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# Functional Education of Monocytes During Infection

## **Abstract**

Tissue-infiltrating Ly6Chi monocytes play important protective roles during infection, including inflammatory cytokine secretion and pathogen killing. Here we show that during acute gastrointestinal infection with the protozoan parasite *Toxoplasma gondii*, recruited monocytes not only contributed to parasite control, but also regulated pathologic immune responses to commensal microbes via secretion of the lipid mediator prostaglandin E2 (PGE2). Priming of monocytes for regulatory function preceded systemic inflammation and was initiated prior to bone marrow egress. Natural killer (NK) cell-derived IFN- $\gamma$  promoted a regulatory program in monocyte progenitors during development. Early bone marrow NK cell activation was controlled by systemic IL-12 produced by Batf3-dependent dendritic cells (DC) in the mucosal-associated lymphoid tissue (MALT). This work challenges the paradigm that monocyte function is dominantly imposed by local signals following tissue recruitment, and instead proposes a sequential model of differentiation in which monocytes are pre-emptively educated during development in the bone marrow to promote their tissue-specific function.

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FUNCTIONAL EDUCATION OF MONOCYTES DURING INFECTION

Michael Horne Askenase

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

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*“Mentoring is a brain to pick, an ear to listen, and a push in the right direction.”*

Thank you Yasmine, for providing all three. I know I haven't always been an easy mentee, but you've always had an intuition for when I needed reassurance and support, and when I simply needed a swift kick in the behind. It's been an honor to be part of your family these past 5 years. I also owe thanks to my many other mentors that have guided me to this point. John, you are my scientific big brother. Your astonishing intellect and creativity would be intimidating, if they weren't outmatched by your kindness and patience. Carlos and Ana, it was truly a blessing to begin my professional scientific career under your supervision. You taught me to always remember that science is born of philosophy, a lesson that has truly shaped who I am as a scientist. Finally, thank you to Harry Bernheim, who introduced me to immunology and inspired me to study it for the rest of my life.

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# ABSTRACT

## FUNCTIONAL EDUCATION OF MONOCYTES DURING INFECTION

Michael Horne Askenase

Yasmine Belkaid, Ph.D.

Tissue-infiltrating Ly6C<sup>hi</sup> monocytes play important protective roles during infection, including inflammatory cytokine secretion and pathogen killing. Here we show that during acute gastrointestinal infection with the protozoan parasite *Toxoplasma gondii*, recruited monocytes not only contributed to parasite control, but also regulated pathologic immune responses to commensal microbes via secretion of the lipid mediator prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Priming of monocytes for regulatory function preceded systemic inflammation and was initiated prior to bone marrow egress. Natural killer (NK) cell-derived IFN- $\gamma$  promoted a regulatory program in monocyte progenitors during development. Early bone marrow NK cell activation was controlled by systemic IL-12 produced by Batf3-dependent dendritic cells (DC) in the mucosal-associated lymphoid tissue (MALT). This work challenges the paradigm that monocyte function is dominantly imposed by local signals following tissue recruitment, and instead proposes a sequential model of differentiation in which monocytes are pre-emptively educated during development in the bone marrow to promote their tissue-specific function.

# Table of Contents

<b>Acknowledgements .....</b>	<b>iii</b>
<b>Abstract .....</b>	<b>v</b>
<b>Table of Contents .....</b>	<b>vi</b>
<b>List of Figures .....</b>	<b>x</b>
<b>List of Publications .....</b>	<b>xiii</b>
<b>CHAPTER 1: Introduction to the Mononuclear Phagocyte System in Health and Disease .....</b>	<b>1</b>
<i>Macrophages.....</i>	<i>6</i>
<i>Dendritic Cells.....</i>	<i>12</i>
<i>Monocytes .....</i>	<i>16</i>
<i>Infection-Induced Remodeling of Hematopoiesis .....</i>	<i>26</i>
<i>Oral Toxoplasma gondii Infection.....</i>	<i>28</i>
<b>CHAPTER 2: Ly6C<sup>hi</sup> Monocytes Acquire Regulatory Capacity Prior to Tissue Entry During Infection .....</b>	<b>32</b>
<i>Abstract.....</i>	<i>32</i>
<i>Rationale.....</i>	<i>34</i>
<i>Results.....</i>	<i>39</i>
Systemic alteration of Ly6C <sup>hi</sup> monocytes prior to tissue recruitment.....	39
IFN- $\gamma$ remodels the blood monocyte compartment during infection .....	47
<i>Discussion .....</i>	<i>52</i>



<b>CHAPTER 3: Monocyte Progenitors in the Bone Marrow are Conditioned by IFN-<math>\gamma</math> During Infection .....</b>	<b>57</b>
<i>Abstract</i> .....	57
<i>Rationale</i> .....	58
<i>Results</i> .....	59
Ly6 <sup>Chi</sup> monocytes are functionally primed in the bone marrow.....	59
IFN- $\gamma$ controls the transcriptional program of monocyte progenitors .....	66
<i>Discussion</i> .....	74
<b>CHAPTER 4: BM-Resident NK Cells Educate Monocyte Progenitors in Response MALT-Derived IL-12 .....</b>	<b>77</b>
<i>Abstract</i> .....	77
<i>Rationale</i> .....	78
<i>Results</i> .....	79
NK cell production of IFN- $\gamma$ in the BM educates monocyte progenitors.....	79
IL-12 Produced by <i>Batf3</i> -dependent DC in the MALT Drives IFN- $\gamma$ production .	86
<i>Discussion</i> .....	94
<b>CHAPTER 5: Discussion and Conclusions .....</b>	<b>97</b>
<i>Re-examining the role of monocytes in acute inflammatory responses</i> .....	97
<i>Anti-inflammatory properties of monocytes during systemic inflammation and cancer</i> .....	103
<b>CHAPTER 6: Materials &amp; Methods .....</b>	<b>108</b>
<i>Mice</i> .....	108
<i>T. gondii Parasite and Infection Protocol</i> .....	108
<i>Yersinia pseudotuberculosis Infection Protocol</i> .....	109

<i>Plasmodium yoelii</i> Infection Protocol.....	109
Tissue Preparation and Immune Cell Isolation.....	110
Flow Cytometry .....	111
<i>Ex vivo</i> Stimulation of Ly6C <sup>hi</sup> Monocytes and Detection of TNF- $\alpha$ and IL-10 .....	112
Serum Cytokine and Enzyme Measurements .....	113
Pathology Assessment.....	113
Generation and Infection of Mixed WT & <i>Ifngr1</i> <sup>-/-</sup> Bone Marrow Chimeras .....	113
Ly6C <sup>hi</sup> Monocyte and cMoP Purification by FACS.....	114
<i>Ex vivo</i> Stimulation of FACS purified Ly6C <sup>hi</sup> Monocytes for PGE <sub>2</sub> Detection .....	114
<i>Ex vivo</i> Stimulation of FAC-Purified Ly6C <sup>hi</sup> Monocytes for NanoString Analysis .....	115
Gene Expression Analysis by NanoString nCounter Technology .....	115
Pathway Analysis of Gene Expression Data.....	117
<i>In vivo</i> Administration of IFN- $\gamma$ .....	117
<i>In vivo</i> Administration of IL-12p70 .....	117
<i>In vivo</i> Blockade of IFN- $\gamma$ .....	118
<i>In vivo</i> Blockade of IL-12p70 .....	118
<i>In vivo</i> NK Cell Depletion .....	118
Intracellular Detection of IFN- $\gamma$ .....	119
Immunofluorescence imaging.....	119
<i>Ex vivo</i> Differentiation of Monocytes and Macrophages.....	120

<i>Ex vivo Detection of IL-12p70 from Tissue Preparations</i> .....	121
<i>Statistical Analysis</i> .....	121
<b>Bibliography</b> .....	<b>122</b>

## List of Figures

<b>Figure 1: Development of the Mononuclear Phagocyte System. ....</b>	<b>5</b>
<b>Figure 2: Ly6C<sup>hi</sup> monocytes suppress neutrophil-mediate pathology during oral <i>T. gondii</i> infection. ....</b>	<b>36</b>
<b>Figure 3: Ly6C<sup>hi</sup> monocytes produce PGE<sub>2</sub> and prevent lethal immunopathology.....</b>	<b>37</b>
<b>Figure 4: Dual Function of Ly6C<sup>hi</sup> monocytes during <i>T. gondii</i> infection. ....</b>	<b>38</b>
<b>Figure 5: Ly6C<sup>hi</sup> monocytes acquire a MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> Phenotype in the SILP during <i>T. gondii</i> infection. ....</b>	<b>42</b>
<b>Figure 6: Infection alters the phenotype and composition of the blood monocyte compartment. ....</b>	<b>43</b>
<b>Figure 7: Blood Ly6C<sup>hi</sup> monocytes acquire regulatory features during infection. ....</b>	<b>44</b>
<b>Figure 8: Changes to blood monocytes precede systemic inflammatory signals and weight loss.....</b>	<b>45</b>
<b>Figure 9: Changes to blood monocytes precede intestinal pathology and parasite dissemination. ....</b>	<b>46</b>
<b>Figure 10: Alterations to the blood monocyte compartment are a common feature of Th1-polarized immune responses to infection. ....</b>	<b>49</b>
<b>Figure 11: IFN-<math>\gamma</math> controls the phenotype and composition of the blood monocyte compartment. ....</b>	<b>50</b>
<b>Figure 12: Cell-intrinsic IFN-<math>\gamma</math> signaling is required for infection-induced alterations to the blood monocyte compartment.....</b>	<b>51</b>
<b>Figure 13: Ly6C<sup>hi</sup> monocytes acquire a MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> Phenotype in the BM during <i>T. gondii</i> infection. ....</b>	<b>62</b>
<b>Figure 14: IFN-<math>\gamma</math> drives regulatory capacity of BM monocytes. ....</b>	<b>63</b>

**Figure 15: Changes to BM monocytes persist after resolution of acute inflammation. .... 64**

**Figure 16: *T. gondii* infection alters BM monocyte responses to bacterial stimuli. .... 65**

**Figure 17: Phenotype of Monocyte Progenitors is altered during infection. .... 68**

**Figure 18: cMoP display an Interferon-responsive gene signature during infection. .... 69**

**Figure 19: Cell-intrinsic IFN- $\gamma$  signaling is required for alterations to cMoP and Ly6C<sup>hi</sup> monocytes in the BM. .... 70**

**Figure 20: IFN- $\gamma$  drives changes to cMoP phenotype and gene expression. .... 71**

**Figure 21: Putative cMoP analyzed during infection do not express core DC signature genes. .... 72**

**Figure 22: cMoP and BM monocytes retain their differentiation potential during infection. .... 73**

**Figure 23: BM NK cells produce IFN- $\gamma$  early during infection. .... 81**

**Figure 24: Clusters of BM NK cells produce IFN- $\gamma$ . .... 82**

**Figure 25: IFN- $\gamma$  producing BM NK cells are located near Ly6C<sup>hi</sup> monocytes. .... 83**

**Figure 26: NK depletion after antibody administration. .... 84**

**Figure 27: NK cell depletion inhibits changes to cMoP and monocyte phenotype during infection. .... 85**

**Figure 28: Systemic IL-12 drives IFN- $\gamma$  production by BM NK cells. .... 88**

**Figure 29: IL-12, but not IL-18 is required for infection-induced changes to monocyte phenotype. .... 89**

**Figure 30: IL-12 administration drives IFN- $\gamma$  production by BM NK cells. .... 90**

**Figure 31: Characterization of *Batf3*<sup>-/-</sup> mice at day 5 of *T. gondii* infection. ....91**

**Figure 32: *Batf3*-dependent DC produce IL-12 and activate BM NK cells during infection. .... 92**

**Figure 33: IL-12 is produced primarily in the MALT early during *T. gondii* infection. .... 93**

**Figure 34: Sequential model of functional priming of monocytes during *T. gondii* infection..... 96**

## List of Publications

The contents of this thesis contain portions of modified text and figures from the following published manuscripts:

- 1. Askenase, M.H.**, Han, S.-J., Byrd, A.L., da Fonseca, D.M., Bouladoux, N., Wilhelm, C., Konkel, J.E., Hand, T.W., Lacerda-Queiroz, N., Su, X.-Z., et al. (2015). Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection. *Immunity* *42*, 1130–1142.
- 2. Grainger, J.R., Askenase, M.H.**, Guimont-Desrochers, F., da Fonseca, D.M., and Belkaid, Y. (2014). Contextual functions of antigen-presenting cells in the gastrointestinal tract. *Immunol. Rev.* *259*, 75–87.
- 3. Grainger, J.R., Wohlfert, E.A., Fuss, I.J., Bouladoux, N., Askenase, M.H.**, Legrand, F., Koo, L.Y., Brenchley, J.M., Fraser, I.D.C., and Belkaid, Y. (2013). Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat. Med.* *19*, 713–721.

## **CHAPTER 1: Introduction to the Mononuclear Phagocyte System in Health and Disease**

The mononuclear phagocyte system of jawed vertebrates is comprised of macrophages, dendritic cells (DCs), and monocytes (Chow et al., 2011a). These cells are specialized to sense signals from their local environment, expressing a wide array of pattern recognition receptors (PRRs) and dedicated cellular machinery for sampling their environment and presenting antigen (Iwasaki and Medzhitov, 2015). In the context of infection, these antigen-presenting cells (APCs) recognize pathogen-associated molecular patterns (PAMPs) via PRRs and become activated, producing inflammatory cytokines and increasing their phagocytic capacity. This results in presentation of microbial antigens to T cells and induction of the adaptive immune response. Activated APCs can also act as immediate effector immune cells, phagocytosing and killing the invading microbe via expression of anti-microbial proteins and toxic free radicals (Wynn et al., 2014).

The critical functions of APCs are not limited to the initiation and shaping of inflammatory responses. They maintain homeostatic function of many tissues via phagocytosis of dead and dying cells, production of growth factors and cytokines that regulate tissue function, and induction of regulatory populations of lymphocytes that inhibit aberrant inflammatory responses (Murray and Wynn, 2011). These anti-inflammatory properties of APCs are particularly critical to tissues that form a barrier with the external environment, such as the gut, skin, and lung. The regulatory milieu of these tissues shapes the function of APCs; resident DCs and macrophages have



developed specialized regulatory mechanisms to ensure tolerance of antigens absorbed at these sites from both the environment and resident commensal microbes (Belkaid and Naik, 2013; Grainger et al., 2014). However, as these barrier sites are also primary points of pathogen entry, resident phagocytes must distinguish between pathogenic and beneficial microbes, and maintain the capacity to initiate a rapid inflammatory response upon infection. Circulating myeloid cells, including monocytes and neutrophils, also play a critical role in controlling invading pathogens at barrier sites (Grainger et al., 2014).

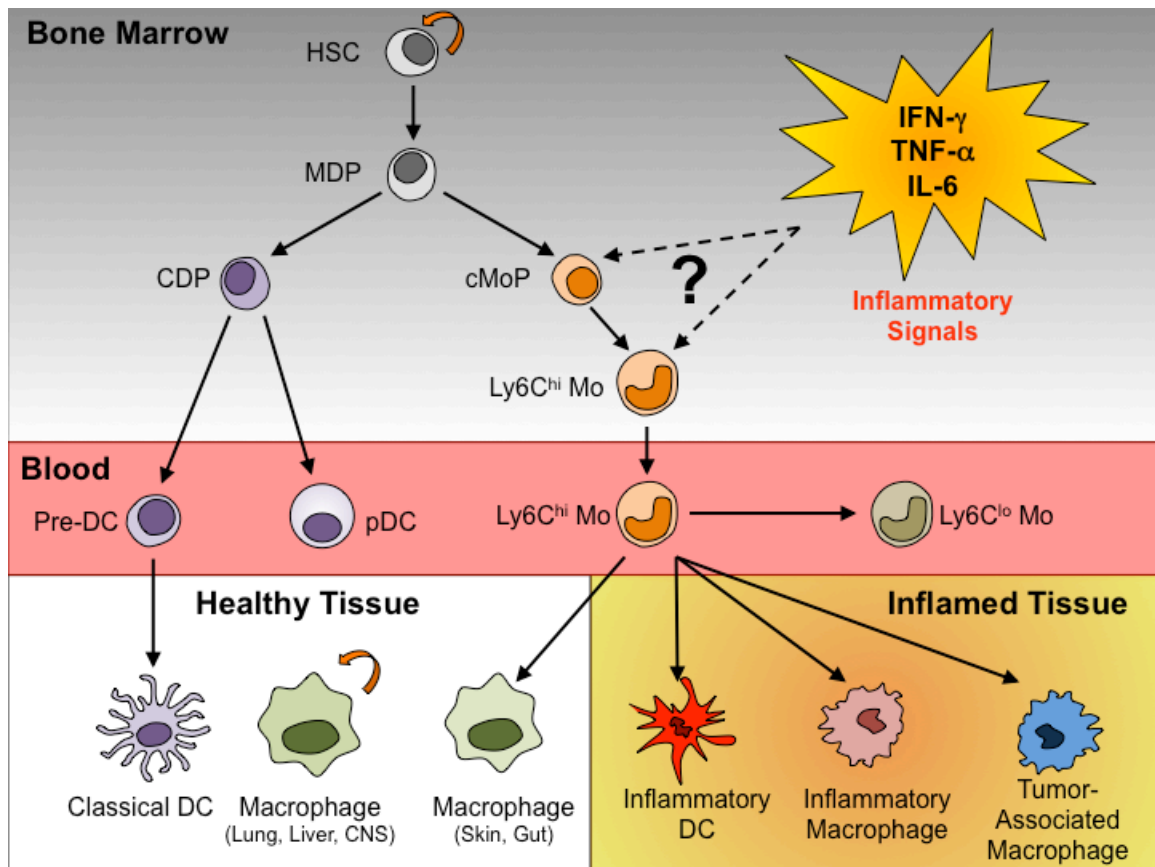
There are no known surface markers that identify and distinguish APC populations across all tissues in the way that the T and B cell receptors are used to identify lymphocyte populations (Chow et al., 2011a). Macrophages, DCs, and monocytes share not only surface markers, but many transcriptional and molecular pathways, making the classification of mononuclear phagocyte populations a difficult task (Murray and Wynn, 2011). Compounding this problem is that as a result of their exquisite sensitivity to their local environment, the morphology, function, and gene expression of tissue-resident macrophages and DCs varies widely between tissues. Recent studies have employed four distinct approaches to assess the relationship between the components of the mononuclear phagocyte system: i) genetic deletion of essential transcription factors expressed during myeloid development and assessment of their effects on the genesis of mature monocyte, macrophage, and DC populations; ii) use of fate-reporter systems to identify the mature progeny of a fluorescent protein-expressing hematopoietic precursor, iii) parabiotic experiments to determine the contribution of circulating progenitors to

tissue-resident DC and macrophage populations, and iv) broad transcriptional analysis and comparison of APC populations throughout the body (Chow et al., 2011a).

From these seminal studies, a new paradigm has slowly emerged: while interrelated and overlapping in gene expression and function, macrophages, DCs, and monocytes represent three separate arms of the mononuclear phagocyte system, with distinct ontogeny, functions, and rates of turnover under steady state conditions. However, the distinction of DCs as a separate lineage from macrophages remains controversial (Gautier et al., 2012; Hume and Freeman, 2014). With the exception of certain resident macrophage populations at barrier sites that are constitutively replenished by monocytes recruited from the population, monocytes do not differentiate into tissue DCs or macrophages under steady-state conditions. DC populations are derived from dedicated precursors constantly generated in the bone marrow (BM) and circulating in the blood, while macrophages in most tissues are self-renewing and not replenished by cells from the circulation (Geissmann et al., 2010). However, it's also apparent that during inflammation, these distinctions become less clear, and monocytes in particular demonstrate the potential for a wide range of fates and functions, including differentiation into DCs and macrophages (**Figure 1**) (Shi and Pamer, 2011).

The overarching goal of the studies described in this dissertation was to identify tissue-specific and systemic signals that control monocyte differentiation and function during infection. To gain a better understanding of the role of monocytes during infection, it is not only necessary to review the current state of knowledge of monocytes,

but also to contrast their development and function to that of other mononuclear phagocyte populations. Thus, in this introduction we first focus on our understanding of macrophages and DCs before fully exploring what is currently known about monocytes during steady-state conditions and infection. Following that, we review how infection can remodel hematopoiesis and increase production of myeloid populations. Finally, we will discuss the function of monocytes, macrophages, and DCs in a model infectious setting: oral infection with the protozoan parasite *Toxoplasma gondii*.



**Figure 1: Development of the Mononuclear Phagocyte System**

Self-renewing HSCs in the BM differentiate into a Monocyte-Macrophage-DC precursor (MDP) that gives rise both to the common DC Precursors (CDP) and common Monocyte Progenitors (cMoP). The CDP gives rise to plasmacytoid DCs (pDC) as well as circulating Pre-DC which replenish classical DC populations in lymphoid and non-lymphoid tissues. cMoP differentiate in the BM into Ly6C<sup>hi</sup> monocytes that exit the BM and enter the circulation. Under steady-state conditions, Ly6C<sup>hi</sup> monocytes differentiate into Ly6C<sup>lo</sup> “patrolling” monocytes as well as resident macrophage populations in the skin and gut. Macrophages in the lung, liver, CNS, and other tissues are seeded by hematopoietic cells early during fetal development and self-renew within these tissues. During inflammation, Ly6C<sup>hi</sup> monocytes can differentiate into a wide range of effector cells in inflamed tissue, including inflammatory DC and macrophages, as well as anti-inflammatory macrophages associated with tumors. Inflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 are known to drive increased monocyte production in the BM, but whether these cells can influence monocyte function remains unknown.

## ***Macrophages***

The study of phagocytes was pioneered by Elie Metchnikoff in the late 19<sup>th</sup> century (Gordon, 2008). Metchnikoff, upon observing the presence of specialized, highly phagocytic cells important for host defense in starfish larvae, categorized these newly identified cells into polynuclear “microphages” (now known as granulocytes), and mononuclear “macrophages”. Metchnikoff also noted the morphological distinctions between the small, round mononuclear phagocytes found in the lymph and blood of vertebrates (“monocytes”), and the larger, morphologically complex macrophages found in tissues. Tissue macrophages are identified by their expression of high levels of CD45, F4/80, and CD11b, and the lack of expression of lymphocyte markers (Gordon et al., 2014). The finding that monocytes isolated from the circulation were capable of differentiating into macrophages both *in vitro* and *in vivo*, led to the proposal by Ralph van Furth that these cells were components of a unified “mononuclear phagocyte system” (Cohn and Benson, 1965; Lewis, 1925; van Furth and Cohn, 1968; van Furth et al., 1972). In this classification, monocytes and macrophages were distinguished primarily by their location and morphology: small, round mononuclear phagocytes found in blood and lymph were known as monocytes, while phagocytes found in solid organs were known as tissue macrophages.

This paradigm persisted for over 40 years, but in recent years our understanding of macrophage ontogeny has been dramatically reshaped. Van Furth himself first noted that, in classical pulse-chase experiments to measure macrophage turnover after

treatment with labeled thymidine, that a proportion of splenic macrophages did not appear to be replenished (van Furth and Diesselhoff-den Dulk, 1984). Later studies utilizing parabiotic mice found that in adult mice, microglia, the resident phagocytes of the central nervous system (CNS) renewed independently of circulating precursors, raising the possibility that these cells were not constitutively replenished by monocytes, although the potential seeding of these populations early during development was not addressed (Ajami et al., 2007; Merad et al., 2002). The concept that tissue resident macrophages develop and are maintained independently of circulating precursors was cemented in 2012, when macrophage populations in the spleen, liver, pancreas, kidney, and lung were all found to develop normally in mice lacking HSCs (**Figure 1**) (Schulz et al., 2012). Using mice deficient in *Myb*, a transcription factor essential for HSC development, a group led by Fredric Geissmann demonstrated that these tissue-resident populations are instead derived from yolk-sac macrophages early in embryonic development. Subsequent studies utilizing gene deletion and fate-mapping reporter mice have demonstrated that microglia are similarly seeded during embryogenesis and are maintained via self-renewal thereafter (Ginhoux et al., 2010). In contrast, macrophage populations in the skin and gut are constitutively replenished by circulating classical monocytes (Tamoutounour et al., 2013; Varol et al., 2009). One unifying trait of resident macrophages throughout the body, however, is their dependence on signaling via CSF-1R, the receptor for CSF-1 (M-CSF) and IL-34 (Davies et al., 2013). These related factors drive homeostatic proliferation of macrophage populations, and are the only known required growth factors for macrophage homeostasis (Jenkins et al., 2013).

Transcriptional control of macrophage development is similarly diverse. While the requirement for PU.1 is shared by nearly all macrophage populations (Schulz et al., 2012), the search for a core macrophage transcriptional program across tissues has been more elusive. Recent computational approaches have predicted that CEBP- $\alpha$ , TCF3, Bach1, and CREG-1 are critical regulators of macrophage transcriptional programs, but the dependence *in vivo* of macrophage development and function on these factors remains unclear (Gautier et al., 2012). Recently, the transcription factor specific for a particular population of tissue-resident macrophages was described: GATA6 is uniquely required for development, homeostasis, and function of peritoneal macrophages (Gautier et al., 2014; Okabe and Medzhitov, 2014; Rosas et al., 2014). The diversity of macrophage origins and the independence of many populations from hematopoietic renewal under steady-state conditions underscore the critical role their local tissue environments place in controlling the development and function of these cells.

The presence of macrophages early during tissue development reflects the essential roles macrophages have been shown to play in development, function, and homeostasis of the tissues in which they reside, as first proposed by Metchnikoff upon their discovery (Wynn et al., 2014). Just as macrophage gene expression and function is dictated by the signals they receive from their environment, macrophages reciprocate by secreting factors that shape the function of the stroma and cells surrounding them, focusing specifically on maintenance of normal tissue turnover and homeostasis. In particular, macrophages have been shown to regulate the differentiation of stem and progenitor cell populations in the liver, intestine, and BM (Boulter et al., 2012; Chow et

al., 2011b; Pull et al., 2005). Furthermore, mice lacking CSF-1 receptor, and thus most peripheral macrophage populations including osteoclasts and microglia, demonstrate substantial defects in bone and neural development (Cecchini et al., 1997; Erblich et al., 2011). As specialized phagocytes, macrophages are also tasked with phagocytosis and removal of apoptotic cells and debris as part of the normal tissue maintenance (Erwig and Henson, 2008). Deficiencies in macrophage apoptosis drives a lupus-like phenotype in mice, and decreased phagocytosis of apoptotic bodies by macrophages has been observed in lupus patients (Baumann et al., 2002; Cohen et al., 2002; Herrmann et al., 1998). Splenic macrophages phagocytose ageing red blood cells and regulate the production of new erythrocytes via heme synthesis and phagocytosis of expelled erythrocyte nuclei, while microglia shape neural development by “pruning” neural synapses, thereby aiding in the formation and organization of new neural circuits (de Back et al., 2014; Paolicelli et al., 2011). The critical activities of macrophages in regulating these changes demonstrate that macrophages are not immune sentinels, hibernating until detecting microbial stimuli, but rather are active regulators of normal tissue physiology.

Macrophages are capable of potent inflammatory and anti-microbial responses, and recognition of PAMPs by toll-like receptors (TLRs) and other innate microbial sensors integrate with cytokine signals to drive macrophage activation. These cells are capable of a remarkable array of activation states depending on the signals received, but the best-characterized are “classical activation”, driven by IFN- $\gamma$  in conjunction with microbial signals including lipopolysaccharide (LPS); and ii) “alternative activation”,



driven by IL-4 and IL-13 signaling (Murray et al., 2014). This bifurcation of macrophage polarization is admittedly an oversimplification of the spectrum of their responses, as a recent study has identified as many as 14 unique macrophage polarization states (Xue et al., 2014). However, this reductionist approach can be useful for understanding the roles these cells play in Th1 and Th2 polarized immune responses.

Macrophages have long been appreciated for their anti-microbial potential, and they are critical effectors of cellular immunity, particularly against intracellular pathogens. Macrophages survey their resident tissues for the presence of pathogenic microbes. Activation of innate microbial sensors, including TLR2, TLR4, and TLR9, in conjunction with IFN- $\gamma$  produced by lymphocytes results in classical macrophage activation, which can be defined by 3 properties: i) secretion of inflammatory cytokines ii) increased phagocytosis, iii) production of intracellular anti-microbial molecules (Mosser and Edwards, 2008). TNF- $\alpha$ , IL-1, and IL-6 produced by macrophages act as alarm signals, initiating inflammatory cascades in nearby hematopoietic and non-hematopoietic cells and driving the recruitment of circulating inflammatory populations including neutrophils and monocytes. As TNF- $\alpha$  and IL-1 can themselves contribute to the classical activation of macrophages, detection of microbial invasion by a few cells can result in inflammatory signals that are propagated in a paracrine fashion throughout the tissue and to draining lymph nodes. Additionally, production of IL-12 and IL-23 by macrophages shapes the induction of the adaptive immune response to infection. Classically activated macrophages act as potent effector cells in their own right; they phagocytose invading pathogens and destroy them via production of toxic nitric oxide,

superoxides, and other anti-microbial factors, although the relative contribution to bacterial and parasite killing by resident macrophages versus recruited monocytes remains unclear and likely varies between tissues (Grainger et al., 2014). Conversely, the production of inflammatory cytokines and dangerous free radicals can result in immunopathology and drive inflammatory disease (Murray and Wynn, 2011). Indeed, the accumulation of classically activated macrophages in white adipose tissue is believed to contribute to chronic immune activation associated with obesity (Wynn et al., 2014).

Alternatively activated macrophages are essential regulators of wound healing (Murray and Wynn, 2011). IL-4 and IL-13 are produced in response to tissue damage and signaling by these Th2 cytokines on tissue-resident macrophages results in the augmentation of pathways contributing to extra-cellular matrix deposition and fibrosis. In addition to sterile injury, IL-4 and IL-13 are robustly produced in response to helminth infection, and alternatively activated macrophages play important roles in the expulsion of these extracellular parasites by supporting the formation of granulomas that control the parasite, by increasing mucus production from epithelial cells, and by augmenting the peristaltic activity of smooth muscle cells in infected tissue (Anthony et al., 2007; Chen et al., 2014). Intriguingly, recent studies have noted that alternative activation induces resident macrophages to rapidly proliferate, and in this context IL-4/13 signaling appears to replace CSF-1 as the critical factor regulating their division and homeostasis (Jenkins et al., 2013; 2011).

In light of the recent discovery that macrophages in most tissues are not derived from monocytic precursors, it's important to reconsider our understanding of macrophage biology. Much of what has been learned about the pathways involved in macrophage activation and their responses to various stimuli has been conducted using human monocytes isolated from peripheral blood or mouse monocytes isolated from the BM and differentiated *in vitro* with CSF-1 to induce what are known as bone marrow-derived macrophages. Recent studies demonstrating the remarkable diversity of macrophage gene expression depending on tissue residence suggests that bone marrow-derived macrophages may not be representative of the populations resident in various tissues (Gautier et al., 2012). The relative role of tissue-resident macrophages versus monocytes in mediating inflammatory responses *in vivo* requires further study. **This issue will be further addressed in our discussion of monocyte function.**

### ***Dendritic Cells***

In 1973, Ralph Steinman and Zanvil Cohn identified a novel population of mononuclear phagocytes in the spleen with much greater capacity to present antigen and activate T lymphocytes than that of splenic macrophages (Steinman and Cohn, 1973). These rare cells (<2% of the total leukocyte pool in the spleen) displayed a unique morphology, with fewer phagosomes than macrophages and distinct long cellular processes, leading Steinman to dub these cells “dendritic cells” (DCs). At this time, DCs were distinguished from macrophages by morphology, as well as their decreased adhesiveness to glass after culture. Subsequent studies revealed that similar to

macrophages, DCs are found not only in lymphoid organs, but in tissues throughout the body (Hart and Fabre, 1981). Upon stimulation by inflammatory cytokines or microbial signals, tissue-resident DCs “mature”, increasing their antigen processing and presentation capability, augmenting expression of co-stimulatory molecules, and migrating to the draining lymph node in a CCR7-dependent manner and prime T cell responses against their captured antigens (Merad et al., 2002; Ohl et al., 2004; Yanagihara et al., 1998). DCs resident in lymphoid tissues are also potent APCs and both resident and migratory DCs play major roles in antigen presentation and the priming of T cell responses.

DCs are identified by their expression of high levels of CD11c and class II MHC molecules, as well as the absence of lymphocyte markers (Geissmann et al., 2010). This has resulted in some disagreement over how to best classify certain tissue-resident APC populations that express CD11c but do not migrate to lymph nodes, and whose function and morphology more closely resembles macrophages than DCs. Recent studies of the gene expression of these cells reveals a lack of expression of DC transcripts, and a transcriptional profile more closely resemble tissue-resident macrophage populations (Gautier et al., 2012; Miller et al., 2012). Additionally, these cells are not derived from DC precursors, and thus are generally accepted to be tissue-resident macrophages, despite their expression of CD11c (Bogunovic et al., 2009; Helft et al., 2010; Tamoutounour et al., 2013).

As a result of the ability to differentiate monocytes into DCs *in vitro*, the predominant view upon the discovery of these cells was that lymphoid and tissue-resident DC populations were constitutively replenished by circulating monocytes (Inaba et al., 1992). The discovery by two groups in 2007 of a common DC precursor (CDP) in the BM that does not give rise to monocytes suggested that *in vivo* DCs constituted an independent lineage from monocytes (**Figure 1**) (Naik et al., 2006; 2007; Onai et al., 2007). Indeed, it is now believed that in the steady-state, the majority of DC populations in tissue and lymph derive from a rare circulating pre-DC population (Liu et al., 2009; 2007). Langerhans cells, a unique DC population found in the epidermis, are the exception, as they are seeded by a precursor during embryonic development and self-renew locally in the skin thereafter (Hoeffel et al., 2012).

DC differentiation is dependent on signaling via the surface receptor Flt3 through binding of its ligand Flt3l, beginning with development from early myeloid progenitors, to the CDP, pre-DC, and mature dendritic cells (Merad et al., 2013). CSF-2 (GM-CSF) also acts as a homeostatic factor for DCs and mice deficient in CSF-2 signaling have decreased numbers of DCs in many tissues (Kingston et al., 2009). However, in contrast to Flt3l, CSF-2 is not absolutely required for DC development. Three general categories of DCs develop from the CDP and seed lymphoid and non-lymphoid tissues throughout the body (Geissmann et al., 2010; Merad et al., 2013). These groups can be distinguished via the network of transcription factors required for their development. Plasmacytoid DCs (pDCs), a circulating population of DCs specialized in type 1 interferon production and with morphology resembling lymphoid cells, differentiate directly from the CDP and

require *PU.1*, *E2-2*, and *Ikaros* for development. CD11b<sup>+</sup> “myeloid” DCs, which include resident populations in lymphoid tissue as well as migratory DCs found in the gut, skin, and lung, develop via the circulating pre-DC in a manner dependent on IRF4 and Notch2. “Lymphoid” DCs, which include CD8 $\alpha$ <sup>+</sup> DCs in lymph as well as CD103<sup>+</sup>CD11b<sup>-</sup> DCs in many tissues, develop from the pre-DC via expression of *IRF8*, *Id2*, and *Batf3*. These latter two groups, referred to collectively as “classical DCs”, can be distinguished from pDCs as well as monocyte-derived DCs by their expression of the transcription factor *Zbtb46*, although these populations develop normally in *Zbtb46*-deficient mice (Meredith et al., 2012; Miller et al., 2012; Satpathy et al., 2012).

The primary function of classical DC populations *in vivo* is the priming of CD4 and CD8 T cell responses, and the nature of the adaptive immune response is dependent on signals received by DCs from microbes and their local tissue environment (Iwasaki and Medzhitov, 2015). In response to these signals, DCs direct T cell responses by producing cytokines and other factors that direct the differentiation of naïve T cells into specific effector and memory lineages (Zhu et al., 2010). DC production of IL-12 directs the differentiation of Th1 cells, while IL-23, and IL-6 secretion induces Th17 differentiation. DCs can also induce regulatory T cells (Treg), a process particularly important for maintenance of tolerance at barrier sites, via secretion of retinoic acid (RA) and activation of latent TGF- $\beta$  (Hall et al., 2011; Worthington et al., 2011). As the primary directors of adaptive immune responses, DCs constitutively mediate the balance between regulatory responses important for maintaining tolerance and preventing

chronic inflammation, while remaining poised to induce appropriate inflammatory responses upon infection by pathogens.

## ***Monocytes***

While long considered primarily as a precursor population for mature DCs and macrophages, studies in recent years have clearly established that circulating monocytes are a distinct mature mononuclear phagocyte population with unique functions. The classical definition of monocytes as mononuclear phagocytes found in the circulation that have not entered tissue must be reconsidered. Recent studies have reported cells with a monocytic transcriptional signature present in many tissues and provided evidence that certain populations of monocytes re-enter the circulation after migrating in and out of solid tissue (Becher et al., 2014; Jakubzick et al., 2013; Tamoutounour et al., 2013). Additionally, the spleen hosts a substantial population of monocytes that rapidly mobilize at the onset of inflammation (Swirski et al., 2009). While under steady-state conditions these cells do not contribute to most tissue macrophage and classical DC populations (with the previously noted exceptions of certain populations of skin and intestinal macrophages), monocytes are rapidly recruited to sites of inflammation to complement the function of resident APC populations (Shi and Pamer, 2011). In these settings, monocytes demonstrate a remarkably diverse range of function in host defense and immunoregulation. Upon entry into inflamed tissue, monocyte-derived cells can differentiate into dendritic cells and macrophages, and demonstrate the potential for both inflammatory and regulatory functions. In this context, macrophages may represent

a sort of patrolling APC reservoir, recruited to sites where local APC populations are insufficient or unable to meet the immunological demands of the tissue.

The circulating monocyte pool in both mice and humans is primarily composed of two populations: a “classical” monocyte population (CD14<sup>+</sup>CD16<sup>-</sup> in humans, Ly6C<sup>hi</sup>CD115<sup>+</sup>CD11b<sup>+</sup> in mice) that is recruited to inflamed tissue, and a “patrolling” population (CD14<sup>lo</sup>CD16<sup>+</sup> in humans, Ly6C<sup>lo</sup>CD115<sup>+</sup>CD11b<sup>+</sup> in mice) that rolls along the endothelium and has been speculated to represent the resident macrophage of the circulatory system (Auffray et al., 2009). Recently, the presence of an “intermediate” monocyte population in humans has been noted. This population is poorly understood, but early reports suggest that it may contribute to inflammatory pathology during infection and autoimmunity (Passos et al., 2015; Wong et al., 2012). It is unclear if a homologous subset exists in mice. Transcriptional analysis of classical and patrolling human and mouse monocyte subsets between species have shown strong conservation between the species (Ingersoll et al., 2010). One important difference between mice and humans however, is the relative abundance of these populations in the circulation: classical monocytes represent ~95% of the circulating pool in humans, but only about 50% in mice (Ginhoux and Jung, 2014). For the rest of this section, the discussion will focus on mouse monocytes, whose development and function is better characterized.

Monocytes differentiate from a BM-resident Lin<sup>-</sup>CD115<sup>+</sup>cKit<sup>+</sup>Flt3<sup>+</sup>Ly6C<sup>lo</sup> progenitor shared with DCs known as the monocyte-macrophage-DC precursor (MDP) (**Figure 1**) (Auffray et al., 2009). This population differentiates into a proliferating Lin<sup>-</sup>



CD115<sup>+</sup>cKit<sup>+</sup>Flt3<sup>-</sup>Ly6C<sup>hi</sup>CD11b<sup>-</sup> population committed to monocyte differentiation known as common monocyte progenitors (cMoP) before maturing into Lin<sup>-</sup>CD115<sup>+</sup>cKit<sup>-</sup>Ly6C<sup>hi</sup>CD11b<sup>+</sup> mature monocytes (Hettinger et al., 2013). Ly6C<sup>hi</sup> monocytes exit the BM in a CCR2-dependent fashion and enter the blood, where they circulate with a half-life of less than 24 hours (Yona et al., 2013). The absolute number of blood Ly6C<sup>hi</sup> monocytes follows a circadian rhythm in accordance with the gene *Bmal1*, with numbers peaking 4 hours after the beginning of the light cycle (Nguyen et al., 2013). However, this oscillation was abolished during infection with *Listeria monocytogenes* as a result of increased CCL2 levels in the serum, suggesting that inflammatory stimuli can direct Ly6C<sup>hi</sup> monocyte abundance in the periphery. Increasing evidence suggests that Ly6C<sup>hi</sup> monocytes differentiate into Ly6C<sup>lo</sup> monocytes, although whether this differentiation occurs in the circulation or upon re-entry to the BM is still unclear, as there is experimental evidence for both possibilities (Varol et al., 2008; Yona et al., 2013). The half-life of Ly6C<sup>lo</sup> monocytes is approximately 2 days, although their lifespan increases substantially after depletion of Ly6C<sup>hi</sup> monocytes, perhaps indicating that these cells can be longer lived in the absence of replenishment of their niche by new cells differentiated from Ly6C<sup>hi</sup> cells (Yona et al., 2013).

Both Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes constitutively express CSF-1 receptor (CD115), and their development and homeostasis is dependent on CSF-1 signaling (Ginhoux and Jung, 2014). Ly6C<sup>lo</sup> monocytes also express high levels of the fractalkine receptor CX3CR1 and depend on this receptor for homeostasis as well; in the absence of this receptor they have a shorter lifespan and display markers of apoptosis (Landsman et al.,

2009). Transcriptional control of monocytes is not yet thoroughly understood. PU.1 is required for their development, and there is increasing evidence that KLF4, c-Maf, and MafB support monocyte fate by inhibiting granulocyte differentiation (Auffray et al., 2009). Ly6C<sup>lo</sup> monocytes are absent in mice lacking NR4A1 (Nur77), but whether this transcription factor is important for their differentiation from Ly6C<sup>hi</sup> monocytes or is required for the survival of Ly6C<sup>lo</sup> populations is unclear (Hanna et al., 2011).

There is little evidence that Ly6C<sup>lo</sup> non-classical monocytes leave the circulation and enter tissue, and it's theorized that their primary function is to patrol the vasculature and maintain the health and integrity of the endothelium (Carlin et al., 2013). Supporting this theory of their function, recent studies have found that non-classical monocytes “crawl” along the endothelium in an LFA-1-dependent manner in both mice and humans (Auffray et al., 2007; Collison et al., 2015; Cros et al., 2010). Additionally, these cells are recruited to atherosclerotic plaques in both species, and Nur77-deficient mice lacking Ly6C<sup>lo</sup> monocytes have increased development of atherosclerosis when fed a high fat diet (Haka et al., 2012; Hanna et al., 2012; Tacke et al., 2007). Whether these cells inhibit or contribute to the growth of atherosclerotic plaques, remains debated however (Randolph, 2009). Additionally, Ly6C<sup>lo</sup> monocytes express many receptors and pathways associated with clearance of apoptotic debris, and are more highly phagocytic than circulating Ly6C<sup>hi</sup> monocytes (Ginhoux and Jung, 2014; Ingersoll et al., 2010).

Ly6C<sup>hi</sup> monocytes are rapidly recruited to inflamed tissues, and early studies of these cells found that they were prodigious producers of inflammatory cytokines,

including TNF- $\alpha$ , IL-6, and IL-1 (Shi and Pamer, 2011). As a result, they are often referred to as “inflammatory monocytes” and, upon recruitment to tissue, as “inflammatory macrophages” (Gordon and Taylor, 2005). However, recent studies have revealed that these cells are highly plastic upon entry into tissue, capable of differentiating into DCs as well as macrophages, and performing a diverse range of inflammatory, regulatory, and anti-microbial functions (Shi and Pamer, 2011). Therefore, as we explore the differentiation and functions of these cells upon tissue recruitment, we will instead refer to them as “classical”, rather than “inflammatory” monocytes.

While CCR2 is the only chemokine receptor known to be required for their egress from BM, recruitment of Ly6C<sup>hi</sup> monocytes to tissues is mediated by many receptors, including CCR1, CCR2, CCR5, and CX3CR1 (Serbina and Pamer, 2006; Shi and Pamer, 2011). Indeed, while CCL1 (MIP1- $\alpha$ , binding CCR1 and CCR5), CCL2 (MCP-1, binding CCR2), and CCL7 (MCP-3, binding CCR2 and CCR7) are all potent chemotactic factors for monocytes, the signals driving Ly6C<sup>hi</sup> monocyte trafficking during inflammation are highly redundant, and inhibition of these signals individually generally causes only partial inhibition of monocyte recruitment to inflamed tissue (Jia et al., 2008; Tacke et al., 2007; Zhong et al., 2004). Ly6C<sup>hi</sup> monocytes in the spleen require angiotensin signaling to exit this tissue, while signaling by this molecule is dispensable for BM egress (Swirski et al., 2009). Additionally the surface molecules L-selectin and ICAM-1 have been shown to play important roles in monocyte recruitment into tissues (Shi et al., 2010; Tedder et al., 1995).

In the absence of inflammation, Ly6C<sup>hi</sup> monocytes not only differentiate into Ly6C<sup>lo</sup> monocytes, but also migrate to the intestine and skin, where they differentiate into tissue-resident macrophages (**Figure 1**) (Bogunovic et al., 2009; Tamoutounour et al., 2013; Varol et al., 2009). It's notable that these organs host large communities of commensal microbes, and the unique ontogeny of macrophages in these tissues may be the result of the tonic low level of inflammation induced by the resident microbiota (Belkaid and Naik, 2013). Upon ablation of Langerhans cells or microglia, Ly6C<sup>hi</sup> monocytes differentiate into these cell types and fill in the missing niche, demonstrating that these cells do indeed have the capacity to replenish these populations after inflammation (Ginhoux et al., 2006; Mildner et al., 2007). However, the targeted ablation techniques used in these studies are non-physiological, and monocyte-derived cells are not found in the brain after the acute phase of experimental autoimmune encephalitis (EAE) (Yamasaki et al., 2014). Thus the question of whether monocytes are a major source of tissue-resident macrophages outside of the skin and gut remains an open question, particularly in light of the limited exposure to inflammatory stimuli inherent to keeping mice under specific pathogen-free conditions.

Ly6C<sup>hi</sup> monocytes are capable of potent microbicidal function, and mediate protection against a wide range of bacterial, fungal, and protozoan pathogens (Dunay et al., 2010; Espinosa et al., 2014; Peters et al., 2004; Saeed et al., 2012; Serbina et al., 2003; Sponaas et al., 2009). In many infections, monocytes are the principal effector cells responsible for phagocytosis and killing of the pathogen (Shi and Pamer, 2011). Inflammatory cytokines and innate PRR signaling activate recruited monocytes,

resulting in expression of TNF- $\alpha$  and inducible nitric oxide synthase (iNOS). In many infections, the expression of these factors by monocytes coincides with their expression of CD11c. CD11c-expressing monocyte-derived cells are critical to defense against the bacteria *Listeria monocytogenes* and the fungus *Aspergillus fumigatus*, among others, and have hence been named TNF- $\alpha$  and iNOS producing DCs (TIP-DCs) (Espinosa et al., 2014; Serbina et al., 2003). However, the gene expression and function of monocyte-derived cells in these settings more closely resembles those of classically activated macrophages, and thus may more accurately be described as monocyte-derived inflammatory macrophages (Chow et al., 2011a; Grainger et al., 2013). Additionally, monocyte-derived dendritic cells capable of CCR7-dependent migration to lymph nodes, IL-12 production, and priming of T cell responses have also been observed during infection with the protozoan parasite *Leishmania major* (León et al., 2007). Innate sources of inflammatory cytokines are critical to the activation of these cells. NK cells in particular, have been shown to be a critical source of an initial burst of IFN- $\gamma$  responsible for guiding monocyte function during the early stages of infection (Coombes et al., 2012; Goldszmid et al., 2012; Kang et al., 2008; Newman et al., 2006). Likewise, production of IL-12 by activated monocytes may further activate NK cells, creating a positive feedback loop resulting in highly activated monocyte-derived cells with microbicidal function.

As potent producers of inflammatory cytokines, monocytes also contribute to pathologic responses in many inflammatory diseases. CD14<sup>+</sup> classical monocytes accumulate in the intestinal mucosa of patients with inflammatory bowel disease, where they produce high levels of TNF- $\alpha$ , IL-1, and IL-6, and monocytes-derived DCs isolated

from the blood and lymph nodes of lupus patients produce high levels of IL-1 and IL-6 (Kamada et al., 2013; Rodriguez-Pla et al., 2014; Rugtveit et al., 1997). Studies utilizing mouse models support the pathologic role of monocytes in the pathology of inflammatory diseases. Ly6C<sup>hi</sup> monocytes enter the CNS during EAE, where they express an inflammatory gene signature and drive progression of the disease (Ajami et al., 2011; Yamasaki et al., 2014). Recruited Ly6C<sup>hi</sup> monocytes are also primary mediators of the intestinal inflammation induced by DSS administration, a murine model of inflammatory bowel disease, and pathology is greatly reduced in CCR2-deficient mice (Bain et al., 2013; Rivollier et al., 2012; Zigmond et al., 2012). Thus, the potent inflammatory potential of monocyte populations is a dual edged sword: these cells are essential for protection against infection with a broad array of pathogens, but drive increased pathology in the context of immune dysregulation and autoimmunity.

In recent years, new studies have established that Ly6C<sup>hi</sup> monocytes can also serve anti-inflammatory functions, resulting in a shift in our understanding of the primary function of this phagocyte population. Recruited monocytes become alternatively activated and assist in tissue repair after muscle and liver damage via expression of transforming growth factor  $\beta$  (TGF- $\beta$ ) and matrix metalloproteases as well as phagocytosis of apoptotic bodies (Arnold et al., 2007; Ramachandran et al., 2012; Zigmond et al., 2014b). Nitric oxide, in addition to its microbicidal properties, is a potent inhibitor of T cell responses, and monocyte-derived nitric oxide has been shown to inhibit T cell proliferation during infection and graft versus host disease (Abrahamsohn and Coffman, 1995; D'Aveni et al., 2015; Sponaas et al., 2009).

Many of these factors also direct chemotaxis of neutrophils, and monocytes and neutrophils can be found in close proximity at foci of infection or inflammation (Grainger et al., 2013; Molloy et al., 2013; Ostrand-Rosenberg and Sinha, 2009; Wantha et al., 2013). Ly6C<sup>hi</sup> monocytes regulate inflammatory neutrophil responses to translocating bacteria during intestinal *Toxoplasma gondii* (*T. gondii*) infection via production of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and CCR2-deficient mice succumb to lethal immunopathology during this infection (Grainger et al., 2013). PGE<sub>2</sub> is an eicosanoid with diverse roles in the induction and resolution of inflammation, however in this context, monocyte-derived PGE<sub>2</sub> inhibits oxidative burst and inflammatory cytokine production, demonstrating a critical regulatory role for monocytes during infection. Recruited monocytes have also been shown to increase expression of COX-2, the rate-limiting enzyme in PGE<sub>2</sub> synthesis in EAE and acute liver injury, suggesting that these cells may play regulatory roles in autoimmunity and sterile inflammation in addition to responding to infection (Yamasaki et al., 2014; Zigmond et al., 2014b).

Ly6C<sup>hi</sup> monocytes are recruited to tumors, and demonstrate anti-inflammatory function in this context as well. A mixed pool of monocytes and granulocytes, termed “myeloid-derived suppressor cells”, have long been known to inhibit tumor-specific T cell responses via secretion of PGE<sub>2</sub>, IL-10 and nitric oxide (Nagaraj et al., 2013; Ostrand-Rosenberg and Sinha, 2009). Due to the heterogenous nature of these populations, the cellular source of these factors has been debated, but a recent study found that the suppressive functionality of these cells is mediated by Ly6C<sup>hi</sup> monocytes (Cortez-Retamozo et al., 2012; Haverkamp et al., 2014). Supporting this finding, Ly6C<sup>hi</sup>

monocytes have been shown to differentiate into anti-inflammatory tumor associated macrophages in lung and mammary gland cancers, and monocyte depletion decreased tumor progression in these studies (Cortez-Retamozo et al., 2013; Franklin et al., 2014).

The diverse roles monocytes play in immune responses to infection, injury, and cancer have complicated the study of these cells. This difficulty is compounded by the lack of known markers to distinguish monocyte-derived APCs from resident populations. Down-regulation of Ly6C and CCR2 often occurs rapidly after tissue entry, and at this point monocyte-derived populations become indistinguishable phenotypically from tissue-resident macrophages and/or DCs (Auffray et al., 2009). Broad transcriptional analyses represent an alternative approach to classify APC populations during infection, and have already demonstrated that monocyte-derived DCs, while sharing a CD11c<sup>+</sup>MHCII<sup>hi</sup> surface phenotype, do not express core DC transcripts shared by all populations of classical DCs (Miller et al., 2012). The factors that determine the fate and function of recruited monocytes in inflammatory settings remains poorly understood, but the prevailing paradigm is that these processes are determined by local signals received after tissue recruitment (Bain and Mowat, 2014; Ingersoll et al., 2011; Serbina et al., 2008).

The recent discovery that recruited monocytes are the predominant APC population in a wide range of inflammatory and regulatory immune responses has generated keen interest into understanding the processes that guide their differentiation



and function. However, at this juncture many critical outstanding questions remain including:

- What factors drive regulatory versus inflammatory functions by monocytes?
- Do signals received prior to tissue recruitment influence monocyte function?
- How plastic are these cells? Can they switch from an anti-microbial gene expression program (classical activation) to one favoring wound healing and resolution of inflammation (alternative activation), or are these activities mediated by distinct waves of monocyte recruitment?

**In this dissertation, we will examine the first two questions using acute intestinal infection with *Toxoplasma gondii* as a model of monocyte differentiation and function in an inflammatory setting.**

### ***Infection-Induced Remodeling of Hematopoiesis***

Profound changes to hematopoiesis have been described in multiple settings of inflammation, including those driven by infection and cancer (Belyaev et al., 2010; Chou et al., 2012; Cortez-Retamozo et al., 2012; MacNamara et al., 2011; Serbina et al., 2009). In particular, inflammatory signals can drive increased BM output of myeloid cells, known as “emergency myelopoiesis”, a critical feature of the host response to injury or infection (Takizawa et al., 2012). Host-derived cytokines, most notably IFN- $\gamma$ , can remodel hematopoiesis in inflammatory settings via their effects on the proliferation and

differentiation of early BM progenitor populations (**Figure 1**) (de Bruin et al., 2014). IFN- $\gamma$  signaling activates quiescent HSCs, favoring programs of differentiation and inhibiting self-renewal (Baldrige et al., 2011; Herndler-Brandstetter et al., 2011; Zhao et al., 2010). Furthermore, IFN- $\gamma$  favors the differentiation of myeloid cells from multipotent progenitors, resulting in dramatic increases in BM of monocytes (Belyaev et al., 2010; MacNamara et al., 2011). IL-6 has also been shown to favor myeloid differentiation from erythromyeloid progenitor populations, resulting in augmented production of monocytes and erythrocyte depletion (anemia) during infection (Chou et al., 2012). Systemic cytokine signals may also influence the function of cells in the BM. A recent study reported that during viral infection localized to the lung, systemic type I interferon was able to prime BM monocytes to rapidly kill the virus (Hermesh et al., 2010).

In addition to being the primary site of hematopoiesis, the BM is home to populations of mature memory T cells, innate lymphoid cells, plasma cells, macrophages, and stromal cells (Becker et al., 2005; Glatman Zaretsky et al., 2014; Herndler-Brandstetter et al., 2011; Tokoyoda et al., 2009). These cells support and direct HSC function, both under steady-state conditions and during inflammation. HSCs reside in a carefully controlled niche; nearby Tregs, macrophages, and stromal cells provide growth factors and cytokines that support HSC renewal (Mendez-Ferrer et al., 2010). During infection, these supporting populations can detect microbial products and drive HSC differentiation into monocytes and granulocytes, as well as mobilizing HSCs to the spleen and other sites where they can participate in extramedullary hematopoiesis (Chow et al., 2011b; Shi et al., 2011). CD4 T cells in the BM have also been shown to

direct hematopoietic responses via IFN- $\gamma$  production in response to MyD88-dependent signals received during infection (Zhang et al., 2013). This suggests that mature lymphocyte populations are maintained in the BM in part to control hematopoietic responses to infection. However, studies to date have focused on responses to systemic infection, and whether BM-resident lymphocytes can direct hematopoiesis during a localized infection in a distal tissue has not been explored.

### ***Oral Toxoplasma gondii Infection***

*Toxoplasma gondii* is an obligate intracellular protozoan parasite with a complex life cycle including a sexual phase hosted in a wide range of feline species (Munoz et al., 2011). The asexual phase of *T. gondii*'s life cycle infects a wide range of hosts, including humans and mice. Oocysts containing sporozoites are ingested by these species and infect the intestinal mucosa. While *T. gondii* infection is considered asymptomatic in most immunocompetent humans, infection in mice induces a robust Th1 polarized immune response, and this pathogen has long been utilized as a model infection to study the immune response to intracellular pathogens in mice (Munoz et al., 2011). There are at least three recognized categories of *T. gondii* strains: Type I, II, and III, with type II and III demonstrating decreased virulence in mice relative to type I strains (Sibley et al., 2009). Intraperitoneal injection or oral administration (by gavage) of Type II strains at low doses results in a transient acute phase of infection, followed by the establishment of a relatively quiescent chronic infection at immunoprivileged sites, including the brain, eyes, and muscle. High dose infection induces lethal immunopathology during the acute

phase of infection (Munoz et al., 2011). **To examine the signals controlling Ly6C<sup>hi</sup> monocyte development and function in inflammatory settings at barrier tissues, we have employed oral infection with a low dose (10 bradyzoite cysts) of Type II strain Me49 of *T. gondii*.**

Oral infection with *T. gondii*, the natural route of infection, induces pronounced acute inflammation in the ileum of the small intestine resembling that seen in Crohn's disease (Liesenfeld, 2002). Infection is associated with a pronounced dysbiosis in the composition of the intestinal microbiota, resulting in a predominance of highly inflammatory gamma-proteobacteria including *Escherichia coli* (Heimesaat et al., 2006; Molloy et al., 2013; Raetz et al., 2013a). Infection causes the elimination of the mucus layer normally separating the microbiota from the intestinal epithelium, leading to extensive epithelial damage and bacterial translocation across the epithelial barrier (Hand et al., 2012; Heimesaat et al., 2007; Molloy et al., 2013). The enormous potential inflammatory stimulus of these translocating *E. coli* requires the immune response to mediate a delicate balance: it must generate sufficient inflammatory responses to control parasite infection and contain the translocating microbes while also preventing lethal immunopathology (Grainger et al., 2013; 2014).

*T. gondii* infection potently activates DCs in the small intestine and mesenteric lymph nodes (MesLN), resulting in robust IL-12 production and initiation of a type 1 immune response characterized by high levels of IFN- $\gamma$  (Gazzinelli et al., 1994; Liu et al., 2006; Scott and Hunter, 2002). In particular, *Batf3*-expressing DCs, including

CD103<sup>+</sup>CD11b<sup>-</sup> migrating DCs in the small intestine as well as CD8 $\alpha$ <sup>+</sup>CD11b<sup>-</sup> DCs in the MesLN have recently been shown to be the critical producers of IL-12 in this context (Mashayekhi et al., 2011). Profilin expressed by the parasite induces activation of these DCs via TLR11 and TLR12, acting synergistically with stimulation of TLR2 and TLR4 by parasite-derived glycosphosphatidylinositols (Debierre-Grockiego et al., 2007; Raetz et al., 2013b; Yarovinsky et al., 2005). DC-derived IL-12 potently activates NK cells and primes Th1-polarized CD4 and CD8 T cell responses (Gazzinelli et al., 1991; 1993; Khan et al., 2006). Production of IFN- $\gamma$  by both NK cells and CD4 T cells is essential for control of the acute phase of *T. gondii* infection (Gazzinelli et al., 1994; Khan et al., 2006; Schariton-Kersten et al., 1996).

Ly6C<sup>hi</sup> monocytes are essential effectors in low-dose oral *T. gondii* infection, and CCR2-deficient mice succumb to infection (Dunay et al., 2008; Grainger et al., 2013). IFN- $\gamma$  activates recruited monocytes, which phagocytose and kill the pathogen via production of nitric oxide, superoxides, and a class of proteins encoded by interferon response genes (IRGs) (Collazo et al., 2001; Howard et al., 2011; Schariton-Kersten et al., 1997; Zhao et al., 2009). These IRGs directly interface with parasite proteins and control parasite replication through mechanisms that remain to be fully elucidated. Recruited monocyte-derived cells dominate the macrophage response, becoming the predominant APC population in the intestine (Grainger et al., 2013).

The inflammatory response needed to control *T. gondii* infection of the small intestine is counterbalanced by novel regulatory mechanisms that arise to limit immunopathology. This is particularly critical in light of the fact that *T. gondii* infection results in the collapse of the intestinal Treg population, a crucial mediator of tolerance in the steady-state (Oldenhove et al., 2009). To help fill this void, Tbet<sup>+</sup> CD4 T cells, in addition to producing IFN- $\gamma$ , secrete the anti-inflammatory cytokine IL-10 which acts on APC populations to decrease their activation during infection (Jankovic et al., 2007). Recruited neutrophils assist in control of bacterial translocation not only by phagocytosis, but also by emigration to the intestinal lumen where they form intraluminal cellular casts that contain *E. coli* and assist in their expulsion from the gut (Molloy et al., 2013). Monocytes, in addition to killing the parasite, provide essential regulatory functions to limit inflammation. Monocyte-derived PGE<sub>2</sub> inhibits neutrophil inflammatory responses to translocating bacterial microbes, decreasing their production of TNF- $\alpha$  and superoxide (Grainger et al., 2013). Pharmacological inhibition of COX-2 (an essential enzyme for PGE<sub>2</sub> synthesis during inflammation) results in lethal immunopathology during the acute phase of infection, as does inhibition of monocyte recruitment in CCR2-deficient mice. Thus, monocytes play a critical dual role in oral *T. gondii* infection. They are responsible for containment and killing of the parasite, as well as for preventing neutrophil-mediated lethal immunopathology. **The primary goal of this study is to identify the signal(s) that mediate the complex balance between the inflammatory and regulatory functions of monocytes during oral *T. gondii* infection.**

## **CHAPTER 2: Ly6C<sup>hi</sup> Monocytes Acquire Regulatory Capacity Prior to Tissue Entry During Infection**

### ***Abstract***

Ly6C<sup>hi</sup> monocytes perform multiple roles upon recruitment to tissue, and the differentiation and effector function of these cells can be shaped by the local tissue environment, both at steady-state and during inflammation (Bain et al., 2013; Tamoutounour et al., 2013; Zigmond et al., 2012). For instance, production of anti-inflammatory factors, such as IL-10, by Ly6C<sup>hi</sup> monocytes has been described upon their recruitment to the healthy gut and inflamed skin, as well as during muscle repair (Arnold et al., 2007; Bain et al., 2013; Egawa et al., 2013). Additionally, Ly6C<sup>hi</sup> monocytes can give rise to immunosuppressive tumor-associated macrophages in cancer (Franklin et al., 2014). From these studies, a paradigm has emerged proposing that local tissue signals are largely responsible for the acquisition of appropriate function and fate of Ly6C<sup>hi</sup> monocytes following tissue entry (Bain and Mowat, 2014; Ingersoll et al., 2011; Serbina et al., 2008). In this study, we examine when, where, and how monocyte function is imprinted during infection. Key findings include:

- 1. Ly6C<sup>hi</sup> monocytes express a unique MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> phenotype during *T. gondii* infection.**
- 2. Acquisition of this phenotype by monocytes is associated with acquisition of regulatory functions and occurs early during infection,**

**prior to both systemic inflammation and monocyte recruitment to the small intestine.**

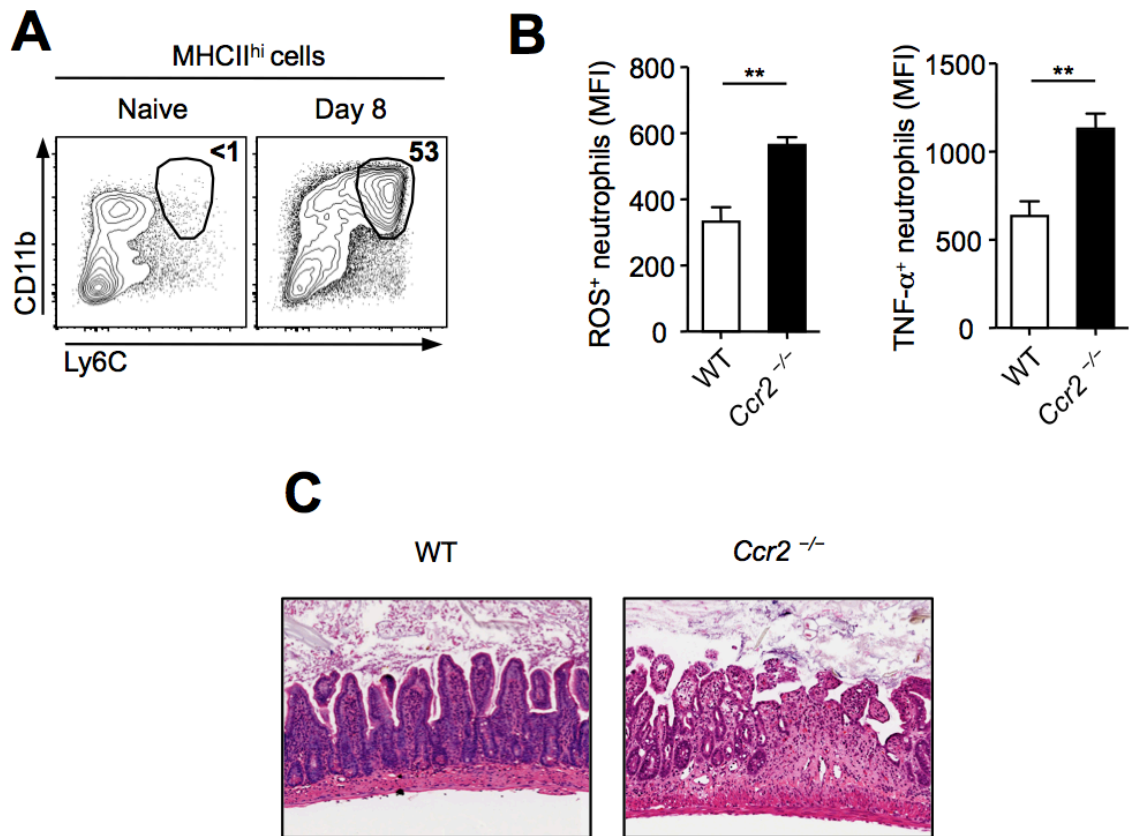
- 3. Changes to the phenotype and composition of the blood monocyte compartment are a common feature of Type 1 polarized infections.**
- 4. IFN- $\gamma$  signaling on blood monocytes controls their phenotype.**



## ***Rationale***

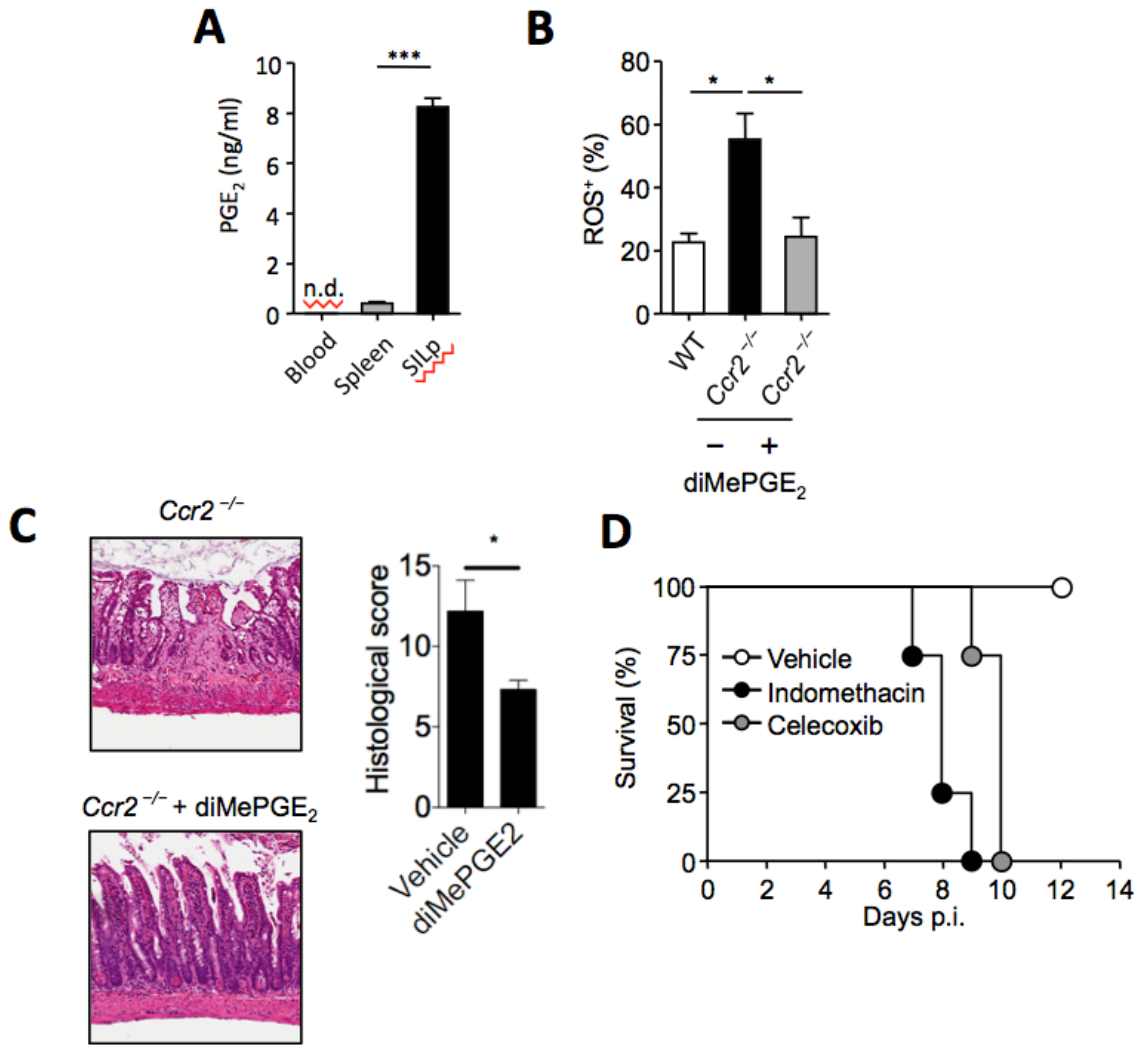
Oral infection with *Toxoplasma gondii* results in small intestinal inflammation characterized by the robust recruitment of Ly6C<sup>hi</sup> monocytes and neutrophils (**Figure 2A**). Although previous work has described a predominantly anti-microbial role for recruited monocytes in this setting (Dunay et al., 2008), we found that infection of CCR2-deficient mice, which have greatly diminished numbers of circulating monocytes, resulted in increased neutrophil activation in the small intestine lamina propria (SILP) and heightened intestinal pathology when compared to WT controls (**Figure 2B, 2C**), suggesting a previously unrecognized anti-inflammatory role for monocytes in responses to infection. In support of this notion, we found that monocytes produced the lipid mediator prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), a factor previously demonstrated to inhibit neutrophil activation (Wertheim et al., 1993), upon encounter with commensal microbes in the small intestine (**Figure 3A**). Administration of dimethyl-PGE<sub>2</sub> to infected CCR2-deficient mice reverted neutrophil activation to levels observed in WT mice and decreased infection-induced tissue pathology (**Figure 3B, 3C**). Conversely, pharmacological inhibition of PGE<sub>2</sub> production in WT mice prevented survival of the acute phase of infection (**Figure 3D**). In light of these and other supporting findings (Grainger et al., 2013), we concluded that recruited Ly6C<sup>hi</sup> monocytes not only controlled *T. gondii* infection, but also prevented the onset of infection-induced lethal immunopathology by inhibiting neutrophil inflammatory responses to translocating commensal microbes via PGE<sub>2</sub> signaling (**Figure 4**).

Production of regulatory factors is a common feature of APC populations in the gut, suggesting a potential role for the local cytokine milieu in driving the observed novel anti-inflammatory properties of monocytes in this infectious setting (Grainger et al., 2014). However rapid PGE<sub>2</sub> production by monocytes in this setting is critical, and may require that monocytes receive systemic signals that induce this regulatory function prior to tissue entry. Pre-emptive induction of regulatory responses may be particularly important upon loss of integrity of barrier tissues, as this can result in systemic bacterial dissemination and the potential for septic shock, which is largely mediated by cytokines produced by monocytes (Cuenca et al., 2011). Thus we initiated studies to assess the timing, location, and identity of the signals driving PGE<sub>2</sub> production by monocytes during oral *T. gondii* infection.



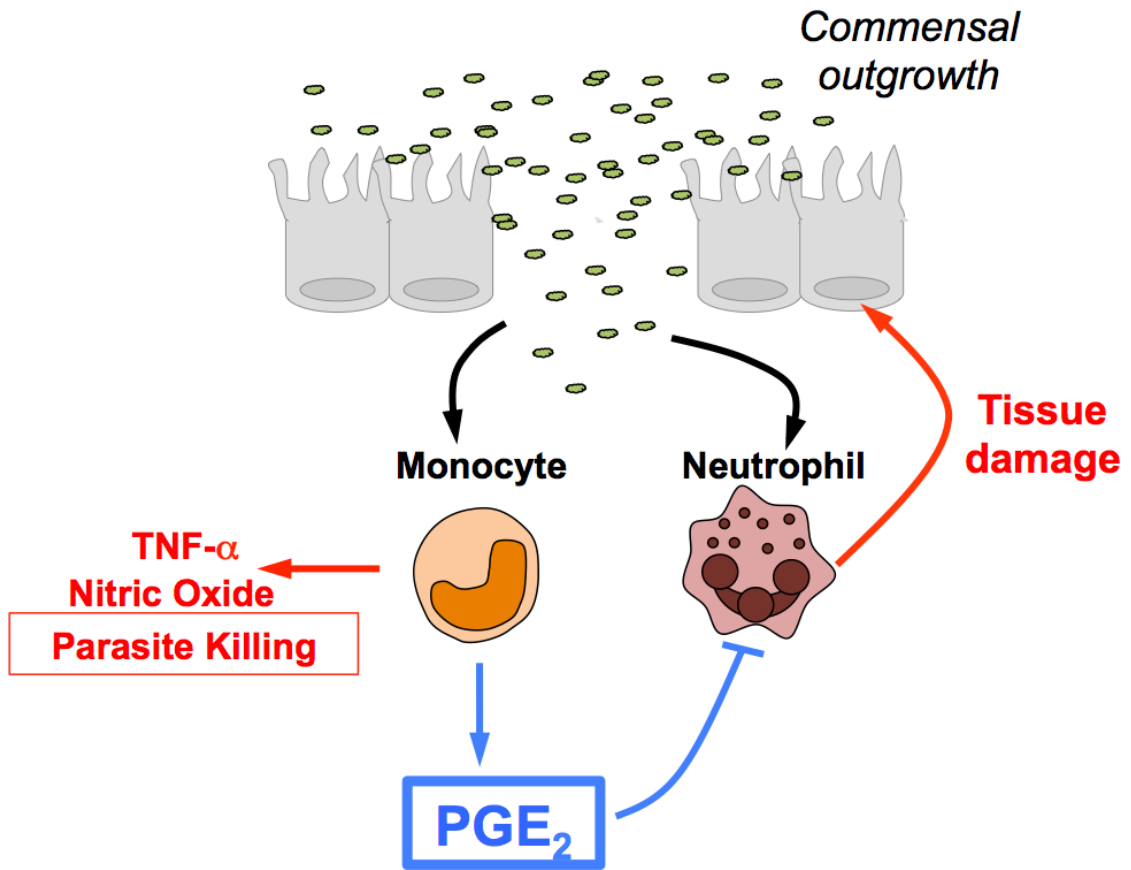
**Figure 2: Ly6C<sup>hi</sup> monocytes suppress neutrophil-mediated pathology during oral *T. gondii* infection.**

**(A)** Flow cytometric assessment of recruited monocytes (Ly6C<sup>hi</sup>CD11b<sup>hi</sup>) in SILP of naïve or day 8 infected mice. Cells are gated on live MHCII<sup>hi</sup> cells. Numbers adjacent to gates represent percentage of cells in gate. **(B)** WT and *Ccr2*<sup>-/-</sup> mice were infected with *T. gondii*. At day 8 of infection, SILP cell from suspensions were cultured *in vitro* and assessed for neutrophil production of ROS and TNF-α production. **(C)** Representative H&E stained sections of small intestine from day 8 infected WT and *Ccr2*<sup>-/-</sup> mice (Scale bar = 100 μm). Data are representative of three independent experiments, n = 3 per group. Statistical comparisons were performed using unpaired student's *t* tests. \*\*: p<0.01



**Figure 3: Ly6<sup>Chi</sup> monocytes produce PGE<sub>2</sub> and prevent lethal immunopathology.**

**(A)** *Ex vivo* PGE<sub>2</sub> production by Ly6<sup>Chi</sup> monocytes sorted from the blood, spleen, or SILP of day 8 *T. gondii* infected mice. **(B-C)** WT or *Ccr2*<sup>-/-</sup> mice were orally infected with *T. gondii*. *Ccr2*<sup>-/-</sup> mice were treated with 16,16-dimethyl (diMe) PGE<sub>2</sub> or vehicle from days 6-8 post infection. **(B)** Neutrophil production of ROS at day 8 post-infection. **(C)** Representative H&E staining of small intestine from infected *Ccr2*<sup>-/-</sup> mice treated with diMePGE<sub>2</sub> or vehicle (Scale bar 100  $\mu$ m). Graph depicts histological scoring. **(D)** Survival curve of animals treated with COX-2 inhibitor (indomethacin or celecoxib) and vehicle treated controls on days 6-8 post infection. Data are representative of at least two independent experiments, n = 4 per group. Statistical comparisons were performed using unpaired student's *t* tests. \*\*: p<0.01



**Figure 4: Dual Function of Ly6C<sup>hi</sup> monocytes during *T. gondii* infection.**

*T. gondii* infection results in epithelial damage and outgrowth of commensal  $\gamma$ -proteobacteria. Breach of the epithelial barrier during infection causes translocation of commensal microbes into the small intestine lamina propria, and recruited Ly6C<sup>hi</sup> monocytes respond to bacterial signals by activating both inflammatory and anti-inflammatory pathways. Production of TNF- $\alpha$  and nitric oxide contributes to parasite killing by monocytes. Production of PGE<sub>2</sub> inhibits activation of neutrophils by commensal microbes and prevents lethal neutrophil-mediated tissue damage.

## **Results**

### **Systemic alteration of Ly6C<sup>hi</sup> monocytes prior to tissue recruitment**

To assess whether the potential for regulatory function was imposed by signals in the mucosal environment or acquired prior to tissue recruitment, we first explored the possibility that functional alterations to monocytes were coupled with a distinct surface phenotype. As previously described (Grainger et al., 2013), all Ly6C<sup>hi</sup> monocytes from *T. gondii* infected SILP expressed MHCII (**Figure 5A, 5B**). Moreover, in contrast to their naïve counterparts, Ly6C<sup>hi</sup> monocytes from infected mice expressed high levels of stem cell antigen 1 (Sca-1) (**Figure 5B**). Unlike monocytes described in other acute inflammatory settings (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2013; Zigmond et al., 2012; 2014a), Ly6C<sup>hi</sup> monocytes did not express fractalkine receptor (CX3CR1) (**Figure 5B**). Thus, monocyte regulatory properties in the gut are associated with a previously un-described phenotypic signature, an observation that provided us with the opportunity to track when and where monocyte regulatory priming occurred.

This cellular phenotype was detectable in the blood as early as day 4 post-infection and adopted by all Ly6C<sup>hi</sup> monocytes by day 6 (**Figure 6A-C**). At this time-point, all circulating Ly6C<sup>hi</sup> monocytes exhibited a CX3CR1<sup>-</sup>Sca-1<sup>+</sup>MHCII<sup>+</sup> phenotype (**Figure 6C**). In addition to Ly6C<sup>hi</sup> monocytes, the blood is home to a population of patrolling Ly6C<sup>lo</sup> monocytes important for repair and maintenance of the endothelium

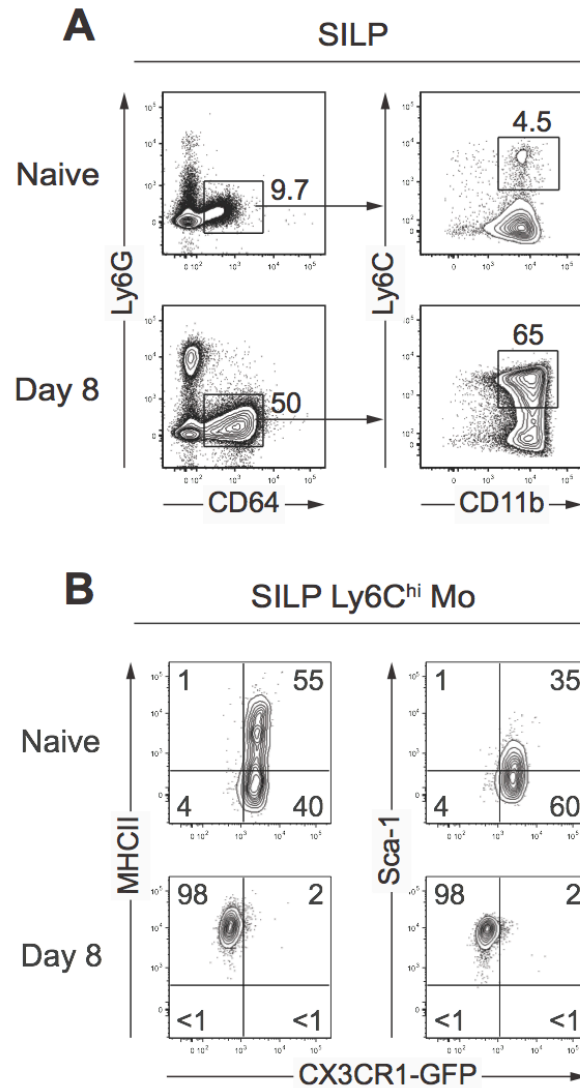
(Carlin et al., 2013). Concurrent with changes to their phenotype, the Ly6C<sup>hi</sup> monocyte subset rapidly became the dominant monocyte population present in the circulation during infection (**Figure 6D**). The loss of Ly6C<sup>lo</sup> monocytes may be the result of the observed decrease in expression of *Nr4a1* (*Nur77*) during infection (**Figure 6E**), as this transcription factor is required for the development and/or maintenance of Ly6C<sup>lo</sup> monocytes (Hanna et al., 2011).

As in the gut, these phenotypic changes in the blood compartment were associated with the acquisition of regulatory responses. Making use of IL-10 BAC-in transgene (10BiT) reporter mice (Maynard et al., 2007), we found that the potential to express IL-10 was increased in Ly6C<sup>hi</sup> monocytes following infection (**Figure 7A**). Furthermore, Ly6C<sup>hi</sup> monocytes isolated from the blood of infected mice produced greater quantities of IL-10 and PGE<sub>2</sub> upon stimulation with lysate derived from a murine commensal *E. coli* (**Figure 7B, 7C**). *E. coli* lysate represents a relevant source of stimulation for monocytes homing to the gut, since during acute mucosal infections the gut becomes dominated by  $\gamma$ -proteobacteria, such as *E. coli*, that contribute to inflammation (Heimesaat et al., 2006; Molloy et al., 2013; Raetz et al., 2013a).

Surprisingly, the onset of systemic alterations to Ly6C<sup>hi</sup> monocytes was initiated at a time preceding monocyte recruitment to the SILP (**Figure 8A**) and prior to any detectable pathology or inflammation as evidenced by (i) significant increase in systemic IFN- $\gamma$  or TNF- $\alpha$  (**Figure 8B**), (ii) alterations in serum levels of liver enzymes (**Figure 8C**), or (iii) changes in weight (**Figure 8D**). Systemic monocyte changes also preceded

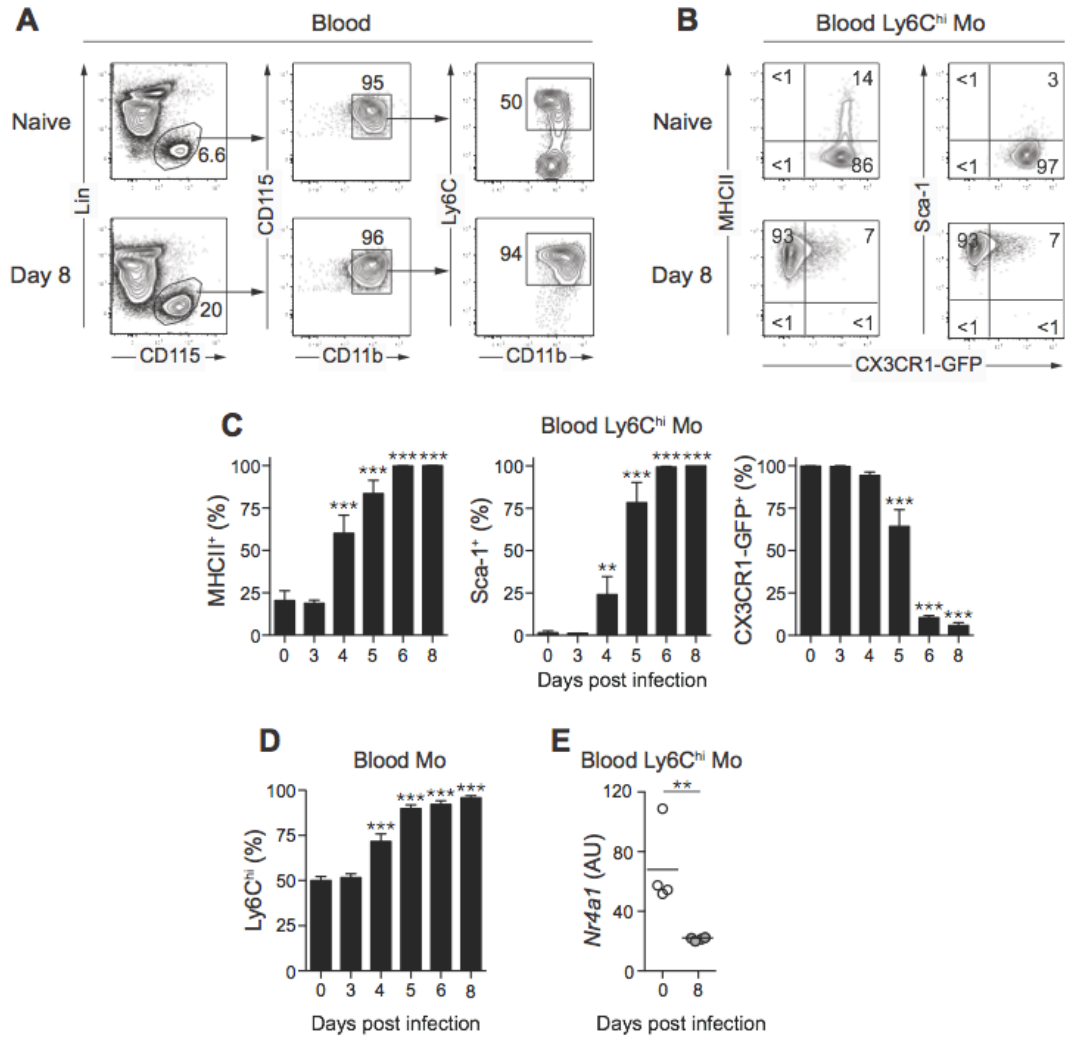
the onset of intestinal pathology (**Figure 9A**) and were not the consequence of parasite dissemination since, at this timepoint, *T.gondii* was restricted to the SILP and mesenteric lymph nodes (mesLN), and was undetectable in the blood or BM compartment (**Figure 9B**) (Coombes et al., 2013). Thus, following infection, early phenotypic alterations to monocytes are associated with profound changes to their function in both the target tissue and in the blood compartment.





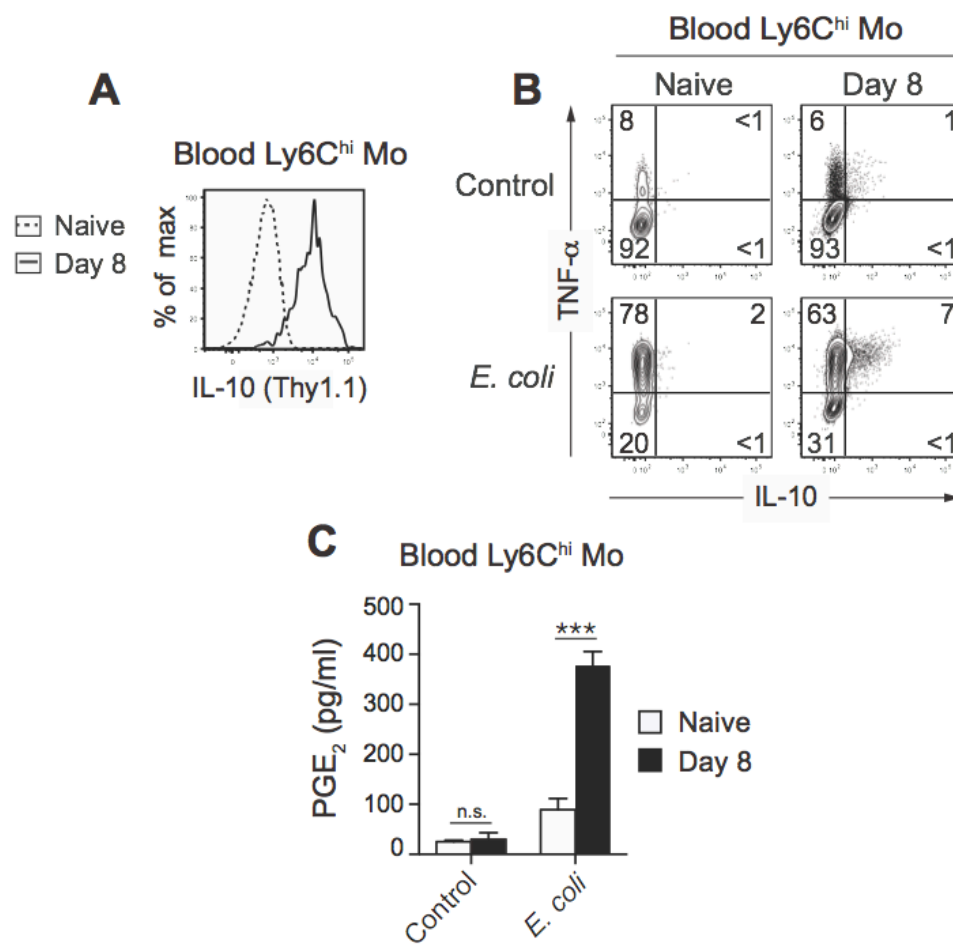
**Figure 5: Ly6C<sup>hi</sup> monocytes acquire a MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> Phenotype in the SILP during *T. gondii* infection.**

CX3CR1-GFP mice were infected per-orally with *T. gondii*. **(A)** Gating strategy to identify Ly6C<sup>hi</sup> monocytes (Mo) in small intestine lamina propria (SILP) by flow cytometry. Plots are initially gated on Live CD45<sup>+</sup> cells, doublets excluded. **(B)** Flow-cytometric analysis of Ly6C<sup>hi</sup> monocytes present in the SILP of naïve animals or at day 8 post-infection. Numbers represent the mean proportion of cells within defined gates among all samples.



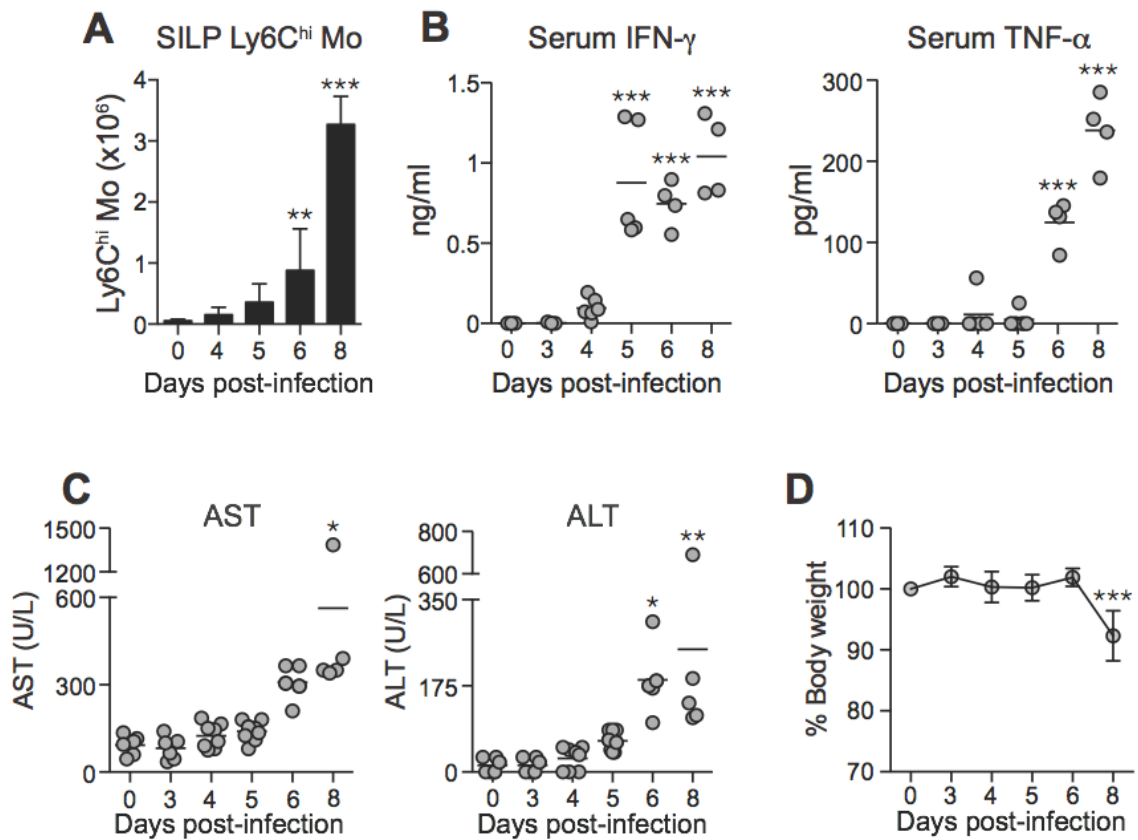
**Figure 6: Infection alters the phenotype and composition of the blood monocyte compartment.**

CX3CR1-GFP mice were infected per-orally with *T. gondii*. **(A)** Gating strategy to identify Ly6C<sup>hi</sup> monocytes in whole blood by flow cytometry. Flow plots are initially gated on Live CD45<sup>+</sup> cells, doublets excluded. Granulocytes and lymphocytes were excluded using a Lineage (Lin) mixture containing anti-Ly6G, Siglec F, TCR- $\beta$ , NK1.1, and B220. **(B-C)** Phenotype of blood Ly6C<sup>hi</sup> monocytes at defined time-points p.i. as measured by flow cytometry. **(D)** Frequency of blood Ly6C<sup>hi</sup> monocytes within the total blood monocyte compartment. **(E)** Expression of *Nr4a1* (*Nur77*) Ly6C<sup>hi</sup> monocytes as assessed by real-time PCR. Numbers in (A) and (B) represent the mean proportion of cells within defined gates among all samples. Error bars represent one standard deviation. Data are representative of at least two independent experiments, n = 3-6 per group (A-D), or a single experiment (E). Statistical comparisons were performed using one-way ANOVA (C,D) or unpaired student's *t* test adjusted for multiple comparisons (E). \*\*: p<0.01, \*\*\*: p<0.001.



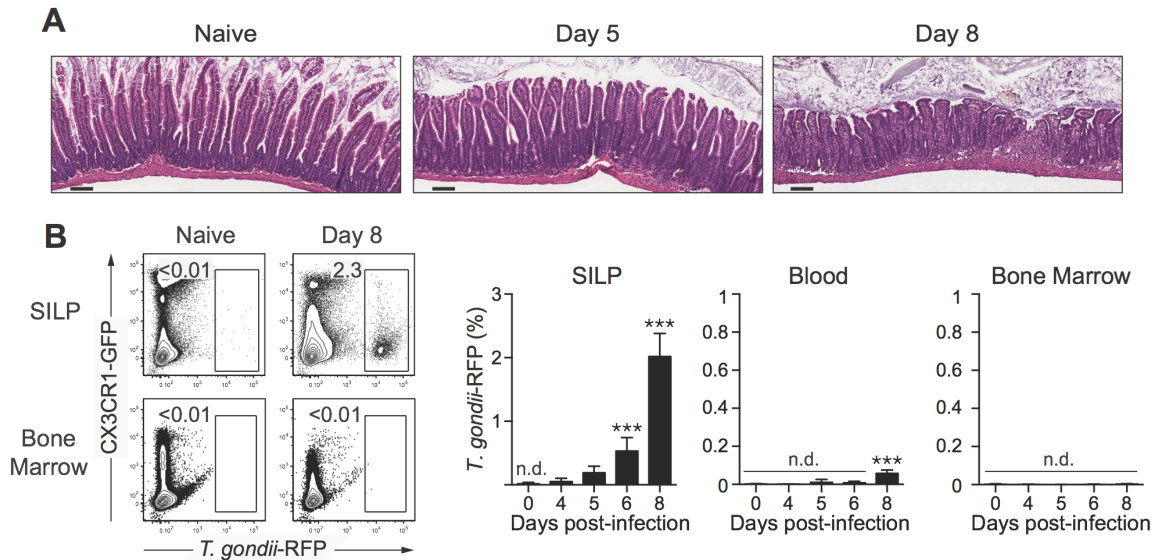
**Figure 7: Blood Ly6C<sup>hi</sup> monocytes acquire regulatory features during infection.**

**(A)** Thy1.1 expression by blood Ly6C<sup>hi</sup> monocytes isolated from naïve and day 8 *T. gondii* infected 10BiT mice. **(B)** IL-10 and TNF- $\alpha$  production by blood Ly6C<sup>hi</sup> monocytes from naïve and day 8 infected WT mice cultured in the presence or absence of *E. coli* lysate. **(C)** *Ex vivo* PGE<sub>2</sub> production by Ly6C<sup>hi</sup> monocytes sorted from the blood of naïve or day 8 *T. gondii* infected mice and cultured in the presence or absence of *E. coli* lysate. Numbers in (B) represent the mean proportion of cells within defined gates among all samples. Error bars represent one standard deviation. Data are representative of at least two independent experiments, n = 3-6 per group. Statistical comparisons were performed using student's *t* test adjusted for multiple comparisons. \*\*\*: p<0.001.



**Figure 8: Changes to blood monocytes precede systemic inflammatory signals and weight loss.**

(A) Accumulation of monocytes in the SILP during *T. gondii* infection. (B) Serum levels of IFN- $\gamma$  and TNF- $\alpha$  in *T. gondii* infected mice. (C) Serum levels of liver enzymes in *T. gondii* infected mice. (D) Weight loss of mice after infection. Data are representative of at least two independent experiments, n = 3-6 per group (A-B), or pooled results from two independent experiments (C-D). Statistical comparisons were performed using one-way ANOVA. \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.



**Figure 9: Changes to blood monocytes precede intestinal pathology and parasite dissemination.**

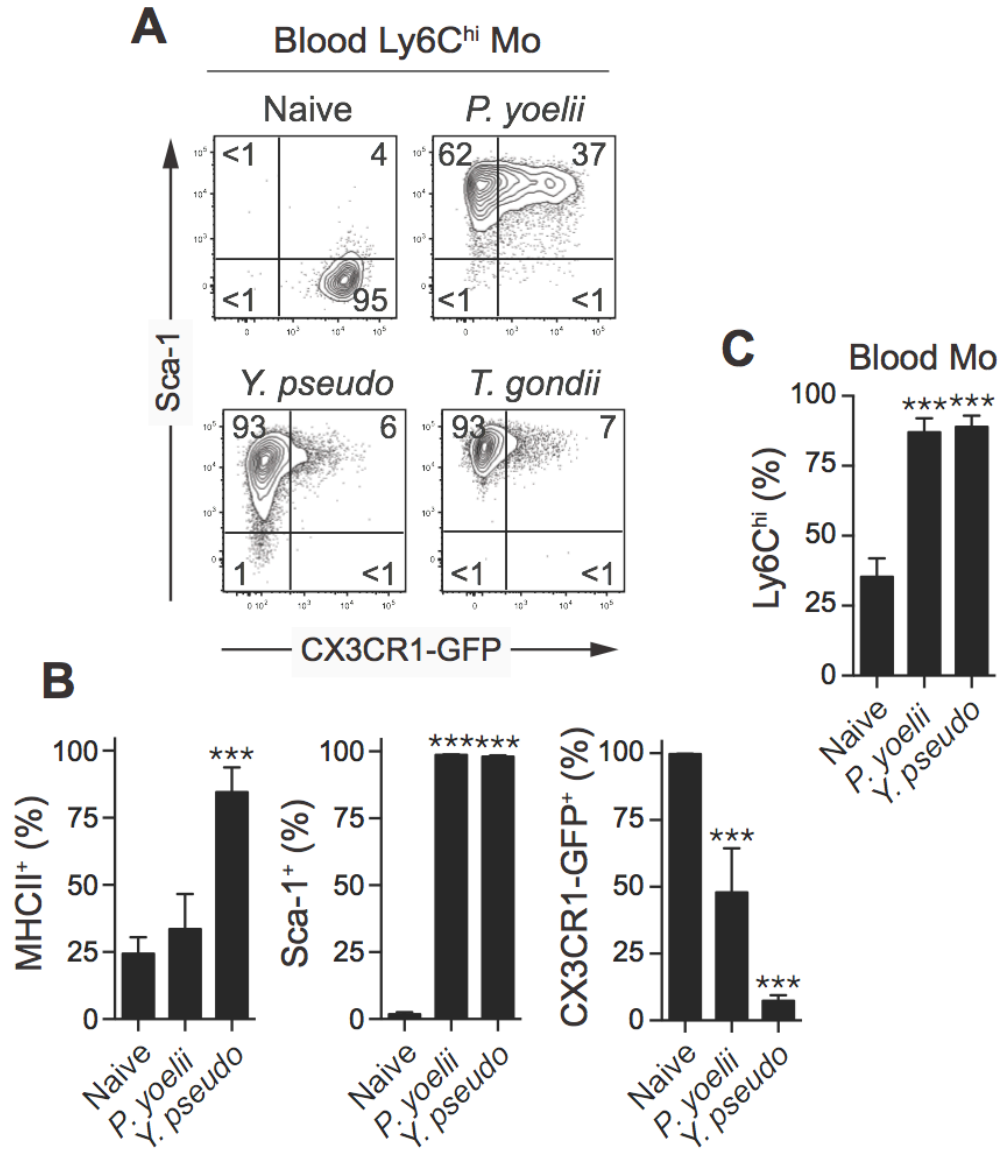
(A) Small intestines were collected at defined time-points following infection and assessed by histology. Images of ileum are representative of 3 mice per group. Scale bar = 100 mm. (B) The presence of *T. gondii* in SILP, blood, and BM was assessed at various time-points post-infection by flow cytometry. RFP<sup>+</sup> events represent cells infected with RFP-expressing parasite. Error bars represent one standard deviation. Data represent pooled results from two independent experiments. Statistical comparisons were performed using one-way ANOVA. n.d.: Not Detected, \*\*\*: p<0.001.

## IFN- $\gamma$ remodels the blood monocyte compartment during infection

We next assessed whether acquisition of the MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> phenotype by monocytes was a common response to infection. To this end, mice were infected with *Yersinia pseudotuberculosis* and *Plasmodium yoelii*, pathogens known to induce type 1 immune responses (Doolan and Hoffman, 1999; Logsdon and Meccas, 2006). In both settings, Ly6C<sup>hi</sup> monocytes acquired a similar phenotype to that observed during *T. gondii* infection and became the dominant blood monocyte subset (**Figure 10A-C**). This observation supported the idea that monocyte regulatory priming was likely driven by a canonical mediator of host defense employed during these responses, rather than by interaction with a specific pathogen. Indeed, following intraperitoneal injection of recombinant IFN- $\gamma$  for three days, blood Ly6C<sup>hi</sup> monocytes ubiquitously expressed Sca-1 and MHCII and demonstrated decreased expression of CX3CR1 (**Figure 11A, 11B**). Moreover, IFN- $\gamma$  increased the proportion of circulating Ly6C<sup>hi</sup> monocytes (**Figure 11C**).

Blockade of IFN- $\gamma$  during *T. gondii* infection prevented these phenotypic and subset alterations (**Figure 12A, 12B**). To assess whether this phenomenon was the consequence of cell intrinsic or extrinsic responses to IFN- $\gamma$ , we generated mixed BM chimeras with WT cells and cells lacking IFN- $\gamma$  receptor (*Ifngr1*<sup>-/-</sup>) that were subsequently infected with *T. gondii*. Changes to monocyte surface phenotype as well as alterations in proportion of Ly6C<sup>hi</sup> monocytes were dependent on cell intrinsic IFN- $\gamma$  receptor signaling (**Figure 12C, 12D**). Preliminary data suggests that IFN- $\gamma$  signaling

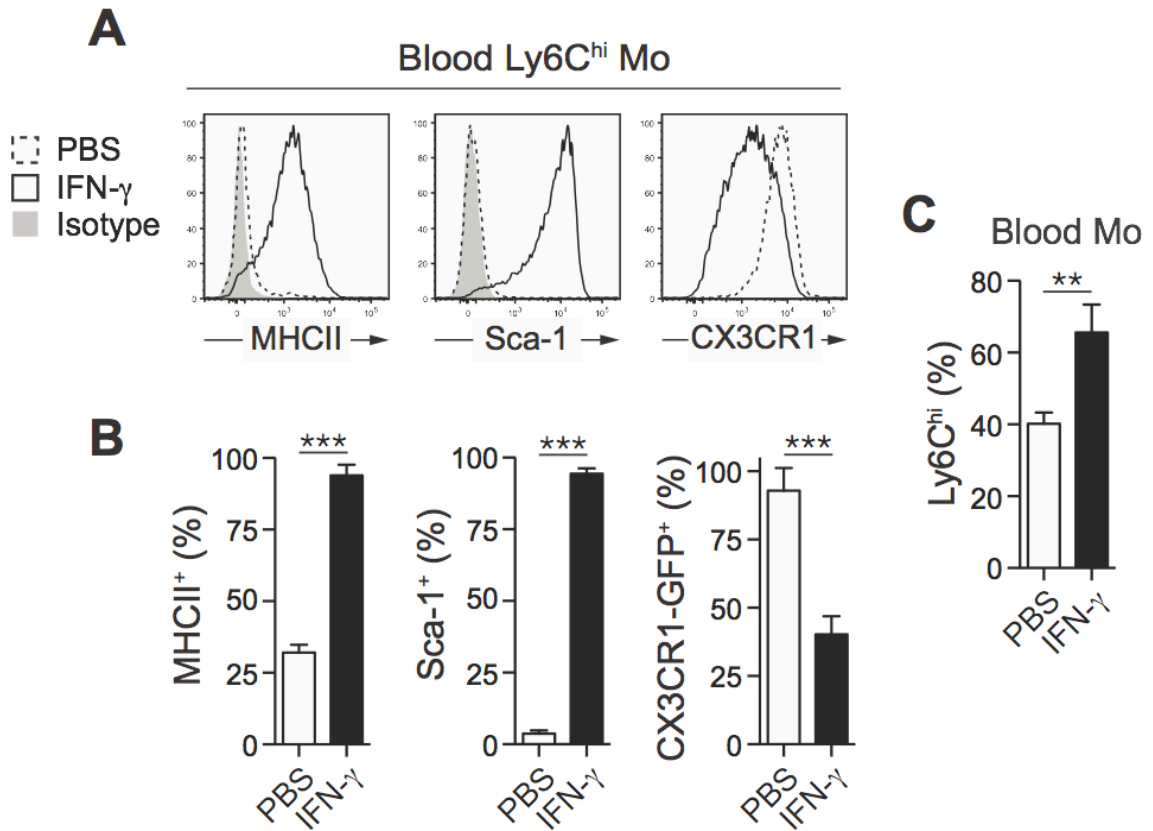
on Ly6C<sup>hi</sup> monocytes also decreases expression of *Nr4a1* (**Figure 12E**), implicating IFN- $\gamma$  as the primary signal responsible for the loss of Ly6C<sup>lo</sup> patrolling monocytes observed during *T. gondii* infection.



**Figure 10: Alterations to the blood monocyte compartment are a common feature of Th1-polarized immune responses to infection.**

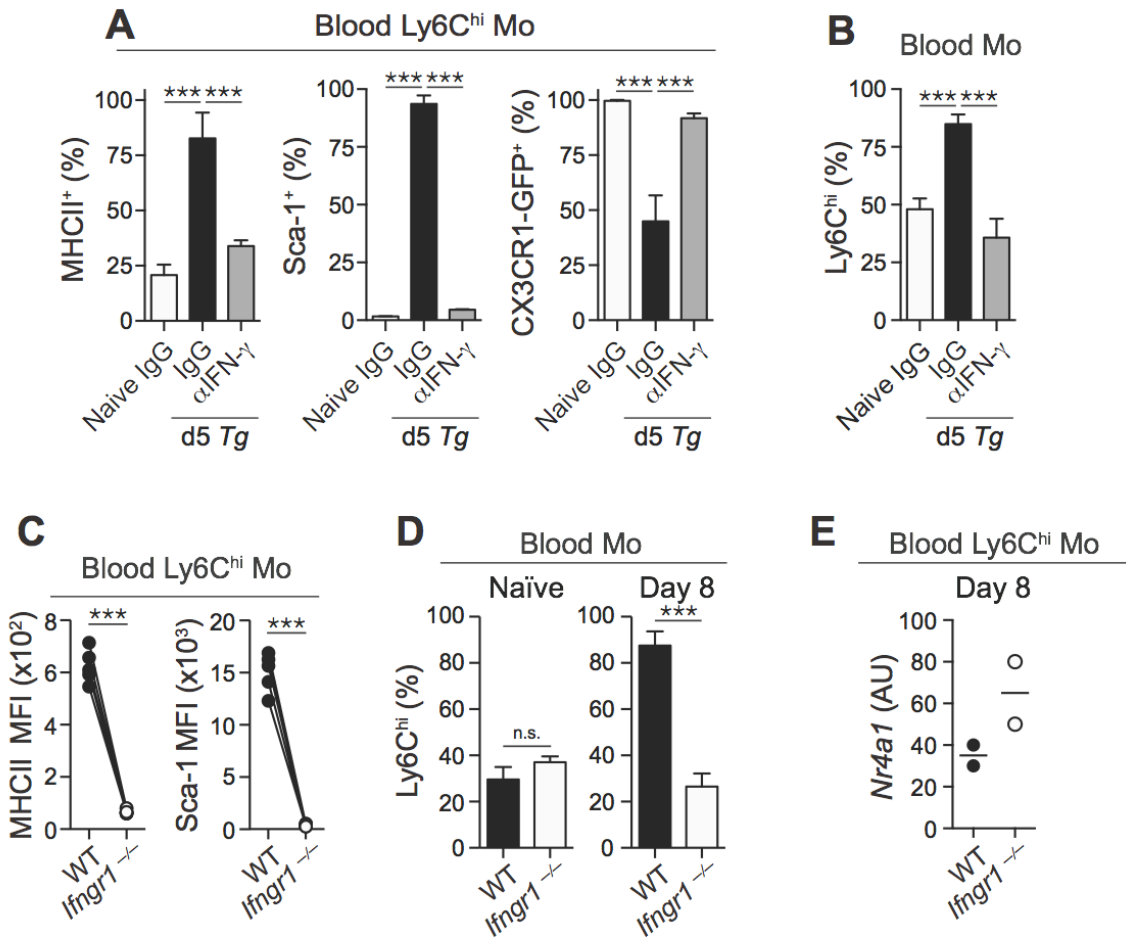
CX3CR1-GFP mice were infected intravenously with *P. yoelii* infected red blood cells, per-orally with *Y. pseudotuberculosis*, or per-orally with *T. gondii*. **(A-B)** Phenotype of blood Ly6C<sup>hi</sup> monocytes following infection with *P. yoelii* (day 4 p.i.), *Y. pseudotuberculosis* (day 5 p.i.) and *T. gondii* (day 8 p.i.). **(C)** Frequency of blood Ly6C<sup>hi</sup> monocytes within the total monocyte compartment at time-points described in (A). Error bars represent standard deviation. Data are representative of at least two independent experiments, n = 3-5 per group. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*\*: p<0.01, \*\*\*: p<0.001.





**Figure 11: IFN- $\gamma$  controls the phenotype and composition of the blood monocyte compartment.**

(A-C) CX3CR1-GFP mice were administered IFN- $\gamma$  or PBS once per day for three consecutive days. (A-B) Phenotype of blood Ly6C<sup>hi</sup> monocytes assessed by flow cytometry. (C) Frequency of Ly6C<sup>hi</sup> monocytes within the total blood monocyte compartment. Data are representative of at least two independent experiments, n = 3-5 per group. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*\*: p<0.01, \*\*\*: p<0.001.



**Figure 12: Cell-intrinsic IFN- $\gamma$  signaling is required for infection-induced alterations to the blood monocyte compartment.**

(A-B) CX3CR1-GFP mice were infected with *T. gondii* and treated with anti-IFN- $\gamma$  Ab or isotype control (IgG). (A) Phenotype of blood Ly6C<sup>hi</sup> monocytes. (B) Frequency of Ly6C<sup>hi</sup> monocytes within the total blood monocyte compartment. (C-E) Chimeric mice comprised of equal numbers of WT CD45.1<sup>+</sup> and *Ifngr1*<sup>-/-</sup> (CD45.2<sup>+</sup>) leukocytes were infected or not with *T. gondii*. (C) Mean fluorescent intensity (MFI) of MHCII and Sca-1 expression by WT and *Ifngr1*<sup>-/-</sup> blood Ly6C<sup>hi</sup> monocytes measured by flow cytometry at 8 days post-infection. Values of WT and *Ifngr1*<sup>-/-</sup> cells from the same host are joined by a line. (D) Proportion of blood Ly6C<sup>hi</sup> monocytes in total blood monocytes within WT and *Ifngr1*<sup>-/-</sup> compartments in naïve and infected mice. (E) Expression of *Nr4a1* (*Nur77*) by WT and KO Ly6C<sup>hi</sup> monocytes as assessed by real-time PCR. Error bars represent standard deviation. Data are representative of at least two independent experiments, n = 3-5 per group (A-D), or a single experiment (E). Statistical comparisons were performed using unpaired student's *t* test, or paired *t* test adjusted for multiple comparisons. \*\*\*: p<0.001.

## ***Discussion***

Based on steady-state paradigms as well as defined inflammatory models, it is often assumed that the tissue microenvironment is largely responsible for determining the function of recruited cells (Bain and Mowat, 2014; Ingersoll et al., 2011; Serbina et al., 2008). Thus, upon initiating these studies, we hypothesized that monocytes acquired regulatory functions during *T. gondii* infection in response to signals received in the intestinal microenvironment, a tissue milieu rich in cytokines favoring tolerance such as TGF- $\beta$  and IL-10 (Grainger et al., 2014). Indeed, monocytes recruited to the intestine under steady-state conditions differentiate into tissue-resident macrophages that constitutively secrete IL-10 and fail to effectively produce inflammatory cytokines in response to microbial signals (Smith et al., 2010). The findings in this chapter challenge the paradigm that local factors dictate monocyte function. During *T. gondii* infection Ly6C<sup>hi</sup> monocytes acquire increased capacity to produce IL-10 and PGE<sub>2</sub> in the blood, prior to recruitment to the intestine (**Figures 7-9**). Notably, acquisition of regulatory features by monocytes is not associated with the loss of inflammatory function, and blood Ly6C<sup>hi</sup> monocytes remained robust producers of TNF- $\alpha$  in response to *E. coli* lysate, including those secreting IL-10.

IL-10 production was restricted to ~7% of blood monocytes, and these cells may represent a subpopulation within the total Ly6C<sup>hi</sup> monocyte pool. However, we did not observe any unique phenotypic markers expressed by monocytes producing IL-10. IL-10 producers also displayed the highest expression of TNF- $\alpha$ , suggesting that IL-10

secretion is not isolated to a unique subset of monocytes, but rather that it is produced as a cell-intrinsic feedback mechanism in highly activated macrophages. In support of this interpretation, IL-10 production by macrophages after LPS stimulation has been shown to occur in response to sequential autocrine/paracrine signaling by type I interferons and IL-27 (Iyer et al., 2010). PGE<sub>2</sub> signaling also promotes IL-10 expression by myeloid populations (Cheon et al., 2006; MacKenzie et al., 2013), and may contribute to the surprising production of this cytokine in blood monocytes during *T. gondii* infection. PGE<sub>2</sub> production was measured in the supernatant of monocyte cultures, and thus whether production of this important regulatory factor is a common feature of all blood Ly6C<sup>hi</sup> monocytes or is restricted to a subset of cells remains unclear. Recent studies have uncovered apparent heterogeneity within human classical monocytes (Wong et al., 2012), and functional subsets may also exist within Ly6C<sup>hi</sup> monocytes, both under steady-state conditions and during inflammation. In this context, it is important to note that the observed increase in PGE<sub>2</sub> production Ly6C<sup>hi</sup> monocytes during inflammation constituted less than a 4-fold change, and an alternative explanation of our findings is that *T. gondii* infection induces the expansion of an already-present regulatory monocyte population, as recently described in the context of bone marrow transplant (D'Aveni et al., 2015). Single cell RNA-seq analysis of monocyte populations during infection would provide a clearer analysis of the composition of the Ly6C<sup>hi</sup> monocyte compartment and deliver insight into the mechanisms by which regulatory properties emerge in this population during infection.

Acquisition of regulatory functions by Ly6C<sup>hi</sup> monocytes was associated with a previously un-described MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> phenotype. While increased MHCII expression by myeloid cells is a common response to stimulation, Sca-1 is best known as a marker of early hematopoietic populations. However, upregulation of this marker is associated with inflammatory conditions, and *in vitro* treatment with IFN- $\gamma$  has been shown to increase expression of Sca-1 by leukocytes (Hsu et al., 2014). In accordance with this study, we find that Sca-1 expression by monocytes is dependent on IFN- $\gamma$  signaling, and acquisition of the MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> phenotype allowed us to track the education of monocytes during infection, both spatially and temporally. Unexpectedly, alterations to monocyte phenotype in the circulation preceded liver and intestinal pathology or detectable serum levels of TNF- $\alpha$  (**Figures 7-9**) The finding that changes to monocyte phenotype were driven by IFN- $\gamma$  suggests that one of the functions of this polarizing cytokine may be to pre-emptively educate myeloid populations early during infection to dictate their function upon tissue recruitment and microbial encounter. **The role of sequential host-derived signals in directing the composition and quality of the myeloid immune response during infection is explored in greater depth in Chapter 5.**

We were surprised to observe the complete loss of CX3CR1-GFP expression by Ly6C<sup>hi</sup> monocytes in the SILP and blood. CX3CR1 expression is commonly used to identify monocytes recently recruited to tissue, and transgenic mice expressing Cre recombinase under control of the CX3CR1 reporter have recently been utilized in a fate-reporter system to identify monocyte-derived cells in many settings (Bain et al., 2014;

Rivollier et al., 2012; Yona et al., 2013). The findings here demonstrate that these strategies must be used with great caution when analyzing the mononuclear phagocyte compartment during inflammation, particularly in the presence of IFN- $\gamma$ . The consequences of decreased CX3CR1 expression during infection by bacterial and protozoan pathogens are not clear. CX3CR1 signaling (via its ligand fractalkine) supports monocyte homeostasis, and blockade of this pathway induces monocyte apoptosis and increases the turnover of these populations (Landsman et al., 2009). Inhibition of CX3CR1 signaling may be an adaptive mechanism that supports the increased production of monocytes by the BM during infection. Under this hypothesis, increased turnover of Ly6C<sup>hi</sup> monocytes “makes room” for fresh monocytes emerging from the BM and allows the rapid reset of the myeloid system to return to steady-state conditions upon the resolution of infection or inflammation. Study of the turnover of the monocyte compartment during inflammation is complicated by the dynamic nature of these responses. The absolute number of cells in the blood in this context could be influenced by increased influx from the BM, changes to their lifespan in response to inflammatory factors, increased efflux into inflamed tissues, or a combination of all three causes. Thus, a true understanding of how monocyte homeostasis is altered during inflammation will require pulse-chase experiments with DNA-labeling factors, such as BrdU, and simultaneous analysis of monocyte populations in the BM, blood, and any inflamed tissues.

A recent publication has described the ability of Ly6C<sup>hi</sup> monocytes to enter tissue, acquire antigen, and reenter the lymphatic and circulatory systems with a unique

transcriptional signature (Jakubzick et al., 2013). Therefore we could consider the possibility that monocytes producing IL-10 and PGE<sub>2</sub> have acquired these functions in the SILP before returning to the blood compartment. Additionally, our findings demonstrate the requirement for IFN- $\gamma$  for the phenotypic changes to monocyte populations during infection, but whether IFN- $\gamma$  signaling directly drives the acquisition of regulatory function by these cells remains unclear. Although we did not observe live parasite outside of the MALT at early time-points (**Figure 9B**), *T. gondii* infection may still result in systemic dissemination of parasite-derived products capable of inducing regulatory function in Ly6C<sup>hi</sup> monocytes. **In Chapter 3, to address these outstanding questions we assess the effect of infection on the phenotype and gene expression of non-circulating monocyte progenitors and directly assess the role of factors derived from the host, parasite, and commensal bacteria in driving PGE<sub>2</sub> production by monocytes.**

## **CHAPTER 3: Monocyte Progenitors in the Bone Marrow are Conditioned by IFN- $\gamma$ During Infection**

### ***Abstract***

The findings described in **Chapter 2** reveal that during infection, IFN- $\gamma$  directs monocyte phenotype and function in the circulation. Previous studies have noted the profound effects of IFN- $\gamma$  on hematopoiesis, in particular driving increased production of monocytes and neutrophils (de Bruin et al., 2014). In this chapter, we examine changes to Ly6C<sup>hi</sup> monocytes and their progenitors in the BM, the primary site of hematopoiesis, during infection. Our key findings include:

- 1. Ly6C<sup>hi</sup> monocytes in the BM are functionally primed early during infection to acquire regulatory function.**
- 2. Bacterial-derived stimuli drive PGE<sub>2</sub> production by monocytes, but parasite-derived stimuli do not.**
- 3. IFN- $\gamma$  signaling induces a novel gene expression program in monocyte progenitors.**
- 4. This program results in altered responsiveness to LPS stimulation by BM monocytes.**



## ***Rationale***

Increased BM output of inflammatory cells, known as “emergency myelopoiesis”, is a critical feature of the host response to injury or infection. This process can be driven by systemic inflammatory factors and/or pathogen-derived products acting on precursor cells (Takizawa et al., 2012). Systemic signals may also influence the function of cells in the BM. Long-term changes to monocyte function favouring microbicidal potential following infection have been documented in a process termed “trained immunity” (Cheng et al., 2014; Quintin et al., 2012), and a recent study reported that during viral infection localized to the lung, systemic type I interferon drove expression of anti-viral genes by BM monocytes (Hermesh et al., 2010). Therefore, we examined whether monocytes acquired a transcriptional program directing regulatory function during development in the BM.

## **Results**

### **Ly6C<sup>hi</sup> monocytes are functionally primed in the bone marrow**

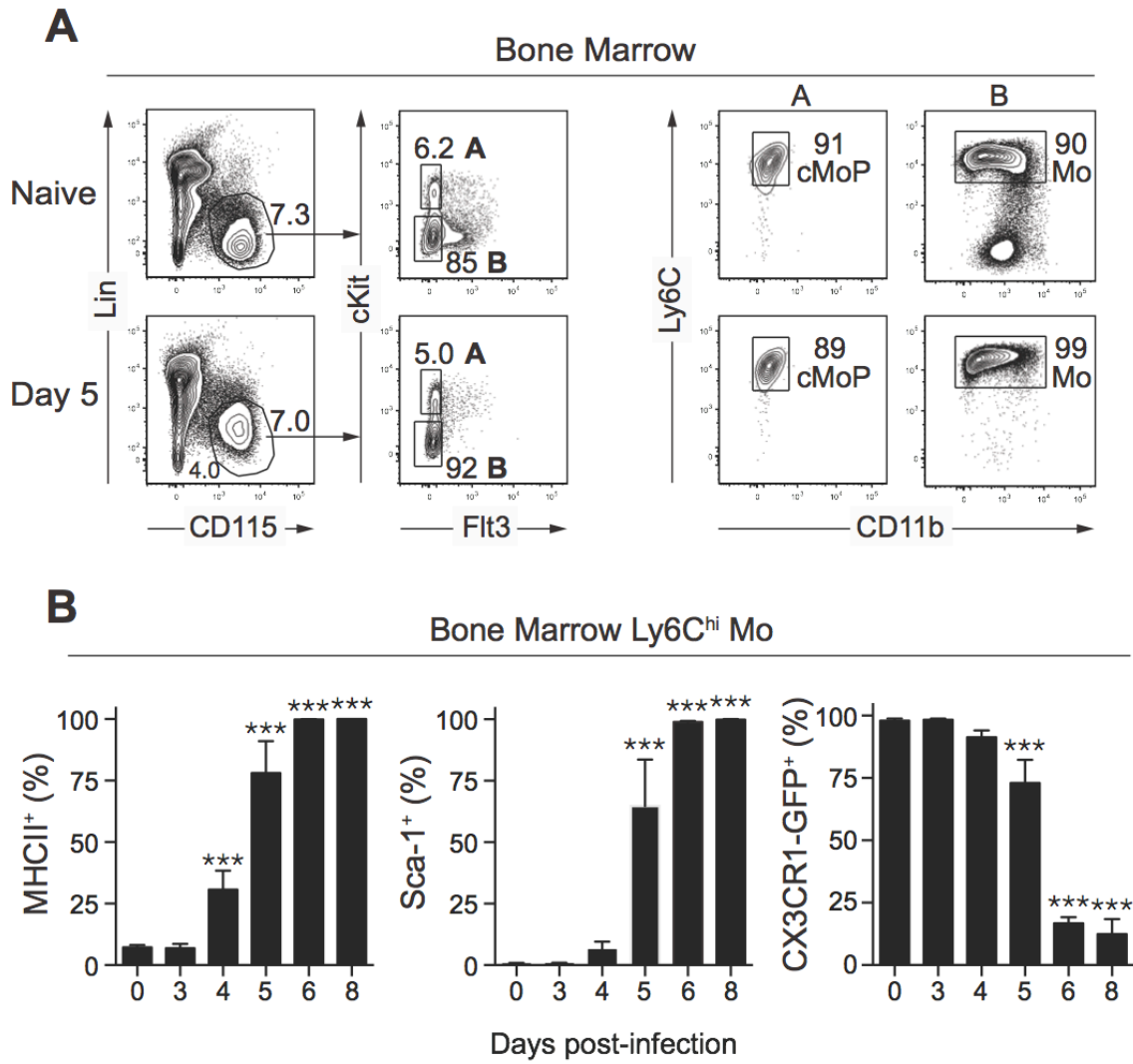
Our results thus far support the possibility that monocytes are primed for regulatory function prior to BM egress. To explore this possibility, we assessed the phenotype of BM monocytes and their responsiveness to microbial stimuli. Ly6C<sup>hi</sup> monocytes in the BM acquired the MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> phenotype as early as day 5 post-infection (**Figure 13A, 13B**). Monocytes isolated from BM at day 5 post-infection and stimulated *ex vivo* with various bacterial or parasite-derived ligands had already acquired enhanced capacity to produce PGE<sub>2</sub> in response to several bacterial ligands compared to cells isolated from naïve mice (**Figure 14A**). Functional alteration was restricted to bacterial ligands, as monocytes were poorly responsive to *Toxoplasma* antigen (STAg) and *T. gondii*-derived profilin (**Figure 14A**). The acquisition of this key regulatory function by BM monocytes was dependent on signaling by IFN- $\gamma$ , as *in vivo* neutralization of IFN- $\gamma$  during infection returned PGE<sub>2</sub> production by BM monocytes to levels similar to that observed in naïve mice (**Figure 14B**). Furthermore, administration of IFN- $\gamma$  to naïve mice was sufficient to drive increased PGE<sub>2</sub> production by BM monocytes, and *ex vivo* IFN- $\gamma$  treatment of monocytes isolated from naïve mice enhanced PGE<sub>2</sub> production (**Figure 14C, 14D**). Altered surface phenotype and enhanced PGE<sub>2</sub> production by monocytes persisted at 24 days post-infection (**Figure 15 A-C**), past the acute phase of disease (Grainger et al., 2013; Molloy et al., 2013). It is important to note that following acute *T. gondii* infection, the parasite establishes a low-

level chronic infection localized to the muscle tissue and the brain. Whether these persistent changes are dependent on the initial priming event, or on low levels of IFN- $\gamma$  circulating in the blood during the chronic stage of infection remains unclear. Ly6C<sup>hi</sup> monocytes from ARE-Del<sup>-/-</sup> mice, which lack a critical regulatory sequence on *Ifng* mRNA and thus constitutively express low levels of IFN- $\gamma$ , also have increased MHCII and Sca-1 expression, as well as increased capacity for PGE<sub>2</sub> production (**Figure 15D-F**) (Hodge et al., 2014). Thus, the acquisition of regulatory features by monocyte populations may extend to settings of chronic inflammation, such as cancer. **We will discuss this intriguing possibility in Chapter 5.**

To more comprehensively explore the early consequences of infection on the function of monocytes, sorted BM Ly6C<sup>hi</sup> monocytes from naïve or d5 *T. gondii* infected animals were cultured in the presence or absence of LPS, and mRNA expression of 490 myeloid genes was assessed using the NanoString platform. Principle component analysis revealed that monocytes from *T. gondii* infected animals demonstrated a transcriptional program distinct from those isolated from naïve controls, when cultured in media alone as well as upon LPS stimulation (**Figure 16A**). To gain a clearer understanding of how *T. gondii* infection altered monocytes responsiveness to bacterial stimuli, we compared the gene expression of untreated Ly6C<sup>hi</sup> monocytes from naïve animals to that of each of the other groups (naïve + LPS stimulated, *T. gondii* infected untreated, *T. gondii* infected + LPS stimulated) (**Figure 16B**). Our analysis revealed that in untreated monocytes from infected animals, a greater than 2-fold change in expression of 33 genes was observed (**Figure 16B, purple circle**). Moreover, following

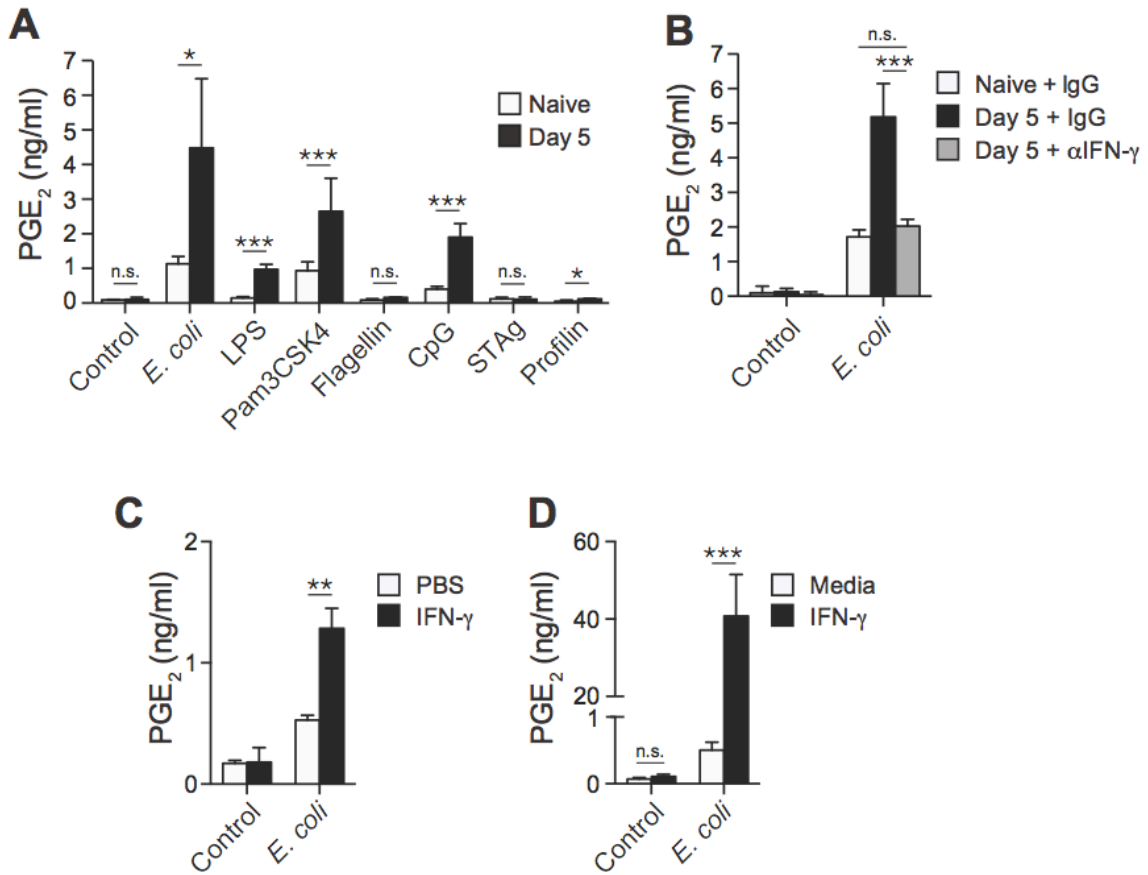
infection, the responsiveness of BM monocytes to LPS stimulation was dramatically altered. In total, 88 common genes were differentially expressed upon LPS stimulation in monocytes from both naïve and infected animals (**Figure 16B, green circle**), but an additional 30 genes were altered in expression only in monocytes from infected animals (**Figure 16B non-overlapping green region, 16C**).

In support of our functional read out, expression of *Ptgs2*, the gene that encodes COX-2, a key enzyme controlling PGE<sub>2</sub> production during inflammation (Ricciotti and FitzGerald, 2011), was induced in response to LPS stimulation in monocytes from infected mice (**Figure 16C**). Further supporting the idea that monocytes were primed for increased regulatory function prior to BM egress, gene expression of *Cd200*, which encodes a surface protein that suppresses innate immune cell function, and *Socs3*, which encodes a negative regulator of inflammatory cytokine signaling was also increased (Carow and Rottenberg, 2014; Snelgrove et al., 2008). Monocytes from infected mice also augmented the expression of genes encoding pro-inflammatory factors upon LPS stimulation, including IL-1 $\beta$  and IL-12p40, suggesting a generally increased responsiveness to bacterial ligands. These results imply that early during infection and prior to BM egress, monocytes are primed for both regulatory and effector responses following exposure to bacterial stimuli.



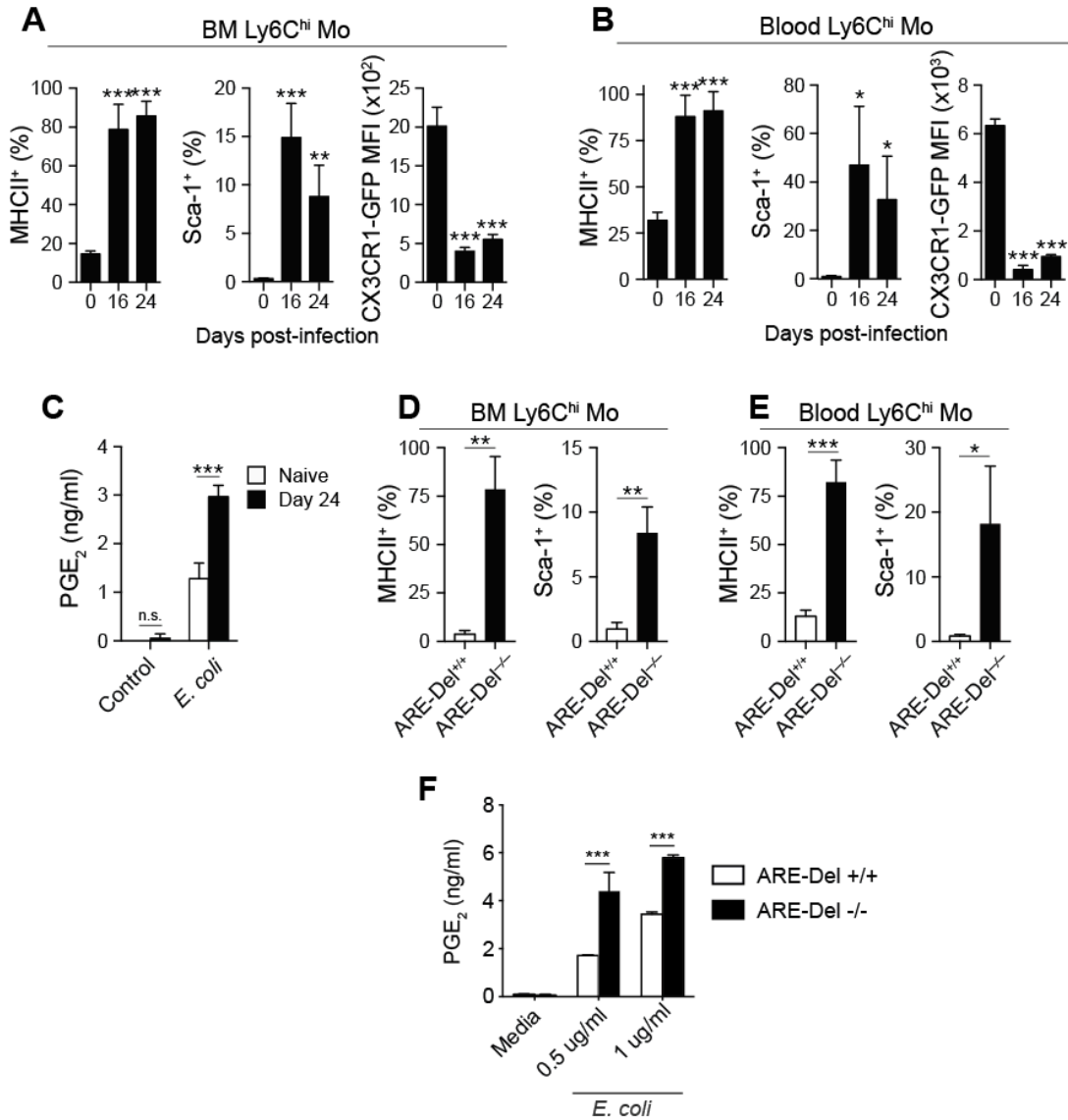
**Figure 13: Ly6C<sup>hi</sup> monocytes acquire a MHCII<sup>+</sup>Sca-1<sup>+</sup>CX3CR1<sup>-</sup> Phenotype in the BM during *T. gondii* infection.**

CX3CR1-GFP mice were infected per-orally with *T. gondii*. **(A)** Gating strategy to identify Ly6C<sup>hi</sup> monocytes in bone marrow by flow cytometry. Plots are initially gated on Live CD45<sup>+</sup> cells, doublets excluded. Granulocytes and lymphocytes were excluded using a Lineage (Lin) mixture containing anti-Ly6G, Siglec F, TCR- $\beta$ , NK1.1, and B220. Numbers represent the mean proportion of cells within defined gates among all samples. **(B)** Flow-cytometric analysis of Ly6C<sup>hi</sup> monocytes present in the bone marrow of naïve animals or at day 8 post-infection. Numbers represent the mean proportion of cells within defined gates among all samples. Data are representative of at least two independent experiments, n = 3-5 mice per group. Statistical comparisons were performed using one-way ANOVA. \*\*\*: p<0.001.

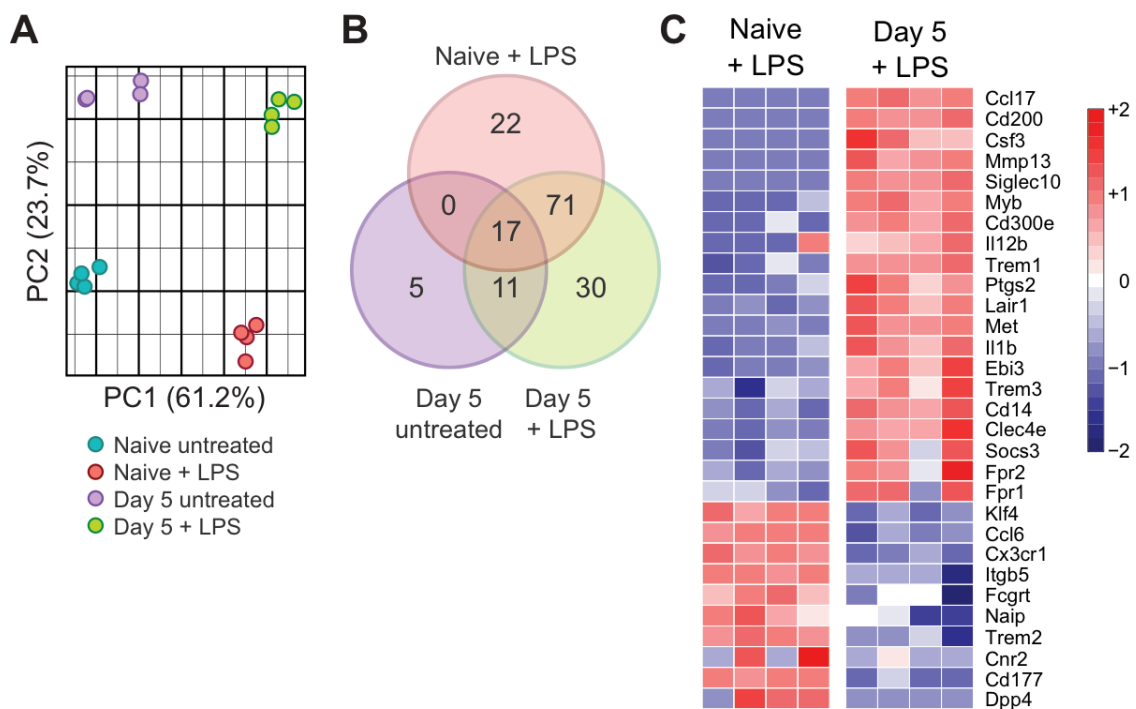


**Figure 14: IFN- $\gamma$  drives regulatory capacity of BM monocytes.**

**(A)** *Ex vivo* PGE<sub>2</sub> production by Ly6C<sup>hi</sup> monocytes sorted from BM of naïve or day 5 *T. gondii* infected mice and cultured with or without *E. coli* lysate, Soluble *Toxoplasma* antigen (STAg), or various TLR ligands. **(B)** WT mice were infected with *T. gondii* and treated with anti-IFN- $\gamma$  Ab or isotype control. Ly6C<sup>hi</sup> monocytes were sorted from BM of naïve and infected mice and assessed *ex vivo* for PGE<sub>2</sub> production in response to *E. coli* lysate. **(C)** *Ex vivo* PGE<sub>2</sub> production by Ly6C<sup>hi</sup> monocytes sorted from BM of CX3CR1-GFP mice treated with IFN- $\gamma$  or PBS once per day for three consecutive days. **(D)** *Ex vivo* PGE<sub>2</sub> production by BM Ly6C<sup>hi</sup> monocytes sorted from naïve WT mice, cultured for 6 hours with or without IFN- $\gamma$ , and subsequently stimulated with *E. coli* lysate. Error bars represent standard deviation. Data are representative of two or more independent experiments, n = 3-5 replicates per group. Statistical comparisons were performed using unpaired student's *t* test, adjusted for multiple comparisons. \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.



**Figure 15: Changes to BM monocytes persist after resolution of acute inflammation.** CX3CR1-GFP mice were infected per-orally with *T. gondii* and assessed at late time points after acute inflammation had resolved. **(A-B)** Phenotype of BM and blood Ly6C<sup>hi</sup> monocytes were assessed by flow cytometry. **(C)** *Ex vivo* PGE<sub>2</sub> production by BM Ly6C<sup>hi</sup> monocytes sorted from naïve or infected mice at day 24 post-infection and stimulated with *E. coli* lysate. **(D-E)** MHCII and Sca-1 expression by Ly6C<sup>hi</sup> monocytes in BM and blood of ARE-Del<sup>-/-</sup> mice and WT controls. **(F)** *Ex vivo* PGE<sub>2</sub> production by BM Ly6C<sup>hi</sup> monocytes sorted from naïve ARE-Del<sup>-/-</sup> mice. Error bars represent one standard deviation. Data are representative of at least two independent experiments, n = 3-5 mice per group. Statistical comparisons were performed using one-way ANOVA or unpaired student's *t* test adjusted for multiple comparisons. \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.



**Figure 16: *T. gondii* infection alters BM monocyte responses to bacterial stimuli.**

Ly6C<sup>hi</sup> monocytes were sorted from BM of naïve or day 5 *T. gondii* infected WT mice. Sorted monocytes were cultured for 6 hours in the presence or absence of LPS, and gene expression assessed using the NanoString platform. **(A)** Principle component analysis comparing gene expression of untreated and LPS stimulated monocytes from naïve and infected mice. Plot represents clustering of samples in a 2-dimensional matrix of principle components 1 and 2. **(B)** Gene expression of untreated naïve monocytes from (C) was compared to each of the other three groups. Numbers in each section of the Venn diagram represent the number of genes altered in expression between that group and untreated naïve controls, with overlapping regions representing genes changed in more than one group. **(C)** Heat maps representing the relative expression of the 30 genes changed only in monocytes from infected mice upon LPS stimulation. Columns represent biological replicates. Statistical comparisons were performed using Welch's *t* test adjusted for multiple comparisons.



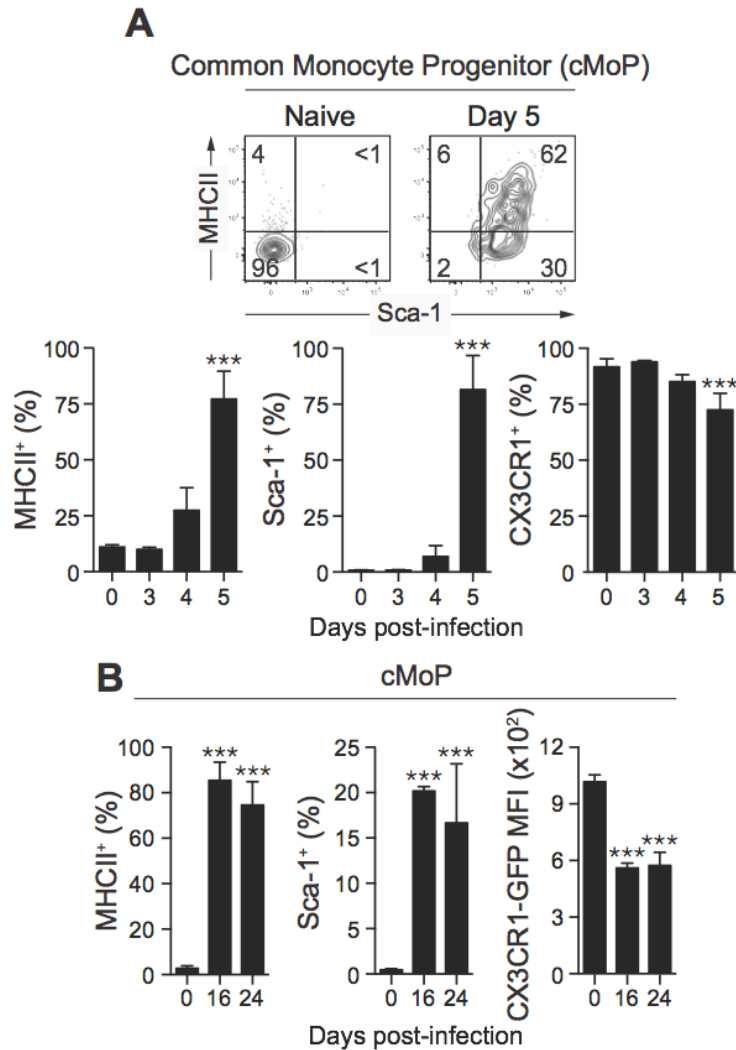
## **IFN- $\gamma$ controls the transcriptional program of monocyte progenitors**

Monocytes are constitutively generated in the BM, developing through a series of well-defined stages including the granulocyte monocyte precursor (GMP), monocyte dendritic cell precursor (MDP), and the more recently described common monocyte progenitor (cMoP) (Fogg et al., 2006; Hettinger et al., 2013). Notably, we found that early during the infection, cMoP and BM monocytes began to acquire the distinct phenotype associated with acquisition of regulatory function (**Figure 13A, 17A**). These phenotypic changes persisted following resolution of the acute phase of the infection (**Figure 17B**).

To assess how mucosal infection changed the transcriptional response of monocyte progenitors in the BM, we analyzed the transcriptional profile of cMoP from naïve versus *T. gondii* infected mice at day 5 post-infection. Pathway analysis revealed a distinct interferon signature, marked by increased expression of 8 transcriptional regulators, including *Stat1*, *Stat2*, and *Irf7* (**Figure 18**). Furthermore, a broad suite of anti-microbial factors were increased in expression, including *Gbp2* and *Ifi47*, intracellular defense factors critical for resistance to *T. gondii* infection (Collazo et al., 2001; Degrandi et al., 2013). In agreement with our flow cytometric characterization, expression of transcripts for MHCII (*H2-Aa*, *H2-Ab1*, *H2-Eb1*) and Sca-1 (*Ly6a*) were increased during infection, while expression of *Cx3cr1* was decreased. These results reveal that the transcriptional program of monocyte progenitors during infection can be dramatically altered in the BM prior to terminal differentiation and egress.

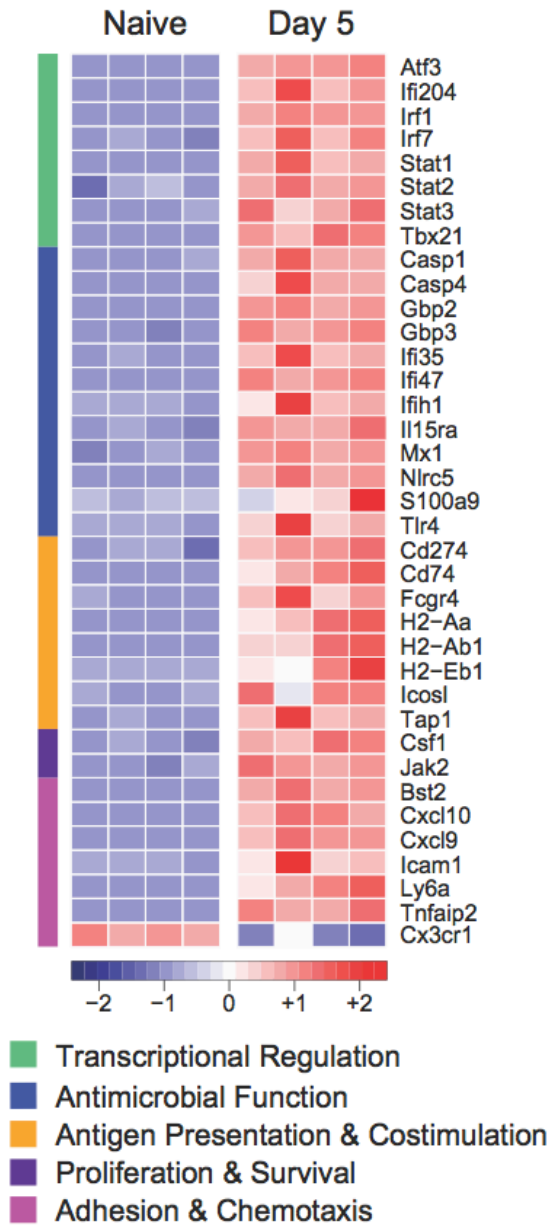
Administration of IFN- $\gamma$  to naïve mice was sufficient to increase expression of MHCII and Sca-1 and decrease expression of CX3CR1 (**19A, 19B**). Acquisition of this phenotype during *T. gondii* infection was impaired in cells lacking a functional IFN- $\gamma$  receptor (**Figure 19C, 19D**) and following IFN- $\gamma$  blockade (**Figure 20A, 20B**). Additionally, treatment with anti-IFN- $\gamma$  abolished the transcriptional program adopted by cMoP during infection (**Figure 20C**). Thus, IFN- $\gamma$  can directly shape the transcriptional program and function of monocytes during their development.

Despite the transcriptional changes induced by infection, cMoP remained committed to the monocyte lineage during infection as evidenced by the sustained expression of *Irf8*, a transcription factor critical for monocyte development (**Figure 21A**), and lack of expression of transcripts associated with the DC lineage such as *Zbtb46*, *Flt3*, and *Id2* (Kurotaki et al., 2013; Merad et al., 2008) (**Figure 21B**). Furthermore, when cultured under monocyte differentiation conditions (Hettinger et al., 2013), MHCII<sup>+</sup>Sca-1<sup>+</sup> putative cMoP from infected mice differentiated into Ly6C<sup>hi</sup>CD11b<sup>hi</sup>cKit<sup>-</sup> mature monocytes (**Figure 22A**). Likewise, when cultured under conditions favoring macrophage differentiation (Hettinger et al., 2013), MHCII<sup>+</sup>Sca-1<sup>+</sup> putative monocytes differentiated into Ly6C<sup>lo</sup>F4/80<sup>hi</sup> macrophages (**Figure 22B**). These macrophages differentiated from MHCII<sup>+</sup>Sca-1<sup>+</sup> monocytes lacked expression of MHCII or Sca-1, making them phenotypically identical to those derived from naïve animals (**Figure 22C**). This suggested that expression of MHCII and Sca-1 required sustained IFN- $\gamma$  signaling.

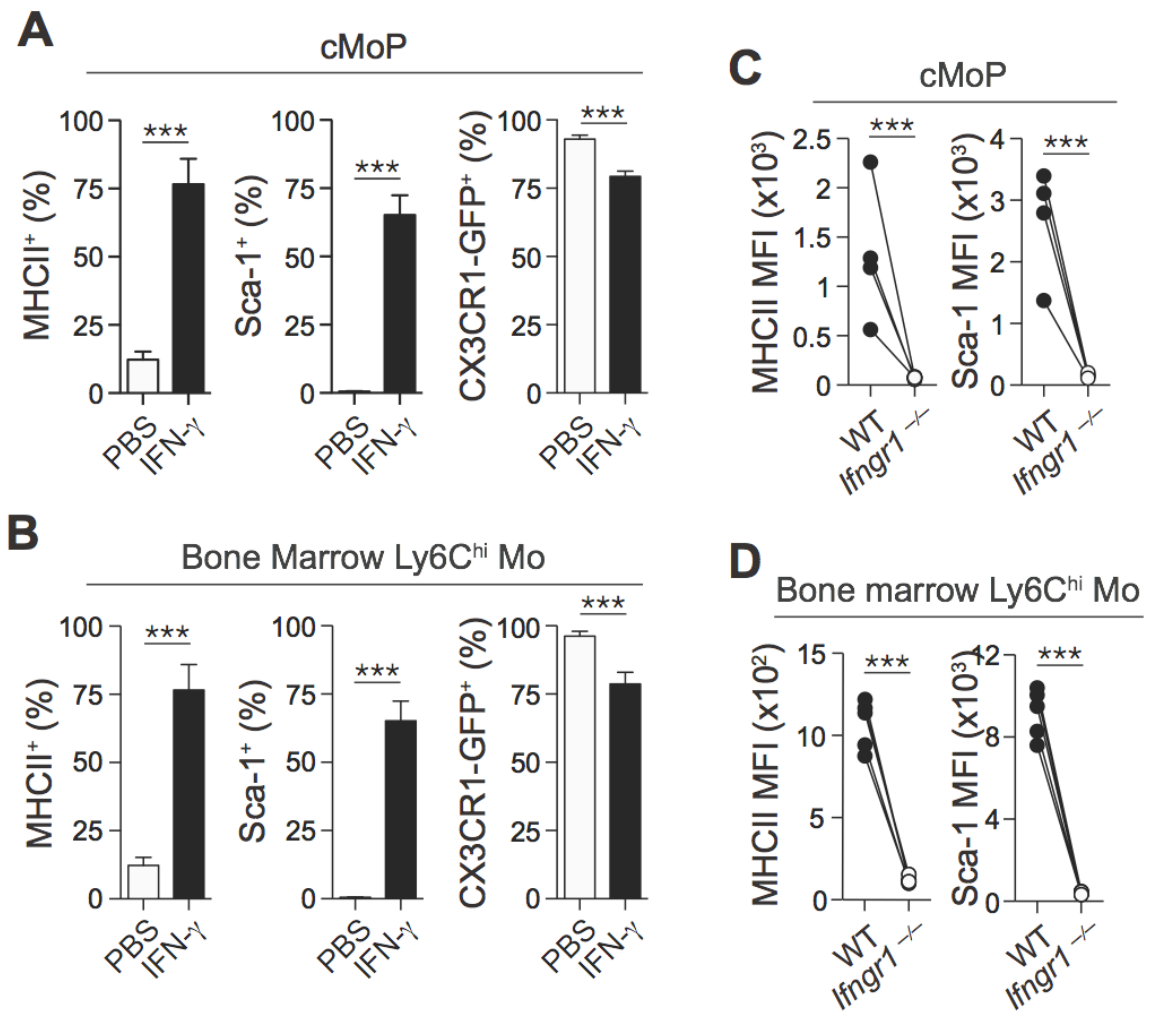


**Figure 17: Phenotype of Monocyte Progenitors is altered during infection.**

CX3CR1-GFP mice were infected per-orally with *T. gondii* and assessed at defined time points post-infection. **(A-B)** Phenotype of BM Ly6C<sup>hi</sup> monocytes and cMoP were assessed by flow cytometry. Numbers represent the mean proportion of cells within defined gates among all samples. Error bars represent one standard deviation. Data are representative of at least two independent experiments, n = 3-5 mice per group. Statistical comparisons were performed using one-way ANOVA. \*\*\*: p<0.001.

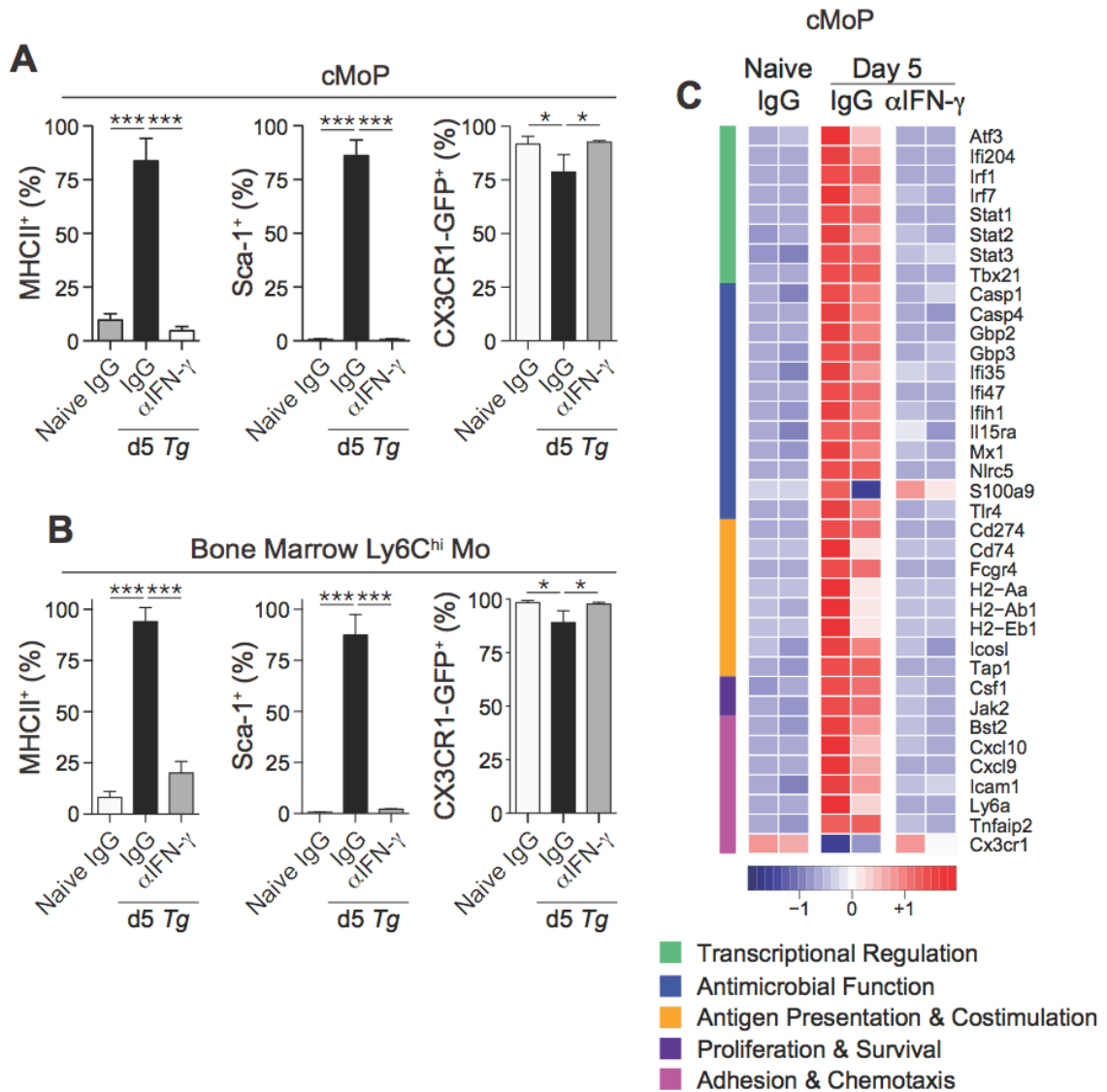


**Figure 18: cMoP display an Interferon-responsive gene signature during infection.** cMoP were sorted from the BM of naïve or day 5 infected mice, and gene expression was assessed by NanoString. Heat maps represent the relative expression of 37 genes by cMoP selected by pathway analysis as being affected by IFN- $\gamma$  signaling from naïve and infected WT mice.



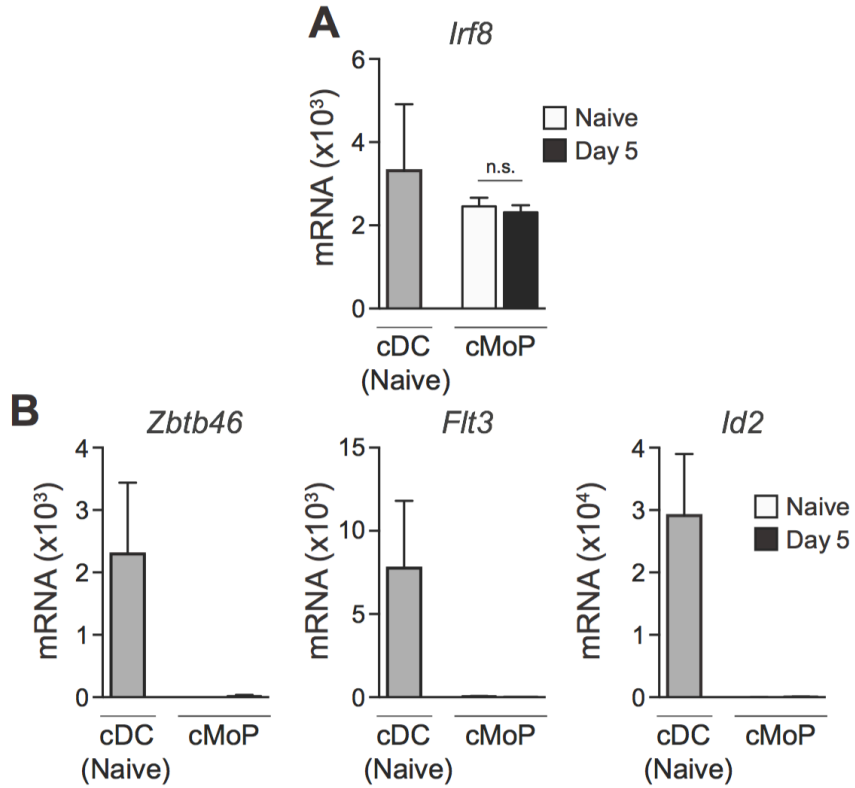
**Figure 19: Cell-intrinsic IFN- $\gamma$  signaling is required for alterations to cMoP and Ly6C<sup>hi</sup> monocytes in the BM.**

(A-B) CX3CR1-GFP mice were administered IFN- $\gamma$  or PBS once per day for three consecutive days. Mice were sacrificed eighteen hours after the final injection, and MHCII and Sca-1 expression by cMoP and Ly6C<sup>hi</sup> monocytes in the BM were assessed. (C-D) Mean fluorescence intensity (MFI) of MHCII and Sca-1 expression by WT and *Ifngr1*<sup>-/-</sup> cMoP and BM Ly6C<sup>hi</sup> monocytes from day 8 *T. gondii* infected mice. Values of WT and *Ifngr1*<sup>-/-</sup> cells from the same host are joined by a line. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3-5 mice per group. Statistical comparisons were performed using unpaired (A,B) or paired student's *t* test (C,D). \*\*\*: p<0.001.



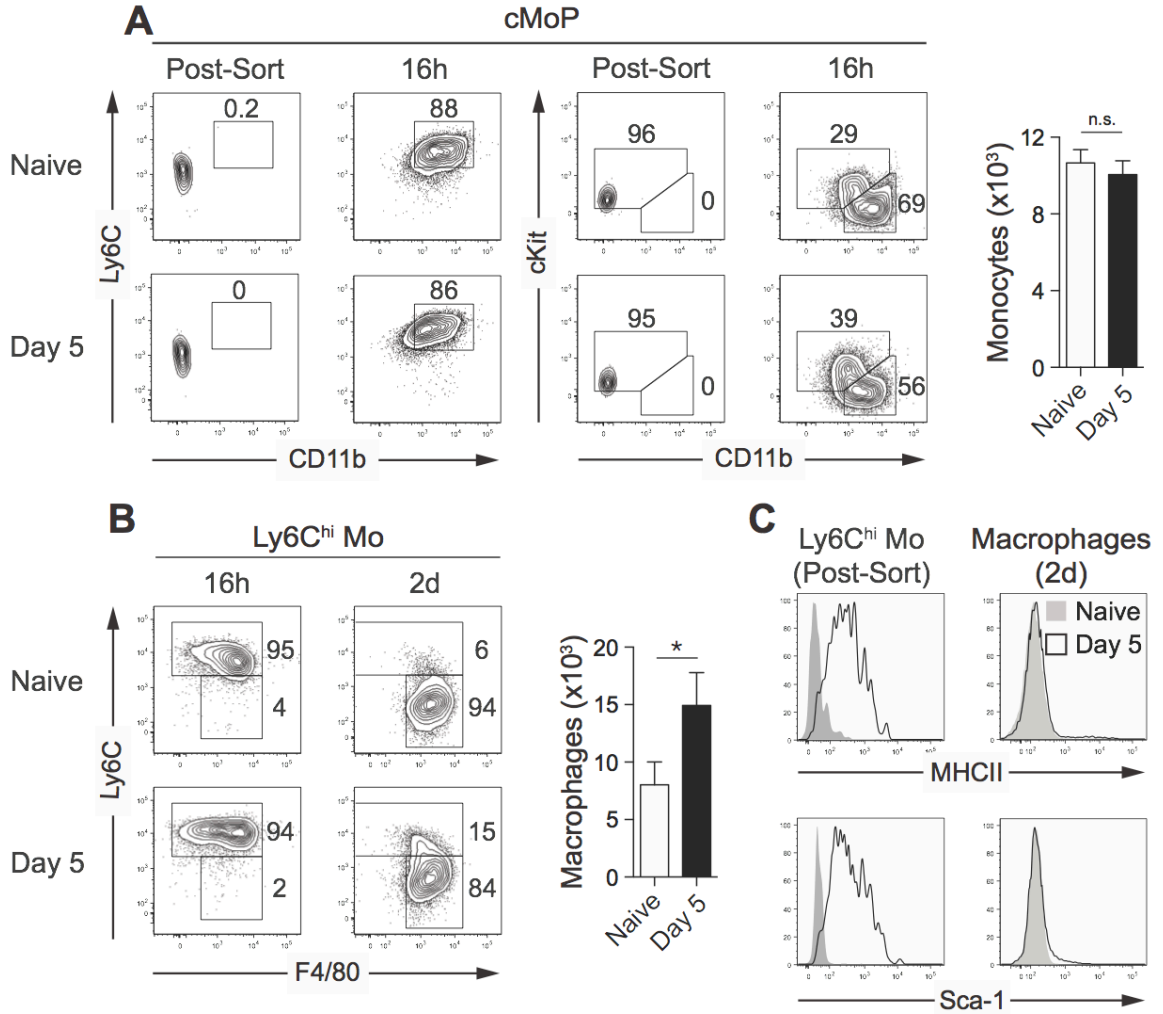
**Figure 20: IFN- $\gamma$  drives changes to cMoP phenotype and gene expression.**

CX3CR1-GFP mice were infected and administered anti-IFN- $\gamma$  Ab or isotype control. **(A-B)** Expression of MHCII and Sca-1 by cMoP and Ly6C<sup>hi</sup> monocytes in the bone marrow was assessed at day 5 post-infection. **(C)** On day 5 post-infection, monocytes were FACS sorted from these mice as well as naïve controls treated with isotype antibody, RNA was purified from these cells, and gene expression was assessed by NanoString. Heat maps represent the relative expression of genes known to be regulated by IFN- $\gamma$ , as described in Figure 14. Error bars represent one standard deviation. Data are representative of two or more independent experiments (A-B), n = 3-5 mice per group. Columns represent biological replicates of 2 pooled samples (C). Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*: p<0.05, \*\*\*: p<0.001.



**Figure 21: Putative cMoP analyzed during infection do not express core DC signature genes.**

**(A-B)** *Irf8*, *Zbtb46*, *Flt3*, and *Id2* expression by classical dendritic cells (cDC) sorted from the cervical lymph nodes of naïve WT mice and cMoP from naïve and day 5 *T. gondii* infected WT mice were compared utilizing the NanoString platform. cDC were sorted based on the following markers: Lineage<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>hi</sup>CD24<sup>-</sup>CD11b<sup>+</sup>CD103<sup>+</sup> (Lineage: Ly6G, Siglec F, TCR- $\beta$ , NK1.1, and B220) Error bars represent one standard deviation. Data are representative of pooled samples from a single experiment.



**Figure 22: cMoP and BM monocytes retain their differentiation potential during infection.**

cMoP and Ly6C<sup>hi</sup> monocytes were sorted from BM of naïve and day 5 *T. gondii* infected WT mice. For purification from infected mice, Sca-1-MHCII<sup>-</sup> cells were excluded. **(A)** cMoP were cultured under conditions promoting monocyte differentiation. After 16 hours in culture, cells were assessed for acquisition of monocyte phenotype (Ly6C<sup>hi</sup>cKit-CD11b<sup>hi</sup>) by flow cytometry. **(B)** Ly6C<sup>hi</sup> monocytes were cultured under conditions promoting macrophage differentiation. At defined time-points after culture, cells were assessed for acquisition of macrophage phenotype (Ly6C<sup>lo</sup>F4/80<sup>hi</sup>) by flow cytometry. **(C)** MHCII and Sca-1 expression was compared between Ly6C<sup>hi</sup> monocytes immediately post-sort (left) and Ly6C<sup>lo</sup>F4/80<sup>hi</sup> macrophages after 2 days in culture (right). Error bars represent one standard deviation. Numbers represent the mean proportion of cells within defined gates among all samples. Data are representative of pooled samples from two independent experiments, n= 4. Statistical comparisons were performed using unpaired student's *t* test. \*: p<0.05.



## ***Discussion***

To the best of our knowledge, we describe here for the first time transcriptional reprogramming of myeloid progenitors in the BM during infection. Host-derived and microbial signals associated with infection drive increased production of monocytes and granulocytes by the BM compartment in a process termed “emergency myelopoiesis” (Takizawa et al., 2012). However, whether increased production of monocytes can be coupled with changes to their function remains unknown. In this chapter, we find that IFN- $\gamma$  remodels the transcriptional program of monocyte progenitors, resulting in altered responsiveness to bacterial stimuli by their monocyte progeny. In particular, pre-emptive priming by IFN- $\gamma$  imparts Ly6C<sup>hi</sup> monocytes with increased capacity to produce PGE<sub>2</sub>, a regulatory function required to prevent lethal inflammatory responses to the intestinal microbiota. While most of the bacterial stimuli examined (with the exception of flagellin) were potent inducers of PGE<sub>2</sub> production by monocytes, signals derived from *T. gondii* did not result in PGE<sub>2</sub> secretion (**Figure 14A**). However, in agreement with previous studies, we found that monocytes remain highly responsive to *Toxoplasma* antigen during infection (Goldszmid et al., 2012; Hunter and Sibley, 2012). Thus, the inability of monocytes to produce PGE<sub>2</sub> in response to parasite-associated signals indicates that activation of this pathway is a specialized response to bacterial stimuli.

The capacity to produce both inflammatory and regulatory factors is ideally adapted for the complex role Ly6C<sup>hi</sup> monocytes play during oral *T. gondii* infection. Early IFN- $\gamma$  signalling on monocyte progenitors engages a transcriptional program that

optimizes downstream monocytes for the control of intracellular pathogens by increasing their expression of an array of defined anti-microbial factors (**Figure 18**). While the priority upon encounter with *Toxoplasma* by monocytes is elimination of the pathogen, translocating bacteria induce a more balanced approach. Although this study focuses on the production of PGE<sub>2</sub> by Ly6C<sup>hi</sup> monocytes due to its previously described importance in preventing lethal immunopathology (Grainger et al., 2013), the overall response of monocytes upon encounter with *E. coli* includes secretion of inflammatory factors, including TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-12. Indeed, we find that functional priming by IFN- $\gamma$  in the BM augments expression of *Il12b* and *Il1b* (**Figure 16C**). The contrast between the purely inflammatory and anti-microbial functions of monocytes in response to *T. gondii* and their balanced responsiveness to *E. coli* emphasizes the sophisticated plasticity of these cells. Upon entry to the small intestine, the function of each recruited monocyte is likely to differ depending on the balance of stimuli it receives from the parasite versus translocating bacteria. Thus as a whole, the recruited population can dynamically adapt to provide an optimal response to both the infection and the associated outgrowth of *E. coli*.

We propose that factors that augment monocyte production during infection can act in tandem to appropriately educate myeloid cell function prior to bone marrow egress. Together, our results support the idea that early sensing of IFN- $\gamma$  by cMoP and

monocytes in the bone marrow has profound consequences on the subsequent function of Ly6C<sup>hi</sup> monocytes, particularly their capacity to generate regulatory responses toward products derived from the microbiota. **The potential mechanisms by which IFN- $\gamma$  regulates gene expression by cMoP and monocytes are discussed in Chapter 5.**

## **CHAPTER 4: BM-Resident NK Cells Educate Monocyte Progenitors in Response to MALT-Derived IL-12**

### ***Abstract***

The findings described in **Chapter 3** reveal that *T. gondii* infection results in profound changes to the transcriptional program of monocyte progenitors in the BM. In addition to being the primary site of hematopoiesis, the BM is home to stable populations of mature lymphocytes and macrophages (Becker et al., 2005; Glatman Zaretsky et al., 2014; Herndler-Brandstetter et al., 2011; Tokoyoda et al., 2009). During infection, these supporting populations can direct the differentiation of progenitor populations during inflammation via local production of cytokines and growth factors (Chow et al., 2011b; Shi et al., 2011). In this chapter, we assess the role of BM-resident lymphocytes in education of monocyte progenitors during infection. Key findings include:

- 1. BM-resident NK cells produce IFN- $\gamma$  early during infection.**
- 2. Depletion of NK cells inhibits education of Ly6C<sup>hi</sup> monocytes and their progenitors.**
- 3. IL-12 produced by *Batf3*-dependent DC in the MALT drives NK cell activation and IFN- $\gamma$  production.**

## ***Rationale***

We hypothesized that NK cells in the BM were driving the early education of monocyte progenitors during *T. gondii* infection via local IFN- $\gamma$  production. Activated NK cells have been found to form clusters in close association with Ly6C<sup>hi</sup> monocytes, priming monocytes for anti-microbial function via IFN- $\gamma$  secretion (Coombes et al., 2012; Goldszmid et al., 2012; Kang et al., 2008). NK cells are essential early producers of IFN- $\gamma$  during *T. gondii* infection (Hunter et al., 1995; Munoz et al., 2011; Scharon-Kersten et al., 1996), and BM-resident lymphocytes have previously been shown to respond to systemic infection by producing IFN- $\gamma$  and driving myelopoiesis (MacNamara et al., 2011; Schürch et al., 2014; Zhang et al., 2013). Therefore, we examined whether BM-resident NK cells and T cells produced IFN- $\gamma$  during *T. gondii* infection and sought to identify the signals driving the activation of these cells.

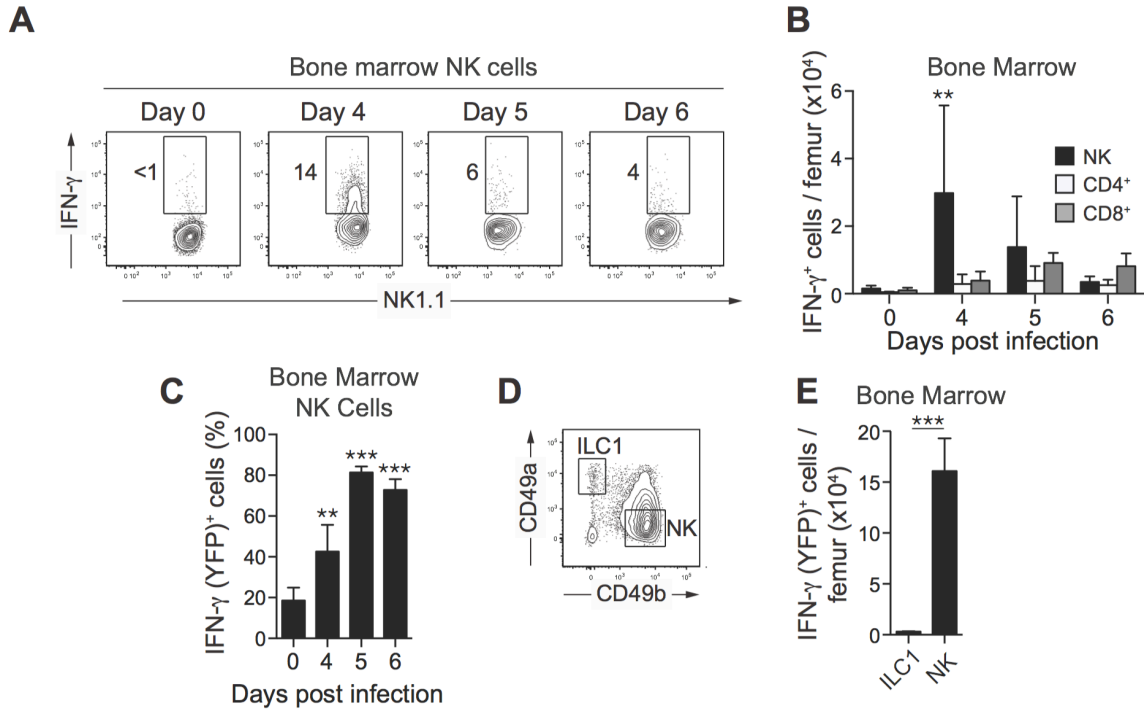
## **Results**

### **NK cell production of IFN- $\gamma$ in the BM educates monocyte progenitors**

The extent of cMoP and monocyte education as early as day 5 post-infection and prior to systemic inflammation raised the possibility that these changes may be triggered by local IFN- $\gamma$  production in the BM. Indeed, phenotypic alterations to BM monocytes during *T. gondii* infection preceded significant increases in serum levels of IFN- $\gamma$  (**Figure 8B, 13B**). The BM is home to mature immune cells capable of producing IFN- $\gamma$ , such as T cells and innate lymphoid cells (ILC), including classical NK cells (Becker et al., 2005; Klose et al., 2014). To identify the early source of IFN- $\gamma$  in the BM during infection, we tracked the production of this cytokine by flow cytometry using direct *ex vivo* staining and IFN- $\gamma$  reporter mice (Reinhardt et al., 2009), as well as by imaging. We found that BM-resident NK cells produce IFN- $\gamma$  as early as day 4 post-infection (**Figure 23A-C**). At this time-point, NK cells were the dominant source of IFN- $\gamma$  in the BM, with minimal contribution from T cells or type 1 ILC (**Figure 23D, 23E**), while at day 5, IFN- $\gamma$  production was also observed in T cells (**Figure 23B**). Histological analysis at day 5 post-infection demonstrated IFN- $\gamma$  producing NK cells in close proximity to BM monocytes (**Figure 24, Figure 25**).

To further assess whether NK cell-derived IFN- $\gamma$  could directly impact cMoP, we treated mice with anti-NK1.1 depleting antibody. This treatment resulted in nearly

complete ablation of the NK compartment in most tissues, while NK cells in the BM were decreased by approximately 75% (**Figure 26**). NK depletion during infection significantly inhibited the early acquisition of Sca-1 and MHCII by BM cMoP and Ly6C<sup>hi</sup> monocytes, and reversed changes to CX3CR1 expression (**Figure 27A, 27B**). NK depletion also reduced changes to monocyte phenotype in the blood, although the effects were less pronounced than on BM populations, suggesting that other sources of IFN- $\gamma$  may further educate monocytes outside of the BM (**Figure 27C**).

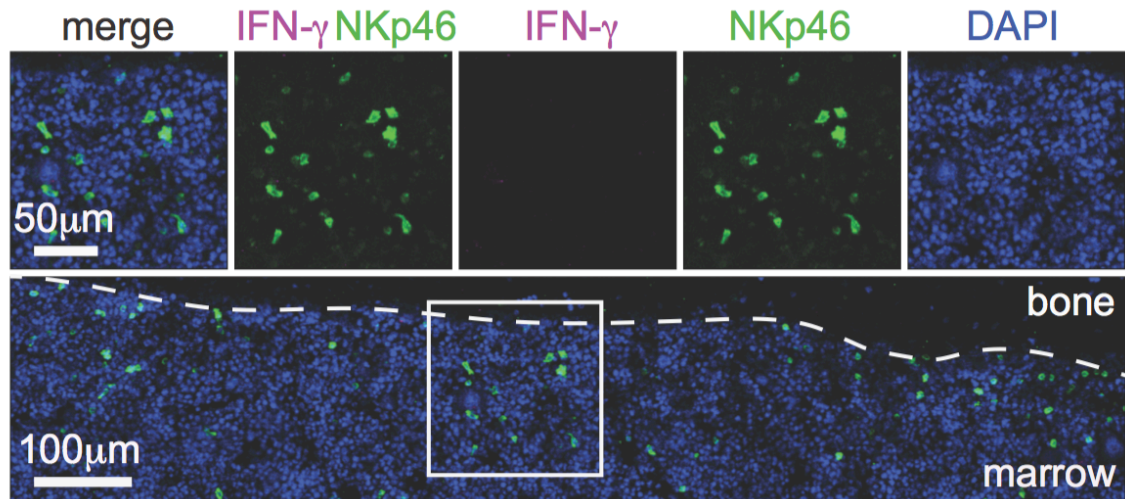


**Figure 23: BM NK cells produce IFN- $\gamma$  early during infection.**

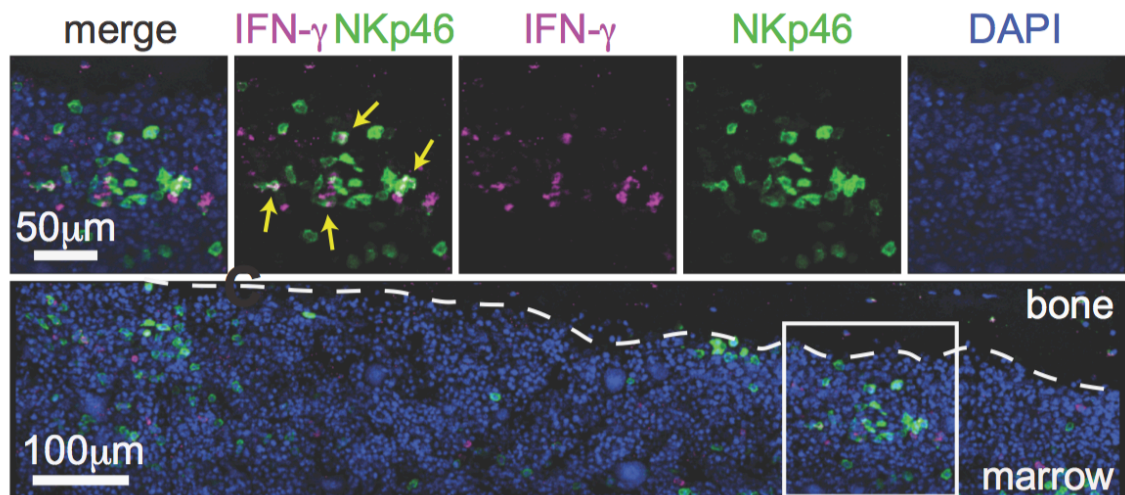
(A) WT mice were infected with *T. gondii*. At defined time-points, lymphocyte IFN-g production was assessed by flow cytometry. Plots are gated on TCR- $\beta$ -NK1.1+DX5+ NK cells. (B) Absolute numbers of lymphocytes producing IFN- $\gamma$  in the BM as assessed in (A). (C) IFN- $\gamma$  YFP mice were infected and BM lymphocytes were assessed for YFP expression at defined time-points post-infection. (D) Gating strategy for type I ILC and NK cells in the BM. Dot plot is gated on CD45+TCR- $\beta$ -NK1.1+ cells. (E) Absolute numbers of IFN- $\gamma$  producing Type I ILC and NK cells at day 5 post-infection as assessed in (D). Error bars represent standard deviation. Data are representative of two or more independent experiments, n = 3-5 mice per group. Statistical comparisons were performed using one-way ANOVA (B,C) or student's *t* test (E). \*\*: p<0.01, \*\*\*: p<0.001.



## Naive



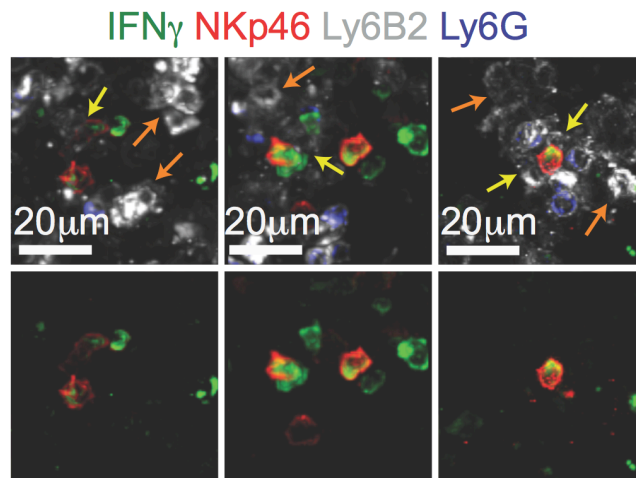
## Day 5



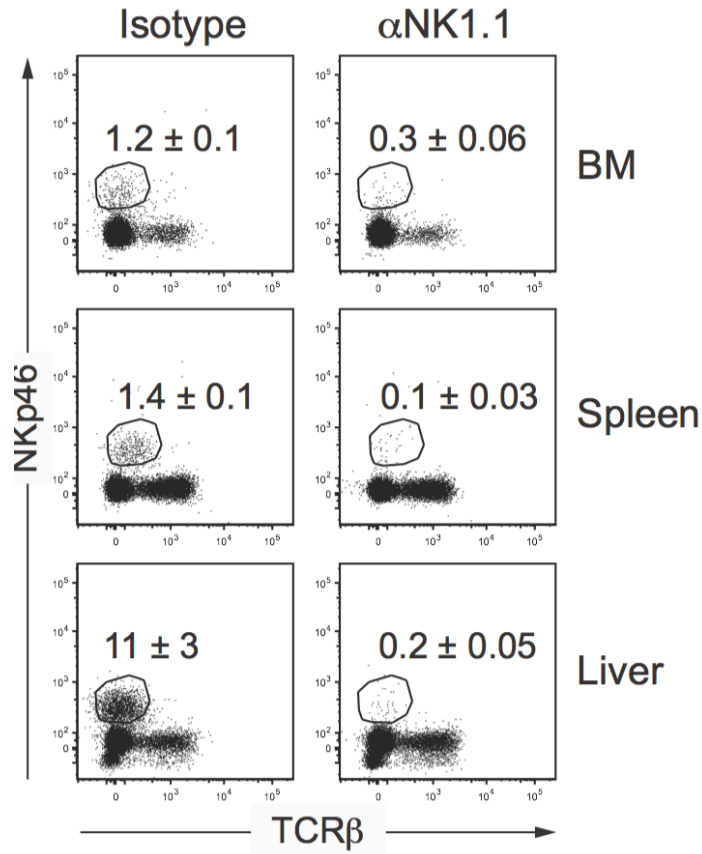
**Figure 24: Clusters of BM NK cells produce IFN- $\gamma$ .**

Confocal images of BM of naïve and day 5 *T. gondii* infected IFN- $\gamma$  YFP mice. Yellow arrows indicate NK cells producing IFN- $\gamma$ .

Day 5

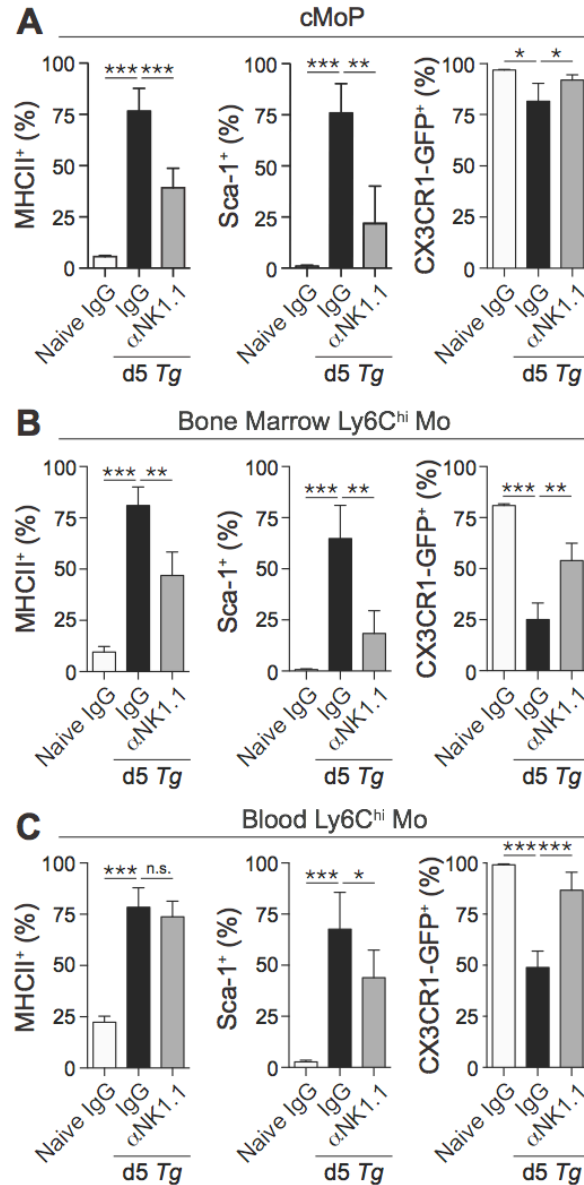


**Figure 25: IFN- $\gamma$  producing BM NK cells are located near Ly6C<sup>hi</sup> monocytes.** Confocal images of BM of IFN- $\gamma$  YFP mice at day 5 post-infection. Ly6G-Ly6B2<sup>+</sup> monocytes are in contact with (yellow arrows) or in close proximity to (orange arrows) NK cells producing IFN- $\gamma$ .



**Figure 26: NK depletion after antibody administration.**

CX3CR1-GFP mice were infected with *T. gondii* and administered NK depleting antibody (anti-NK1.1) or isotype control. At day 5 post-infection, single cell suspensions were prepared from BM, spleen, and liver, and were assessed for the presence of NK cells. Dot plots are gated on live CD45<sup>+</sup> cells. Numbers represent mean frequency of cells within each gate +/- one standard deviation.



**Figure 27: NK cell depletion inhibits changes to cMoP and monocyte phenotype during infection.**

(A-C) CX<sub>3</sub>CR1-GFP mice were infected with *T. gondii* and administered NK depleting antibody (anti-NK1.1) or isotype control. Naïve controls were administered isotype control antibody. Expression of MHCII, Sca-1, and CX<sub>3</sub>CR1-GFP by cMoP and Ly6C<sup>hi</sup> monocytes in the BM and blood was assessed at day 5 post-infection. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3-5. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.

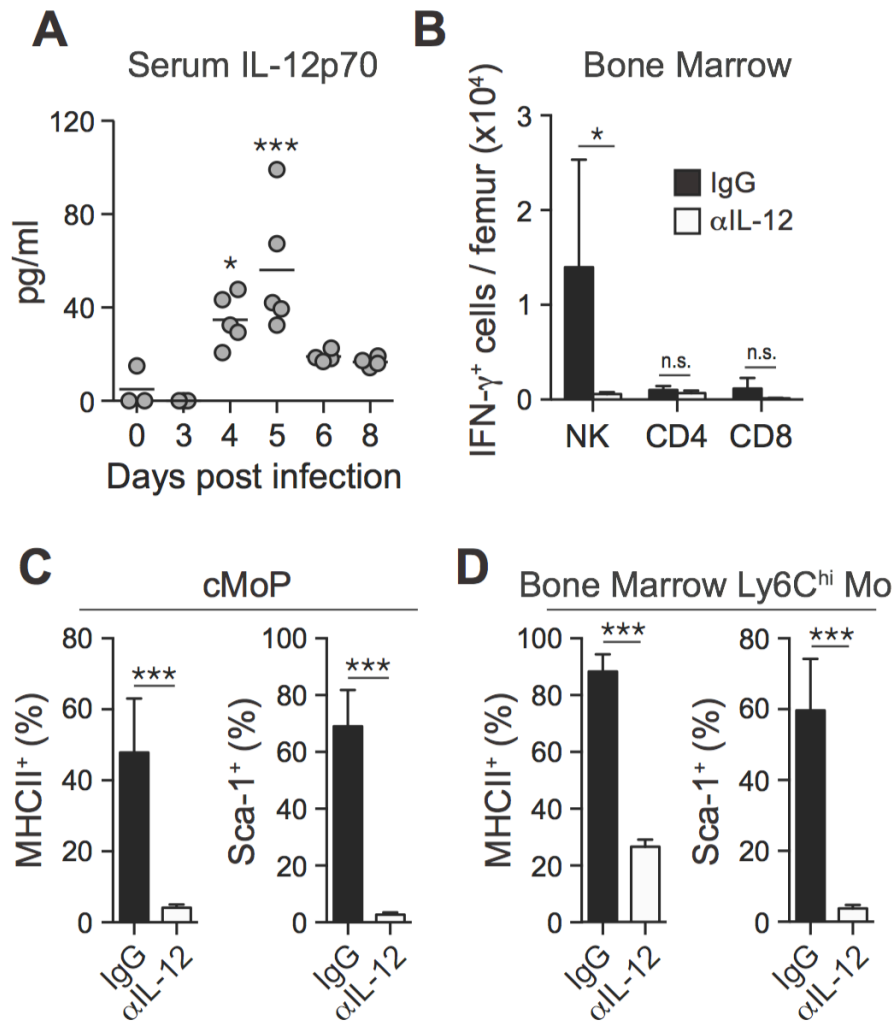
## **IL-12 Produced by *Batf3*-dependent DC in the MALT Drives IFN- $\gamma$ production**

*T. gondii* is a potent inducer of IL-12, a cytokine involved in NK cell activation (Trinchieri, 2003). Following infection, a rapid increase in IL-12p70 was detectable in the blood that coincided with NK activation in the BM (**Figure 28A**). Antibody blockade of IL-12 prevented IFN- $\gamma$  production by BM-resident NK cells and impaired MHCII and Sca-1 expression by cMoP and monocytes (**Figure 28B-D**). Early alterations in cMoP and monocytes were likewise abolished in IL-12p35 deficient animals, but not in mice deficient in IL-18, another potent stimulator of IFN- $\gamma$  production from NK cells (Scharton-Kersten et al., 1996) (**Figure 29A, 29B**). Moreover, administration of recombinant IL-12 alone was sufficient to drive IFN- $\gamma$  production by NK cells and phenotypic alterations to cMoP and monocytes (**Figure 30A, 30B**).

Having determined that IL-12 was critical for IFN- $\gamma$  production by NK cells and for priming of monocytes and their progenitors in the BM, we next assessed the tissue and cellular source of IL-12 during *T. gondii* infection. Previous work revealed a dominant role for *Batf3*-dependent DC in IL-12 production during intra-peritoneal *T. gondii* infection (Mashayekhi et al., 2011). Strikingly, in *Batf3* deficient mice that are devoid of CD8a<sup>+</sup> lymphoid-resident DC and migratory CD103<sup>+</sup>CD11b<sup>-</sup> DC (**Figure 31**) (Edelson et al., 2010; Hildner et al., 2008), IL-12 was not increased in the serum upon infection and early NK cell activation was not detected (**Figure 32A, 32B**).

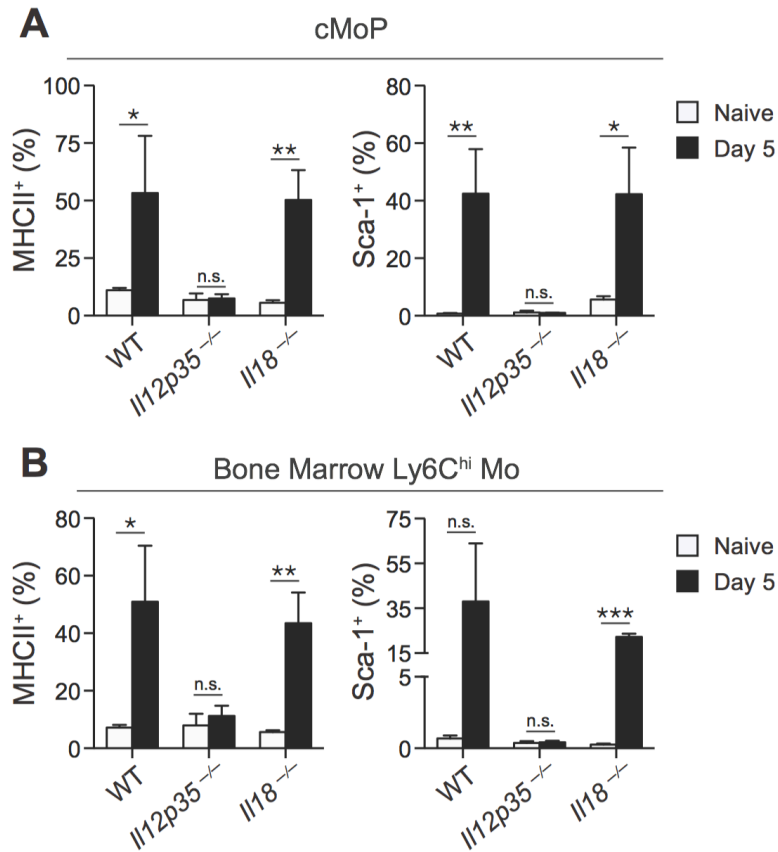
Furthermore, reduced NK activation in these mice was associated with impaired acquisition of the Sca-1<sup>+</sup> MHCII<sup>+</sup> phenotype by BM cMoP and monocytes (**Figure 32C, 32D**).

In contrast to the spleen and BM, the mesLN compartment produced detectable levels of IL-12p70 post-infection (**Figure 33A**). Additionally, transcriptional analysis of *Batf3*-dependent DC in the mesLN and spleen revealed a significant increase in *Il12b* expression in mesLN DC but not splenic DC post-infection (**Figure 33B**). Although we cannot completely exclude the possibility of a transient and discrete contribution of the BM, our present results, in agreement with previous studies (Oldenhove et al., 2009; Washino et al., 2012), support the idea that the mucosal-associated lymphoid tissue (MALT) is the dominant source of early IL-12 following per-oral *T. gondii* infection.



**Figure 28: Systemic IL-12 drives IFN- $\gamma$  production by BM NK cells.**

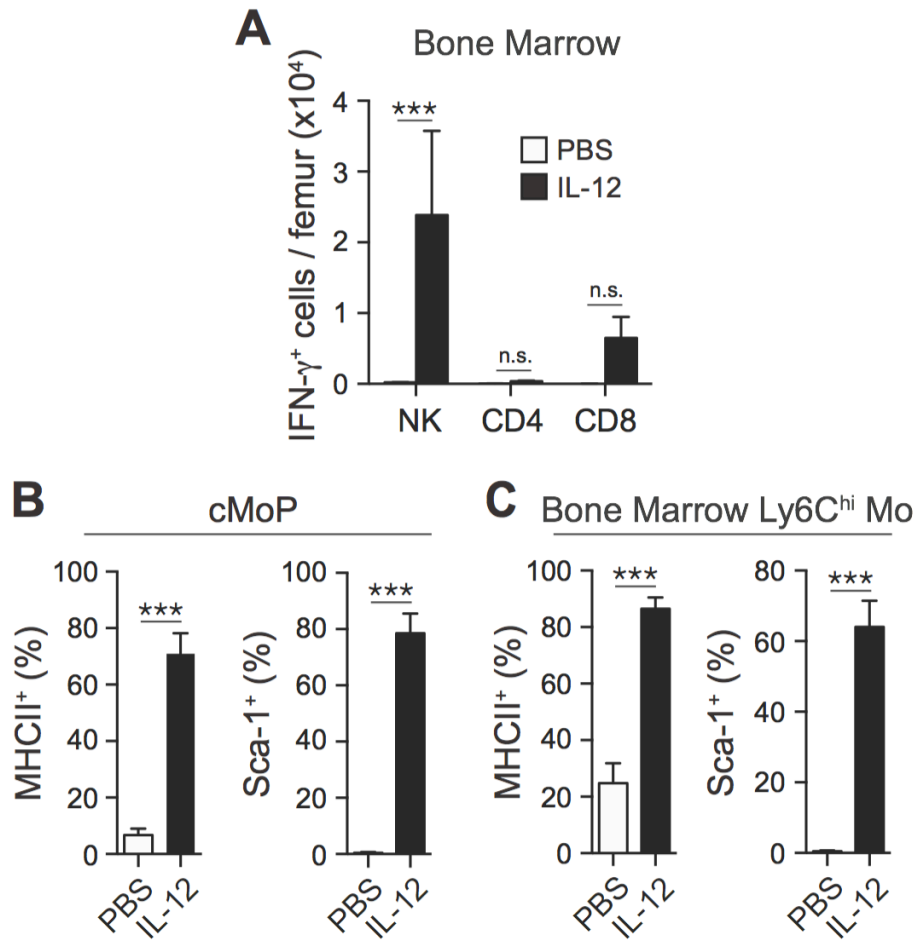
(A) Serum IL-12p70 levels after infection with *T. gondii*. (B-C) WT mice were infected with *T. gondii* and treated with anti-IL-12p70 Ab or isotype control. (B) Absolute numbers of IFN- $\gamma$  producing lymphocytes at day 5 post-infection. (C-D) MHCII and Sca-1 expression by cMoP and Ly6C<sup>hi</sup> monocytes in the BM at day 5 post-infection. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3-5. Statistical comparisons were performed using one-way ANOVA (A), or unpaired student's *t* test adjusted for multiple comparisons (B-D). \*: p<0.05, \*\*\*: p<0.001.



**Figure 29: IL-12, but not IL-18 is required for infection-induced changes to monocyte phenotype.**

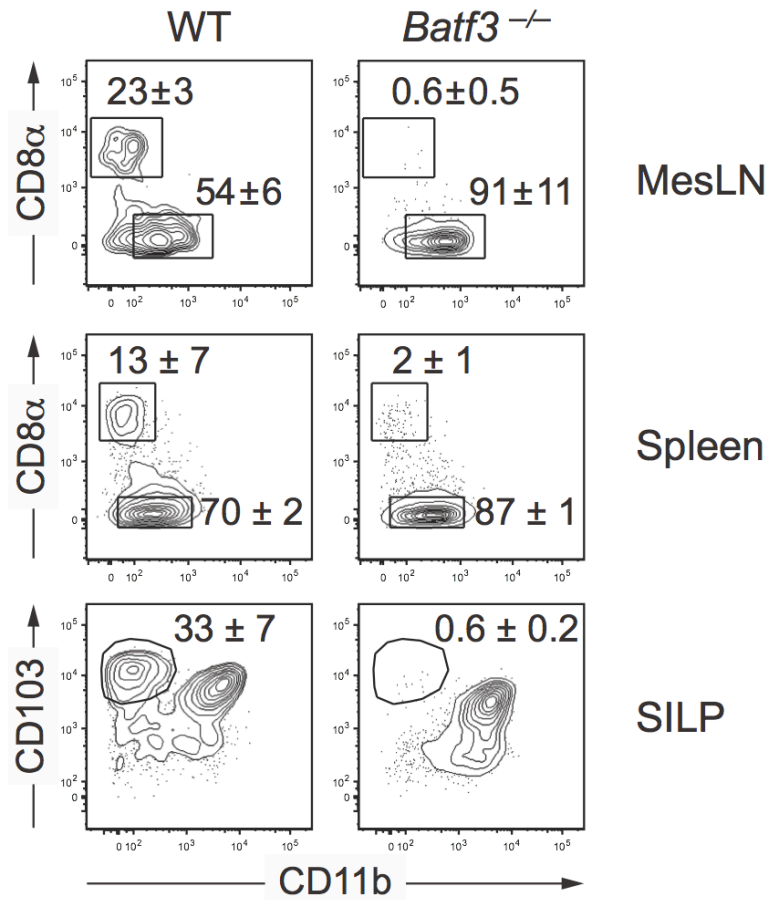
**(A-B)** MHCII and Sca-1 expression by cMoP and BM monocytes of *Il12p35*<sup>-/-</sup> mice, *Il18*<sup>-/-</sup> mice and WT controls naïve and at day 5 post-infection. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3-5. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.





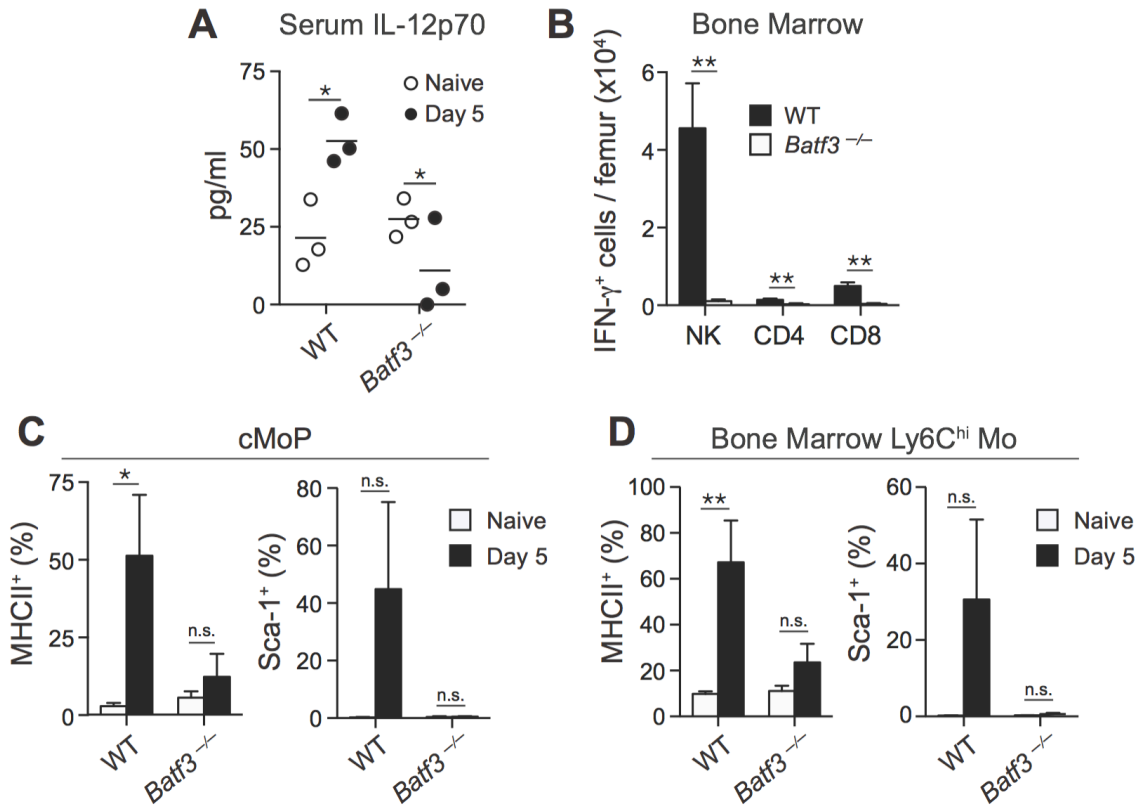
**Figure 30: IL-12 administration drives IFN- $\gamma$  production by BM NK cells.**

WT mice were administered IL-12 or PBS once per day for two days. **(A)** Absolute numbers of lymphocytes producing IFN- $\gamma$  in the BM. **(B-C)** MHCII and Sca-1 expression by cMoP and Ly6C<sup>hi</sup> BM monocytes. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3-5. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*\*\*, p<0.001.



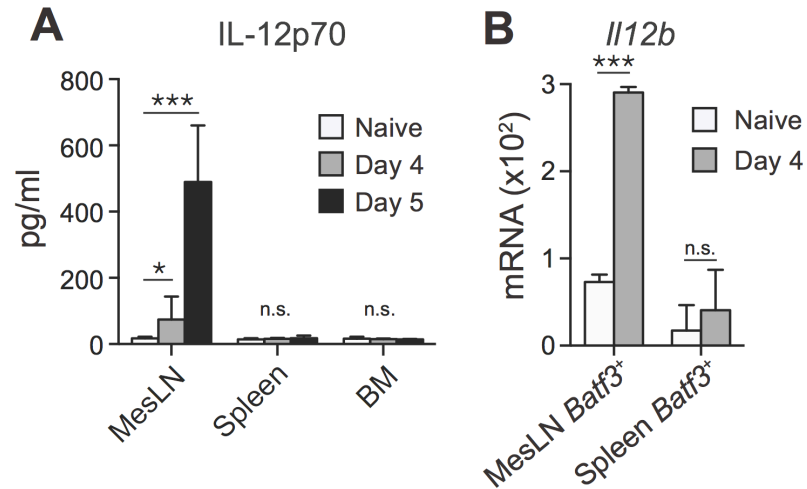
**Figure 31: Characterization of *Batf3*<sup>-/-</sup> mice at day 5 of *T. gondii* infection.**

*Batf3*<sup>-/-</sup> mice and WT controls were infected and the presence CD8α<sup>+</sup> and CD11b<sup>+</sup> resident DC in the mesLN and spleen, as well as CD103<sup>+</sup>CD11b<sup>-</sup> DC in the SILP, were assessed at day 5 post-infection. Dot plots are gated on TCR-β<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>int</sup> resident DC (mesLN and spleen) or TCR-β<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>hi</sup> migratory DC (SILP). Numbers represent mean frequency of cells gated in this manner +/- standard deviation.



**Figure 32: *Batf3*-dependent DC produce IL-12 and activate BM NK cells during infection.**

(A) Serum IL-12p70 in *Batf3*<sup>-/-</sup> and WT mice, naïve and day 5 post-infection. (B) Absolute numbers of lymphocytes producing IFN- $\gamma$  in BM of *Batf3*<sup>-/-</sup> mice and WT controls at day 5 post-infection. (C-D) MHCII and Sca-1 expression by cMoP and Ly6C<sup>hi</sup> monocytes in the BM of *Batf3*<sup>-/-</sup> mice and WT controls at day 5 post-infection. Error bars represent one standard deviation. Data are representative of two or more independent experiments, n = 3. Statistical comparisons were performed using unpaired student's *t* test adjusted for multiple comparisons. \*: p<0.05, \*\*: p<0.01.



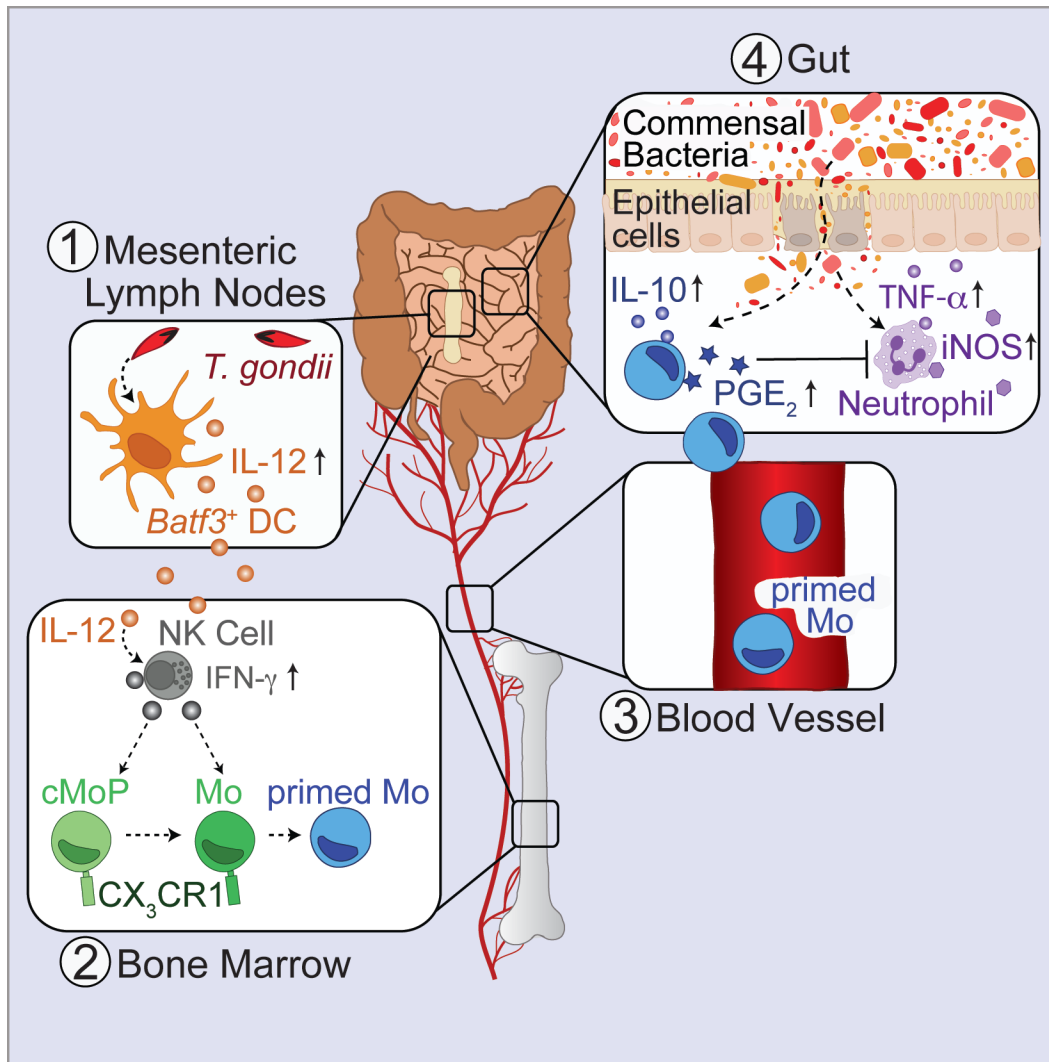
**Figure 33: IL-12 is produced primarily in the MALT early during *T. gondii* infection.** (A) WT mice were infected per-orally with *T. gondii* and IL-12 production was assessed at various time points after infection. 10% of the total cells from each tissue were cultured *ex vivo* for 16 hours and cell supernatants were assessed for IL-12p70. (B) *Batf3*-dependent DC were sorted from the MesLN and spleen of naïve and day 4 *T. gondii* infected WT mice and assessed for expression of *Il12b* by NanoString. Error bars represent one standard deviation. Data are representative of two or more independent experiments  $n = 3-5$  (A), or represent 3 samples, each pooled from 2 mice (B). Statistical comparisons were performed using unpaired student's *t* test corrected for multiple comparisons. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$ .

## ***Discussion***

We propose a model in which mucosal infection can result in early IL-12 production in the MALT, which acts in a systemic manner to activate NK cells in the BM, resulting in IFN- $\gamma$  production in this primary hematopoietic tissue. Although we cannot dismiss a potential role of systemic IFN- $\gamma$  later in this process, our findings suggest that early IFN- $\gamma$ -producing NK cells present in the BM rapidly prime Ly6C<sup>hi</sup> monocytes to respond in a regulatory manner upon their arrival at barrier sites. Collectively, these data reveal that monocyte functional education is a sequential process in which discrete signals act in defined spatially and temporally segregated niches (**Figure 34**). Specifically, *T. gondii* infection drives robust IL-12 production by *Batf3*-dependent DC in the infected MALT (Signal 1). IL-12 emanating from the MALT reaches the BM, where it activates resident NK cell populations, resulting in the local production of IFN- $\gamma$  (Signal 2) and education of proliferating myeloid progenitors. This results in altered responsiveness of Ly6C<sup>hi</sup> monocytes to commensal bacteria upon recruitment to the GI tract (Signal 3), most notably an increased capacity to produce PGE<sub>2</sub>, which limits neutrophil activation at this site and prevents lethal immunopathology.

A number of recent studies have noted that upon infection, activated NK cells form clusters in close association with Ly6C<sup>hi</sup> monocytes, priming these cells via IFN- $\gamma$  production and thus enhancing their anti-microbial function (Coombes et al., 2012; Goldszmid et al., 2012; Kang et al., 2008). In each of these studies, the activation of NK cells was proposed to occur in response to local IL-12 signaling. Here we show that this

activation process can occur in the hematopoietic environment in response to IL-12 emanating from a distally infected tissue. In addition to their critical role in collecting antigen and organizing the initiation of adaptive immune responses, one of the primary functions of peripheral lymph nodes may be to act as an alarm system for highly virulent infections, particularly at barrier sites, providing systemic warning signals to mature immune populations in the BM. BM-resident lymphocytes in turn direct the development of rapid and optimally tuned effector cells via local production of IFN- $\gamma$ . NK cell subsets with decreased cytotoxic function but increased capacity for cytokine secretion have been identified in humans and mice (Hayakawa and Smyth, 2006; Poli et al., 2009). Intriguingly, these specialized cytokine-producing NK cells have been attributed immunoregulatory properties, although the mechanisms underlying this function remain unclear. One intriguing possibility is that the BM is enriched for cytokine-producing NK cells with minimal cytotoxic potential whose primary function is to dynamically regulate hematopoietic development. BM-resident NK cells have, to this point in time, primarily been considered a progenitor population for peripheral NK cells (Kim et al., 2002), and the gene expression and function of these cells is yet to be explored. Further study of NK cells in the BM is required to assess their relationship to NK populations in the periphery and their ability to modulate hematopoiesis in a broad range of inflammatory responses.



**Figure 34: Sequential model of functional priming of monocytes during *T. gondii* infection.**

The cascade driving regulatory priming of Ly6C<sup>hi</sup> monocytes during *T. gondii* infection begins in the MLN (1) where CD8 $\alpha$ <sup>+</sup> DC produce IL-12 in response to signals from the parasite. The IL-12 acts on bone marrow resident NK cells (2) to induce production of IFN- $\gamma$  locally, initiating the regulatory priming of cMoP and monocytes in the bone marrow. Mature primed monocytes enter the vasculature (3) and are subsequently recruited to the inflamed gut (4) where they respond to signals from the commensal microbiota by producing regulatory PGE<sub>2</sub> and IL-10, limiting neutrophil activation in the tissue.

## **CHAPTER 5: Discussion and Conclusions**

In light of the findings described in the previous chapters, it is important to reconsider our understanding of how the innate immune response, and mononuclear phagocytes in particular, respond to acute inflammation and infection. In the first part of this chapter, we will examine how our study informs the current paradigm concerning the role of monocytes and macrophages in inflammatory settings. Additionally, we have uncovered a novel mechanism by which monocytes can acquire the capacity to produce regulatory mediators during acute infection in response to defined inflammatory cues. Emergence of novel monocyte-derived populations with regulatory functions have been described in other systemic inflammatory settings, and we will discuss the potential effects of these cells on tumor development and susceptibility to secondary infection in the second half of this chapter.

### ***Re-examining the role of monocytes in acute inflammatory responses***

In the last few years, the distinction between the individual components of the mononuclear phagocyte system has become clearer as scientists have identified the distinct developmental pathways that generate monocytes, macrophages, and DCs. The unique ontology of each of these populations informs us about their function, and our study has extended this analysis to include an understanding of how signals influencing monocyte development likewise direct their ultimate function in tissue during infection. We posit that the remarkable functional diversity of monocytes during inflammation is



facilitated by their constitutive and rapid differentiation from BM progenitors, while the slower turnover and permanent tissue residence of macrophages is indicative of their primary function as regulators of normal tissue function.

As immunologists, guided by illuminating experiments on macrophage polarization *in vitro*, we have increasingly focused on the role of macrophages in host defense and wound healing. We believe it is time we reconsider this perspective and return to Metchnikoff's understanding of the primary role of these cells: to phagocytose dying cells and cellular debris and to support the normal function of the primary cells comprising each organ and maintain tissue homeostasis. As a result of self-renewal within tissue, the epigenetic programming of macrophages is predominantly shaped by signals present in their local microenvironment (Gautier et al., 2012; Gosselin et al., 2014). This results in incredible diversity in the gene expression programs, morphology, and functions of these cells across different tissues. Individual macrophage populations acquire distinct specialized functions associated with the unique requirements of the tissue in which they reside, such as neuronal pruning by microglia, or directing of hepatocyte regeneration by Kupffer cells in the liver (Gordon and Taylor, 2005). While this careful epigenetic control of macrophage function is essential for supporting normal organ function and maintaining tissue homeostasis, it may hamper their ability to acquire the full range of activation states necessary to mediate immunity against intracellular and extracellular viral, protozoan, fungal, and bacterial pathogens. The ability to enact the broad range of effector responses needed to protect against these diverse pathogens necessitates a less differentiated phagocyte, such as a monocyte,

whose epigenetic and transcriptional identity has not been molded throughout development to perform tissue-specific functions.

In fact, the majority of our understanding of macrophage activation and polarization has been guided by *in vitro* experiments assessing the secretory and transcriptional responses to various stimuli of macrophages differentiated from murine BM monocytes (Bone marrow derived macrophages, BMDM), or human peripheral blood mononuclear cells (PBMC-derived macrophages). While easy to isolate and differentiate *in vitro*, it has become increasingly clear that these monocyte-derived macrophages are not an accurate surrogate for tissue-resident macrophages, differing greatly in their morphology and responsiveness to stimulation (Chow et al., 2011a; Geissmann et al., 2010; Murray et al., 2014). Rather, these studies more accurately model the ability of monocytes to acquire distinct transcriptional and proteomic profiles in response to various combinations of host and pathogen-derived factors (Xue et al., 2014). Indeed, more recent work has revealed that recruited Ly6C<sup>hi</sup> classical monocytes are the predominant effector phagocytes in protection against many systemic and localized infections (Dunay et al., 2008; Espinosa et al., 2014; Hermesh et al., 2010; Quintin et al., 2012; Serbina et al., 2012). Likewise, recruited monocytes have recently been shown to differentiate into alternatively activated macrophages and contribute to wound healing responses in liver, heart, and skeletal muscle tissue (Arnold et al., 2007; Dal-Secco et al., 2015; Lavine et al., 2014; Nascimento et al., 2014). Unfortunately, no unique phenotypic marker has been identified to clearly distinguish monocyte-derived and resident phagocytes (Ly6C is sometimes used, but this is downregulated by

monocytes rapidly upon tissue entry) (Bain et al., 2013; Tamoutounour et al., 2013; Yona et al., 2013). This has necessitated the use of fate-mapping reporters and CCR2-deficient mice to establish the relative contributions of these populations. Similarly, the origin and identity of phagocyte populations in human studies are often difficult to determine. Nonetheless, emerging evidence of monocyte and macrophage responses during inflammation continues to favor a new paradigm in which macrophages are primarily responsible for maintaining normal tissue function, while upon the onset of inflammation, recruited monocytes become the predominant APC population mediating both inflammatory and regulatory effector responses.

The most notable aspect of monocyte development is the incredibly rapid turnover of the circulating monocyte pool: the half-life of a Ly6C<sup>hi</sup> monocytes in the blood is less than 24 hours (Yona et al., 2013). This rapid and constant monocyte replenishment requires enormous energy expenditure by the host, and therefore must confer important advantages over an alternative strategy in which monocytes are replenished more slowly and circulate for a longer period. One such advantage may be that a system that constitutively produces large numbers of phagocytes is better adapted to dynamically increase the production of these cells as demand increases during inflammation. Emergency myelopoiesis is a common feature of infection, suggesting that in these settings, increased output of monocytes from the BM provides improved protection to the host (Takizawa et al., 2012). A recent study elegantly investigated this premise in a zebrafish model of mycobacterial infection by modulating both the virulence of the pathogen as well as the availability of circulating monocytes during

infection. Intriguingly, more virulent strains of mycobacterium required more rapid replenishment of macrophages by circulating monocytes to form a protective granuloma, and inhibition of CSF-1-driven increases in myelopoiesis during infection resulted in granuloma necrosis, loss of control of the infection, and death (Pagán et al., 2015). Infection with less virulent strains were controlled even in CSF-1-deficient mice, suggesting that emergency myelopoiesis may have evolved primarily to support host control of highly virulent infections. In agreement with this concept, a recent study found that decreased myeloid output by the BM was strongly associated with age-related susceptibility to infection in mice and humans (Nacionales et al., 2015). Thus, the preponderance of rapidly proliferating myeloid progenitors in the BM may be energetically excessive under steady-state conditions, but important for increased monocyte production needed to control acute infection.

Our findings in this dissertation suggest an additional advantage conferred by the development and turnover of circulating monocyte populations. In addition to increasing the number of monocytes entering the circulation during infection, the rapid turnover of this population allows the innate immune system to swiftly alter the transcriptional program of myeloid progenitors, generating effector cells ideally tuned to perform the functions needed in particular immune settings. The early stages of monocyte development are characterized by rapid proliferation of myeloid progenitors, and the associated DNA replication may provide an ideal opportunity for epigenetic modification in response to infection-induced signals. We have noted a distinct IFN- $\gamma$ -dependent mechanism by which monocyte function is educated during BM development, but it is

likely that other host and pathogen derived signals can also modulate myeloid immune responses by acting on hematopoietic progenitors. As discussed in chapter 2, many inflammatory and homeostatic cytokines, including TNF- $\alpha$ , IL-6, CSF-1, CSF-2, and IL-3, can augment myelopoiesis (Chow et al., 2011b; de Bruin et al., 2014; Shi et al., 2011; Takizawa et al., 2012), and these factors may simultaneously shape the transcriptional program of myeloid progenitors to dictate ultimate effector function.

Our results confirm and provide clarification to longstanding *in vitro* studies describing the effects of sequential IFN- $\gamma$  and TLR stimulation on classical macrophage activation (Murray et al., 2014). We have demonstrated that this ordered sequence of host and pathogen-derived signals occurs naturally during an *in vivo* immune response to infection and has important consequences for the ultimate function of monocytes, including induction of regulatory function. IFN- $\gamma$  signaling has recently been shown to reshape the epigenetic landscape of monocytes via Stat1 and IRF1 occupation of enhancers and promoters of key inflammatory genes, including *Tnf*, *Il6*, and *Il12b*. Stat1 and IRF1 binding at these sites drives histone acetylation and increased chromatin accessibility (Qiao et al., 2013). Chromatin remodeling augmented NF $\kappa$ B binding and transcription of these genes upon TLR engagement, revealing a key mechanism for the long-observed phenomenon that prior priming of macrophages with IFN- $\gamma$  increases their subsequent responses to stimulation. Intriguingly, occupation of the *Il10* locus by Stat1 requires sustained IFN- $\gamma$  signaling (Ivashkiv, personal correspondence), suggesting

that acquisition of regulatory functions by monocytes may be a common feature of not only acute inflammation, but also chronic inflammation and cancer.

### ***Anti-inflammatory properties of monocytes during systemic inflammation and cancer***

In the studies described in this dissertation, we have described the surprising induction of regulatory function in Ly6C<sup>hi</sup> monocytes by the inflammatory cytokine IFN- $\gamma$ . Intriguingly, engagement of these anti-inflammatory pathways was not limited to settings of acute inflammation, as we observed that monocytes isolated from mice in the chronic phase of *T. gondii* infection maintained an increased capacity to produce PGE<sub>2</sub>, as did transgenic mice expressing low levels of IFN- $\gamma$  (**Figure 15C, 15F**). These results suggest that constitutive low-level inflammation, such as that observed during cancer, may be sufficient to prime monocytes for regulatory function. Indeed, cancer in mice and humans is associated with the development of anti-inflammatory MDSCs (Marvel and Gabrilovich, 2015; Ostrand-Rosenberg and Sinha, 2009). MDSCs are a heterogeneous pool of cells, including monocytes and granulocytes, found both locally at tumor sites as well as in the systemic circulation during cancer. The prevailing belief is that this mixed lineage represents the aberrant development of immature and undifferentiated myeloid cells that arises as a result of growth factors secreted by developing tumors (Marvel and Gabrilovich, 2015; Ostrand-Rosenberg and Sinha, 2009). While it's clear that these cells can promote tumor growth and metastasis, the study of MDSCs has been complicated by a lack of distinction made in many studies between the role of monocytic and

granulocytic cells in these processes, a shortcoming noted in many recent reviews on the topic (Cuenca et al., 2011; Ostrand-Rosenberg and Sinha, 2009; van Ginderachter et al., 2010). Studies addressing this issue have found that the majority of the tumor-promoting potential of MDSCs is provided by monocytic cells (Mo-MDSC), with disagreement arising over whether granulocytic MDSC also demonstrate anti-inflammatory properties (Haverkamp et al., 2014; Movahedi et al., 2008; Youn et al., 2008).

The monocytes we observe arising during *T. gondii* infection closely resemble Mo-MDSC, not only in their acquisition of anti-inflammatory properties, but also in the transcriptional regulators driving their development and function. Inflammatory cytokines contribute to Mo-MDSC development, and in particular, the combination of IFN- $\gamma$  and LPS signaling by bacterial ligands has been shown to drive the differentiation of Mo-MDSC from BM progenitors, both *in vitro* and *in vivo* (Delano et al., 2007; Greifenberg et al., 2009; Movahedi et al., 2008). Stat1, activated by IFN- $\gamma$ , and Stat3 are critical transcriptional regulators of Mo-MDSC development, and we observed increased expression of each of these factors by monocyte progenitors during *T. gondii* infection in an IFN- $\gamma$ -dependent manner (**Figure 18, Figure 20C**). Monocyte progenitors also upregulated expression of S100A9, a calcium signaling protein required for Mo-MDSC development (Cheng et al., 2008). Many regulatory factors expressed by monocytes during *T. gondii* infection upon microbial encounter are also expressed by MDSCs. iNOS (Nos2) expression has long been implicated in the profound capacity of Mo-MDSC to suppress T cell proliferation and inhibit anti-tumor responses via increased consumption

of l-arginine (Condamine and Gabrilovich, 2011). We observed high levels of *Nos2* expression upon LPS stimulation of BM monocytes, and Ly6C<sup>hi</sup> monocytes in the lung and spleen have previously been demonstrated to suppress T cell responses during *T. gondii* infection via nitric oxide production (Candolfi et al., 1994; Voisin et al., 2004). Moreover, in recent years, IL-10 and PGE<sub>2</sub> have been implicated in the anti-inflammatory properties of Mo-MDSC. IL-10 inhibits the priming of tumor-specific T cell responses by suppressing DC activation, and PGE<sub>2</sub>-expressing MDSC have been found to drive tumor growth and metastasis in a mammary tumor model (Donkor et al., 2009; Hu et al., 2011). PGE<sub>2</sub> also promotes the development of Mo-MDSC, suggesting that autocrine/paracrine production of this factor by monocytes may reinforce their anti-inflammatory program, particularly as they enter the prostaglandin-rich tumor microenvironment (Eruslanov et al., 2010; 2011; Sinha et al., 2007; Xiang et al., 2009; Zhang et al., 2009). Pharmacological inhibition of PGE<sub>2</sub> signaling in these studies resulted in decreased MDSC accumulation and tumor progression, demonstrating the importance of PGE<sub>2</sub> in promoting tumor growth and metastasis. The similarities in the development, gene expression, and function between Mo-MDSC and the monocytes we observe during *T. gondii* infection suggest that Mo-MDSC simply constitute a regulatory monocyte population that develops in response to the chronic inflammatory signals associated with cancer, primarily IFN- $\gamma$ .

The emergence of regulatory monocytes has also recently been described in other inflammatory settings. G-CSF administration prior to bone marrow transplant induced the generation of a CD34<sup>+</sup> classical monocyte population that inhibited activation of



graft versus host allogeneic T cell responses via production of nitric oxide in both mice and humans (D'Aveni et al., 2015). Monocyte-derived nitric oxide has likewise been implicated in suppression of inflammatory responses to infection (Abrahamsohn and Coffman, 1995; Ahvazi et al., 1995; Voisin et al., 2004), and monocytes in these settings resemble Mo-MDSC. Thus, we propose that the acquisition of anti-inflammatory properties by Ly6C<sup>hi</sup> monocytes is a conserved mechanism to limit immunopathology during both acute and chronic inflammation.

Epigenetic reprogramming of monocytes following a primary infection that results in subsequent protection against secondary infection has recently been documented in a process termed “trained immunity” (Cheng et al., 2014; Quintin et al., 2012; Saeed et al., 2014). While the authors of these studies postulated that  $\beta$ -glucan signalling directly on monocytes was responsible for the long-lasting protection observed after initial infection with *Candida albicans*, the short half-life of mature monocytes suggests that epigenetic changes conferring protection more likely occurred in long-lived progenitor populations. The observed alterations to monocyte function in these studies were driven by profound changes to cellular metabolism (Cheng et al., 2014; Saeed et al., 2014). Activation of Hif-1 $\alpha$  and mTor in response to stimulation by  $\beta$ -glucan resulted in increased glycolysis and decreased oxidative phosphorylation. In agreement with previous studies, the conversion to glycolytic metabolism was associated with increased cytokine production and responsiveness to stimulation by PAMPs (Cheng et al., 2014; Everts et al., 2014; 2012). Intriguingly, a similar Hif-1 $\alpha$ -mediated switch to glycolytic metabolism has been shown to be required for development of Mo-MDSC (Corzo et al.,

2010). Thus, the long-term acquisition of anti-inflammatory features by monocytes in chronic inflammatory settings such as cancer may likewise be mediated by alterations to the epigenetic landscape of hematopoietic progenitors and the resulting alterations to cellular metabolism.

We have found that transcriptional reprogramming of monocyte progenitors in the BM can impart regulatory functionality to their progeny upon arrival at sites of inflammation, and that this programming can be dynamically regulated by BM-resident lymphocyte populations in response to signals received from the periphery during infection. The potential diversity in monocyte function and their central role in both inflammatory and regulatory immune processes makes these cells attractive targets for immunomodulatory therapy in inflammatory disease and cancer. However, the epigenetic changes that monocytes undergo in these settings are not well understood, and further study is required to elucidate the mechanisms that control their acquisition of regulatory features. New techniques have recently been developed that allow analysis of chromatin remodeling in increasingly small cell populations, and ATAC-seq analysis of tissue-infiltrating regulatory monocytes, as well as cMoP and other early myeloid progenitors in the bone marrow, will provide insight into how signals received both during development and upon tissue recruitment direct monocyte differentiation and function.

## CHAPTER 6: Materials & Methods

### ***Mice***

All mice were bred and maintained under pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. All experiments were performed under an animal study proposal approved by the NIAID Animal Care and Use Committee. Gender- and age-matched mice between 6-14 weeks of age were used. C57BL/6 (WT) mice were purchased from Taconic Farms or The Jackson Laboratory. B6.129S7-*Ifngr1<sup>tm1Agt</sup>/J* (*Ifngr1<sup>-/-</sup>*), B6.129P2-*Il18<sup>tm1Aki</sup>/J* (*Il18<sup>-/-</sup>*), B6.129S1-*Il12a<sup>tm1Jm</sup>/J* (*Il12p35<sup>-/-</sup>*), B6.129S(C)-*Batf3<sup>tm1Kmm</sup>/J* (*Batf3<sup>-/-</sup>*), and B6.129P(Cg)-*Ptprca<sup>a</sup> Cx3cr1<sup>tm1Litt</sup>/LittJ* (CX3CR1-GFP) mice were purchased from The Jackson Laboratory. In some experiments bone marrow chimeras were generated. 10BiT reporter mice (Maynard et al., 2007) were kindly shared by Dr. Dragana Jankovic and Dr. Alan Sher with the permission of Dr. Casey Weaver. B6.129S4-*Ifng<sup>tm3.1Lky</sup>/J* (GREAT) mice were kindly shared by Dr. Vanja Lazarevic.

### ***T. gondii Parasite and Infection Protocol***

ME-49 clone C1 of *T. gondii* (provided by Dr. Michael Grigg, NIAID/NIH) was obtained by electroporation of the parental ME-49 type II strain (ATCC 50840) with red fluorescent protein (RFP) and was used for production of tissue cysts in C57BL/6 mice. Tissue cysts used in experiments were obtained from female mice that were per-orally

inoculated with 10 cysts 2-3 months earlier. Animals were euthanized and their brains removed and homogenized in 1 ml of PBS pH 7.2. Cysts were counted on a fluorescent microscope. For experiments, mice were infected by intragastric gavage with 10 cysts of ME-49 C1.

### ***Yersinia pseudotuberculosis Infection Protocol***

*Y. pseudotuberculosis*, strain IP2777 (serogroup I) (Simonet and Falkow, 1992) was generously provided by Dr. Igor Brodsky. *Y. pseudotuberculosis* was grown overnight at 28°C in 2X YT (Quality Biological) medium. Bacterial suspension was washed and resuspended in PBS pH 7.2 and the concentration was adjusted to  $5 \times 10^7$  CFU/mL as measured by optical density. For the infection, mice were fasted for 12 hours prior infection and infected by intragastric gavage with  $1 \times 10^7$  CFU in 200  $\mu$ L.

### ***Plasmodium yoelii Infection Protocol***

The parasite *Plasmodium yoelii nigeriensis* N67C was initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4, <http://www.mr4.org>) and was maintained as previously described (Li et al., 2011). Experimental infections were initiated by intravenous inoculation of  $1 \times 10^6$  red blood cells (iRBCs) suspended in 100  $\mu$ L phosphate buffer saline (PBS). Naïve mice receiving an equivalent number of uninfected RBCs served as a negative control group. Parasitemia was monitored daily by examination of Giemsa-stained thin tail blood smears.

## ***Tissue Preparation and Immune Cell Isolation***

Cells from bone marrow, spleen, liver, mesenteric lymph nodes (mesLN), and small intestine lamina propria (SILP) were prepared as previously described (Chou et al., 2012; Sun et al., 2007). Blood was acquired by cardiac puncture and immediately suspended in RPMI 1640 supplemented with 3% FBS (Jackson ImmunoResearch), 100 U/mL penicillin (Cellgro), 100 µg/ml streptomycin (Cellgro), 20mM HEPES (Cellgro), and 5 mM EDTA (Quality Biological) on ice. Suspensions were washed and resuspended in ACK Lysing Buffer (Lonza) for 3 minutes on ice, twice. Suspensions were then washed and resuspended in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM HEPES, 2 mM glutamine (Media Tech Inc), 1 mM Sodium Pyruvate (Mediatech), nonessential amino acids (Cellgro), and 50 mM of β-mercaptoethanol (Complete Medium).

When assessing DC populations by flow cytometry or assessing IL-12 production *ex vivo*, bone marrow, spleen, and mesLN were minced and incubated at 37°C for 25 minutes in complete media (with the exception of FBS, which was excluded) supplemented with 50 µg/ml liberase TL (Roche) and 0.025% DNase I (Sigma-Aldrich) prior to manual tissue disruption.

## ***Flow Cytometry***

Single cell suspensions from SILP, blood, and bone marrow were incubated with an anti-Fc $\gamma$ III/II receptor and mouse serum (Jackson Immunoresearch) and fluorochrome-conjugated antibodies against surface markers in PBS containing 1% FBS (FACS Buffer) for 15 minutes on ice and then washed. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Sigma-Aldrich) was used to exclude dead cells, except for SILP suspensions, for which cells were stained with Live/Dead Fixable Blue Cell Stain Kit (Invitrogen) prior to fixation. B220 (CD45R, RA3-6B2), CD4 (RM4-5), CD11b (M1/70), CD24 (M1/69), and CD45 (30-F11), CD64 (X54-5/7.1), and CD68 (30-F11) were purchased from Biolegend. CD8 $\alpha$  (53.6.7), CD11c (N418), CD34 (RAM34), CD49b (DX5), CD103 (2E7), CD115 (CSF-1R, AFS98), cKit (CD117, ACK2), Flt3 (CD135, A2F10), Ly6C (HK1.4), MHCII (I-A/I-E, M5/114), NK1.1 (PK136), and Sca-1 (D7), TCR- $\beta$  (H57-597) were purchased from eBioscience. Ly6G (1A8) and Siglec F (E50-2440) were purchased from BD Biosciences.

For SILP suspensions, cells were stained for surface markers, then washed twice with FACS buffer, and fixed in a solution of 2% paraformaldehyde (Electron Microscopy Sciences). To more precisely identify recruited monocytes in this tissue, cells were in some cases stained intracellularly with fluorochrome-conjugated antibodies against CD68 for 60 minutes in FACS buffer containing 0.5% saponin. For examination of transcription factors, cell suspensions from bone marrow were subsequently treated with the Foxp3 staining kit (eBioscience) in accordance with the manufacturer's instructions

and stained for 20 minutes at room temperature with fluorochrome-conjugated antibody against Eomes (DAN11Mag, eBioscience). Cell acquisition was performed on an LSRII machine using FACSDiVa software (BD Biosciences). For each sample, at least 300,000 events were collected. Data were analyzed with FlowJo software (TreeStar). The lineage antibody mixture for excluding lymphocyte and granulocyte populations was comprised of B220, TCR- $\beta$ , NK1.1, Ly6G, and Siglec F.

### ***Ex vivo Stimulation of Ly6C<sup>hi</sup> Monocytes and Detection of TNF- $\alpha$ and IL-10***

For *ex vivo* cytokine detection, single cell suspensions from blood were cultured in complete medium at  $5 \times 10^5$ - $1 \times 10^6$  cells/well in a 96-well round-bottom plates in the presence of 1  $\mu$ g/mL Brefeldin A (GolgiPlug, BD Biosciences). In some cases, single cell suspensions were stimulated with commensal or parasite-derived ligands in combination with Brefeldin A. After 3 hours, cells were stained for surface markers, then washed twice with FACS buffer, and fixed in a solution of 2% paraformaldehyde (Electron Microscopy Sciences). Prior to fixation Live/Dead Fixable Blue Cell Stain Kit (Invitrogen) was used to exclude dead cells. Cells were then stained with fluorochrome-conjugated antibodies against TNF- $\alpha$  (MP6-XT22, BD Biosciences) and IL-10 (JES5-16E3, eBioscience), or their appropriate isotype controls in the presence of anti-Fc $\epsilon$ III/II receptor for 60 minutes on ice in FACS buffer containing 0.5% saponin.

## ***Serum Cytokine and Enzyme Measurements***

Concentrations of TNF- $\alpha$ , IL-12p70, and IFN- $\gamma$  in serum samples or culture supernatants were measured by FlowCytomix bead assay (eBioscience) according to the manufacturer's instructions. Liver aspartate transaminase (AST) and alanine aminotransferase (ALT) in serum samples were measured using commercially available kits (Boehringer Mannheim).

## ***Pathology Assessment***

C57Bl/6 mice were euthanized at day 4-8 post-infection, along with naïve controls. Small intestines were removed and sections of the jejunum and ileum immediately fixed in a 10% formalin solution. Paraffin-embedded sections were cut longitudinally at 0.5  $\mu$ m and stained with hematoxylin and eosin. At least 4 sections were assessed, spanning a total tissue depth of at least 40 microns.

## ***Generation and Infection of Mixed WT & *Ifngr1*<sup>-/-</sup> Bone Marrow Chimeras***

Bone marrow was extracted from hind legs of B6.129S7-*Ifngr1*<sup>tm1Agt</sup>/J (*Ifngr1*<sup>-/-</sup>) and WT animals and T cell depleted using CD90.2 microbeads (Miltenyi Biotec). 6 week old CD45.1 animals were lethally irradiated and reconstituted with 2 x 10<sup>6</sup> WT cells and 2 knockout bone marrow cells. Animals were maintained on antibiotics for up to one week after reconstitution. After waiting 8 weeks for immune reconstitution, these mice were



infected by intragastric gavage with 15 cysts of ME-49 C1. In order to reach a comparable level of intestinal parasitemia as C57Bl/6 mice infected with 10 bradyzoite cysts, 15 cysts were used for these infections.

### ***Ly6C<sup>hi</sup> Monocyte and cMoP Purification by FACS***

Cell suspensions of naïve or *T. gondii* infected SILP, blood, or BM were incubated with mixtures of monoclonal antibodies containing anti-Fc $\gamma$ III/II, mouse serum, Rat IgG (Jackson ImmunoResearch), and 7-AAD viability staining solution (eBioscience) for 15 minutes on ice. After staining, cell suspensions were washed, filtered, resuspended in complete medium without phenol red and sorted on a BD FACS Aria II.

### ***Ex vivo Stimulation of FACS purified Ly6C<sup>hi</sup> Monocytes for PGE<sub>2</sub>***

#### ***Detection***

Purified Ly6C<sup>hi</sup> monocytes were cultured in complete RPMI at a concentration of 150,000 cells/mL (for monocytes derived from BM) or 30,000 cells/mL (for monocytes derived from blood) in a 96-well round-bottom tissue culture plate. In some cases, cells were stimulated with *E. coli* lysate (1  $\mu$ g/ml), Soluble *Toxoplasma* antigen (STAg) (1  $\mu$ g/ml), or commercially available TLR ligands Pam2CSK4 (Invivogen, 500 nM), LPS (Enzo Life Sciences, 500 ng/ml), Flagellin (Invivogen, 500 ng/ml), CpG (5'TCCATGACGTTCTGAT3'), Integrated DNA Technologies, 500 nM), or Profilin (Alexis Biochemicals, 500 ng/ml) for 18 hours. STAg was prepared as previously described

(Grunvald et al., 1996). Three to five replicates of pooled supernatants from 4-5 mice were assayed for PGE<sub>2</sub> using an enzyme-immunoassay (EIA) (Cayman Chemicals), as per manufacturers' instructions. In some experiments, cells were incubated with 20 ng/ml recombinant IFN- $\gamma$  (BioLegend) for 6 hours and washed prior to stimulation with *E. coli* lysate.

### ***Ex vivo Stimulation of FAC-Purified Ly6C<sup>hi</sup> Monocytes for NanoString Analysis***

Ly6C<sup>hi</sup> monocytes FACS sorted from the BM of uninfected mice or *T. gondii* infected mice at day 5 post-infection were cultured for 6 hours in complete RPMI at a concentration of 30,000 cells in 100 mL of complete media in a 96 well v-bottom plate. In some cases, cells were stimulated with 500 ng/ml LPS (Enzo Life Sciences). After 6 hours, cells were washed and resuspended at 2,000 cells/ $\mu$ L of RLT buffer (Life Technologies) for RNA isolation and stored at -80° C.

### ***Gene Expression Analysis by NanoString nCounter Technology***

The nCounter analysis system (NanoString Technologies) was used to assess gene expression by, cMoP, bone marrow Ly6C<sup>hi</sup> monocytes, and various lymphoid-resident DC populations. Briefly, RNA was obtained by lysing the sorted cells (5-10 x 10<sup>3</sup> cells/5  $\mu$ L) in RLT buffer (Qiagen) and then hybridized with the C2566 Mouse Myeloid Panel. Data analysis was performed according to NanoString Technology recommendations.

mRNA counts were processed to account for hybridization efficiency, background noise, and sample content, using the R package NanoStringNorm with arguments: CodeCount = 'geo.mean', Background = 'mean.2sd', SampleContent = 'housekeeping.geo.mean'. Post normalization, genes with mean counts less than 2 standard deviations above the mean count of the highest negative control were excluded, and differential expression of remaining genes was determined using a nonparametric Welch t-test with correction for multiple testing using the BH false discovery rate (FDR) controlling procedure in the multtest package in R. Using those genes with a FDR < 0.05, a heatmap was rendered using the R package pheatmap.

For gene expression analysis of *Batf3*-dependent DC populations in the mesLN and spleen, DC were FACS sorted from each tissue. Lin<sup>-</sup>CD64<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>hi</sup> cells were analyzed for expression of CD103, CD11b, and CD8 $\alpha$ . From the mesLN, CD11b<sup>-</sup>CD103<sup>-</sup>CD8 $\alpha$ <sup>hi</sup> resident DC and CD11b<sup>-</sup>CD103<sup>+</sup>CD8 $\alpha$ <sup>int</sup> migratory DC populations were pooled together for gene expression analysis due to the low number of cells collected from this tissue. From the spleen, CD11b<sup>-</sup>CD8 $\alpha$ <sup>hi</sup> resident DC were used for gene expression analysis. Gene expression was assessed using the nCounter analysis system as described for cMoP and monocytes.

For gene expression analysis comparing cMoP to classical DC (cDC), Lin<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>hi</sup>CD24<sup>-</sup>CD11b<sup>+</sup>CD103<sup>+</sup> migratory DC were FACS sorted from skin draining

lymph nodes and assessed for gene expression analysis as described for cMoP and monocytes.

### ***Pathway Analysis of Gene Expression Data***

Data were analyzed using Ingenuity Pathway Analysis software (IPA, Qiagen) with the following parameters: FDR<0.05, Fold Change >2.0, and “experimentally observed observations only”. Upstream analysis was used to identify potential mediators of differences in gene expression between cMoP from naïve and infected mice. Predicted upstream mediators from this analysis were filtered by  $p < 0.05$  and ranked by Z-score.

### ***In vivo Administration of IFN- $\gamma$***

Mice were administered 5  $\mu\text{g}$  of recombinant carrier-free IFN- $\gamma$  (BioLegend) in 200  $\mu\text{l}$  sterile PBS by intraperitoneal (i.p.) injection once each day for 3 days. Mice were sacrificed 16 hours after the final injection.

### ***In vivo Administration of IL-12p70***

Mice were administered 1  $\mu\text{g}$  of recombinant carrier-free IL-12 (kindly provided by Dr. Stan Wolf, Genetics Institute, Cambridge) in 200  $\mu\text{l}$  sterile PBS by intraperitoneal (i.p.) injection once each day for 2 days. Mice were sacrificed 8 hours after the final injection.

### ***In vivo Blockade of IFN- $\gamma$***

*T. gondii* infected mice were administered 1 mg of  $\alpha$ IFN- $\gamma$  (R4-6A2, BioXcell) or isotype control in 200  $\mu$ l sterile PBS by i.p. injection once each day at days 2, 3, and 4 post-infection. Mice were euthanized 16 hours after the final injection (day 5 post-infection).

### ***In vivo Blockade of IL-12p70***

*T. gondii* infected mice were administered 1 mg of  $\alpha$ IL-12p70 (C17.8) or isotype control in 200  $\mu$ l sterile PBS by i.p. injection once each day at days 0, 2, 3, and 4 post-infection. Mice were euthanized 16 hours after the final injection (day 5 post-infection).

### ***In vivo NK Cell Depletion***

*T. gondii* infected mice were administered 600  $\mu$ g of  $\alpha$ NK1.1 (PK136) purified from hybridoma by the NIAID Custom Antibody Services Facility or isotype control in 200  $\mu$ l sterile 2X PBS by tail vein intravenous injection once each day at days 1, 2, and 3 post-infection. Mice were euthanized 48 hours after the final injection (day 5 post-infection). NK depletion was assured by flow cytometry assessment of NKp46<sup>+</sup> cells in bone marrow, spleen, and liver.

## ***Intracellular Detection of IFN- $\gamma$***

For *ex vivo* IFN- $\gamma$  detection, cell suspensions from BM were cultured at  $5 \times 10^5 - 1 \times 10^6$  cells/well in 96-well round-bottom plates in the presence of  $1 \mu\text{g/mL}$  Brefeldin A (GolgiPlug, BD Biosciences). After 3 hours, cells were stained for surface markers, then washed twice with FACS buffer and fixed in a solution of 2% paraformaldehyde (Electron Microscopy Sciences). Prior to fixation, Live/Dead Fixable Blue Cell Stain Kit (Invitrogen) was used to exclude dead cells. Cells were then stained with PE-conjugated antibody against IFN- $\gamma$  (XMG1.2, eBioscience) or isotype control in the presence of anti-Fc $\epsilon$ III/II receptor for 60 minutes on ice in FACS buffer containing 0.5% saponin.

## ***Immunofluorescence imaging***

For immunofluorescence staining of whole-mounted tissues and frozen sections of BM, we adapted a previously published protocol (Kunisaki et al., 2013). Femoral bones were perfused with Periodate-lysine-paraformaldehyde (PLP) fixation buffer. Post perfusion femurs were fixed for at least 10 hours in PLP-buffer at 4°C, incubated in 30% sucrose overnight, embedded in optical cutting temperature compound (OCT) (Sakura Finetek) over dry ice and stored at -80°C. Bones were cut on a cryostat until the BM was fully exposed and then harvested by melting the OCT. Open bones were blocked/permeabilized with 10% normal rabbit serum, 10% normal mouse serum, 10% Fc-block and 0.25% Triton X-100 for 2 hours at room temperature, stained with anti-GFP/YFP (Life Technologies), goat anti-NKp46 (AF2225, R&D Systems), anti-Ly6G

(1A8, BD Biosciences), and anti-Ly6B2 (7/4, AbD Serotec) overnight at 4°C while shaking. Bones were then washed with PBS and incubated for 2 hours at room temperature in secondary antibody AF647 rabbit anti-goat IgG. After antibody staining, bones were washed and nuclei were stained with DAPI (Sigma-Aldrich). Stained bone was imaged in a chambered cover glass using a Leica TCS SP8 confocal microscope. Images were processed using Imaris Bitplane software.

### ***Ex vivo Differentiation of Monocytes and Macrophages***

Bone marrow Ly6C<sup>hi</sup> monocytes and cMoP were sorted from naïve and d5 *T. gondii* infected WT mice. Sca-1<sup>-</sup> MHCII<sup>-</sup> cells from infected mice were excluded, so that all monocytes and cMoP sorted from infected animals expressed Sca-1 and/or MHCII.

For experiments assessing differentiation of cMoP into monocytes, 10,000 cMoP from naïve and infected mice were cultured in 200 µl of complete medium supplemented with 20 ng/mL IL-6, 20 ng/ml IL-3, 10 ng/ml IL-3, 50 ng/ml SCF, and 10 ng/ml LIF (all cytokines from Peprotech). After 16h in culture, the cells were stained with antibodies to surface antigens and assessed by flow cytometry. For differentiation of monocytes into macrophages, 30,000 Ly6C<sup>hi</sup> bone marrow monocytes from naïve and infected mice were cultured in 200 µl of complete medium supplemented with 20 ng/ml CSF-1. After 16 hours and 2 days in culture, the cells were stained with antibodies to surface antigens and assessed by flow cytometry.

### ***Ex vivo Detection of IL-12p70 from Tissue Preparations***

MesLN, spleen, and BM were minced and enzymatically digested at 37°C for 25 minutes in complete media (with the exception of FBS, which was excluded) supplemented with 50 µg/ml liberase TL (Roche) and 0.025% DNase I (Sigma-Aldrich) to improve extraction of DC populations. Following digestion, cell suspensions were generated from these tissues. Ten percent of the total cells from each tissue were cultured in 25 µl of complete medium for 18 hours and IL-12p70 was detected in the supernatant by Enhanced Sensitivity Cytometric Bead Array (BD Biosciences).

### ***Statistical Analysis***

Groups were compared with Prism software (GraphPad). When two experimental groups were compared, a 2-tailed unpaired Student's *t* test was used to determine statistical significance. For data generated using mixed BM chimeras, a 2-tailed paired Student's *t* test was used to determine statistical significance when comparing WT and *Ifngr1*<sup>-/-</sup> cells.



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