced under TLR7 conditions exhibited characteristics that rendered them better to sense and eliminate ssRNA viruses through increased inflammatory cytokine secretion. Specifically modifying myelopoiesis for each pathogen encounter may be a goal of emergency myelopoiesis; therefore the generation of specialized progeny is a conceivable component of this pathway. To this end, recent work suggests that vaccinated mice have more responsive myeloid cells upon rechallenge than naïve mice, providing a potential linkage between how inflammatory signaling may alter hematopoiesis in a long-lasting manner[101]. Inflammatory monocytes become microbicidal during infection, so it is also possible that development from myeloid-committed progenitors under TLR-driven conditions may exhibit increased expression of components of the NADPH oxidase or iNOS. Alternatively, inflammatory monocytes also can have immunoregulatory capacities, and therefore TLR-differentiated cells may exhibit suppressive properties such as prostaglandin synthesis or arginase-1 expression[102]. Supporting this possibility, suppressive myeloid cells have been observed with chronic viral infections like HIV[103] and HCV[104], two diseases associated with TLR7 and Type I IFN signaling. Future studies investigating gene expression and behavior of mature myeloid cells that are derived from CMP or GMP cultured with TLR7 or MCSF will be useful in addressing these possibilities.

To benefit the host, the immune system must eradicate pathogens without undue immunopathology or initiating autoimmunity. During infection, myeloid cells must be produced rapidly and leave the bone marrow to home to sites of infection. When this system aberrantly functions, myeloid cells

can suppress proper immune responses or exacerbate autoimmune diseases like SLE, in which dying neutrophils have been implicated in promoting disease. TLR7 and type I IFN signaling have been shown to play roles in both beneficial and deleterious immune responses and these outcomes, whether good or bad, can be traced to perturbations in myeloid homeostasis. Our findings in the mouse suggest that the dysregulated myeloid development loop in SLE in which pDC generate TLR7-dependent type I IFN in response to aberrant neutrophil function and this type I IFN in turn acts to is continuously generate neutrophils in the BM may be short circuited by small molecule inhibitors to PI3K and mTOR that are targeted to myeloid-committed progenitors. However, our findings do not explain the entire suite of clinical literature regarding how type I IFN and TLR7 may modulate myeloid homeostasis. For example, many MS, HCV and cancer patients are treated with recombinant type I IFN, but this has not been reported to cause massive myeloid expansion, though a detectable increase in myeloid cells is in some cases observed. This could be for a number of reasons, particularly as these clinical manifestations are of complex etiology and pathogenesis, but may depend upon whether myeloid progenitor cells receive a concurrent TLR signal. An important next step would be to validate our work regarding myeloid development in humans to investigate whether our findings in the mouse model are conserved in humans. Indeed, $CD34^+$ human progenitors express TLR and TLR signaling can predispose them to differentiate into myeloid cells but the precise mechanisms underlying these responses have not been investigated[8,10,37]. The in vitro culture systems we have in place are amendable to human studies. Future studies in primary human $CD34^+$ progenitors present an exciting avenue to validate and extend our murine work and also make an impact on human health.

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