Identification and isolation of five developmental stages in GM-CSF-stimulated murine bone marrow culture.

by

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Abstract

Dendritic cells (DCs) are highly potent cells that link the innate immunity with the adaptive. However, these cell types (and their precursors) are extremely rare in vivo. Considerable work has been done to map the development and function of DCs that develop in the absence of inflammation. However, due to their conditional development and short life-span, our understanding of the development and function of inflammatory-derived DCs is lacking. DCs generated from granulocyte/macrophage-colony stimulating factor (GM-CSF) stimulated murine bone marrow have been used for nearly half a century as a model for studying DC functions. However, recent studies have shown these cells to be considerably more heterogenous than previously believed. In studying this heterogeneity, we found five developmentally distinct populations that could be identified and isolated based on expression of three cell surface molecules: Ly6C, CD115 (CSF-1R), and CD11c. We further characterized these populations based on gene expression profiles, cell surface marker expression, and response to molecules which can initiate an immune response. These characterizations suggest the five identified populations correspond to five discreet developmental stages identified in vivo: Common Myeloid Progenitors (CMP), Granulocyte/Macrophage Progenitors (GMP), Monocytes, Monocyte-derived macrophages (moMac), and Monocyte-derived DC (moDC). Furthermore, we have identified a subset of moMacs which possess the capacity to give rise to moDCs, thus termed moDC precursors (moDP).

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"Acknowledgements" is such a cold term for the appreciation I have for people who guided me through this accomplishment. The next 120 pages are filled with cold terminology (except the joke about embryology…); so, I am going to take this opportunity to warmly express my deepest appreciation. These people will forever be a part of who I am.

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List of Abbreviations

CD	cluster of differentiation
cDC	Conventional DC
CDP	Common DC Precursor
CLP	Common Lymphoid Progenitor
cMoP	Common Monocyte Progenitor
CMP	Common Myeloid Progenitor
DC	Dendritic Cell
Flt3L	FMS-like tyrosine 3 ligand
G-CSF	Granulocyte- colony stimulating factor
GM-CSF	Granulocyte/Macrophage-colony stimulating factor
GMP	Granulocyte/Macrophage Progenitor
HSC	Hematopoietic Stem Cell
M-CSF	Macrophage- colony stimulating factor
MDP	Macrophage/DC Progenitor
moDC	monocyte-derived DC
moMac	monocyte-derived Macrophage
pDC	plasmacytoid DC

Chapter 1

Literature Review

Introduction

Before the 20th century, immunology was largely a study of "how to make people exempt from diseases". Considering how the term "immunology" is still synonymous to developing vaccines, this theme has clearly been maintained. However, like the centralized role dendritic cells (DCs) play in translating a stimulus for the immune system, immunology has established itself as a centralized field of science, translating knowledge across nearly every other field to the context of human biology.

At the turn of the 20th century, biologists noticed there was a biological recognition of "self." In other words, the body could tell if something was part of the body (self) or if it was something foreign (non-self). Even if we set aside the philosophical implications of this discovery, this discovery was remarkable, and it would begin to set the foundation of modern medicine.

Ilya Mechnikov is commonly attributed as the father of cellular immunology[1]. After several unsuccessful suicide attempts, Mechnikov resigned himself to study embryology--arguably, a fate worse than death. Like many scientists, Mechnikov began his work by poking something (a star fish larvae) with a stick (from a tangerine tree). He began to notice that mobile

cells would rush to a site of invasion and speculated (correctly) that these cells take up and digest the invading pathogen. These cells would soon be termed “phagocytes”[1]. In addition to changing the world, this discovery also renewed Mechnikov’s optimism in life (Immunology: 1; Drudgeries of Life: $1x10^{80}$).

Since Mechnikov’s time, the tools we have used to study cell types have improved; however, the principles have remained unchanged. That is to say, when we want to study immunology, we usually start with “poking” our specimen with a stick. The stick could range from global (heat[2–4], diet[5–7], infections, etc.) to local (cytokines[8–12], growth factors[10,11,13], etc.), but the end goal is ultimately the same: initiate the immune response.

The beauty (and frustration) of immunology associated with infection is that it needs to be initiated. In other words, its default state is “off” (commonly referred to as a “steady-state” as it is more similar to a state of maintenance). Furthermore, an immune response is typically very short-lived[9,14], but it is also very busy and very precise. As is the trend in biology, there is no technique for “turning on” one immunological cell, or even one *type* of immunological cell. The immune system relies on a magnitude of interactions to fine tune its response. As a result, much of our knowledge of immunology has come from comparing how a cell behaves in a steady-state to how it behaves in its “on state” (discussed here as inflammation)[15–20]. However, this focus ignores the cells that develop exclusively during inflammation. Inflammatory cells share progenitors with steady-state cells, but ultimately, they only develop in the presence of abundant inflammatory signals, whereas steady-state cells are present all the time but only activated in the presence of inflammatory signals.

To be fair, these inflammatory cells do not make themselves easy to study. They are very short-lived, and they are excoriatingly similar to their steady-state counter parts. Although these

problems make studying inflammatory cells difficult, there has been a considerable amount of work done to understand the role that these fully differentiated cells play in immunity[12,21–25]. However, one of the largest gaps in our knowledge is the developmental pathway between progenitor cell and fully-developed cell (**Figure 1.1**).

Modern technologies (such as cellular barcoding[26–29] and single-cell RNAseq[30–32]) have made it possible to track and study rare populations. While these techniques have made it possible to study these rare populations, they have not fixed the problem of impractically low cell yields, which is a limiting factor when working to understanding the functional capacity of cells. The goal of this project was to develop a strategy for identifying and isolating large numbers of inflammatory cells at distinct stages in development (from the earliest myeloid committed progenitor to a fully developed inflammatory DC).

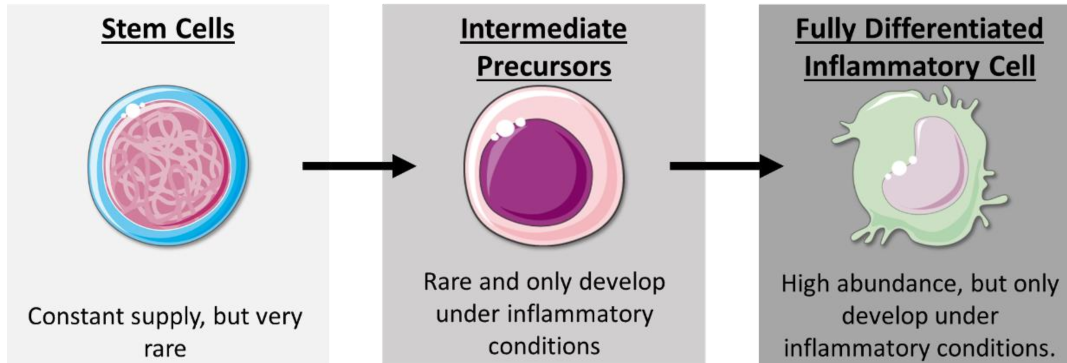

Figure 1.1 Pros and Cons of the three major stages in inflammatory development. (Left) Stem cells are maintained in the bone marrow; however due to their incredibly low numbers, comprehensive functional analysis is lacking. (Right) Fully differentiated inflammatory cells are at a high abundance. However, developmental studies are difficult due to inflammatory-dependent development and short life-span. (Center) Intermediate precursors are both rare and only develop under inflammatory conditions. As such, the largest gaps in our knowledge of immunological development rest here.

Hematopoiesis

Steady-state developmental pathways have been well characterized and continue to be a focus in the field. All hematopoietic cells (cells of the blood) go through hematopoiesis, which starts with pluripotent hematopoietic stem cells (HSCs)[33,34]. As HSCs develop, they reach a stage (multipotent progenitor; MPP) in which they will begin to undergo myelopoiesis or lymphopoiesis (the pathway for developing lymphocytes). Traditionally, each developmental stage following MPPs has been defined by a loss of potency. In other words, as cells move through stages in development, they lose the ability to develop into other cells. For example, MPPs can enter myelopoiesis and become a common myeloid progenitor (CMP) which is able to give rise to all myeloid cells but not lymphocytes, or MPPs can enter lymphopoiesis and become a common lymphocyte progenitor (CLP) which is able to give rise to all lymphocyte cells but not myeloid cells[35].

As mentioned above, this project focuses on the development of myeloid cells (myelopoiesis), more specifically granulocyte-macrophage colony-stimulating factor (GM-CSF) driven myelopoiesis. CMPs are the earliest myeloid committed stage, and they can give rise to megakaryocytes-erythrocyte progenitors (MEPs; leading to erythrocyte and platelets) or granulocyte-macrophage progenitors (GMPs)[36]. In the presence of granulocyte colony-stimulating factor (G-CSF), GMPs have the capacity to give rise to granulocytes. In the presence of GM-CSF, GMPs give rise to monocytes, which will ultimately give rise to DCs and macrophages[36].

The step from GMP to monocyte is currently a hot topic of study, and over the last decade, several substages — macrophage/DC progenitor (MDP)[37–39] and common monocyte progenitor (cMoP)[40,41] — have been identified. However, there is a large gap in understanding

what is happening from monocyte to its fully differentiated progeny. One of the factors that makes this step hard to study is that monocytes are the first stage to leave the bone marrow[42,43]. Therefore, in addition to being a relatively low yield cell type, they are decentralized.

Much of the work that has been done in understanding these pathways has been "snap-shot" analysis. While the previously mentioned cell types are almost certainly "true" cell types, the means by which they have been identified has been by studying their stage as a population. In other words, most analysis has been done by isolating a group of phenotypically similar cells. The limitation of this is that there is often a mixture of cells just entering a developmental stage and cells ready to exit the stage, branching into multiple pathways.

The use of cellular bar-coding and single-cell RNAseq has addressed this issue, but this technology is still relatively new and not always practical. The strategy discussed here was designed with the goal of isolating the earliest myeloid progenitor (CMP) and restricting their developmental signaling to GM-CSF, with the intention of removing tangent developmental pathways. As a result, cells that survive and develop may not be homogenous, but they are following a common signaling pathway.

Cells of Immunology

As mentioned above, one of the earliest decisions in hematopoiesis is the differentiation toward lymphocytes and myeloid cells. Although the focus of this text will be on the myeloid pathway, many of the myeloid cells have an intimate interaction with lymphocytes; the most important being communication between DCs and T cells.

T cells are traditionally described as a class of lymphocytes that develop in the thymus and share the CD3 surface marker. However, beyond these simple distinctions, they represent

cell types of a diverse functional repertoire. Most generally, they are known for their central role in cell-mediated immunity, orchestrating the adaptive and innate immunity. However, these cells remain in a “dormant” (naïve) state in the lymph nodes until they are presented with antigens in the context of major histocompatibility complex II (MHCII).

Dendritic cells are largely characterized by their ability to activate naïve T cells. Rather than have every naïve T cell travel to the site of trauma, DCs take up (phagocytose) particles at the site and migrate to a local draining lymph node where it can present processed antigen to naïve T cells[44–46]. Because of this, DCs are said to be the link between innate and adaptive immunity.

However, DCs’ role is not limited to presentation of antigen on their MHCII. They also produce several signals (in the form of cytokines and cell surface proteins known as “co-stimulatory” molecules) that are necessary for influencing the response of the T cell[21–23]. In other words, in addition to presenting antigen to T cells, DCs also provide the context of the antigen. In doing so, the T cell will know how to respond even though it never directly interacts with the naïve antigen.

While the type of antigen (for example, viral antigen or bacterial antigen) certainly influences the DCs’ signaling, there is evidence that immature, non-differentiated cells respond differently, suggesting that signaling functions are stage dependent. Particularly interesting, even the earliest myeloid cells (CMPs) can detect danger signals through several receptors (toll-like receptors, TLRs; NOD-like receptors, NLRs)[47,48] and respond through cytokine production and changes in development[49,50]. However, because these non-differentiated cells are rare and thus difficult to identify for functional analysis, there is a major gap in our understanding of the role these cells play in an inflammatory response.

Role of Myeloid Cells in the Immune Response

Like T cells, myeloid cells represent a group of cells that are functionally diverse, making them difficult to generalize them beyond their lineage. In fact, some of the myeloid cells (such as erythrocytes) have a very limited direct role in the immunological response. However, most of the myeloid cells are major players in the innate immune response. Unlike naive lymphocytes (which remain in the lymph nodes until activated), myeloid cells can be found residing in peripheral or lymphoid tissue (termed "resident" cells) or traveling through the circulatory system ("migratory" cells)[51–54]. When trauma is detected, resident myeloid cells and local endothelial cells release chemokines to recruit circulating lymphocytes. These migratory cells are quick to localize to the site, where they are activated by the local signaling networks (both cell-to-cell and cytokine-driven).

Myeloid cells are of particular interest in medical research because: 1) they are the first responders to trauma, reaching the site in as little as minutes compared to the days it takes lymphocytes, and 2) myeloid cells (specifically, DCs) leave the site of trauma and migrate to a local draining lymph node, where they will activate and recruit lymphocytes, initiating the adaptive response[51–53]. These two characteristics are medically relevant because the time it takes to mount the adaptive response and how well the innate system can limit the spread of a pathogen during that time are the two most critical factors in surviving many diseases.

This text will primarily focus on development of myeloid DCs. As mentioned above, much of our understanding of these cells come from analysis of steady-steady conditions. Steady-state development is believed to be regulated by Flt3L (FMS-like tyrosine 3 ligand), a cytokine and growth factor that is critical for development of myeloid and lymphoid cells[12,13,55–

[58]. In the presence of Flt3L, hematopoietic stem cells (HSCs) lead to the development of a common dendritic cell precursor (CDP), which ultimately gives rise to two types of steady-state DCs: plasmacytoid DCs and cDCs (which represent several DC subtypes)[59–61]. On the other hand, in the presence of GM-CSF, the developmental pathway is less clear. GM-CSF leads to the development of monocytes, which have the capacity to give rise to macrophages and DC[16,62,63]. GM-CSF deficient mice were shown to have normal levels of steady-state DCs, where Flt3L deficient mice had severely depleted DC levels[64]. This has led to the general acknowledgement of distinct state-dependent dendritic cell lineages. This discussion will largely focus on the GM-CSF driven linages.

The Inflammatory State

GM-CSF signaling largely leads to inflammation, the body's immediate response to what it considers to be danger[23–25,55,63]. Inflammation is triggered by infection or damage to host tissue and leads to the accumulation of cells to the affected site. Briefly, during infection induced inflammation, tissue resident cells, such as macrophages or mast cells, quickly detect the presence of damage through TLR or NOD (nucleotide-binding-oligomerization-domain)-like receptors (NLRs)[65]. This initial recognition leads to the production of inflammatory mediators, including chemokines (which act to recruit other cells) and cytokines (which regulate the cellular response). After a quick clearance of infection, there is a switch to an anti-inflammatory response, inhibiting the recruitment of granulocytes and promoting the recruitment of monocytes which will aid in removal of cellular debris[66,67]. If this quick response (commonly referred to as an "acute inflammatory response" fails to clear the infection, the anti-inflammatory response will be delayed. Instead, macrophages and eventually lymphocytes are recruited to the site of

inflammation. It is important to note that there are other avenues for inducing inflammation, ranging from psychological stress to diet. Interestingly, "developed nation inflammation" is becoming more common than infection induced inflammation; however, it is poorly understood and is outside the scope of this project.

The role of steady-state DCs during inflammation is most easily described as a "mixed bag". However, due to the variety of DC subtypes, it would be more appropriate to describe them as a "mixed bag of mixed bags"[68]. In general, DCs are mildly phagocytic during steady-states. This allows them to sample their environment for the presence of dysbiosis[69,70]. However, much of their functionality is inhibited, preventing an unnecessary immune response while maintaining systemic homeostasis[71,72]. In fact, there is evidence that DCs will actively repress T cells that recognize peptides that the DC encountered in the absence of inflammatory signals[73,74]. The game changes when DCs receive an inflammatory signal. The most common example are DCs that survey the microbiota of the gut. Under healthy conditions, this survey prevents a harsh inflammatory response to the commensal bacteria. However, under dysbiosis, DCs receive inflammatory signals in combination to the bacterial samples, resulting in further disruption.

The role of inflammatory DCs remains poorly understood, although it is well-established that they develop from monocytes. Like steady-state DCs, inflammatory DCs seem to play many roles, some which oppose others. These specialized subtypes might represent one cell lineage but different activation stimuli (for example, LPS-stimulated DC versus CpG-stimulated DC) or entirely separate developmental lineages (steady-state DCs versus inflammatory DCs)[75–77]. The goal of this study is to provide a strategy for isolating distinct developmental stages which will be critical in understanding if development of inflammatory subtypes is due to a fully developed

cell responding to stimuli or due to the multiple developmental pathways resulting in multiple cell types.

Development of Immune Cells

Nearly all the components of the blood originate from the hematopoietic stem cell (HSC) located in the bone marrow. As HSCs develop into new cell types, they give rise to progressively less pluripotent cells. Eventually, a stage is reached in which the cell is lineage committed. In other words, it can give rise to only one *type* of cell; however, within each type, there may be multiple branches of development, referred to as *subtypes*. For example, T helper cells are a type of cell that can differentiate toward several different subtypes, such has T helper 1 or T helper 2[76].

Advances in RNA sequencing and flow cytometry has led to a considerable amount of work done in mapping the hematopoiesis developmental tree[30–32]. Much of the current work in the field still relies on these methods, as they are relatively inexpensive and reliable. These have yielded extensive knowledge of which nodes or developmental stages are present during hematopoiesis, but they are limited in helping to understand the path each cell takes when developing from one stage to the next.

Much of our early knowledge of development is derived from techniques involving the isolation and cultivation of a single cell, followed by analyzing the resulting colony. This expanded upon with the development of genetically modified mice, but ultimately, very few could track the development of individual cells. This issue was addressed by the development of cellular barcoding[26–29]. This is a process of tagging cells of interest with unique, genetic sequences inserted by a retrovirus. While this technique will likely prove to be incredibly useful

in the future, it only provides information about the developmental pathways the cells follow. Therefore, it relies on previous knowledge of the stages present.

Moreover, even with advances that are allowing scientists to track individual cells, there remains a deficiency in our knowledge of the functional capacity of these cells. As a network of complex inter-cellular relationships, immunological functions largely rely on interactions with other cells. In other words, while devising methods for tracking individual cells will likely yield impressive data, there is still a demand for methods of generating large quantities of distinct stages that can be used in functional analysis.

Major Stages in GM-CSF-driven Myeloid Development

Extensive research has gone into understanding the distinct stages between a hematopoietic stem cell (HSC) and fully differentiated cells (**Figure 1.2**). The scope of this work will largely focus on the well-established stages from HSC to monocyte-derived DCs (moDCs): HSCs, GMPs, monocytes, and moDCs. Additionally, several recently discovered monocyte-associated stages (macrophage/DC progenitor, common monocyte progenitors) will be outlined below. While these are not among the stages directly analyzed in this project, it is relevant to understand the related work being done in the field in order to fully appreciate the contribution of this project.

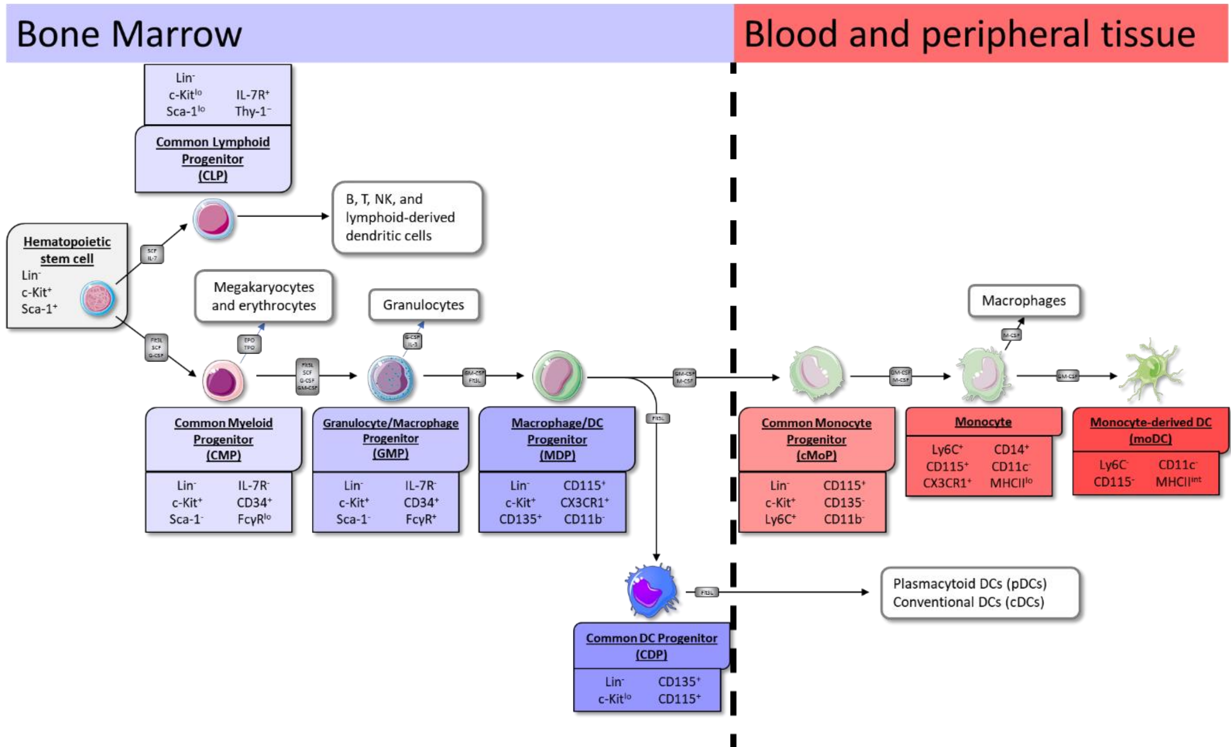

Figure 1.2 Major stages in dendritic cell development.

Hematopoiesis originates from a HSC, which can self-renew and give rise to multilineage progenitor cells: the CLP which can generate cells of the lymphoid lineage: B, T, NK, and lymphoid-derived dendritic cells (DCs) and the CMP which give rise to cells of the myeloid lineage. In the presence of Flt3L or GM-CSF, CMPs develop toward a GMP, losing the ability to give rise to megakaryocytes and erythrocytes, and GMPs give rise to MDPs. In the presence of Flt3L, MDPs differentiate toward CDPs, which remain in the bone marrow and act as a reservoir for pDCs and cDCs. In the presence of GM-CSF, MDPs delineate toward monocytes, which act as a circulating reservoir for monocyte-derived macrophages and DCs.

Lin, lineage; SCF, stem cell factor; IL, interleukin, Flt3L, FMS-related tyrosine kinase 3 ligand; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; TPO, thrombopoietin; EPO, erythropoietin; NK, natural

Hematopoietic Stem Cells (HSCs)

All blood cells are derived from an anxiety-inducingly small number of stem cells called hematopoietic stem cells. Interestingly, the work that lead to the discovery of these pluripotent cells began in the aftermath of the Hiroshima and Nagasaki bombing in 1945. Exposure to low radiation level for extended periods of times left many with compromised hematopoietic systems, and these individuals were unable to mount a functional immune response largely due to an inability to generate sufficient leukocytes[78]. In the early 1950s, studies showed that shielding a single bone drastically decreases the symptoms. Scientist quickly discovered that injection of healthy bone marrow into a mouse (who had received a lethal dose of radiation) was often sufficient to dramatically increase survival. In fact, one of the most commonly practiced treatments for intense irradiation is still bone marrow transplantations[79].

Eventually, these cells were isolated by repeatedly transferring cells from one lethally irradiated organism to another, selecting only cells that were able to produce colonies of other cells. However, with developments in antibody generation and purification, HSC were quickly characterized by their cell surface marker profiles. The most commonly used profile is the KLS phenotype: c-Kit (CD117)$^{+}$Lineage^{-}Sca-1^{+}[80]. However, it is estimated that only 10% of KLS HSCs are legitimate HSCs, whereas the other 90% are cells preparing to enter the next stage in development. Other strategies have been developed to increase the enrichment and purity of HSCs, most commonly the use of CD34 and Flk-2 to differentiate long-term HSCs (CD34^{-}Flk-2^{-}) from short-term HSCs (CD34^{+}Flk-2^{+})[81]. While, many researchers have been willing to sacrifice purity for

yield, there is a constant demand (especially in clinical applications) for methods of generating highly purified HSCs for treatments involving bone marrow reconstitution.

The two essential features of an HSC are its ability to self-renew and its ability to have the capacity to give rise to all other blood cells[82,83]. As cells become more differentiated, both traits are gradually lost. Therefore, it is critical to maintain a stem cell population that can replenish cells that are unable to renew themselves. It might stand to reason that isolating a single HSC would be sufficient, as they (in vivo) make many copies of themselves (a process termed cellular expansion). However, there has been very little success in artificially inducing HSC expansion[82,84]. Although there is a considerable amount of knowledge in relation to the various stem cell factors present in the bone marrow (such as SCF (stem cell factor) which is recognized by c-Kit, thrombopoietin which drives differentiation toward megakaryocytes, and various colony-stimulating factors and interleukins), these molecules seem to drive differentiation rather than expansion[83]. However, transfer of a single HSC into a new host results in 1,000-100,000-fold expansion in addition to differentiation[85]. While there are many ongoing studies into the proposed mechanism behind this, such as the Wnt/fzd/beta-catenin signaling pathway, there is still a considerable amount of work to be done before these cells can be efficiently generated in vitro[86,87].

Common Lymphoid Progenitor (CLP)

In the late 1990s, a group from Stanford University identified a progenitor that only gave rise to lymphoid cells (B, T, and NK cells)[88]. While this cell type had been theorized since the discovery of HSCs, it had remained elusive. The group reported a

$Lin^{-}IL\text{-}7R^{+}Thy\text{-}1^{-}Sca\text{-}1^{lo}c\text{-}Kit^{lo}$ population that lacked myeloid differentiation potential. The key differentiating attribute was the expression of the receptor for IL-7, a cytokine which is essential for B and T cell development and initiating the differentiation toward a lymphoid lineage[89]. This was the first stage in hematopoiesis to be well established.

Common Myeloid Progenitor (CMP)

Two years after the discovery of CLPs, the same group published a report showing the identification of a common myeloid progenitor (CMP) which did not give rise to lymphoid cells[36,90]. This cell type could be identified by their $lineage^{-}$ $Sca\text{-}1^{-}c\text{-}Kit^{+}$ profile, and the lack of IL-7R made them easily distinguishable from CLPs. However, like the heterogeneity observed in HSCs (long term versus short term), it became clear that there was heterogeneity within the lineage- $Sca\text{-}1^{-}c\text{-}Kit^{+}$ population. Fortunately, cells that share this phenotype were common enough to be efficiently subtyped. This led to the establishment of the $CD34^{+}Fc\gamma R^{lo}$ phenotype as bona fide CMPs, $CD34^{+}Fc\gamma R^{+}$ as GMPs, and $CD34^{-}Fc\gamma R^{lo}$ as megakaryocyte-erythrocyte progenitors[36]. Moreover, these cells could give rise to myeloid lineages (megakaryocyte/erythrocyte, granulocyte, and monocyte/macrophage/ DC) and not lymphoid cells. Luckily, there has been more success in identifying global regulators of CMPs than there has for HSC or CLPs[91,92]. Two critical factors to CMP regulation are transcription factors PU.1[93–95] and CCAAT/enhancer-binding protein-α (C/EBP-α)[96].

PU.1 is one of the most significant transcription factors involved in the myeloid pathway. Its expression is required for development from HSC to CMP and is induced upon GM-CSF and M-CSF stimulation[93]. Additionally, PU.1 plays an active role in late

stage differentiation. Similarly, C/EBP-α is necessary for regulating the initiation of myelopoiesis (generation of myeloid cells)[96]. While its expression has been shown to be essential to GMP development (discussed below), C/EBP-α deficient mice have been shown to have significantly decreased development of all myeloid cells.

Granulocyte/Macrophage Progenitor (GMP)

As previously mentioned, granulocyte/macrophage progenitors (GMPs) were discovered when it became clear that some fractions of the "CMP-like" cells were unable to give rise to all myeloid lineages when in the presence of various lineage dependent growth factors. Researchers found that the $CD34^{+}Fc\gamma R^{+}$ fraction of CMP-like cells were insensitive to growth factors that drive megakaryocyte-erythrocyte development (erythropoietin and thrombopoietin) and did not give rise to any megakaryocyte-erythrocyte cell types[36]. However, they responded strongly to G-/M-/GM-CSF, giving rise to granulocytes and macrophage-like cells.

These cells depend on expression of C/EBP-α in CMPs for development and upregulate C/EBP-ε when committing to granulocyte (neutrophil, basophil, and eosinophil) development[92,97]. In humans, GMP regulation seems to be highly reliant on Flt3L signaling[98]. However, in murine models, Flt3 signaling appears to have little impact[99]. Maintaining high levels of PU.1 expression will initiate the expression of interferon response factor-8 (Irf8)[100], which plays a role in blocking the granulocyte potential. Previous studies have shown that $Irf8^{-/-}$ mice exhibit high levels of neutrophil and decreased monocyte/macrophage/DC development[101].

Macrophage/DC Progenitor (MDP)

Much of the early work to map the developmental pathway of DCs used the GM-CSF model developed by the Steinman group[102]. As technology in cell isolation and flow cytometry advanced, scientists began to discover unique DC subtypes that are present in the steady-states. These would soon be termed conventional dendritic cells (cDCs)[103]. In an effort to discover if monocytes serve as a reservoir for all (conventional and inflammatory) DCs, mice were injected intracutaneously with monocytes that had phagocytosed fluorescent particles[104]. After several days, the fluorescent cells were found in the draining lymph nodes. This was unsurprising as DCs are uniquely specialized for homing to the lymph nodes after taking up particles[105]. However, it was surprising that these cells did not share the phenotype of previously described lymph node homing cDCs. While these fluorescent cells displayed high major histocompatibility markers (MHCI and MHCII) and costimulatory molecules (CD86) similar to that observed in activated cDCs, these monocyte-derived cells exhibited low levels of CD11c (a cell surface marker found on nearly all DCs)[104]. This lad to the distinction between cDC and moDCs and began the search for a cell type that serves as a progenitor for cDCs and monocytes.

This lead to the question "Where are cDCs coming from?". Fogg et al set out to find a cell type that could respond to inflammatory growth factors (M-CSF and GM-CSF) and steady-state growth factors (Flt3L), was not lineage committed, and had passed the GMP stage. They found a cell that makes up 0.5% of the mononuclear cells in bone marrow and exhibited a $Lin^{-}CX3CR1^{+}CD11b^{-}CD115^{+}$ $cKit^{+}CD135^{+}$ phenotype[106]. These cells could give rise to cDCs and macrophage-like but not lymphoid cells or

granulocytes. While this was an exciting breakthrough, the low cell yield and complex phenotype has largely left the functional capacity of these cells untouched.

Common Monocyte Progenitor (cMoP)

The common monocyte progenitor (cMoP) is among the most recent progenitors to be discovered[38]. In studying cDC development, a common DC progenitor (CDP; discussed below) was found downstream of MDPs that could give rise to all the cDC subsets but not monocytes. Therefore, scientists set out to determine if there was a similar cell type on the monocyte side of the fork that gave rise to monocytes, macrophages, and moDCs. They identified a cell type with a similar phenotype Lin^{-}c-$Kit^{+}CD115^{+}$ $Ly6C^{+}CD11b^{-}$ $CD135^{-}$ [38]. Of particular interest was the loss of CD135, the receptor for the steady-state growth factor Flt3L. These cells exclusively gave rise to monocyte and monocyte-derived cells but failed to generate cDCs[38,40,41]. However, studying these cells presents a similar problem to MDPs: they are very rare and short-lived. Like MDPs, there is very little understanding in how these cells interact and respond to their environment.

Monocytes

Monocyte are among the best characterized, non-terminally differentiated cells of the immune system[107]. It is not a coincidence that they are also large, long-living, and abundant (relative to other leukocytes)[108]. Unique in immunology, the phenotype of monocytes is rarely disputed. Known for their classic $Ly6C^{hi}CD115^{+}$ phenotype, monocytes are easily identified microscopically (due to their size) and cytometrically (due to very few cell types sharing their phenotype).

These cells develop from GMPs with high PU.1 expression (often due to M- and GM-CSF)[109–111]. Unlike CMPs and GMPs which act more as transitional stages for cells, monocytes are maintained effector cells. In other words, when cells reach the monocyte stage, they stop and wait for environmental stimuli before proceeding toward monocyte-derived DCs (moDCs) or monocyte-derived macrophages (moMacs)[112,113]. To accomplish this, monocytes enter circulation but do not proliferate until receiving an inflammatory signal[23,25]. As effector cells, monocytes have a repertoire of chemokine and adhesion receptors that allow them to easily monitor their environment and migrate toward sites of inflammation. In the absence of activation signals, these cells exhibit mild phagocytic and pro-inflammatory capacities[16].

The combination of being easily identifiable and delayed transitional stages attribute monocytes with a unique "intermediate checkpoint" characteristic. As such, monocytes are often used as a landmark of sorts for many developmental studies. Unfortunately (but somewhat fortunate for me), there is a significant gap in our understanding of what happens after monocyte activation. Circulating monocytes are recruited in a CCR2-dependant manner[43,114] and rely heavily on MyD88 signaling[115,116], but it remains unclear what drives monocytes toward moDC and moMac differentiation.

Monocyte-derived DCs (moDCs)

As the name implies, moDCs are derived from circulating monocytes[117]. Importantly, when Ly6C^{high} monocytes are transferred to a host undergoing a robust inflammatory response, they give rise to a unique DC subtype that can be found at the site of inflammation and in the spleen[104,118,119]. However, when the same type of monocyte is transferred to a healthy host, none of their progeny are detected in lymphoid organs[104]. This suggests monocytes selectively give rise to "inflammatory DCs" (a term used interchangeably with "monocyte-derived DCs").

Unfortunately, moDCs and steady-state DC subtypes have remarkably similar phenotypes. Over the last decade, several differences have been noted: moDCs generally have lower CD11c, higher CD11b, and lack CD4 and CD8[120]. Functional studies have shown moDCs to be a "jack of all trades." Where steady-state DCs are often highly specialized (discussed below), moDCs have an arsenal of functions that would otherwise require several steady-state DC subtypes: broad range of pathogen-recognition receptors (toll-like receptors 2, 3, 4, 7, and 9), repertoire of cytokines (IL-12, IL-23, type 1 interferons), ability to activate CD8 and CD4 T cells, and ability to cross-present antigen[64,117,118,121]. Because of their incredibly broad functional spectrum, moDCs have served as a model for understanding DC functions for nearly half a century and are heavily studied for their use in vaccine delivery.

Steady-State DC Subtypes

Unlike the transient nature of inflammatory DCs, steady-state DCs populations are maintained to be constitutively present. As a result, there has been a significant

volume of work generated about the development, functions, and subtypes of these DCs. It should be emphasized that inflammatory DC and steady-state DCs are developmentally separate, and, while there may be significant overlaps in function, they have very different roles in the immunological response[122]. However, due to the difficulty presented in studying inflammatory cells, the collection of knowledge available for steady-state DCs serves as an invaluable reference for studying inflammatory DCs.

Common DC Progenitor (CDP)

As mentioned above, steady-state DCs deviate from the inflammatory pathway at the stage of MDPs. In the absence of GM-CSF, the steady-state growth factor Flt3L drive MDPs toward steady-state DC development[56,61,123]. Where GM-CSF stimulated MDPs to develop toward cMoPs, Flt3L drives the development of the common DC progenitors (CDPs)[61]. These cells are identified by their $Lin^{-}CD115^{+}CD135^{+}c\text{-}kit^{lo}$ phenotype, and as the name implies, CDPs only give rise to dendritic cells; however, it gives rise to two general subtypes: plasmacytoid DCs (pDCs) and conventional DCs (cDCs)[60].

Plasmacytoid DCs (pDCs)

pDCs received their name due to their morphological similarity to the antibody-producing plasma cells generated during the adaptive immune response[68,124]. However, rather than producing antibodies, pDCs produce high levels of type I interferon. These cells exist in circulation, and are most sensitive to stimulation by foreign nucleic acids. Upon stimulation, pDCs produce large amounts of interferon-α and begin upregulating

the characteristics attributed to DCs: long, dendritic arms and antigen processing/presenting mechanisms[124].

Conventional DCs (cDCs)

Unlike the homogeneity of pDC, there are many subtypes of cDCs, and, as the saying goes: “Where there are subtypes, there is very likely to be a multi-potent precursor that serves as a reservoir and regulatory check-point for these subtypes.” For cDCs, this precursor has been termed pre-DC[125,126]. This nomenclature is often confusing as it ca refer to any cell type that serves as an immediate precursor to DCs (for example, many models consider monocytes to be a pre-DC for inflammatory DCs, and naive pDCs are sometimes considered pre-DCs for activated pDCs). However, for the sake of clarity, pre-DCs will be referred to as the immediate precursor to the various cDC subtypes. cDCs are commonly separated into two types: migratory and resident DCs.

Tissue-resident DCs

Resident DCs home to a lymph node where they complete their development and remain for their entire life cycle. These DCs collect antigen that enters through the lymphatics and present it to the local T cells[68]. There are two major subsets of tissue-resident cDCs, which are identified by their CD8 expression[126–128]. $CD8^+$ resident cDCs localize to the T-cell area and marginal zones and are specialized for cross-presentation. $CD8^-$ resident cDCs are often found in the red pulp and move into the T-cell area upon activation, where they specialize in antigen processing for MHCII presentation.

Migratory DCs

On the other hand, migratory DCs are (to the frustration of a non-migratory DC immunologist) often considered the "text-book" DCs[68]. They regularly sample peripheral tissue and migrate to the lymph node to present the antigens they encounter to T cells[128]. Upon activation (for example, when the DC detect the presence of a danger signal such as pathogen-associated molecule patterns) this migratory pattern in upregulated[129,130]; however, even in the absence of a danger signal, these cells regularly home to the lymph node[131]. This is an important factor in maintaining homeostasis and self-tolerance. Most peripheral tissues have their own subsets of migratory cDCs with unique phenotypes and functions[58,120]; however, there is a common split between $CD11b^+$ and $CD11b^-$ migratory cDCs, similar to that observed in $CD8^+$ and $CD8^-$ resident cDCs.

Hypothesis and Goals

The hypothesis of this project is major inflammatory DC developmental stages can be identified and isolated from GM-CSF stimulated murine bone marrow. This hypothesis was addressed with three major goals: First, we developed a strategy for identification and isolation of cells during development based on Ly6C and CD115; second, we mapped developmental pathway and kinetics driven by GM-CSF; and third, we characterized the stages along this pathway and correlated them to cell types identified in vivo, as well as identified the novel moDP stage. Additionally, we provided a framework for future studies into the GM-CSF developmental pathway.

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Chapter 2

Analysis of the developmental stages, kinetics, and phenotypes exhibited by myeloid cells driven by GM-CSF in vitro

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Abstract

The developmental progression of conventional DC has been quite well defined, yet the developmental pathway of monocyte-derived, GM-CSF-driven DC is less well understood. We addressed this issue by establishing an isolation strategy that identifies five distinct GM-CSF derived cell types. Expression of Ly6C and CD115 (Csf-1R) was used to identify and isolate four populations. One of the populations could be further separated based on CD11c expression, distinguishing five populations. We further defined these cells based on expression of transcription factors and markers of early and later stages of myeloid development. These discreet developmental stages corresponded well with previously defined populations: Common Myeloid Progenitors (CMP), Granulocyte/Macrophage Progenitors (GMP), Monocytes, as well as Monocyte-derived macrophages (moMacs) and Monocyte-derived DC (moDC). Finally, within the moMac population we also identified a moDC precursor (moDP) that could be distinguished from moMac and moDC based on their level of MHC class II expression and developmental plasticity.

Introduction

Dendritic cells (DC) are central to the establishment of adaptive immune responses and offer great promise as vehicles for vaccination and therapies for a variety of diseases [1-4]. Culture of cytokine differentiated DC from mouse bone marrow has also enabled the study of the molecular mechanisms utilized by these cells for pathogen recognition, antigen processing and presentation, and T cell priming. Large numbers of DC can be generated by culturing bone marrow in cytokines such as Flt3L or GM-CSF [5-8]. DC generated from mouse bone marrow in culture with GM-CSF (GMDC) phenotypically and functionally reflect inflammatory DC in vivo elicited by a variety of infections [9-11]. The developmental progression of cells differentiated in Flt3L has been well studied [12-16], yet the development and differentiation of GM-CSF-driven, or monocyte-derived DC (moDC), is less well understood. Thus, the developmental stages at which specific phenotypes and functions are acquired during this process remain to be determined.

The developmental program of DC occurs through a set of sequential steps, at each of which, the cells express a unique profile of transcription factors and characteristic cell surface markers [13,17,18]. Several developmental progenitors and precursors of conventional DC have been identified, including MDP (monocyte-macrophage dendritic progenitor) [19-21] and CDP (common dendritic precursor) [22-25]. In the developmental pathway of GM-CSF-driven or monocyte-derived DC (moDC), the early stages of development include common myeloid progenitors (CMP) [26,27], which give rise to granulocyte macrophage progenitors (GMP) [15,28], followed by monocytes. A precursor of monocytes and macrophages but not dendritic cells (cMoP) has also been identified in the bone marrow [19], yet its place in the GM-CSF-

driven differentiation pathway remains to be determined. Furthermore, while moDC are known to derive from monocytes [29], the later developmental checkpoints that have been identified in cDC, known as preDCs have not been identified for this lineage. Specifically, it is not clear if there is a correlate of the preDC in the inflammatory DC lineage between monocytes and IDC.

Thus, with this study, we set out to better define the sequential development of myeloid cells on the path to DC differentiation driven by GM-CSF in vitro. We have developed a sorting strategy based on the expression of two key phenotypic/functional markers (Ly6C and CD115). This strategy has enabled identification of five developmentally distinct cell stages, which represent CMP, GMP, Monocytes, and two more differentiated $CD11c^{+}MHCII^{+}$ cell types, moMac (a population resembling GM-Macs recently described by Helft, et al [30]) and traditional moDC. We also observed DC precursor activity in the population that shares the phenotype of moMac, and have termed this cell type moDP (Monocyte-derived DC Precursor). Adoptive transfer studies confirm that this GM-CSF driven developmental progression is also observed in vivo. This advance in our understanding of moDC development will support the use of these cells in the development of clinical therapies providing better ways to isolate and identify specific developmental stages with ideal functional characteristics.

Materials and Methods

Mice

This work is approved by an in full compliance with the Institutional Care and Use Committee of Auburn University regarding the use of animals. C57BL/6 and B6.SJL-$Ptprc^{a}$ $Pepc^{b}$/BoyJ mice were purchased from Jackson Laboratories. Mice were euthanized prior to bone marrow harvest by CO_2 narcosis in accordance with the rules established by the 2013 American Veterinary

Medical Association (AVMA) Guidelines on Euthanasia. To ensure irreversibility of the euthanasia process, cervical dislocation was performed following CO_2 euthanization.

DC propagation

Bone marrow DC were generated as previously described [5]. Briefly, bone marrow was removed from the femurs and tibias of C57BL/6 mice. Following red cell lysis, cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, 2-mercaptoethanol, and 10ng/mL of recombinant granulocyte/macrophage-colony stimulating factor at a density of $1x10^6$ cell/mL. Cells were incubated at 37°C in 5% CO_2 and fed with fresh media every two days.

Flow cytometry

Fluorescently conjugated antibodies against mouse surface antigens were used to measure expression. Anti-Ly6C (clone HK1.4), anti-MerTK (clone DS5MMER), anti-CD34 (clone HM34), and anti-I-A^b (clone AF6-120.1), were obtained from eBioscience. Anti-CD115 (clone AF598), anti-Sca-1 (clone E13-161.7), Ly6G (clone HK1.4), anti CD64 (clone x54-517.1), and anti-CD11b (clone 1D4B) were obtained from Biolegend. Anti-CD117 (clone 2B8), anti-CD14 (clone RMC5-3), anti-CD16/32 (clone 2.4G2), anti-Gr-1 (clone RB6-7C5), anti-CD172a (clone 1D4B), anti-CD11c (clone HL3), anti-CD40 (clone 3/23), anti F4/80 (clone T45-2342), and anti-CD86 (clone GL1) were obtained from BD. The level of fluorescence was determined by flow cytometry using a BD Accuri™ C6 flow cytometer and analyzed using FlowJo® software. Fluorescence Minus One (FMO) controls were generated by staining cells with only Ly6C and CD115 and measuring the fluorescence in the un-stained channel.

Cell sorting was performed on day 3 or day 5 based on a panel of either Ly6C, CD115, and CD11c expression, or CD11b, MHC II, and CD11c expression. All cell sorting was performed

using a Cytomation MoFlo® XDP High-Speed Cell Sorter. Double sort analysis was performed by isolating $Ly6C^{-}CD115^{+}$ cells on day 3, culturing in GM-CSF supplemented medium, and isolating the downstream $Ly6C^{-}CD115^{+}$ and $Ly6C^{-}CD115^{-}$ populations 6 days post initial sort. Gating strategy for exclusion of doublets and sorting is depicted in **Figure 2.1**.

Magnetic-associated cells sorting was performed using Anti-APC Multisort Kit (Milteyi Biotec; #130-091-255), Ly6C-APC (eBiosciences; clone HK1.4), CD115 Microbead Kit (Milteyi Biotec; #130-096-354), and LD Columns (Milteyi Biotec; #130-042-901). Murine bone marrow cells were harvested and cultured for 2 days as previous described. $3x10^{7}$ cells were recovered and sorted according to manufacturer's instructions. Cells were treated with FC blocking buffer and stained with Ly6C-APC and CD115-biotin. After incubation and washing, cells were incubated with anti-APC beads and passed through an LD column. The flow through and retained factions were collected, and anti-APC beads were cleaved with Release Buffer. Both factions were then incubated with Stop Buffer and anti-Biotin beads. The two factions were passed through a second LD column, resulting in 4 factions based on Ly6C and CD115 profiles.

Gene Expression Analysis

RNA was isolated from sorted populations using RNAqueous®-4PCR kit, and qPCR was performed using a custom RT^{2} Profiler PCR array from Qiagen® according to manufacturer's instructions. The following transcripts were analyzed: Id2, Irf8, Irf4, Stat3, Stat5b, Spi1, Nfkb1, Batf3, Gfi1, Cebpa, Ciita, Irf2, Cx3cr1, Tcf7l2, Cebpe, Pecam, Cd34, Kit, Flt3, Relb, Klf4, Zbt46, Runx2, Zfp367, Pml, Csfr3, Rn18s, and Gapdh.

Co-Culture and adoptive transfer experiments to track developing myeloid cells in vitro and in vivo

Bone marrow was harvested from $Ptprc^b$ (CD45.1) mice and cultured in GM-CSF supplemented medium for 1 or 4 days. Cells were sorted based on expression of CD11c, Ly6C, and CD115. In co-culture assays, 10^4 $CD45.1^+$ sorted cells were co-cultured with 10^6 $CD45.2^+$ fresh bone marrow cells supplemented with GM-CSF. Adoptive transfers were performed by intraperitoneal injection of 10^6 sorted $CD45.1^+$ cells suspended in PBS with 200ng of GM-CSF into $CD45.2^+$ mice. Mice received daily injections of 200ng of GM-CSF. Peritoneal lavage was collected every 48 hours.

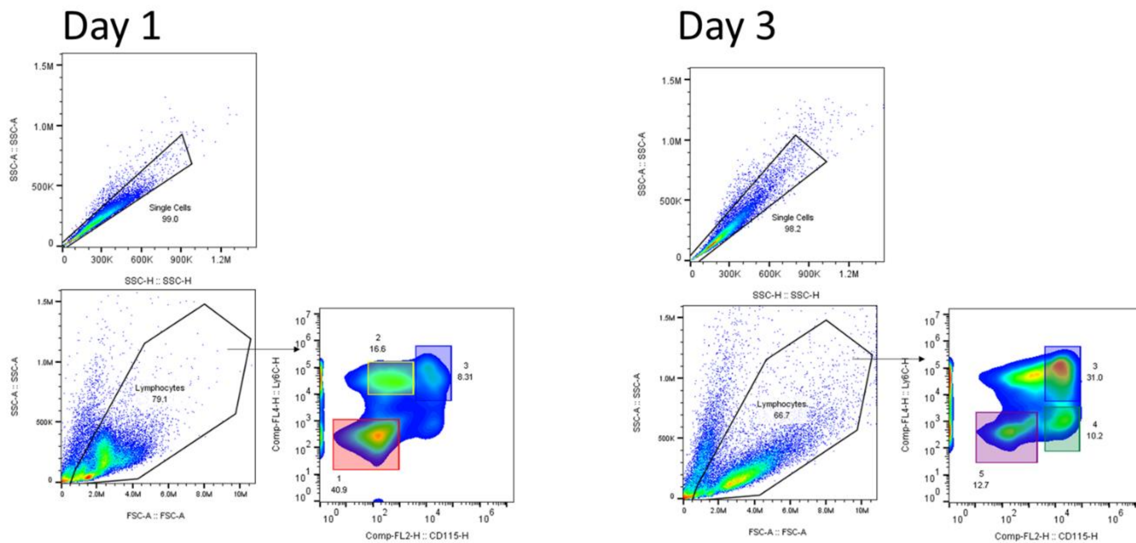

Figure 2.1. General gating strategy for the 5 cell types. Murine bone marrow stained with Ly6C and CD115 was analyzed by flow cytometry over a range of time points. Debris-size and high SSC events were excluded. Early cell types (Ly6C$^-$CD115$^-$, Ly6C$^+$CD115$^-$, and Ly6C$^+$CD115$^+$) were collected and analyzed at early times points when they were most abundant (Day 1 and 2), whereas more developed cells types (Ly6C$^+$CD115$^+$, Ly6C$^-$CD115$^+$, Ly6C$^-$ CD115$^-$) were collected and analyzed at later times points (Day 3-5). A doublet gate was applied during sorting to exclude cells that clump while waiting to be sorted. However, this was not necessary for much of our analysis, as cells were analyzed immediately after filtering.

Results

Differential kinetics of Ly6C and CD115 expression allows for identification of developmentally distinct populations of GM-CSF driven myeloid cells.

To design a strategy for isolating myeloid cells at distinct stages of GM-CSF-driven development and differentiation, we first set out to identify cell surface markers expressed with distinct kinetics during differentiation in vitro. The expression of markers such as CD11b and CD11c increased gradually and remained high through the end of the culture period, making these markers poor candidates for identifying cells at the early stages of development. However, Ly6C and CD115 were transiently expressed and with distinct kinetics. Ly6C expression peaked around day 3 and CD115 at day 5 (**Figure 2.2A**).

Two-parameter analysis of Ly6C vs. CD115 (CSF1-R) expression allowed for isolation of four distinct populations: $Ly6C^{-}CD115^{-}$, $Ly6C^{+}CD115^{-}$, $Ly6C^{+}CD115^{+}$, and $Ly6C^{-}CD115^{+}$ across the seven-day culture period (**Figure 2.2B**). We then monitored the relative frequencies of each of the four populations within the culture over the same time frame (**Figure 2.2C**). The $Ly6C^{-}CD115^{-}$ population was the most common population at day 1, but decreased in frequency through day 5. Interestingly, there was an increase in this population at day 6 and 7. The $Ly6C^{+}CD115^{-}$ population was also abundant on day 1 then it decreased slowly in frequency through day 7. The $Ly6C^{+}CD115^{+}$ population was present at a low frequency initially, but became the predominant population at days 3 through 7. The $Ly6C^{-}CD115^{+}$ population was the least abundant initially, but it grew steadily through day 7 (**Figure 2.2C**).

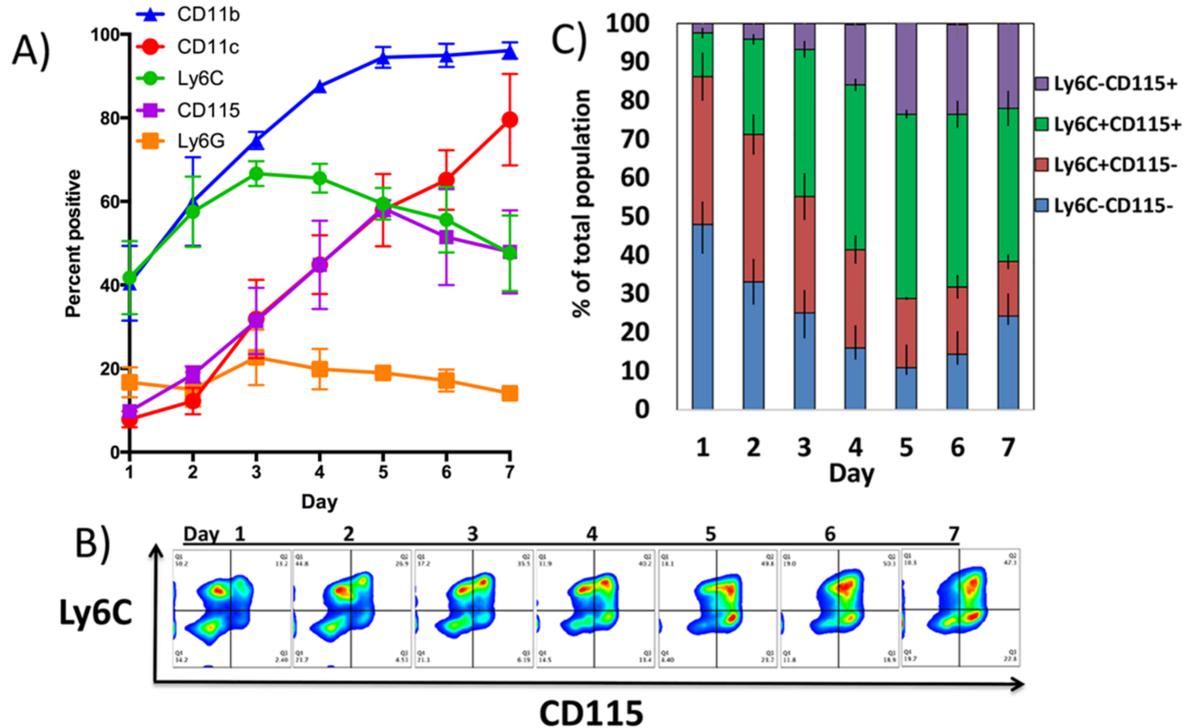

Figure 2.2. Expression of myeloid and dendritic cell markers by GM-CSF driven bone marrow cells over seven days in culture. Bone marrow cells were cultured in GM-CSF for seven days. A) Expression of CD11b, CD115, Ly6C, and CD11c were monitored by flow cytometry each day. The percent of cells expressing each marker is depicted vs. day of culture. The mean and standard deviations of three independent experiments are shown. B) Flow cytometric plots of co-expression of Ly6C vs. CD115 by bone marrow cells cultured in GM-CSF over seven days. C) Compiled data from three independent experiments illustrating the relative percentages of each of the four populations over 7 days of culture.

Myeloid cells express Ly6C and CD115 in a sequential pattern during differentiation

To determine the developmental sequence of Ly6C and CD115 expression on GM-CSF-driven myeloid cell differentiation, cells were sorted on day 3 of culture into four populations based on expression of these markers. Following isolation, the purified populations were re-cultured in GM-CSF supplemented medium to track their subsequent progression (**Figure 2.3)**. Within one day post sorting, a subset of the Ly6C^{+}CD115^{-} population up regulated expression of CD115 (**Figure 2.3A**). After two days, most of these cells now expressed both Ly6C and CD115 with a subset having progressed to the Ly6C^{-}CD115^{+} phenotype. Following a similar pattern, cells initially expressing both markers (Ly6C^{+}CD115^{+}) began to down regulate Ly6C within 48 hours, and a subset of these cells went on to down regulate CD115 by 72h, transitioning to the double negative phenotype (**Figure 2.3B**). Finally, many of the Ly6C^{-}CD115^{+} cells down regulated CD115 within 48 hours, with roughly half of the cells having shifted to double negative by 72h (**Figure 2.3C**).

Upon further examination of the Ly6C^{-}CD115^{-} population, we identified both CD11c^{+} and CD11c^{-} cells with this phenotype. To address the GM-CSF-driven developmental potential of both populations, they were further sorted based on expression of CD11c and re-cultured (**Figure 2.3D**). The Ly6C^{-}CD115^{-} CD11c^{+} population did not change its phenotype and did not proliferate in culture. In fact, within 3 days post sorting, most cells in this population were apoptotic (data not shown). The phenotype of the Ly6C^{-}CD115^{-} CD11c^{-} population did not change until day 7 of culture (3 days post sort), demonstrating primarily proliferative activity during this time (data not shown). Subsequently, these cells first up regulated Ly6C, then went on to co-express CD115, and then down regulated Ly6C. At the late timepoints of 15 and 16

days post sort, many of the cells had progressed to the double negative phenotype, while a majority maintained CD115 expression. Thus, over the course of 16 days of culture, we identified at least five stages of development, ending with two distinct $CD11c^{+}$ populations ($Ly6C^{-}CD115^{-}$ and $Ly6C^{-}CD115^{+}$) (**Figure 2.3D**).

To control for any potential off-target effects of high-speed cell sorting on the cell populations, we utilized a magnetic separation method (MACS, Miltenyi) as an alternative approach. Using a negative selection method, we acquired $Ly6C^{-}CD115^{-}$ cells at 92-96% purity. After separation, these cells were cultured with GM-CSF for 12 days to follow their progression (**Figure 2.4**). Consistent with the results observed in **Figure 2.3,** the cells first up-regulated Ly6C, then CD115, then down regulated Ly6C, and then down regulated CD115 (**Figure 2.4**). Having observed the same pattern of development in the absence of cell sorting, we conclude that sorting did not significantly alter the developmental progression of the cells.

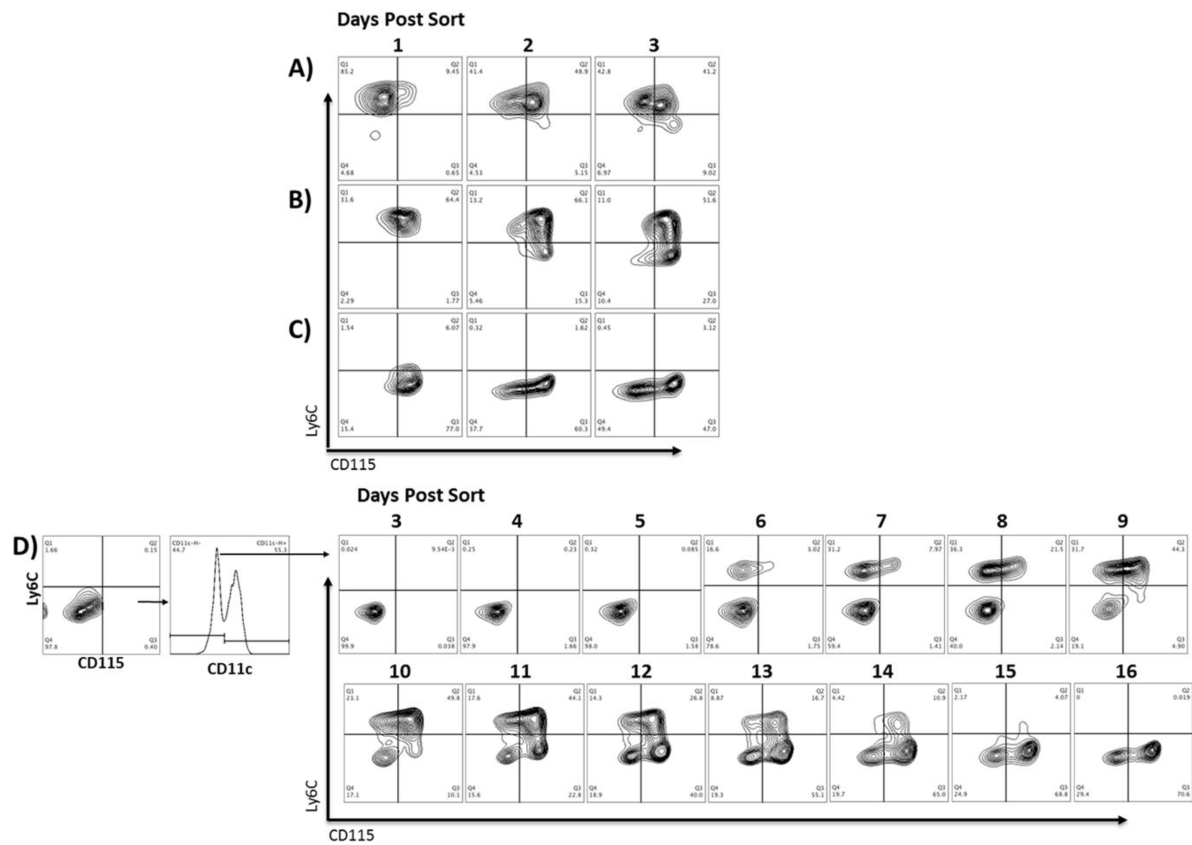

Figure 2.3. GM-CSF differentiated cells isolated based on expression of Ly6C and CD115 transition through a series of successive stages of development. After 3 days of culture in GM-CSF, cells were sorted into four populations based on expression of Ly6C and CD115. Isolated populations were then re-cultured in GM-CSF and re-examined for expression of Ly6C and CD115 on the indicated days. A) Ly6C^{+}CD115^{-} B) Ly6C^{+}CD115^{+} cells C) Ly6C^{-}CD115^{-} cells were sorted and monitored over three days post sorting. D) Ly6C^{-}CD115^{-} cells were first depleted of CD11c^{+} cells to remove contaminating DC. Then they were monitored over 16 days because no progression was evident within the three-day time period of the other populations. Changes in the population became evident at day 7 of culture after sorting and continued through day 16.

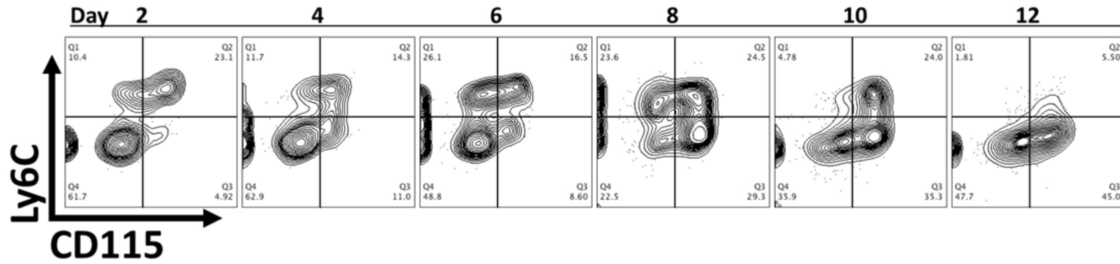

Figure 2.4. Developmental progression of CMP purified by magnetic cell isolation. Murine bone marrow was harvest and cultured as previous described. Ly6C$^-$CD115$^-$ cells were isolated on Day 2 post-harvest by MACS according to manufacturer's protocol. Briefly, $3x10^6$ cells were stained with CD115-biotin and Ly6C-APC, followed by an incubation with anti-APC magnetic beads. Tagged cells were passed through a MS column. The flow through faction was incubated with anti-Biotin magnetic beads and passed through fresh MS column. The flow through contained an enriched Ly6C$^-$CD115$^-$ population. These cells were analyzed for purity and re-cultured in GM-CSF supplemented media.

Developmental progression of GM-CSF driven differentiation in vitro and in vivo

To first determine if the same sequence of phenotypic development was observed in the presence of other bone marrow cells, we isolated 3 of the earlier populations by sorting at day 3 of culture. These CD45.1-expressing cells were then mixed in culture with an excess of congenic (CD45.2) bone marrow cells and GM-CSF for up to 6 days. The expression of Ly6C and CD115 on the CD45.1-expressing cells was measured at days 1, 3, and 6 post sorting (**Figure 2.5A**-D). In a pattern like that observed in **Figure 2.3,** double negative cells first up-regulated Ly6C, then CD115, then the majority of the cells became Ly6C^{-}CD115^{+} by day 6 (**Figure 2.5B**). Likewise, Ly6C^{+}CD115^{-} cells first up-regulated CD115, then the majority of cells displayed a CD115 single positive phenotype by day 6 (**Figure 2.5C**). Ly6C^{+}CD115^{+} cells quickly down-regulated Ly6C, and most cells became double negative by day 6 (**Figure 2.5D**). The one most notable difference between this and our previous experiment was the kinetics of development. In the presence of the other bone marrow cell populations, the developmental sequence proceeded in the same order but did so much more rapidly (Figs 2 and 3).

To determine if a similar GM-CSF-driven developmental sequence of myeloid differentiation was observed in vivo, we used an adoptive transfer system in which congenic donor cells could be tracked in recipient mice. Bone marrow was isolated from mice expressing the CD45.1 isoform and expanded in culture with GM-CSF for 2 or 5 days (to increase the yield of cells at early vs. later stages of development). These cells were then sorted based on expression of Ly6C and CD115 and transferred into mice expressing the CD45.2 isoform. GM-

CSF was administered daily for the indicated time points. Cells were then harvested from the peritoneal cavity and spleen. Expression of Ly6C and CD115 was then measured on CD45.1-expressing cells (**Figure 2.5E-G**). The $Ly6C^{-}CD115^{-}$ cells began to upregulate expression of Ly6C after 4 days in vivo and this population increased at day 6 and day 10 (**Figure 2.5E**). These double negative cells also gave rise to cells with a $Ly6C^{-}CD115^{+}$ phenotype, first appearing at day 6, and increasing at day 10 post transfer. The pattern of expression that was consistently observed was first Ly6C, followed by CD115 in each case, giving rise to a similar progression as observed in vitro (**Figure 2.5E**). To our surprise however, we were unable to recover many cells with the monocyte phenotype ($Ly6C^{+}CD115^{+}$) in these experiments. This could have been due to their highly migratory function or rapid transition to the next stage of development in vivo. To investigate the fate of monocytes in vivo, we transferred in $Ly6C^{+}CD115^{+}$ cells and looked for them at 2 and 4 days post transfer (**Figure 2.5G**). Again, we were not able to recover cells with the $Ly6C^{+}CD115^{+}$ phenotype even at day 2, indicating that they had either migrated out of the site or had all transitioned to the next stage of development.

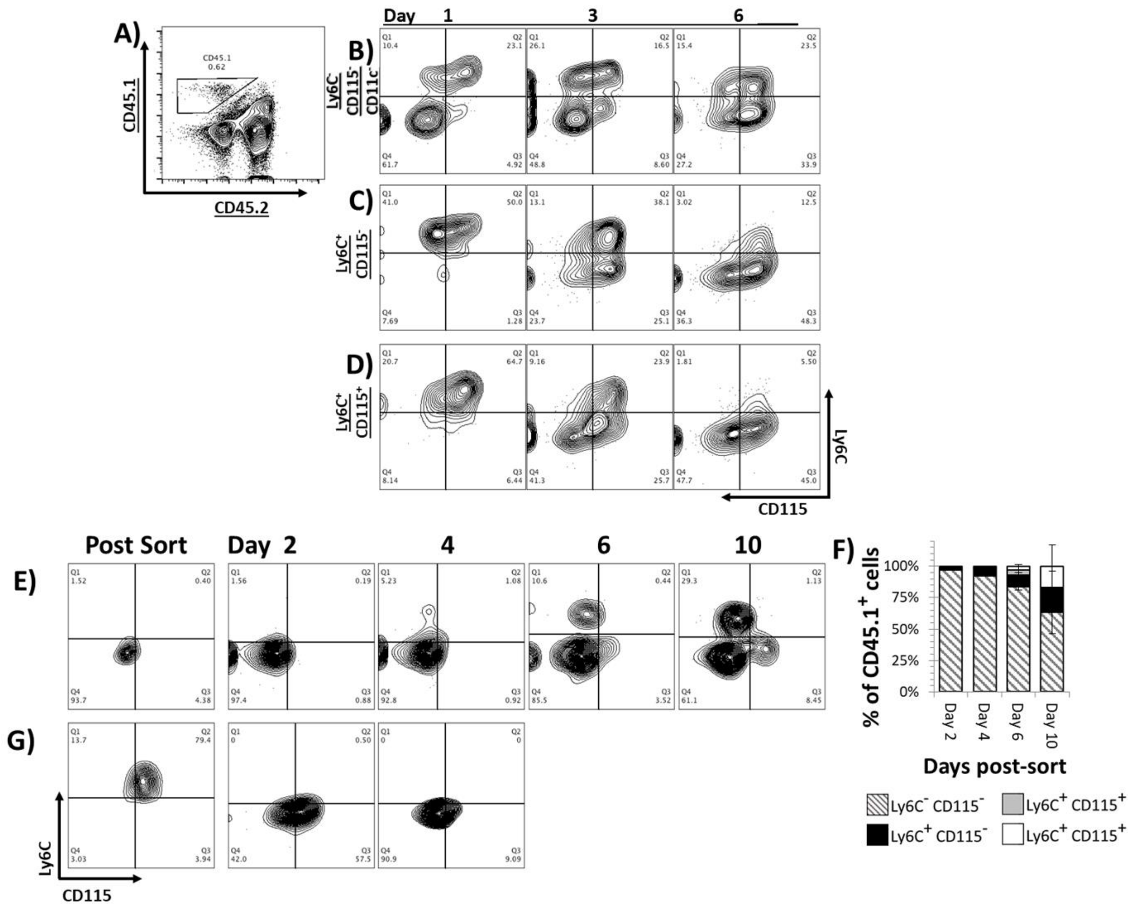

Figure 2.5. Developmental progression of GM-CSF driven differentiation in the presence of feeder cells in vitro or in vivo. Bone marrow was harvested from Ptprc^b (CD45.1) mice, cultured in GM-CSF supplemented medium for 2 or 5 days, and sorted as previously described. **A)** 10^4 CD45.1$^+$ sorted cells were co-cultured with 10^6 CD45.2$^+$ fresh bone cells, and **B-D)** Ly6C/CD115 expression was analyzed for six days by flow cytometry. Adoptive transfers were performed by intraperitoneal injection of 10^6 (CD45.1$^+$) **E)** Ly6C$^-$CD115$^-$ or **G)** Ly6C$^+$CD115$^+$cells into CD45.2 mice, suspended in PBS with 200ng of GM-CSF. **F)** Composition of recovered CD45.1$^+$ cells following CMP adoptive transfer 2, 4, 6, and 10 days post injection compiled from 3 independent experiments. Mice received daily injections of 200ng of GM-CSF. Peritoneal lavage was collected every 48 hours, and donor (CD45.1$^+$) cells Ly6C/CD115 levels were evaluated by flow cytometry.

Phenotypic characterization of each population based on cell surface markers and gene expression

Each of the five populations were phenotypically characterized based on expression of cell surface markers representing several stages of myeloid cell development [31-33] (**Figure 2.6A**) and on patterns of expression of key genes active at different stages of development (**Figure 2.6B**). To identify the earliest progenitor populations, we monitored expression of Sca-1, CD117, and CD34. Flt3L-responsive DC progenitors were tagged with anti-CD135. CD11b was measured as a marker of myeloid commitment, CD16/32 was measured to differentiate CMP (which lack its expression) from GMP and all subsequent populations (which express it). Ly6G was tested to identify granulocytes and their precursors [34]. CD14, CD64, F4/80, and MerTK were tested as markers of macrophages. Markers of later stage DC development included CD172a (SIRP-α) [35], CD11c, CD86 and MHC class II (**Figure 2.6A**). We then measured the expression of 24 targeted genes using a custom qRT-PCR array (**Figure 2.6B**). We selected genes known to be expressed by cells at several stages of myeloid development [31-33]: CMPs (Gfi1, Kit, Cebpa, Flt3, Cd34), GMPs (Csf3r, Cebpe, Spi1), monocytes (Tcf7l2, Cx3cr1, Pecam, Klf4), and during conventional DC development (Irf2, Id2, Irf8, Stat3, Stat5, Nfkb, Relb, Batf3, Irf4, Ciita). Data are depicted as a heat map showing relative expression levels (**Figure 2.6B**).

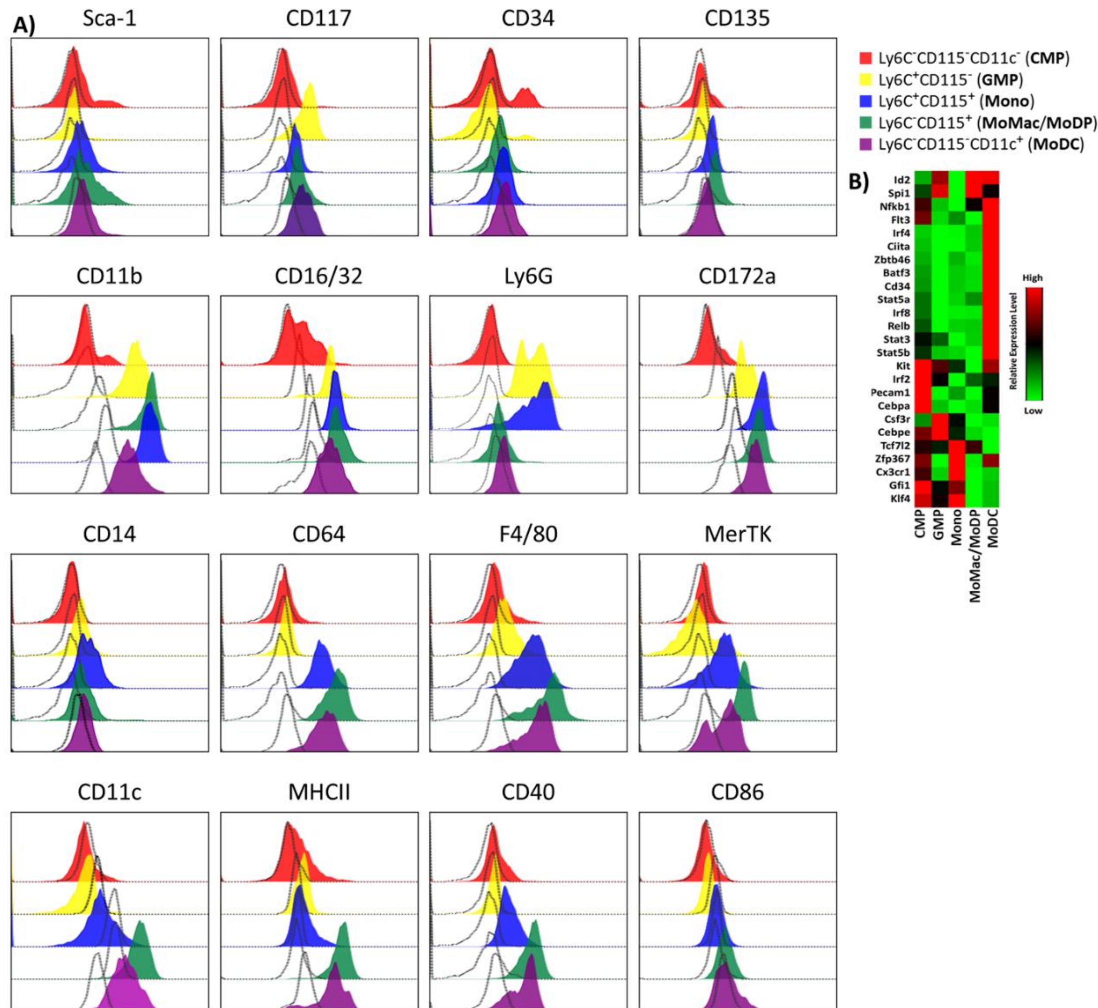

Figure 2.6. Distinct cell surface marker and gene expression profiles in the five stages of inflammatory DC development. **A)** 16 cell surface markers were measured by flow cytometry at each of the five stages of development. Empty histograms represent fluorescence minus-one controls. **B)** After three days of culture in GM-CSF, bone marrow cells were sorted into five populations based on expression of Ly6C, CD115 and CD11c. RNA was purified from each population and analyzed for expression of 25 genes plus controls using a custom qRT-PCR array. Relative levels of expression are depicted by intensity of color on the heat map with red being highest expression and green lowest. Results represent averages from three independent experiments.

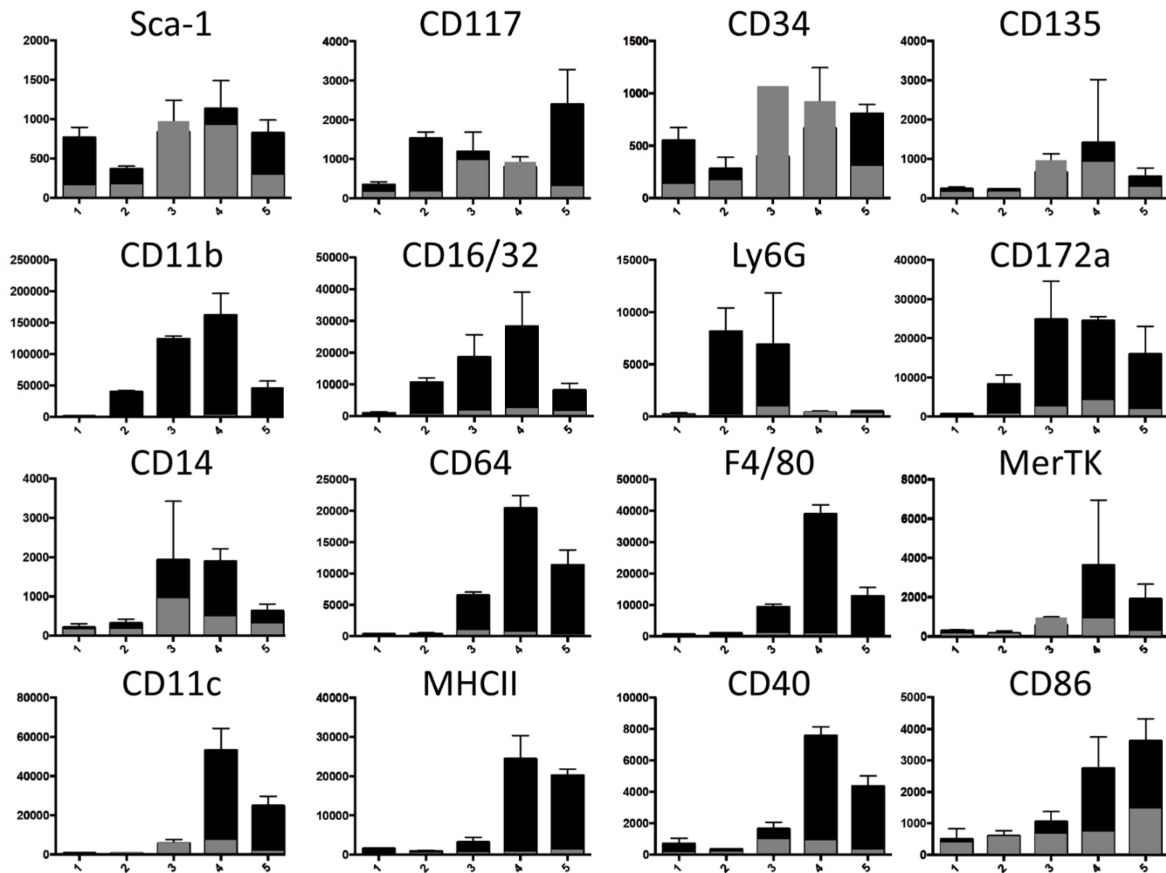

Figure 2.7. Mean Fluorescence Intensity (MFI) of markers commonly expressed by myeloid cells. Black bars indicated MFI of indicated cell surface markers. These are overlaid with gray bars that represent the MFI of the Fluorescence Minus One control. Populations are indicated by 1 (CMP), 2 (GMP), 3 (monocytes), 4 (moMac/MoDP), and 5 (MoDC).

Ly6C$^-$CD115$^-$CD11c$^-$ (CMP): This early population contained a subset of very early progenitors expressing Sca-1, and CD34, with generally lower levels of expression of all of the subsequent markers (**Figure 2.6A** and **Figure 2.7**). These cells were unique in lacking CD11b and expressed low levels of CD16/32, which were expressed by all subsequent populations. They also expressed little or none of the macrophage and DC markers (**Figure 2.6A** and **Figure 2.7**). The gene expression profile of these cells demonstrates expression of genes typical of early myeloid progenitors, monocyte committed cells and Common Myeloid Progenitors: Gfi1 [36], Klf4 [37], Cebpa [38,39], Pecam1 [40], Irf2 [41] and Kit [26] . Taken together, the cell surface profile and gene expression pattern indicates that these Ly6C$^-$CD115$^-$CD11c$^-$ cells most closely correspond to Common Myeloid Progenitors (CMP).

Ly6C$^+$CD115$^-$ (GMP): This population lacked expression of the most of the stem cell markers, except for a small subset that expressed CD34 (**Figure 2.6A** and **Figure 2.7**). This population was also the first to demonstrate higher levels of expression of CD11b, CD16/32, Ly6G, and CD172a than CMP. These cells lacked expression of the macrophage markers CD14, CD64, low F4/80, and low MerTK. These cells also expressed very low levels of the dendritic cell markers, CD11c, MHC class II, CD40 and CD86 (**Figure 2.6A** and **Figure 2.7**). Gene expression analysis revealed expression of Csf3r and Cebpe [42], which are hallmarks of Granulocyte Macrophage Progenitors (GMPs), as well as Spi1 (PU.1) [43] (**Figure 2.6B**). Thus, this Ly6C$^+$CD115$^-$ population closely resembles GMPs.

Ly6C$^+$CD115$^+$ (Monocytes): These cells lacked expression of the stem cell markers Sca-1, CD117, and CD34, and expressed low levels of CD135. The majority of cells in this population also expressed Ly6G yet at a lower frequency than GMP (**Figure 2.6A** and **Figure 2.7**). This population expressed a very low level of CD11c and a high level of CD172a. This

population displayed intermediate CD40 and CD86, yet low level MHC class II. Notably, the $Ly6C^{+}CD115^{+}$ cells were the first population to demonstrate high expression of the macrophage markers, CD64 and F4/80, yet intermediate expression of MerTK. These cells also expressed high levels of monocyte-associated genes Tcf7l2 [44], Klf4, and Cx3cr1 [45] (**Figure 2.6B**). Collectively, the phenotype and gene expression pattern most closely resemble the monocyte cell type.

$Ly6C^{-}CD115^{+}$ (moMac): Cells with this phenotype were negative for stem cell markers and Ly6G expression. Notably, this population expressed the highest levels of macrophage markers F4/80, and MerTK relative to the other four populations. They also expressed high levels of CD11c, MHC II, and CD40 and an intermediate level of CD86 (**Figure 2.6A** and **Figure 2.7**). Upon examination of gene expression, these cells displayed high level expression of only two genes, Spi1 and ID2 [31]. Spi1 (PU.1) is a central transcription factor in myeloid cell and DC development [46,47]. While highly expressed in the $Ly6C^{-}CD115^{+}$ population, Spi1 was also up regulated initially in the $Ly6C^{+}CD115^{-}$ population (**Figure 2.6B**). Based on the phenotype and gene expression patterns, this population most closely resembles monocyte-derived Macrophages (moMac). A similar population, referred to as GM-Macrophages, was recently described by Helft, et al. [30]

$Ly6C^{-}CD115^{-}CD11c^{+}$ (moDC): This final population expressed CD11c and CD172a as well as high levels of MHC class II, CD40, and CD86. However, these cells displayed low levels of the macrophage markers CD14, CD64, F4/80, and MerTK and had a slightly lower level of CD11b expression (**Figure 2.6A** and **Figure 2.7**). This population also expressed high levels of several genes critical for DC function and differentiation including: Ciita (required for transcription of MHC class II genes); Stat5a, Stat5b, and Stat3 (transcriptional regulators of

myeloid differentiation and GM-CSF signaling [31]); Zbtb46 and Batf3, (both critical to DC development [48,49]); and Relb and Nfkb1, both well documented regulators of inflammatory gene expression (**Figure 2.6B**). Also, while we were unable to detect Flt3 expression by flow cytometry on this population, there was a strong signal of its gene expression. Taken together, this population closely reflects monocyte-derived Dendritic Cells (moDC) in phenotype.

The moMac population contains DC-precursors as well as macrophages

We routinely observed that most Ly6C^{-}CD115^{+} cells would ultimately down regulate CD115, taking on the phenotype of monocyte-derived DC (moDC). However, in long-term culture, a subset of Ly6C^{-}CD115^{+} (moMac) persisted, maintaining CD115 expression even out to 16 days (**Figure 2.3, Figure 2.5**). To more definitively address these final stages of development, we performed a two-stage sorting experiment. moMacs were first purified from GM-CSF stimulated bone marrow on day 5, and re-cultured for 6 days in GM-CSF (**Figure 2.8A**) before undergoing a second sort, based on their level of CD115 expression (**Figure 2.8B**, **Figure 2.8C**). 48 hours after the first sort, ~44% of moMacs had downregulated CD115, and this phenotypic distribution did not change over the subsequent 4 days (**Figure 2.8A**). Six days after the first sort, cells that were selected based on low level of CD115 expression maintained that phenotype (**Figure 2.8B**). Likewise, the majority of CD115^{high} cells maintained CD115 expression over this time period (**Figure 2.8C**) yet, a small subset within this population continued to give rise to CD115 low cells (~20% by 4 days).

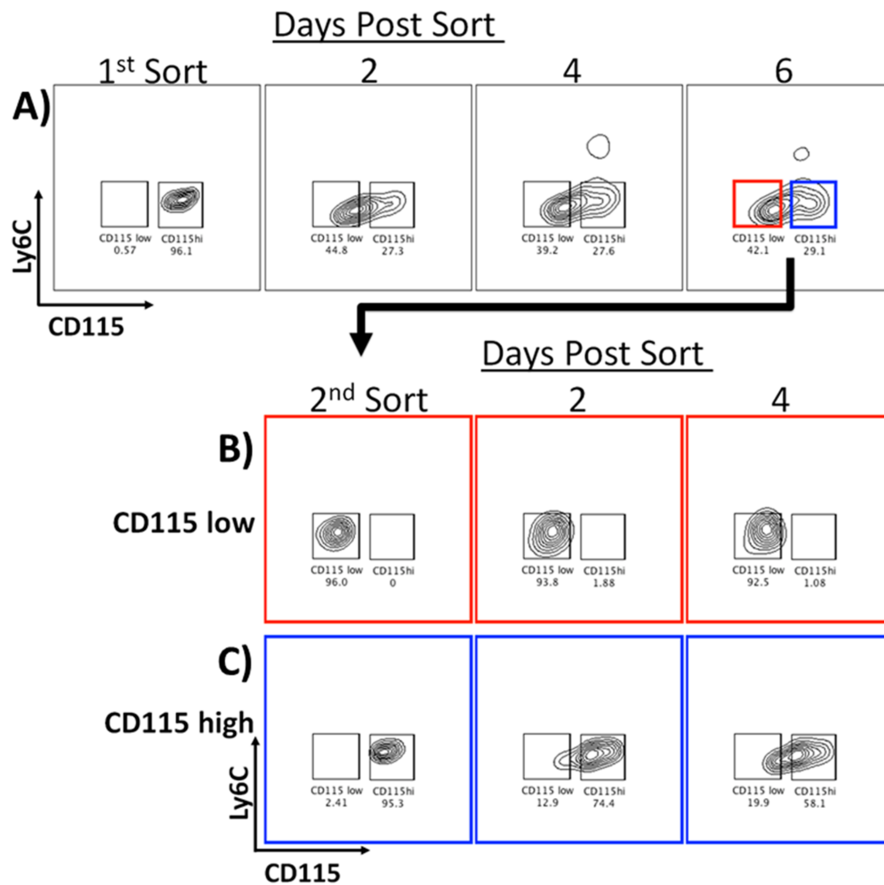

Figure 2.8. Early moMacs give rise to two cell types. **A)** After five days of culture in GM-CSF, moMacs (Ly6C$^-$CD115$^+$) were purified and recultured with GM-CSF. CD115 expression was monitored by flow cytometry for 6 days. Six days post-initial sort, **B)** moDC (Ly6C$^-$CD115$^-$) and **C)** moMacs (Ly6C$^-$CD115$^+$) were purified and recultured with GM-CSF. CD115 expression was monitored by flow cytometry for the next 48 hours.

To determine if the moDC progenitors could be further distinguished from moMacs based on CD11c, CD11b, and MHCII expression, we incorporated a sorting strategy previously published by Helft et al [30]. After five days in culture, 41% of cells were CD11c^{+}. Within this group, there were three CD11b^{+} populations: MHCII^{low} (29%), MHCII^{int} (34.3%), and MHCII^{high} (17.2%) (**Figure 2.9A**).

Post-sort analysis of the MHCII^{Low} population revealed two predominant populations, Ly6C^{+}CD115^{+} and Ly6C^{-}CD115^{+} (**Figure 2.9B**; top panel). Two days after sorting, most MHCII^{Low} cells had upregulated MHCII to an intermediate level (72.6% ± 27.4 on day 2; 62.2% ± 19.6 on day 4) (**Figure 2.6C**; top panel). Additionally, analysis of these cells' Ly6C and CD115 levels four days post sort showed the resulting MHCII^{Int} cells represent a heterogenous mixture of primarily Ly6C^{+}CD115^{+} (33.6% ± 4.6) and Ly6C^{-}CD115^{+} (52.8% ± 9.47) (**Figure 2.9D**). A small subset of MHCII^{Low} cells were also able to give rise to MHCII^{High} cells (9.5% ± 10.7 on day 2; 8.58% ± 6.5 on day 4) (**Figure 2.9C**; top panel). Unlike the MHCII^{Int}, MHCII^{High} were primarily (80.4%±4.81) Ly6C^{-}CD115^{-} (**Figure 2.9D**; top panel). The results of several replicate experiments are graphically illustrated in **Figure 2.9E**.

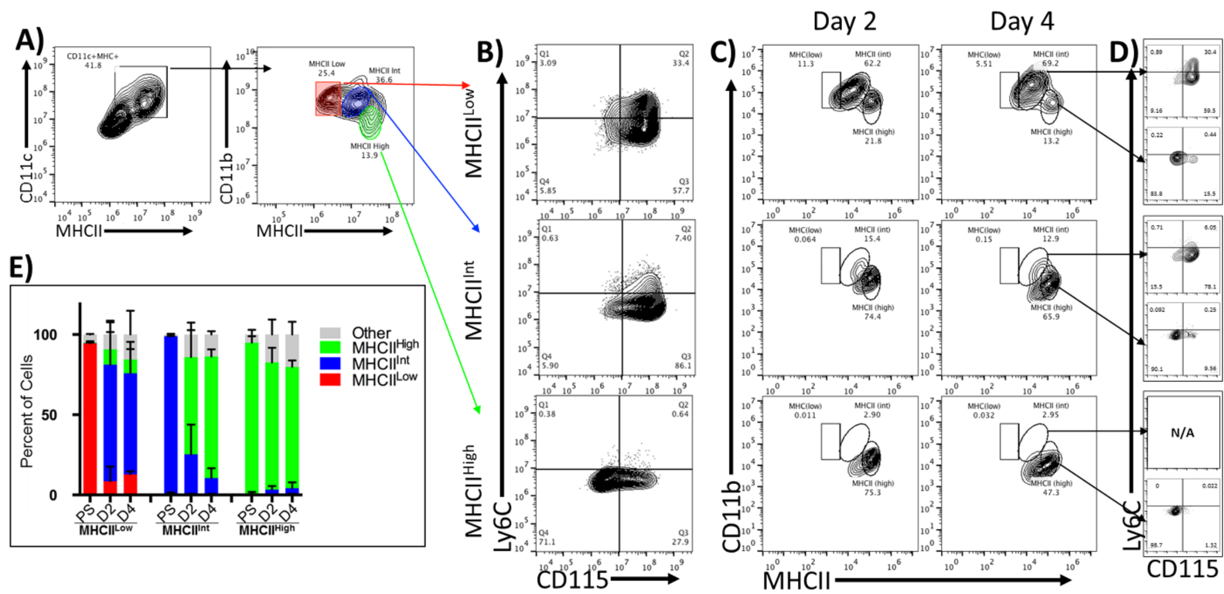

Figure 2.9. MHC class II level distinguishes developmental stages within moMac phenotype. Bone marrow cells were cultured in GM-CSF for 5 days. **A)** CD11c^{+} cells were sorted based on expression of CD11b and MHCII into three populations: MHCII^{Low}, MHCII^{Int}, and MHCII^{High}. **B)** Expression of Ly6C and CD115 were analyzed post sort, and the isolated populations were re-cultured in GM-CSF. **C)** Changes in CD11b and MCHII expression were analyzed on day 2 and 4 post sort by flow cytometry. **D)** Resulting MHCII^{Int} and MHCII^{High} cells on Day 4 were further analyzed by Ly6C and CD115 expression by flow cytometry. **E)** Percent of cells exhibiting MHCII^{Low}, MHCII^{Int}, or MHCII^{High} phenotypes post-sort (PS) and after re-culture for 2 or 4 days.

The isolated $MHCII^{Int}$ cells were primarily of the $Ly6C^{-}CD115^{+}$ phenotype (86.1%), with few exhibiting the $Ly6C^{+}CD115^{+}$ phenotype (7.4%) (**Figure 2.9B**; middle panel). After 48 hours of culture in GM-CSF, nearly all $MHCII^{Int}$ cells had upregulated MHCII to a high level (60.6% ± 21.5 on day 2; 75.75% ± 4.45 on day 4), while only a fraction maintains the $MHCII^{Int}$ phenotype (14% ± 2.7 on day 2; 13.69% ± 2 on day 4) (**Figure 2.9C**; middle panel). The newly developed $MHCII^{High}$ cells primarily consisted of $Ly6C^{-}CD115^{-}$ cells (91.8% ± 2.47), whereas the 72.75% ± 7.57 of those that maintained $MHCII^{High}$ phenotype exhibited a $Ly6C^{-}CD115^{+}$ phenotype (**Figure 2.9D**; middle panel).

Finally, analysis of $MHCII^{High}$ isolated cells showed that they maintained their $MHCII^{High}$ phenotype on day 2 and day 4 (**Figure 2.9C**; bottom panel). Additionally, 97.9% ± 0.14 of these cells exhibited a $Ly6C^{-}CD115^{-}$ phenotype 4 days post isolation. Together, these patterns suggest that, when isolated on day 5, $CD11c^{+}CD11b^{+}MHCII^{low}$ cells act as a progenitor to a terminal $Ly6C^{-}CD115^{+}MHCII^{int}$ population, resembling moMacs. $CD11c^{+}CD11b^{+}MHCII^{int}$ primarily give rise to $Ly6C^{-}CD115^{-}MHCII^{high}$ cells, suggesting these cells have moDC precursor activity and thus we refer to them as monocyte-derived Dendritic Precursor (moDP). Finally, cells with the phenotype $CD11c^{+}CD11b^{+}MHCII^{high}$ tended to maintain high MHCII expression, indicative of a DC phenotype.

Discussion:

Based on these findings, we propose that GM-CSF-driven differentiation of murine bone marrow cells in vitro proceeds through at least five distinct stages: Common Myeloid Progenitor (CMP), Granulocyte/Macrophage Progenitor (GMP), Monocytes, monocyte-Derived Macrophage/monocyte-derived Dendritic Precursor (moMac/moDP) and monocyte-derived DC (moDC) (**Figure 2.10**). Three of the stages of development are "transitional" indicating that by day 6 of culture they are absent or represent only a very small subset of the cells (CMP, GMP, Monocytes). The two dominant populations by day 6 represent differentiated cell types, the phenotypes of which are maintained long term (moMac and moDC). The moMac population was also found to contain a population of moDC precursors (moDP) that shares most phenotypic features with moMac. These cells were distinguishable only by their intermediate level of MHC class II on day 5 and their developmental plasticity (**Figure 2.9**). These data further demonstrate that these isolated populations have distinct expression profiles of key genes and phenotypic markers involved in myeloid and DC development, supporting the notion that they represent distinct stages of the developmental process driven by GM-CSF.

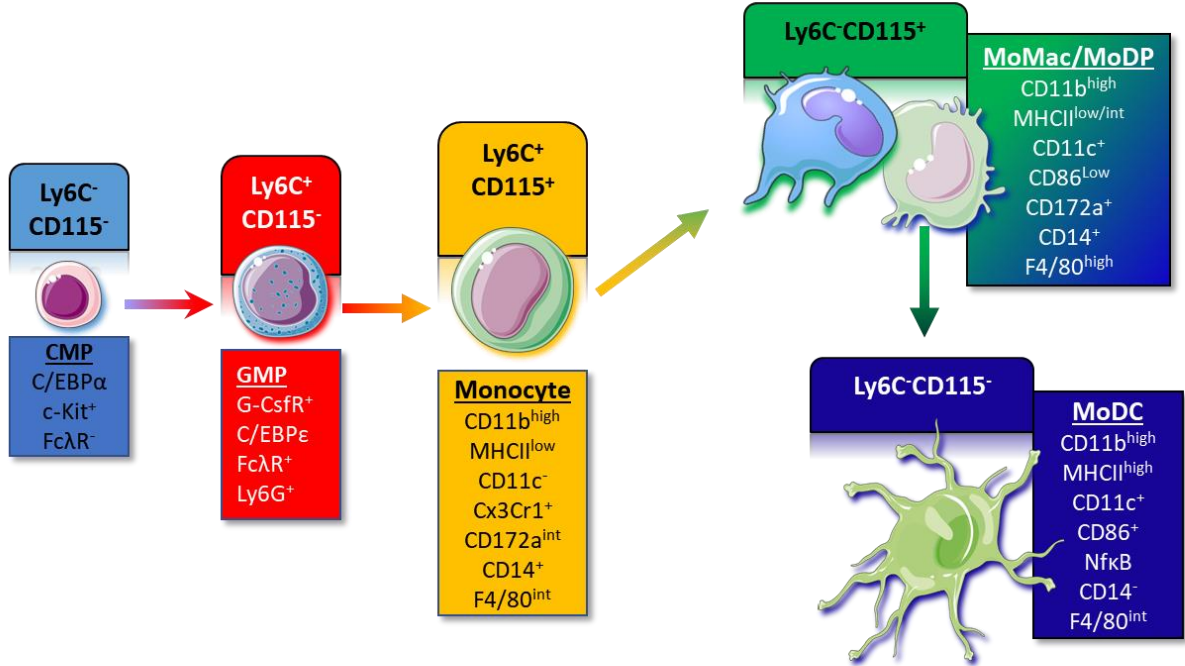

Figure 2.10. Comprehensive model of GM-CSF driven DC development. Transcriptional and phenotypic changes as cells progress through GM-CSF driven development. Common myeloid progenitor (CMP) gives rise to granulocyte/monocyte progenitor (GMP), followed by monocytes, and monocyte-derived macrophages (moMac). moMacs are maintained long term and share a phenotype with a precursor of monocyte-derived DC (moDC). This precursor has been termed monocyte-derived DC progenitor (moDP).

The first population, $Ly6C^-CD115^-CD11c^-$ cells correspond to a common myeloid progenitor (CMP) population based on their ability to give rise to all subsequent populations both in vitro and in vivo (**Figure 2.3, Figure 2.5)**, and their expression of early progenitor markers (Sca-1, c-kit, and CD34) and lack of FcγR [26]. The $Ly6C^+CD115^-$ population appears second in the developmental progression and displays phenotype typical of granulocyte monocyte/macrophage progenitor cells (GMP) based on their expression of CSF3R, CEBPe, Ly6G, and FcγR (**Figure 2.6)** [50].

The third population in the GM-CSF-driven developmental progression is representative of monocytes, expressing both Ly6C and CD115, as well as CX_3CR1. These cells were also the first to demonstrate expression of the macrophage markers, CD64 and F4/80, yet lacked MerTK expression. In contrast, these cells did not express a key marker of DC maturation and function, MHC class II. The gene expression pattern of monocytes was also quite distinct from both GMPs and moMac. Others have shown that during *Listeria monocytogenes* infection and other conditions in which GM-CSF is at high levels in circulation, $Ly6C^{high}$ monocytes differentiate into TNF/iNOS producing DCs (Tip-DC) [11,51,52]. However, new evidence suggests that Tip-DC respond more specifically to M-CSF than GM-CSF [53]. Nonetheless, we found that after TLR triggering, $Ly6C^+CD115^+$ cells were able to produce TNF-α and iNOS (unpublished data).

A recent study by Hettinger, et al., identified a common monocyte progenitor (cMOP) in mouse bone marrow that gives rise to monocytes and macrophages [19]. This population proliferated in response to GM-CSF as well as IL-3, and M-CSF [19]. While some of the phenotypic features of these cells were shared with specific populations identified in our cultures, there were several inconsistencies. As a progenitor cell type, cMOP express CD117 (c-

kit) and lack CD11b, an expression pattern exclusive to CMP in our system. However, cMOP also express CD115, Ly6C, and CX_3CR1, much like the monocytes identified in our system. Thus, cMOP do not directly correspond to any population identified in our cultures. One likely explanation for why these cells were not detected in our system is that cMOP were isolated from whole bone marrow while our cells were treated in culture with GM-CSF for several days, likely selecting for non-progenitor populations. cMOP are also a very rare population and could have been overlooked by our sorting strategy.

Following adoptive transfer of CMP in vivo, we sequentially recovered cells phenotypically resembling all of the developmental stages observed in vitro, with the exception of monocytes. We did however, recover a large number of moMacs by day 10 post transfer, suggesting that these cells might have rapidly transitioned through the monocyte stage, yet were not detected at the timepoints we tested. An alternative explanation is that cells at the monocyte stage of development migrated out of the peritoneal cavity. Thus, we examined the spleen, blood, and bone marrow for the presence of these cells, yet were not able to detect them (data not shown). In support of the notion that monocytes rapidly transition into DC, our in vivo data demonstrate that when cells at the monocyte stage were transferred, they rapidly underwent transformation into moMac and moDC in the recipient, such that virtually no monocytes are detectable after 48h (**Figure 2.5**). These findings are in line with other studies demonstrating the rapid differentiation (~18h) in vivo into DC or macrophages based on available space in the niche. Thus, the kinetics of monocyte differentiation in vivo appear to be more rapid than in vitro [54,55].

The fourth population, $Ly6C^{-}CD115^{+}$ (moMac), contains two populations; one precursor that gives rise to DC, and one macrophage-like cell that is maintained long term (**Figure 2.9**).

These results are generally consistent with a recent report by Helft, et al, with some minor differences [30]. They demonstrated that after six days culture in GM-CSF there were two $CD11c^{+}MHCII^{+}$ populations: one $CD115^{+}$ GM-MACs (similar to moMac), and a second $CD135^{+}$ GM-DC. These cells could also be distinguished based on level of MHC class II and CD11b. When we utilized CD11b and MHC II sorting strategy, we observed 3, not 2, populations: those with low, intermediate, or high level MHCII expression. The MHC^{low} cells reliably corresponded to moMac, retaining an intermediate MHC II expression. The MHC^{int} population demonstrated strong precursor activity, corresponding to moDP. Finally, the MHC^{hi} population maintained their phenotype, corresponding to moDC. While the level of expression of MHC class II serves as a strong predictive factor of cell type, determining the full extent of the differences in these three populations is the focus of ongoing studies. Notably, moMacs are also distinct from $Ly6C^{low}$ monocytes described in the literature in that they have intermediate basal levels of CD86 and have a larger morphology than monocytes (data not shown) [56].

Common dendritic cell precursors (CDPs) and pre-DCs have been identified as giving rise to conventional and plasmacytoid DCs respectively during development. We believe we have now identified a cell type driven by GM-CSF that shares many of phenotypic features of moMacs, yet acts as a precursor of moDC. moDP also share several phenotypic features with CDPs: both express $CD172a^{int}CD115^{+}$, but moDP are $CD11c^{+}$, more similar to pre-DCs. There has been little functional analysis of CDPs, but, unlike moDP, they have been observed to have low MHCII [24]. Unlike CDP and pre-DCs, moDP also express high levels of CD11b. Additionally, it has been previously shown that CDPs do not originate from monocytes [25,57]. Another recent study has demonstrated the presence of one or more monocyte derived DC precursors in the skin (P2MoDC) [58]. However, while both cell types are similar in

developmental status, moDP are distinctly different from P2MoDCs in regard to Ly6C, MHCII, and CD11c expression. There are likely several tissue-specific factors that dictate different phenotypes and functions in vivo that would not be observed in this in vitro system.

As expected, the hallmarks of DC differentiation (**Figure 2.6**) were most highly expressed in population 5, the moDC. Only the moDC expressed transcription factors typical of DC: Zbtb46, Irf4, Irf8, Batf3, as well as other DC-associated molecules such as Flt3, Ciita, Stat5a, and Stat5b. Expression of CD135 (Flt3) has been emphasized as indicative of dendritic cell phenotype or ontogeny [30]. Interestingly, we observed Flt3 expression at the transcript level but not at the cell surface. Perhaps since GM-CSF is the sole driving cytokine in this system, Flt3 expression is accessory to moDC development.

Collectively, these findings offer several novel insights as to the diversity of cell types present in GM-CSF-driven bone marrow cultures and the timing of their progression through the developmental program to become DC. Thanks to our sorting strategy, large numbers of cells can be isolated at each of these stages for further functional analysis. This represents a step forward not only in the study of murine DC differentiation, but likely can be adapted for therapeutic applications of human DC.

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Chapter 3

Generation of Large Numbers of Myeloid Progenitors and Dendritic Cell Precursors from Murine Bone Marrow Using a Novel Cell Sorting Strategy

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Abstract

Cultures of monocyte-derived dendritic cells (moDC) generated from mouse bone marrow using Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) have recently been recognized to be more heterogeneous than previously appreciated. These cultures routinely contain moDC as well monocyte-derived macrophages (moMac), and even some less developed cells such as monocytes. The goal of this protocol is to provide a consistent method for identification and separation of the many cell types present in these cultures as they develop so that their specific functions may be further investigated. The sorting strategy presented here separates cells first into four populations based on expression of Ly6C and CD115, both of which are expressed transiently by cells as they develop in GM-CSF-driven culture. These four populations include Common myeloid progenitors or CMP (Ly6C$^-$CD115$^-$), granulocyte/macrophage progenitors or GMP (Ly6C$^+$CD115$^-$), monocytes (Ly6C$^+$CD115$^+$), and monocyte-derived macrophages or moMac (Ly6C$^-$CD115$^+$). CD11c is also added to the sorting strategy to distinguish two populations within the Ly6C$^-$CD115$^-$ population: CMP (CD11c$^-$) and

moDC ($CD11c^+$). Finally, two populations may be further distinguished within the $Ly6C^-$ $CD115^+$ population based on the level of MHC class II expression. MoMacs express lower levels of MHC class II, while a monocyte-derived DC precursor (moDP) expresses higher MHC class II. This method allows for the reliable isolation of several developmentally distinct populations in numbers sufficient for a variety of functional and developmental analyses. We highlight one such functional readout, the differential responses of these cell types to stimulation with Pathogen-Associated Molecular Patterns (PAMPs).

Introduction

Culturing of murine bone marrow cells with the cytokine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is widely used as a method to generate monocyte-derived dendritic cells (moDC; also known as inflammatory DC) in large numbers [1-5]. These cells have been extremely useful in a variety of studies of dendritic cell (DC) function [6-8]. Typically, these murine bone marrow cells are cultured for 6-8 days and are then used for study of dendritic cell function [5]. These cultures had long been considered mostly homogenous, consisting of a majority of differentiated moDC. More recently, it has become clear that at the end of this 6-8 day culture period, there are indeed many moDC, as well as a large subset of differentiated monocyte-derived macrophages (moMacs) [9-11]. Our own studies have further extended these findings demonstrating that other subsets of less developed cells, such as moDC precursors (moDP) and monocytes, remain in the cultures at low frequency even after 7 days [10]. Thus, studies of DC function using cells generated by this system could reflect the responses of a broader cohort of cell types than previously appreciated.

We have learned a great deal from the study of GM-CSF-generated moDC relating to the function of these cells in the final stages of differentiation[12-14]. However, we understand significantly less about the developmental pathway of these cells[2,15,16] and of how and when they exhibit specific functions, such as responsiveness to Pathogen Associated Molecular Patterns (PAMPs), phagocytosis, antigen processing and presentation[13], and anti-bacterial activity. A protocol for isolation of large numbers of conventional Flt3L-driven DC progenitors and precursors has been reported[17]. Isolation of these distinct populations was achieved using carboxyfluorescein succinimidyl ester (CFSE)-stained bone marrow cells (to track dividing cells)

and culture in Flt3L for 3 days. Cells were then depleted of linage positive cells and sorted into progenitor and precursor populations based on CD11c expression[17]. Another approach by Leenen's group to identify early progenitors of DC in GM-CSF-driven culture was to sort cells based on CD31 and Ly6C [18]. Our initial goal was to create a similar method for obtaining progenitors and precursors of GM-CSF-driven moDC. Due to the specific cell types generated by GM-CSF, we adapted our approach and sorting strategy based on expression of molecules that were expressed at early and later stages of development. We ultimately determined that Ly6C, CD115 (CSF-1 receptor), and CD11c were the best markers for distinguishing these cell types[10]. Here, we present a method for isolation of cells at several distinct stages of development along the pathway of differentiation driven by GM-CSF: Common Myeloid Progenitor (CMP), Granulocyte-Macrophage Progenitor (GMP), monocyte, monocyte-derived Macrophage (MoMac) and monocyte-derived DC (MoDC). The moMac population can be further segregated based on level of MHC class II expression, revealing a moDC precursor population (moDP) [10]. We utilize a high-speed fluorescence-activated cell sorting (FACS) strategy to isolate these 5 populations based on expression of Ly6C, CD115, and CD11c. We then demonstrate the examination of these cells in functional assays revealing their responses to PAMP stimulation.

Method

All animal work was approved by the Auburn University Institutional Animal Care and Use Committee in accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

1. Preparation for Bone Marrow Collection

1.1. Prepare 250mL complete medium by adding a solution of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 µM 2-mercaptoethanol to the top of a 0.22 µm vacuum filter flask unit, and apply vacuum.

Note: Complete medium can be stored at 4 °C for up to 2 months.

1.2. Prepare 70% ethanol solution by mixing 350 mL of 100% ethanol with 150 mL sterile H_2O in a 500-mL flask.

1.3. Set centrifuge to 4 °C.

1.4. Sterilize forceps, scalpel, and scissors in 70% ethanol.

1.5. Using a serological pipette, add 5 mL of complete media to three 60-mm Petri dishes.

1.6. Using a serological pipette, add 30 mL of complete media to a 50-mL conical tube.

2. Collection of Murine Bone Marrow Cells

2.1. Euthanize C57BL/6 mouse by CO_2 narcosis in accordance with the rules established by the 2013 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

2.2. Remove and strip hind legs.

2.2.1. Saturate the hind legs and torso with 75% ethanol, and make shallow cuts through the skin around the hip joint with curved tissue scissors. Using forceps, firmly pull the skin from the hip down toward the ankle, revealing the muscle. Use scissors to remove the skin flap.

2.2.2. Remove the whole hind leg by cutting through the bone just above the femur/hip joint.

Note: In addition to sterilizing the area, the ethanol will aid in providing clean cuts and prevent hair from contaminated the samples.

2.2.3. If the legs will be transferred to a new location for bone marrow harvesting, submerge the legs in complete media.

2.3. Working in a sterile biosafety cabinet, transfer the legs to one of the previously prepared Petri dishes.

2.4. Use scissors to cut the just below the ankle, and carefully remove as much of the muscle and elastic connective tissue as possible. Transfer the cleaned bone to a fresh Petri dish.

Note: Although it is not necessary to remove all the muscle, too much remaining tissue can make it difficult to flush out the bone marrow.

2.5. Separate the femur, knee, and tibia.

2.5.1. Using forceps, hold the leg at the knee and locate the marrow.

Note: Bone marrow should be visible as a faint red line inside the bone cavity at the top of the femur and toward the end of the tibia.

2.5.1.1. Using scissors, make three cuts.

2.5.1.1.1. Cut the tibia just above where the marrow appears to end.

2.5.1.1.2. Cut just below the knee joint.

2.5.1.1.3. Cut just above the knee joint.

2.5.1.1.4. If the hip joint is still connected to the femur, cut just below the hip joint.

2.5.1.2. Return the three fragments to the Petri dish and repeat on other leg.

2.6. Flush bone marrow from femur and tibia.

2.6.1. Fill a 10-mL syringe with complete media from the 50-mL conical tube, and cap with 23-gauge needle.

2.6.2. Holding the bone with forceps above the third Petri dish, insert the needle into the bone canal and push media through, flushing out the cells. Repeat until no more color can be seen through the bone. Refill the syringe with media as necessary.

2.7. Crush the epiphyses.

2.7.1. Still in the second Petri dish, hold the knee cap firmly with forceps, and mash the knees with the tip of the syringe. Continue until the epiphyses are no longer red.

2.8. Using the syringe, transfer the cells from the second and third Petri dish to the 50-mL tube. Breakup up clumps by gently pipetting up and down. Try not to generate bubbles. Centrifuge at 250 x g at 4 °C for 10 min.

2.9. Lyse red blood cells

2.9.1. Remove supernatant with serological pipette, dislodge pellet by flicking, and lyse red blood cells by incubating in 1 mL of ACK (Ammonium-Chloride-Potassium) Lysis Buffer for 1 min at room temperature.

2.9.2. Using a serological pipette, add 40 mL of HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer.

2.10. Using a serological pipette, filter the cells though a 70 µm cell strainer into a new 50-mL conical tube. Centrifuge at 250 x g at 4 °C for 10 min.

2.11. Using a serological pipette, remove supernatant and wash cells with 40 mL of complete media. Centrifuge at 250 x g at 4 °C for 10 min.

Note: At this stage, lineage positive cells can be removed by FACS or magnetic column purification. However, lymphocytes are not maintained long term in culture. Although a large number of lineage positive cells are present in the $Ly6C^{-}CD115^{-}$ population, nearly all are absent by day 5 (**Figure 3.2**)

2.12. Using a serological pipette, remove the supernatant, and culture the bone marrow cells in complete media with 10 ng/mL of recombinant mouse GM-CSF at a density of 10^6 cells/mL.

Note: Typically, 4×10^7 total cells can be harvested after red blood cell lysis. However, expect as little as 2×10^7 for beginners and up to 5×10^7 cells for experienced harvesters.

2.13. Using a serological pipette, transfer the cells to tissue culture plates, and incubate at 37 °C in 5% CO_2.

2.14. Every 48 h, use a serological pipette to remove half of the media and replace with fresh complete media and GM-CSF.

Note: Cultures can be kept up to 9 days. However, composition changes over time. See step 3 for more information.

3. Choosing Day of Sort

3.1. Because population compositions change over time, select a day that yields the highest number of desired cells. **Table 3.1** shows the expected cell yield post sort for each of the populations after 3, 5, and 7 days of culture in GM-CSF per $1x10^7$ cells.

4. Staining strategy

4.1. Use small aliquots of cells to prepare control samples. These should include an unstained control, compensation control samples stained with only one fluorescent antibody each, and fluorescence-minus-one controls in which all antibodies are added except one, to control for non-specific fluorescence in that channel. If using indirect labeling, include primary alone, secondary alone, and both primary and secondary.

Note: Phycoerythrin (PE) and allophycocyanin (APC) tagged antibodies provide distinct separation with minimal bleed over when used together. However, if fluorochrome option are limited, CD115 is expressed at a relatively low level, while Ly6C is expressed at very high levels. Therefore, brighter fluorochromes are desirable for anti-CD115 and anti-Ly6C fluorochromes are chosen to prevent bleed over.

4.2. Prepare 100 mL of FACS wash buffer (FWB) by mixing 97 mL of chilled Dulbecco's phosphate buffered saline (DPBS) with 3 mL of fetal calf serum in a 50-mL conical tube, and place in ice bath.

4.3. Use a pipette to gently, but thoroughly, pipette cells up and down to dislodge any loosely adherent cells.

4.4. Using a serological pipette, transfer cells to a 50-mL conical tube. Centrifuge at 250 x g at 4 °C for 10 min.

4.4.1. If cell volume exceeds 50 mL, transfer cells to the necessary number of tubes and combined at the staining step.

4.5. Gently pour off the supernatant, and wash the pelleted cells by adding 30 mL of FWB with a serological pipette. Centrifuge at 250 x g at 4 °C for 10 min, and repeat wash.

Note: If high cell death is expected, cells can be washed with FWB with as low as 0.5% fetal calf serum (FCS). This will prevent cell clumping.

4.6. Suspend and stain cells per antibody manufacturer's instructions.

4.6.1. Suspend 5×10^7 cells in 1 mL of FWB and add 2 μg each of anti-Ly6C and anti-CD115 in the fluorophores of your choice. **Figure 3.3** was generated using Ly6C-PE and CD115-APC.

4.6.2. To further distinguish CMP from moDC (both are Ly6C$^-$ and CD115$^-$), add 2 μg of anti-CD11c antibodies (CMP are CD11c$^-$; moDC are CD11c$^+$).

4.6.3. Incubate for 30 min on ice.

4.7. Using a serological pipette, add 10 mL of FWB, and centrifuge at 250 x g at 4 °C for 10 min.

4.8. Gently pour off the supernatant, and wash the pelleted cells by adding 30 mL of FWB with a serological pipette. Centrifuge at 250 x g at 4 °C for 10 min, and repeat wash.

4.9. Before suspending cells, flick tube thoroughly to dislodge the pellet. Use a serological pipette to suspend cells at 10^7 cell/mL of FWB, and filter through 35 µm cell filter.

4.10. Use a serological pipette to transfer filtered cells into polypropylene tube, and place on ice until ready to sort.

Note: If polypropylene is unavailable, protein coated (nonfat dry milk or FCS) polystyrene tubes can be used to reduce binding.

5. Set Gates Based on Control Samples

Note: To prevent cell disruption due to the pressure of the high-speed flow stream, use a 100-130 µm nozzle for cell sorting.

5.1. Run the unstained control through the cell sorter, and apply a gate excluding small debris (low forward scatter; FSC) and highly granular (high side scatter; SSC) particles.

5.1.1. If only analyzing later stages (monocytes, moMac/MoDP, and MoDC), apply gating to only include larger cells (high FSC).

5.1.2. If viability stains are being used, use these to exclude stained, non-viable events (an example is illustrated in **Figure 3.1**).

5.2. Run the single fluorescent control samples through the cell sorted, and adjust compensation as needed.

5.3. Run a sample of the multi-labeled sample. Four distinct populations should be clear: $Ly6C^+CD115^-$ (GMPs), $Ly6C^+CD115^+$ (monocytes), $Ly6C^-CD115^+$ (moMacs/moDP), and $Ly6C^-CD115^-$ (CMPs/moDCs). Apply a gate to isolate each of the major four populations.

Note: CMPs and moDC share their $Ly6C^-CD115^-$ phenotype. However, they can be differentiated based on CD11c expression: CMP lack CD11c, whereas MoDCs express CD11c.

6. Collection of Isolated Populations

6.1. Prepare collection tubes by adding enough FCS to achieve at least 20% final concentration when full. For example, if using 5 mL tubes, add 1 mL of FCS before sorting, and remove the tube when it reaches 5 mL total volume.

6.2. To prevent membrane turnover and antibody uptake, keep all samples (mixed and sorted) at 4 °C throughout the sort.

6.2.1. If this is not possible, keep the tube containing the cells to be sorted on ice as much as possible and transfer aliquots to the sorter as needed.

6.2.2. Additionally, transfer sorted samples to ice every 20-30 min.

6.3. After the desired number of cells has been collected, use a serological pipette to transfer the cells to a new conical tube. Centrifuge at 250 x g at 4 °C for 10 min.

6.3.1. Confirm purity with post-sort analysis on small aliquots from each collected population.

6.4. Remove the supernatant, suspend in 10 mL of FWB and centrifuge at 250 x g at 4 °C for 10 min. Repeat for a total of two washes.

Note: It can be difficult to remove all the supernatant without dislodging the pellet. Attaching a pipette tip to a vacuum line can help with removal. If this is not available, it is suggested that the supernatant be collected in a fresh tube in case of pellet dissociation.

6.5. Remove the supernatant after the second wash.

6.6. If user's experimental design dictates the cells be re-cultured, follow steps 2.12 - 2.14. Otherwise, if cells will be used for immediate analysis, prepare cells according to desired protocol. An example of a typical functional analysis is included as **Figure 3.4**.

Representative Results

In effort to keep as many channels available for analysis as possible, viable cells were routinely selected based on forward and side scatter, excluding very small and very granular events (a typical gate is applied to all the dot plots in **Figure 3.1A**). To determine if this gating strategy reliably excluded dead cells, we stained with 7-AAD (7-Amino actinomycin D) (**Figure 3.1B**). 7AAD stains DNA in dead and dying cells due to membrane permeability, and is excluded by viable cells. Viability of GM-CSF cultured bone marrow cells was analyzed immediately post-harvest (PH) and 1, 3, and 5 PH. Cell analyzed immediately PH had approximately 10% of 7-AAD positive cells were present when a typical FSC/SSC gate was applied to freshly isolated cells from the bone marrow (**Figure 3.1**, Post-Harvest), and similar proportion of dead cells was also present at day 1 (~12%) and day 3 (~11%) of culture (**Figure 3.1**, 1 day PH and 3 days PH). By day 5, the number of dead cells within the gate was much reduced to ~5% (**Figure 3.1**, 5 days PH). Thus, using such a viability gate is generally appropriate for sorting on day 5 and after. Therefore, if users are limited in their available parameters, FSC/SSC gating is generally appropriate. However, for more sensitive assays (particularly if they rely on precise cell numbers), we suggest incorporating a viability stain.

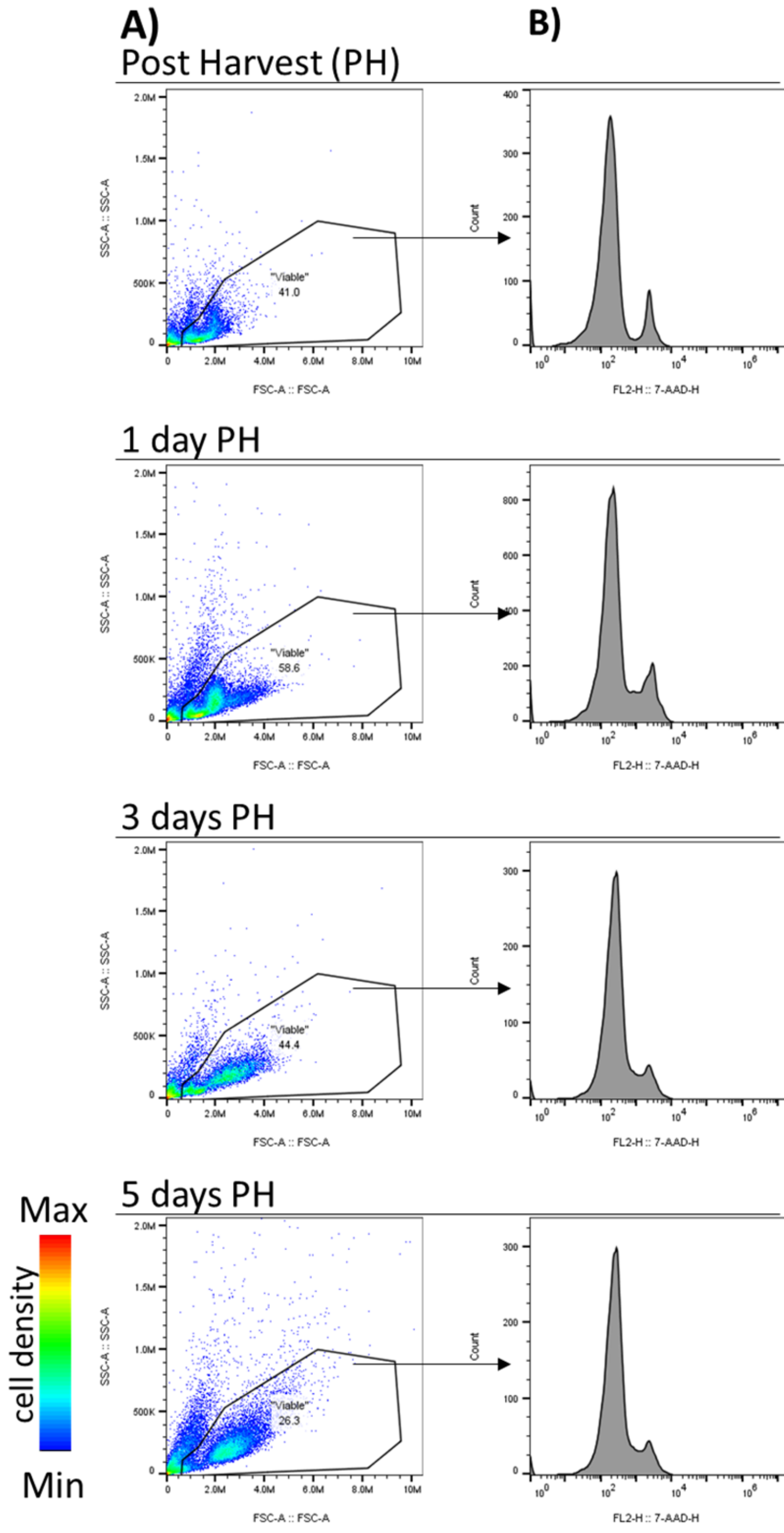

Figure 3.1: Viable cells within Forward vs. Side Scatter gate. Mouse bone marrow was cultured in GM-CSF, and viability was measured using 7-AAD (7-amino-actinomycin D) staining post-harvest (PH) and 1, 3, and 5 days post-harvest. (**A**) Viable cells were selected by applying a gate (Viable) which omitted small and highly granular events based on FSC and SSC. (**B**) Histograms of 7-AAD staining generated from events within Viable (FSC/SSC) gate. Events positive for 7-AAD indicate cells undergoing apoptosis. Arrows indicate Viable gating.

Many protocols for propagation of dendritic cells recommend depletion of lineage positive cells, especially lymphocytes, from bone marrow prior to culture[11,17]. This procedure is thought to increase the purity of the cells recovered upon GM-CSF-mediated differentiation. Typically, cells expressing markers of T cells (CD3), B cells (CD45R or CD19), and NK cells (NK1.1) may be depleted by positive selection using magnetic beads or cell sorting[11,17,18]. However, based on our culturing system, lymphocytes were rarely recovered or detected in our assays. Thus, we sought to assess the longevity of lymphocytes maintained in the Ly6C$^-$CD115$^-$ population among the GM-CSF-driven cells **(Figure 3.2A)**. GM-CSF cultured bone marrow cells (that had not been lineage depleted) were stained with antibodies to CD3, CD45R, and NK1.1 (in the same fluorophore) and measured daily by flow cytometry (**Figure 3.2B**). Within the Ly6C$^-$CD115$^-$ population, CD3/CD45R positive cells persisted strongly through Days 0 – 3 (**Figure 3.2B**). On Day 4, only a few CD3/CD45R positive cells remained, and by Day 5 and 6, there were no CD3/CD45R expressing cells present. Thus, within 4 days of culture in GM-CSF, lineage positive cells were essentially absent and were not detected at all at days 5 and 6 of culture.

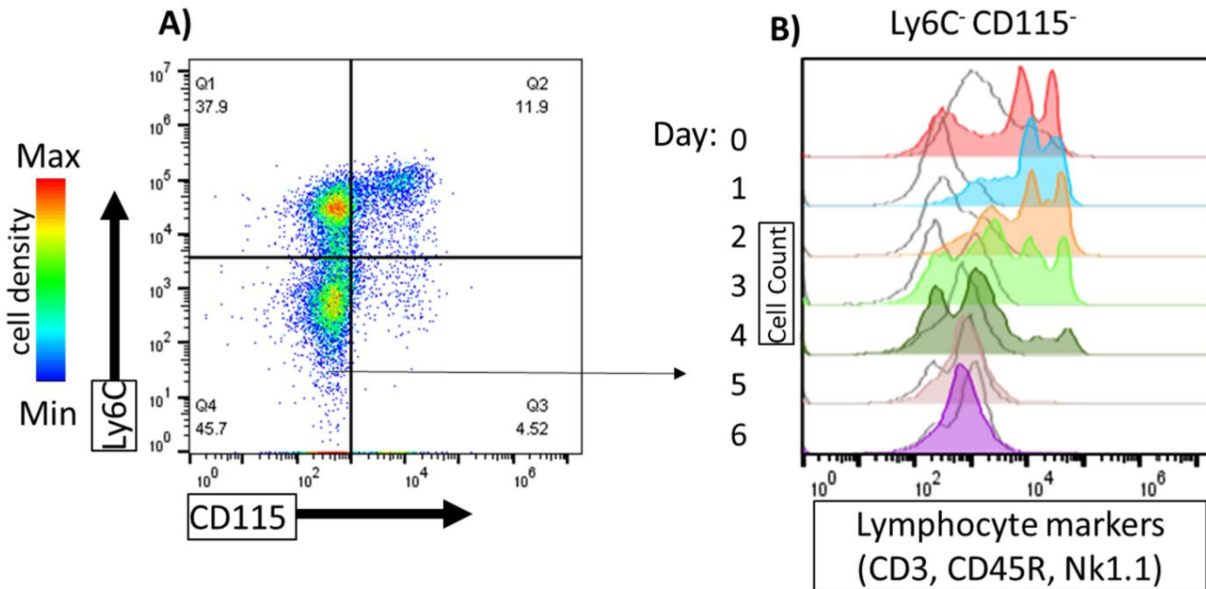

Figure 3.2: Lymphocytes within the Ly6C⁻CD115⁻ population. Mouse bone marrow was cultured in GM-CSF and lymphocyte markers (CD3, CD145R, and NK1.1) were analyzed daily by flow cytometry. (**A**) Cell were stained with Ly6C-PE and CD115-APC to identify Ly6C-CD115- cells. Quadrant gate applied based on single-color controls. Pseudocolor dot plot generated from day 0 (**B**) CD3, CD45R, and NK1.1 expression of Ly6C⁻CD115⁻ cells was analyzed on day of harvest (day 0) until day 6. Cell counts were normalized to mode using FlowJo. Arrow indicate Ly6C⁻CD115⁻ gating.

The composition of the GM-CSF-driven cell culture changes daily in this system as the cells develop and differentiate (**Table 3.1**; **Figure 3.3**). At early time points, the most abundant cells are progenitors and precursors, and at later times the majority of cells are more differentiated[10]. To determine how sorting on different days of culture might affect the subsequent developmental path or kinetics, sorts were performed 3, 5, and 7 days PH (**Figure 3.3A**). The development of the MoMac population ($Ly6C^{-}CD115^{+}$) was then tracked over 2-3 further days in culture (**Figure 3.3B-3D**).

When sorted 3 days PH, only 40% of $Ly6C^{-}CD115^{+}$ cells had decreased CD115 expression within 24 h post-sort (PS) (**Figure 3.3B**). By 48 h PS, the fraction that had down-regulated CD115 was 66%, and by 72 h, 70% of the cells had this phenotype. This phenotypic composition was maintained (~70-72% $Ly6C^{-}CD115^{-}$) even after further days of culture (data not shown). When sorted 5 days PH, ~75% of the cells were $Ly6C^{-}CD115^{-}$, having rapidly down-regulated CD115 within 24 h PS, and ~80% were $CD115^{-}$ after only 48 h. This distribution was maintained after 72 h (**Figure 3.3C**). Finally, when sorted 7 days PH, down-regulation of CD115 was also quite rapid. Within 24 h PS, ~75% of cells had down-regulated CD115 expression, and this trend was maintained after 48 h (**Figure 3.3D**). Interestingly, when sorted at day 7, the overall level of CD115 expression was lower on the cells within the $CD115^{+}$ population.

Thus, these findings indicate that the kinetics of development are somewhat slower when sorting at an early day, such as day 3 sorted cells compared to the more rapid development and differentiation observed after sorting on day 5 or 7. Based on these results, a user seeking larger numbers of moDC should likely sort on day 5 or 7.

		Day 3		Day 5		Day 7	
Phenotype	**Cell type**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
$Ly6C^-CD115^-CD11c^-$	CMP	$3x10^6$	$4x10^6$	$5x10^5$	$1x10^6$	N/a	N/a
$Ly6C^+CD115^-$	GMP	$2x10^6$	$3x10^6$	$2x10^6$	$3x10^6$	N/a	N/a
$Ly6C^+CD115^+$	Mono	$2x10^6$	$3x10^6$	$2x10^6$	$3x10^6$	$5x10^5$	$1x10^6$
$Ly6C^-CD115^+$	MoMac	$5x10^5$	$1x10^6$	$3x10^6$	$4x10^6$	$3x10^6$	$4x10^6$
$Ly6C^-CD115^-CD11c^+$	MoDC	N/a	N/a	$5x10^5$	$1x10^6$	$3x10^6$	$4x10^6$

Table 3.1: Expected minimum and maximum number of cells recovered post-sort per $1x10^7$ cells. CMP (common myeloid progenitor); GMP (granulocyte/macrophage progenitor) Mono (monocytes); MoMac (monocyte-derived macrophage); MoDC (monocyte-derived dendritic cell); N/a (not available); Min (minimum cell yield out of $1x10^7$ cells); Max (maximum cell yield out of $1x10^7$ cells.)

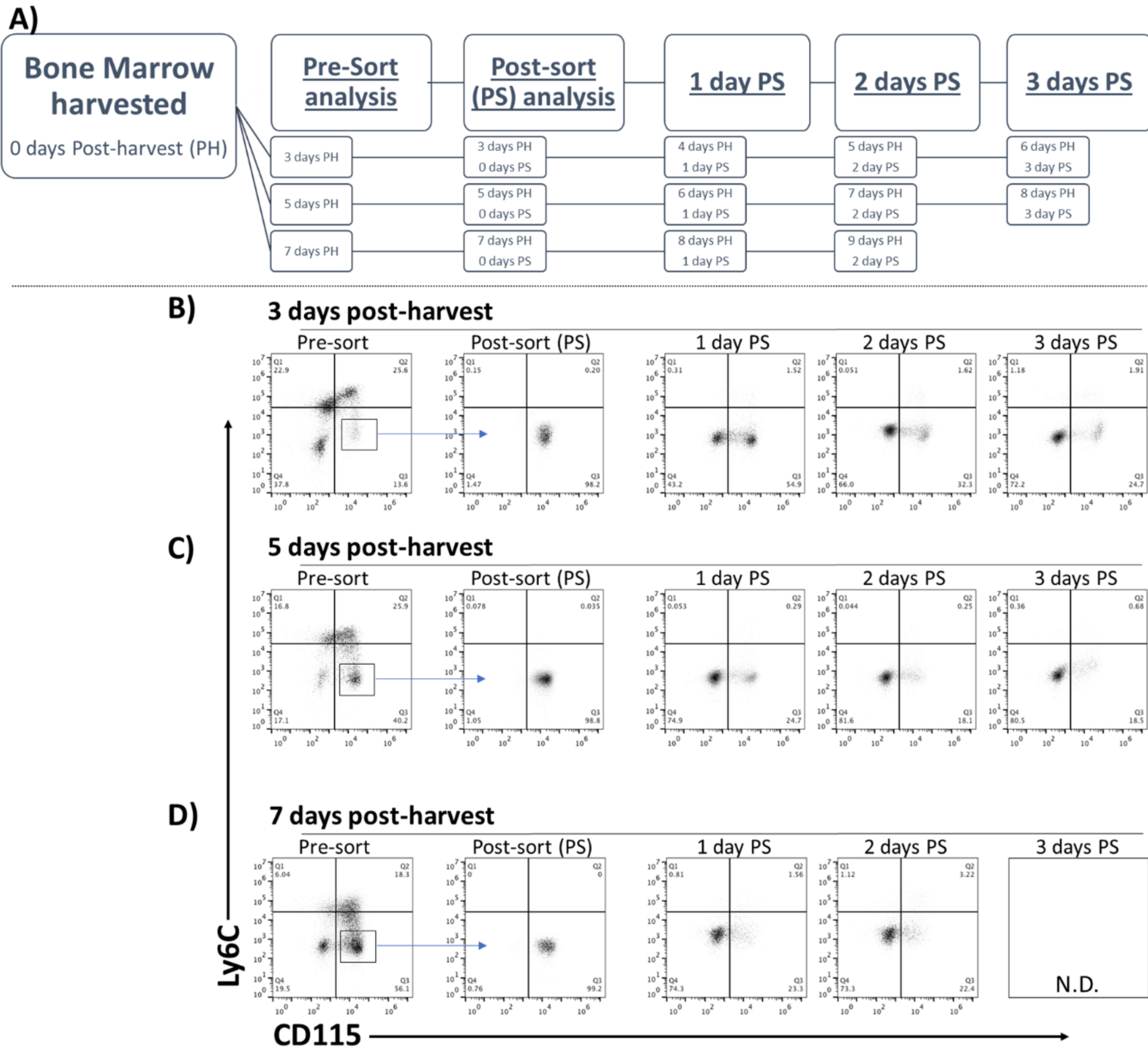

Figure 3.3: Kinetics of development of Ly6C⁻CD115⁺ cells after sorting on different days. (**A**) Mouse bone marrow was harvested and cultured in GM-CSF. Aliquots of $1x10^7$ cells were harvested (**B**) 3, (**C**) 5, or (**D**) 7 days post-harvest (PH). On the indicated days PH, $Ly6C^-CD115^+$ cells were sorted from mixed culture (Pre-sort) and analyzed immediately post-sort (PS). Sorted cells were re-cultured in GM-CSF, and changes in Ly6C/CD115 expression were analyzed daily by flow cytometry. Box and arrow indicate sorting gate.

The maturation response of DC is well established [6,7,12,14]. When treated with a variety of pathogen-associated molecular patterns (PAMPs), immature DC up-regulate the expression of MHC, costimulatory molecules, and pro-inflammatory cytokines, enhancing their T cell-activating capacity[6]. However, it is less clear when developing cells gain the capacity to respond to PAMP stimulation and which feature of DC maturation they might exhibit. To determine each of the sorted populations' response to maturation stimuli, each population was treated shortly after sorting with a cocktail of PAMPs including Poly I:C, LPS, and CpG DNA to trigger TLR3, TLR4, and TLR9, respectively. Cells were treated (or untreated) for 24 h and expression of CD86 and MHCII was measured by flow cytometry **(Figure 3.4A-4B**). Furthermore, IL-12p40/70 and IL-6 production was measured in the supernatants by cytokine array ELISA (**Figure 3.4C-4D**).

CMPs and GMPs expressed very low levels of MHC class II and CD86 in an unstimulated state, and these expression patterns did not change significantly upon exposure to the cocktail of PAMPs (**Figure 3.4A** and **3.4B**). Likewise, the expression of MHC class II by the monocytes was also low and changed little upon PAMP exposure (**Figure 3.4A**). However, the expression of CD86 was moderate on the monocytes 24 h after sorting, and it increased further following 24 h stimulation. We observed higher basal expression of MHC class II and CD86 in the MoMacs and MoDCs, and both populations exhibited a strong induction of these molecules upon PAMP stimulation. In terms of MHC class II expression following stimulation, there was no statistical difference between the CMPs, GMPs, and monocytes, while the MoMacs and MoDCs formed a distinct group. Yet, in terms of CD86 expression, the CMPs and GMPs cells were statistically different than MoMacs and MoDCs. However, monocytes were not different than MoMacs or MoDCs.

We next wanted to assess the relative ability of each of the five populations to produce cytokines in response to TLR stimulation. Thus, we performed a cytokine array on the sorted populations after culture in the presence or absence of the TLR agonist cocktail used above. Cells were cultured with the stimuli for 24 h, and supernatants were collected. We observed very little IL-12p40/70 or IL-6 production by CMPs upon TLR stimulation (**Figure 3.4C-4D**). The second population, GMPs, were unable to produce IL-12p40/70 (**Figure 3.4C**), but these cells produced low levels of IL-6 upon stimulation by PAMPs (**Figure 3.4D**). The highest levels of cytokine production were observed in the latter three populations. Monocytes and MoMacs had very similar patterns and magnitudes of cytokine production. Both greatly increased IL-12p40/70 and modestly increased IL-6 production up stimulation (**Figure 3.4C-4D**). Interestingly, in the presence of PAMPs MoDCs did not increase IL-12p40/70 secretion; however, this population greatly increased IL-6 production (**Figure 3.4C-4D**). These findings indicate that the sorted populations maintained their immunological functions after isolation.

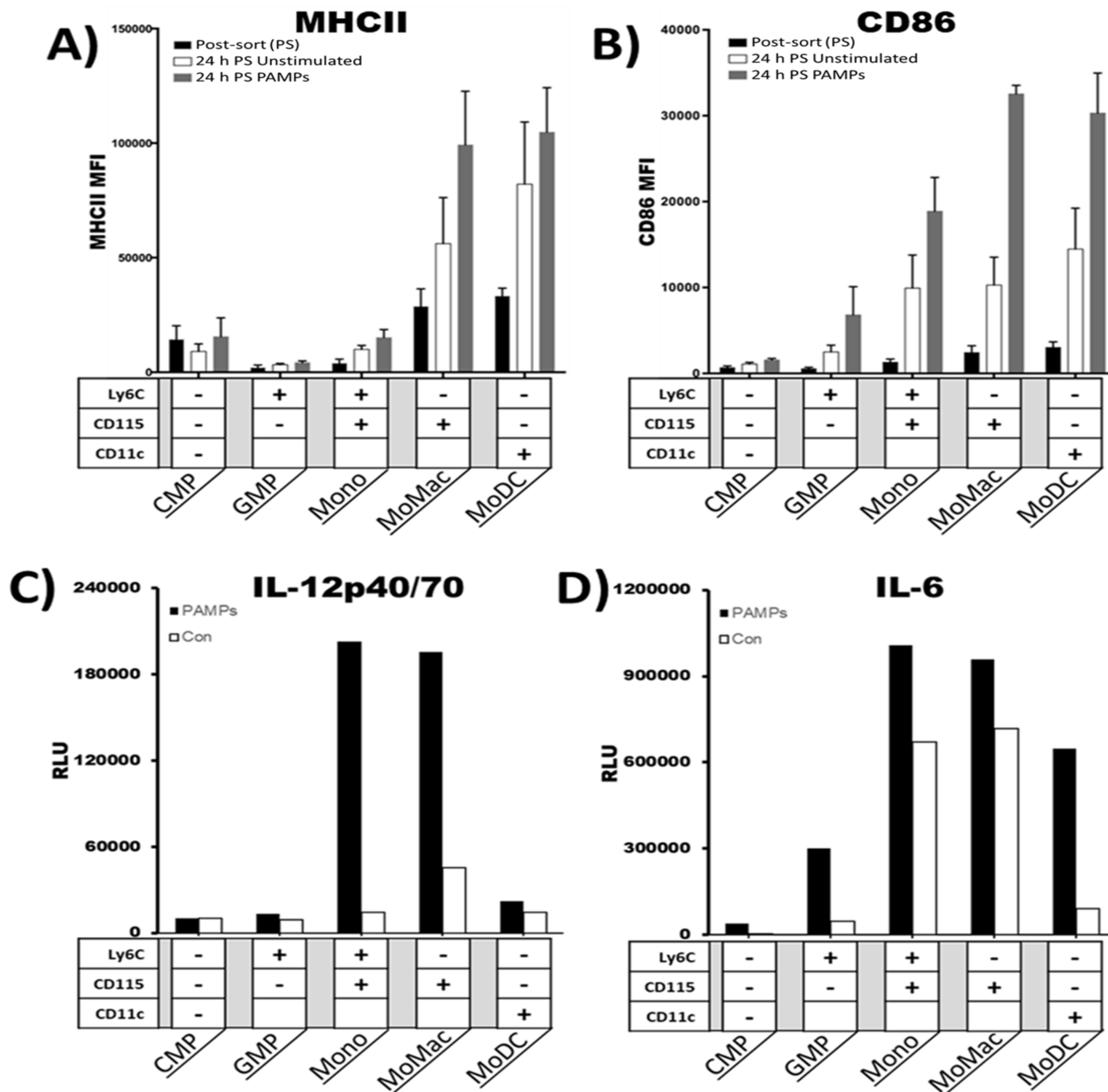

Figure 3.4: Maturation and cytokine expression following TLR stimulation. Cells were sorted into 5 populations on day 3 of culture in GM-CSF. They were then treated with a cocktail of PAMPs (LPS, Poly I:C, and CpG DNA) for 24 h. Mean fluorescent intensity (MFI) of (**A**) MHC class II and (**B**) CD86 was analyzed immediately post-sort (PS) and 24 h with and without PAMPs by flow cytometry. Error bars represent standard deviation of 3-5 replicate experiments. (**C**) IL-12p40/70 and (**D**) IL-6 in supernatants from 3 pooled samples were measured by cytokine array dot blot ELISA after 24 h with and without PAMPs. RLU; Relative light units; -/+ indicate the presence of the cell surface marker for the designated population.

Discussion

This protocol facilitates isolation of GM-CSF-driven progenitor and precursor cell types in numbers sufficient for several types of analyses including biochemical assays, assays of cellular function in vitro, or instillation in vivo. This method represents a significant advance in the field of monocyte-derived dendritic cell development, enabling the reliable isolation and identification of cells early in this pathway of development as well as those differentiated cell types more commonly isolated in prior studies.

Previous protocols for isolation of progenitors and precursors generated from bone marrow in vitro have relied on CFSE-staining to identify proliferative progenitor cells[17] or on staining with CD31 and Ly6C as markers of myeloid cells[18]. The CFSE staining protocol described by Naik was designed for use in generation of Flt3L-driven DC progenitors and precursors[17]. When we attempted this approach in the GM-CSF culture system, we encountered two main issues. The CFSE was somewhat cytotoxic to the developing cells and was so bright (even in divided cells) that it made compensation difficult. This approach proved to be unsuitable for our goals of isolating early cell types (progenitors), as well as cells across the developmental spectrum, from highly proliferative (CMPs/GMPs) to highly developed (MoDC/MoMac). We also tried the sorting strategy for GM-CSF-driven cells described by Leenen's group which was based on CD31 and Ly6C[18]. However, we found that CD31 was problematic. It was expressed at very low levels (making it difficult to resolve the populations for clean sorting) by a vanishingly small subset of cells and only very early during the culture period. In fact, CD31 expression was not detectable past day 2 of culture in GM-CSF[10].

Ly6C, however, was a very useful molecule for separating cells at different stages of development due to its transient pattern of expression[10]. The addition of CD115 enabled us to more closely discern cells at intermediate and later stages of development that was not possible with CD31. We also examined other markers of progenitors such as CD34 and CD117 in hopes of isolating large numbers of these early cells[10]. However, unlike that reported in the Flt3L system, we found that CD34 and CD117 were also expressed on a very small subset of cells and were virtually absent by day 3 of culture[17]. However, these stem cell markers may be useful in future studies to distinguish subsets within the CMP or GMP populations.

There are several potential modifications to the protocol that may affect the yield of desired cell populations. First, the user may choose to apply a viability stain to exclude any dead cells. Based on our observations, the rate of dead cells within a typical forward and side scatter gate are relatively low in general, and only a problem during the first days of culture, coincident with decreases in lineage positive lymphocytes. However, for applications in which cell numbers must be precise, a viability stain will ensure exclusion of dead cells.

A second potential modification is depletion of lineage-positive lymphocytes prior to culture. We have tried this approach to address the small population of lineage positive cells that we regularly observed within the Ly6C^{-}CD115^{-} cell population. We observed that the overall cell yield was slightly lower at days 5 and 6, and the purity was not significantly higher (data not shown). Thus, the purity did not justify the reduced yield. Likewise, if the user plans to sort on day 5 or after, the frequency of lymphocytes within the culture is well below 1% and should not present a problem.

Finally, because this strategy is designed to isolated cells across a large developmental spectrum the user should tailor the timing of their sort to collect as many of the desired

populations as possible. This sorting strategy faithfully yields the cell types indicated regardless of the day of culture on which the cells are sorted. For example, cells with the phenotype $Ly6C^{+}CD115^{-}CD11c^{-}$ are true to the GMP phenotype and function similarly whether they are sorted and isolated on day 3 or 5 of culture. However, the frequency of these cells is much greater on day 3 than 5, so if the goal is isolation of this cell type, sorting at day 3 would be recommended. It is also notable that cells sorted on day 3 progress through the subsequent developmental stages with slower kinetics than if they were sorted on day 5 or 7 (**Figure 3.3**).

While our method allows for the clear delineation and isolation of 5-6 distinct populations along the developmental spectrum, there are likely many more populations or transitional stages along this pathway. Within each of the five described populations there may be several sub-populations at slightly different increments of development. For example, we have observed “distinct” populations with intermediate levels of CD115 and of Ly6C which undergo slightly different patterns of development, in terms of how many moMac and moDC are generated (data not shown). It is also likely that populations previously observed in vivo are present in our culture and sorting system, but these have been difficult to identify due to their very low frequency. Populations such as cMOP[19], MDP[20], or CDP[21] are likely present, but may be obscured within the larger populations. Future studies will be needed to further clarify the numerous developmental stages that may be isolated within this sorting framework with the addition of specific markers of differentiation. The value of this sorting strategy is that it allows consistent isolation of large numbers of developmentally distinct stages during DC ontogeny.

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Chapter 4

Conclusions

There is a limitation in our understanding of inflammatory cell development stemming from an overall lack of well-established models for isolating quantities of cells large enough to be useful for research and clinical applications. Due to the short life-span and overlapping phenotype of inflammatory dendritic cells (DCs), many functional studies rely on attempts to draw direct analogies between steady-state driven development (which has been well-characterized) and inflammatory driven development.

While the data generated from steady-state development serves as an invaluable foundation in guiding our understanding of inflammatory DC development, it is critical that there are mechanisms for studying these cells and their precursors directly. Much of the work being conducted within the inflammatory models is severely limited regarding functional analysis, largely due to an inability to generate the quantities of the cells necessary for effective analysis. Additionally, as new cell types are discovered, the identification strategies are becoming more complex, requiring extensive analysis simply for identification. The combination of low cell yield and heavy preparatory analysis severely limits down steam analysis, resulting in a large

collection of data identifying various cell types and development pathways but lacking significant functional interpretation.

The overall purpose of this study was to develop a strategy for identifying and isolating each of these major stages in inflammatory DC development. This model takes advantage of the well-established GM-CSF culture model which has historically been used to generate large quantities of fully differentiated inflammatory DCs. This will provide a major framework for further work in subtyping the cells in myeloid development.

We hypothesized that stimulation with GM-CSF results in bone marrow cell developing along a pathway with major distinct stages that can be identified using traditional myeloid markers. Our findings suggest that five distinct stages can isolated from GM-CSF stimulated bone marrow cells which share cell surface markers and gene expression patterns similar to stages observed in vivo (CMP, GMP, monocytes, moMacs, and moDCs). Large quantities of cells at these stages are easily identified and isolated based on Ly6C and CD115 expression, and functionality is retained even in isolation.

Identification of Five Developmentally Distinct Stages

When murine bone marrow cells are stimulated with GM-CSF, expression of Ly6C, CD115, and CD11c was used to identify five developmentally distinct stages in linear developmental pathway: $Ly6C^{-}CD115^{-}CD11c^{-}$, $Ly6C^{+}CD115^{-}$, $Ly6C^{+}CD115^{+}$, $Ly6C^{+}CD115^{+}$, and $Ly6C^{-}CD115^{-}CD11c^{+}$ (**Figure 2.10**). Through analysis of phenotypes, developmental kinetics, and functional capacity, we found these cells corresponded to the common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), monocytes, monocyte-derived macrophages (moMacs), and monocyte-derived DCs (moDCs). Within the moMac populations, we have identified the presence of a moDC precursor. These results are a significant

advancement in our understanding of GM-CSF driven DC development as they provide a framework for reliably identifying and isolating large quantities of various stages in development.

Table 4.1 Gene Expressed in Ly6C^{-} CD115^{-} CD11c^{-}	
Gene	**Stage relevant function**
Gfi1	Control self-renewal, quiescence, and differentiation of progenitor cells.[1]
Klf4	Required for monocyte lineage development[2,3]
Cebpa	Required for self-renewal and DC development[4,5]
Pecam1	Adhesion molecule which prevents progenitors from leaving bone marrow[6]
Irf2	Transcription factor required for myeloid DC development[7]
Kit	Receptor for Stem Cell Factor, a cytokine critical in maintaining the stem cell niche[8,9]

Ly6C$^-$CD115$^-$CD11c$^-$

This population can be found upon analysis of freshly harvested bone marrow (day 0) (**Figure 3.2, Figure 4.1**). They expressed cell surface markers associated with very early progenitors (Sca-1, c-kit and CD34) and gene expression patterns typical of early myeloid committed cells (Gfi1, Cebpa, Pecam1, and cKit), as well as genes associated with monocyte/DC committed development (Klf4 and Irf2) (**Figure 2.6, Table 4.1, Figure 4.1**).

These phenotypic patterns indicate that Ly6C$^-$CD115$^-$CD11c$^-$ cells closely correspond to Common Myeloid Progenitors (CMPs). Importantly, these cells lack expression of CD11b (an intermediate myeloid stage marker) and CD16/32 (i.e. FcγR; a cell surface marker found on all myeloid populations except the CMPs) (**Figure 2.6, Figure 4.1**). This population has an overlapping phenotype (Ly6C$^-$CD115$^-$) with the last stage observed -- monocyte-derived DCs (moDCs). CMPs and moDCs can be resolved based on forward scatter properties: CMP have low forward scatter (similar to that attributed with lymphocytes) due to their relatively small size, and moDCs have high forward scatter properties (similar to that attributed to monocytes) (**Figure 3.1A**).

Additionally, this population's identity is supported by their ability to give rise to all subsequent populations (**Figure 2.3D, Figure 4.1**). Upon isolation, this population underwent extensive proliferation and delayed differentiation (relative to the other discussed developmental stages and whole bone-marrow) (**Figure 2.3, Figure 2.2**). When Ptprc^b (CD45.1) CMPs were co-cultured with wild-type CD45.2 feeder cells, differentiation of CD45.1 cells progressed at a normal rate (**Figure 2.5B**). This suggests that these cells self-renew and delay their development until a cellular threshold is reached.

Interestingly, when CMPs were adoptively transferred via intraperitoneal injection, almost no Ly6C$^+$CD115$^+$ (which correspond to monocytes; discussed below) where recovered (**Figure 2.5E**). While this may suggest CMPs do not give rise to monocytes *in vivo*, this conclusion would contradict a significant volume of published research[10–15]. When Ly6C$^+$CD115$^+$ were transferred using the model described above, they quickly downregulated their Ly6C expression (**Figure 2.5G**). We believe the lack of recoverable Ly6C$^+$CD115$^+$ cells from CMPs is due to this fast down-regulation.

This population exhibited no changes in maturation or cytokine expression following a broad range of pathogen-associated molecule patterns (PAMPs) (**Figure 3.4, Figure 4.1**). Interestingly, these cells have very low MHCII mean fluorescent intensity (MFI), but, while the MFI did not change when stimulated, it is higher in CMPs than the following two stages. This inconsistency is likely due to a small portion of contaminating B cells and their precursors[16]. However, in a GM-CSF stimulated culture, this lymphoid populations not maintained longer than 4 days (**Figure 3.2**).

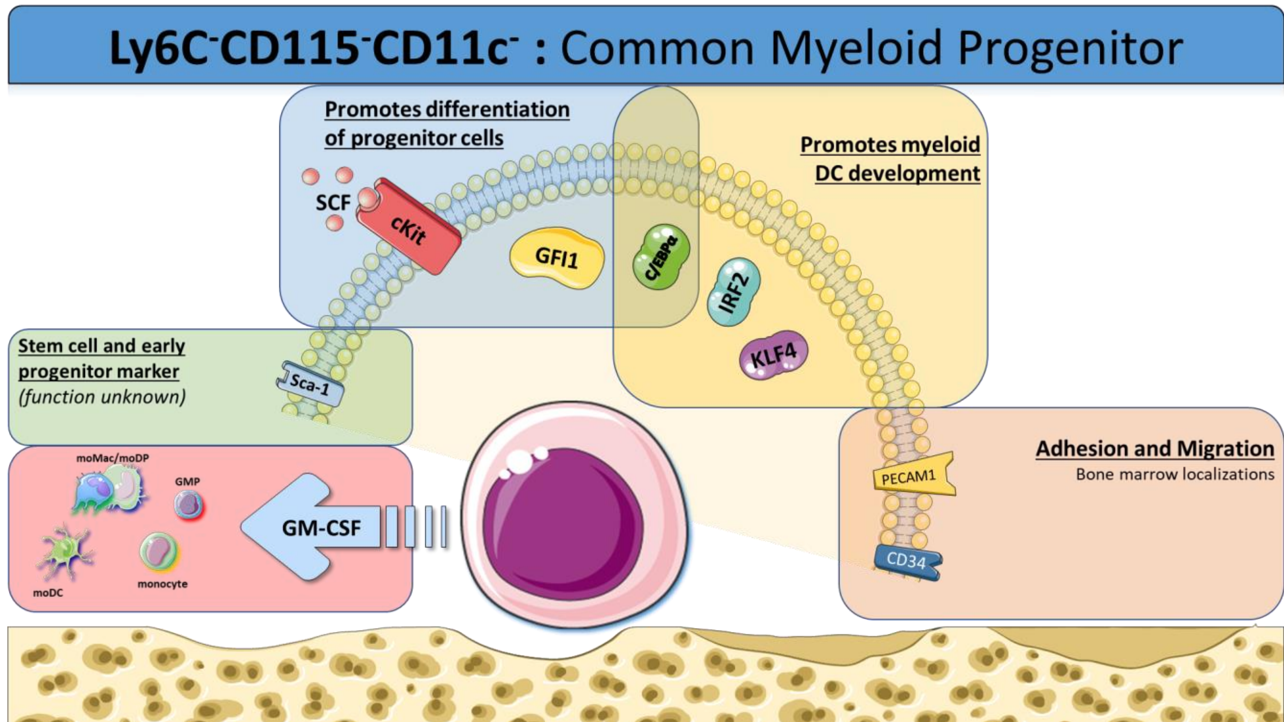

Figure 4.1 Characterization of Ly6C$^-$CD115$^-$CD11c$^-$. This developmental stage most closely resembles the Common Myeloid Progenitor (CMP). This population expresses cell surface markers that as highly associated with the earliest hematopoietic progenitors (SCA-1) and maintain bone marrow localization (PECAM1 and CD34), as well as a variety of factors that induce cellular differentiation over self-renewal. Expression of IRF2 and KLF4 suggest these cells are primed to follow myeloid DC development, even at this very early stage. Lastly, when stimulated with GM-CSF, this stage gives rise to all subsequent populations.

Ly6C$^+$CD115$^-$

Like CMPs, this population is present in freshly isolate bone marrow on day 0 (**Figure 3.2, Figure 4.2**); however, this population lacked expression of stem cell markers and demonstrated expression of intermediate myeloid markers: CD11b, CD16/32, Ly6G, and CD172a (**Figure 2.6A, Figure 4.2**). Additionally, these cells lack or exhibited very low expression of markers associated with macrophages/monocytes (CD14, CD64, F4/80 and MerTK) and DCs (CD11c, MHCII, CD86, and CD40) (**Figure 2.6, Figure 4.2**). These cells were also found to express genes that are hallmarks in granulocyte/macrophage progenitors (GMPs): Csf3r, Gfi1, and Cebpe (**Figure 2.6B, Table 4.2, Figure 4.2**). Additionally, this population expressed DC lineage associated genes Klf4 and Spi. Together, these results suggest Ly6C$^+$CD115$^-$ cells represent GMPs. A notable difference between this stage and previously described GMPs is the expression of CD11b. GMPs harvested from bone marrow are negative for CD11b[17]; however, the GMPs identified in this model are CD11b$^+$. This matched early GM-CSF studies that showed GM-CSF induced the expression of CD11b on bone marrow cultures earlier than cells generated in steady-state conditions[18]. In addition to giving rise to monocytes, GMPs act as a major progenitor for granulocytes. The granulocyte developmental pathway requires signaling through granulocyte colony stimulating factor receptor (CSF3R) and expression of C/EBPε[19]. Although this population expresses genes associated with granulocyte development, their high expression of Spi1, a transcription factor required for all DC development, likely favors myeloid DC development.

Table 4.2 Gene Expressed in Ly6C^{+}CD115^{-}	
Gene	**Stage relevant function**
Gfi1	Control self-renewal, quiescence, and differentiation of progenitor cells.[1]
Klf4	Required for monocyte lineage development[2,3]
Csf3r	Receptor for granulocyte-colony stimulating factor[20]
Cebpe	Transcription factor required for granulocyte development[21]
Spi1	Essential for maintaining composition of myeloid population and required for development of all DC subtypes[21,22]

Isolated GMPs could give rise to down-stream populations, but at a much lower relative rate than isolated CMPs (**Figure 2.3, Figure 4.2**). Because GMPs are isolated from mixed bone marrow, there are GMPs at various sub-stages in their development, including cells primed to become granulocytes[23,24]. When these cells are removed from the growth-rich environment of the bone marrow, they no longer receive the required stimulation and are developmentally halted. Although this population disappears from mixed (**Figure 2.2**) and CMP cultures (**Figure 2.3D**), it is maintained at a high abundance when CMPs are adoptively transferred. This is likely due to the combination of GM-CSF injections and host cytokines driving a neutrophil response[25].

This population was able to weakly upregulate co-stimulatory molecule CD86 expression upon PAMP stimulation and produced a low yield of IL-6 (**Figure 3.4**). While both are much lower than at subsequent stages, these modest increases suggest GMPs acquire functional capabilities beyond their role as a progenitor.

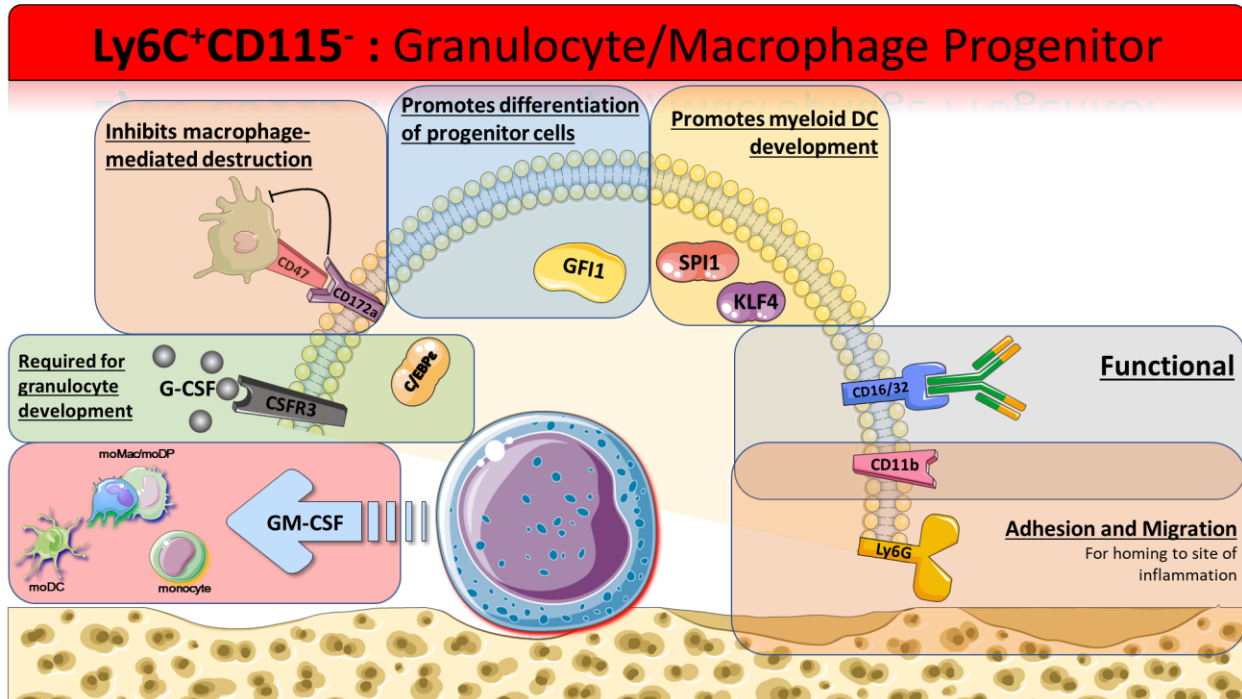

Figure 4.2 Characterization of Ly6C^{+}CD115^{-}. This developmental stage most closely resembles the Granulocyte/Macrophage Progenitor (GMP). This population expresses cell surface markers that are required to granulocytic potential (CFR3 and Ly6G). However, expression of SPI1 and KLF4 suggest the major of these cells are likely to develop along the DC pathways. This stage exhibits limited functional potential through expression of Fc receptors, CD16 and CD32, and complement binding receptor CD11b. Lastly, when stimulated with GM-CSF, this stage can give rise to all the subsequent stages and has lost the potential for CMP renewal.

Ly6C$^+$CD115$^+$

Like GMPs, a subset of this population expressed Ly6G. However, these cells expressed a low level of CD11c and an intermediate level of CD172a, CD40, and CD86 (**Figure 2.6A, Figure 4.3**). Interestingly, this population was shown to be positive for a variety of macrophage markers: CD64, F4/80, and MerTK. However, Ly6C$^+$CD115$^+$ cells expressed high levels of monocyte-associated genes: Tcf7l2, Klf4, and Cx3cr1 (**Figure 2.6B, Table 4.3, Figure 4.3**). KLF4 expression is required for monocyte development, and its expressed was observed in all previous population but not in the subsequent stages discussed below. This trend and the landmark expression of CX3CR1 suggest the population bona fide monocytes. TCF7L2 expression in monocytes is attributed to cells undergoing differentiation, suggesting that this stage represents cells at various substages in development.

This population gave rise to Ly6C$^-$CD115$^+$ cells (**Figure 2.3B**). However, a significant percentage of monocytes in mixed (**Figure 2.2**) and isolated cultures (**Figure 2.3B**) remained at the monocyte stage, whereas all monocytes generated from CMP cultures progressed to the Ly6C$^-$CD115$^+$ phenotype (**Figure 2.3D**). Similar to the GMP stage, monocytes were present in low numbers at day 0 (**Figure 3.2A, Figure 4.3**). While most fully developed monocyte leave the bone marrow, several monocytic stages (monoblast, promonocyte, and immature monocyte) and retained[28]; thus, some were likely primed for steady-state development, but development was halted in the absence of appropriate growth factors.

Table 4.3 Gene Expressed in Ly6C⁺CD115⁺	
Gene	**Stage relevant function**
Tcf7l2	Upregulated on monocytes preparing for macrophage/DC differentiation[26]
Klf4	Required for monocyte lineage development[2,3]
Cx3cr1	Critical for monocyte trafficking[27]

As discussed above, this population could not be recovered by peritoneal lavage following adoptive transfer of CMPs and monocytes (**Figure 2.5E-D**). Unique among the cell types discussed above, monocytes are the first stage to leave the bone marrow and are equipped with receptors to aid in migration. Therefore, this population may leave the intraperitoneal cavity and enter circulation. Spleen, blood, and bone marrow were analyzed for migrating monocytes, but we were unable to detect significant levels. Although we were unable to recover monocytes from the site of injection, subsequent cell stages ($Ly6C^{-}CD115^{+}$ and $Ly6C^{-}CD115^{-}CD11c^{+}$) were recovered following adoptive transfer of monocytes (**Figure 2.5D**). Therefore, the lack of recovery could be due to a rapid *in vivo* transition to downstream cell types.

This population upregulated CD86 24-hours post sort, in the presence PAMPs; however, this trend was also seen in the absence of PAMPs (**Figure 3.4B**). It is likely the dramatic shift we see in basal CD86 expression is due to the rapid rate of monocyte differentiation. Indeed, unstimulated monocytes express CD86 and IL-6 at levels similar to that observed in the subsequent population (**Figure 3.4B, Figure 3.4D, Figure 4.3**). Similar to monocyte identified *in vivo,* this population was also able to produce a robust IL-12p40/70 response (**Figure 3.4C, Figure 4.3**)[29–31].

When cells were sorted based on CD11c, MHCII, and CD11b expression, most monocytes were initially among the $MHCII^{Low}$ phenotype (**Figure 2.9**). Interestingly, the population of monocytes that was able to upregulate their MHCII to intermediate levels was maintained in culture (**Figure 2.9**). This subpopulation likely corresponds to a subset of monocytes that have been found to undergo very little differentiation upon antigen stimulation[32].

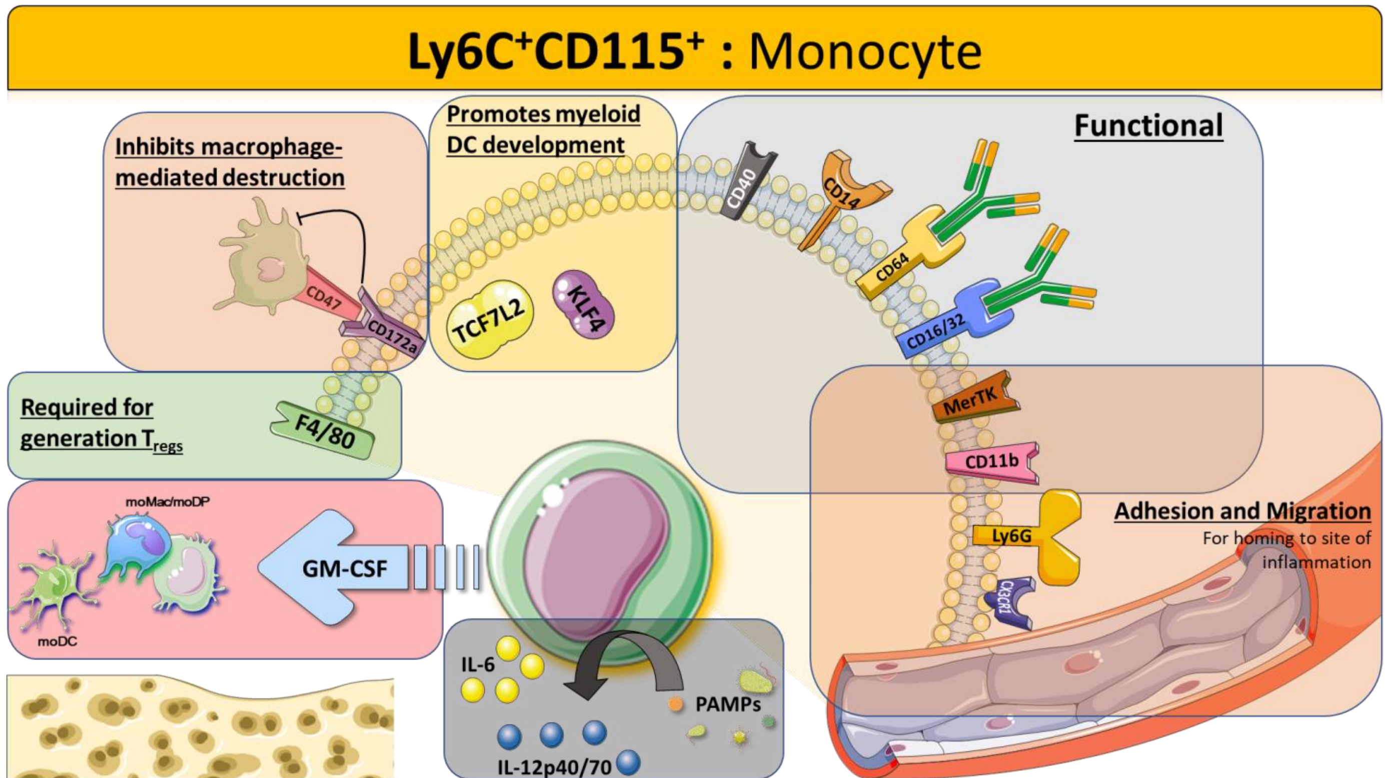

Figure 4.3 Characterization of Ly6C⁺CD115⁺. This developmental stage most closely resembles Monocytes. This population has lost the expression cell surface receptor required for granulocytic development (CFR3), yet retains and upregulates the expression of receptors critical in homing to sites of inflammation (Ly6G and CX3CR1). There is a loss of transcription factors involved in regulation of progenitor cells; however, there is large upregulation in the number of products associated with immunological functions, including intercellular signaling molecule CD40 and Fc receptors. This is the first stage of significant cytokine production in response to pathogen-associated molecular patterns (PAMPs). This is the last stage to express KLF4, a transcription factor required for monocyte development, and the first stage of TCF7L2, a transcription factor which drives differentiation of monocytes toward macrophages and DCs. Lastly, this is the first major stage to egress the bone marrow and enter circulation, evident due to their low numbers in freshly harvested bone marrow.

Ly6C$^-$CD115$^+$

Like their predecessor monocyte, Ly6C$^-$CD115$^+$ cells were negative for stem cell markers; however, they lacked Ly6G expression (**Figure 2.6A, Figure 4.4**). Most notably, these cells expressed the highest levels of macrophage markers (F4/80 and MerTK), as well as high level CD11c, MHCII, and CD40 (**Figure 2.6A, Figure 4.4**). These had relatively high expression of late stage genes: Id2, Spi1, Tcf7l2, and Nkfb1 (**Figure 2.6B, Table 4.4, Figure 4.4**). As discussed above, TCF7L2 is upregulated in monocytes undergoing differentiation; however, previous studies have suggested it is only retained in monocyte derived macrophages and at a significantly higher level than their monocyte precursor[33].

Table 4.4 Gene Expression in Ly6C^{-}CD115^{+}	
Gene	**Stage relevant function**
Id2	Transcription factor which blocks pDC differentiation[34]
Spi1	Required for development of all DC subtypes[22]
Tcf7l2	Upregulated on monocyte-derived cells[26,33]
Nfkb1	Critical for maintain resting state, preventing uncontrolled activation of inflammatory pathways[35]

Therefore, $Ly6C^-CD115^+$ cells most closely represent monocyte-derived macrophages (moMacs)[36,37]. As a terminally differentiated cell, moMacs are maintained in culture long-term (**Figure 2.3D, Figure 2.9**); however, a subset of these cells acts as a precursor to $Ly6C^-CD115^-CD11c^+$ (**Figure 2.3C, Figure 2.9, Figure 4.4**). We believe these cells – termed moDC precursor (moDP) – downregulate TCF7L2. However, moMacs and moDPs' gene expression was analyzed collectively; thus, the relatively lower Tcf7l2 expression is likely due to pooling a few bona fide moMacs (high Tcf7l2 expression) with many moDPs (low Tcf7l2 expression).

When analyzing $CD11c^+CD11b^+$ cells, most $MHCII^{Low}$ and $MHCII^{Int}$ cells are at this stage in development (**Figure 2.9**). When $MHCII^{Int}$ cells were isolated from a mixed culture, the majority of cells expressed high levels of MHCII and down-regulated CD115 expression (**Figure 2.9; middle row**). However, when $MHCII^{Low}$ cells were isolated from mixed cultures, they express intermediate levels of MHCII, never reaching high levels of MHCII and largely retained their $Ly6C^-CD115^+$ phenotype (**Figure 2.9; top row**). This suggests the presence of a cell-dependent feedback loop. IL-6 is expressed at high levels by monocyte and moMac stages (**Figure 3.4D**), and it has been proposed to be a factor in regulating the differentiation toward DCs and macrophages[38]. By enriching $MHCII^{Low}$ monocyte, we may be creating an IL-6 rich environment that favors macrophage development.

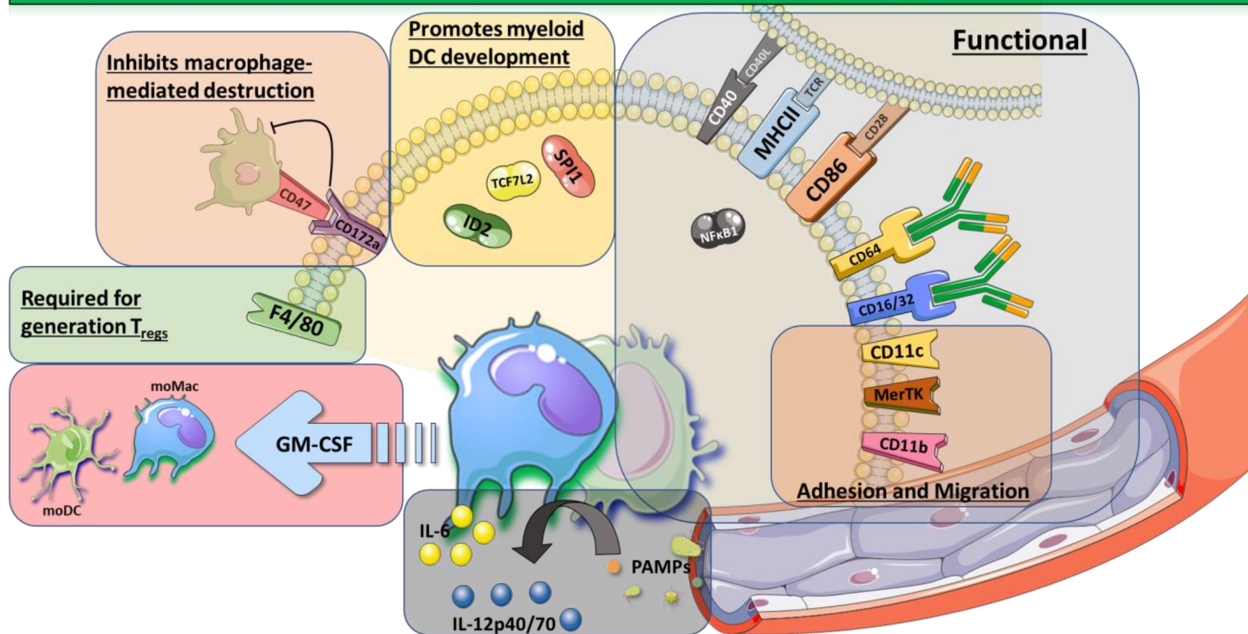

Figure 4.4 Characterization of Ly6C⁻CD115⁺. This developmental stage can be subdivided into two stages based on developmental potential, Monocyte-derived Macrophages (moMac) and Monocyte-derived DC Progenitor (moDP). Although no cell surface molecule was identified to resolve these two stages, moMacs are at a terminally differentiated stage, and moDPs ultimately give rise to monocyte-derived DCs. These two stages upregulate the expression of receptors critical in adhesion and migration, and there is an increase in transcription factors that drive the final stages in DC development. Additionally, these cells exhibit a significant increase in expression of proteins involved in T cell activation. Like the monocyte stage, moMacs/moDPs are able to produce a repertoire of cytokines and chemokines in response to pathogen-associated molecular patterns (PAMPs).

Ly6C$^-$CD115$^-$CD11c$^+$

Similar to moMacs, Ly6C$^-$CD115$^-$CD11c$^+$ cells express CD11c, CD172a, MHCII, CD40, and CD86; however, they displayed low levels of the macrophage markers CD14, CD64, and MerTK (**Figure 2.6A, Figure 4.5**). Interestingly, this population expressed moderate levels of F4/80, a receptor shared among macrophages and CD11b$^+$ DCs that populate the small intestine. This population expressed high levels of genes critical for DC function (Ciita, Stat3, Relb, Nfkb, Cd34) and late stage development (Stat5a, Stat5b, Batf3, Id2, and Irf4 (**Figure 2.6A, Table 4.5, Figure 4.5**). Interestingly, this population had no detectable levels of CD34 when analyzed by flow cytometry but very high Cd34 gene expression. CD34 has been suggested to be required in the migration of mucosal DCs[17,39].

Of the five stages discussed, this is the only one that does not give rise to any other cell type, suggesting terminal differentiation (**Figure 2.8B, Figure 4.5**). This stage most closely resembles monocyte-derived DCs (moDCs)[11,40–42]. Due to the uniqueness of CD34 and F4/80 expression on bona fide DC populations, it is likely that the moDC stage resembled CD11b$^+$ small intestine residing DCs, which rely heavily on BATF3, IRF4, and ID2 regulation[34]. This hypothesis is further supported by functional analysis. CD11b$^+$ subtypes have very low expression of TLR3[17], and GM-CSF generated cells respond very poorly to TLR3 agonist, poly(I:C) (data not shown).

When analyzing CD11c$^+$CD11b$^+$ cells, moDCs were found almost exclusively within the MHCII^{High} population. Furthermore, the data suggest moDCs differentiation is dependent on precursors having reached MHCII^{Int} prior to isolation. Additional studies are required to confirm this; however this could suggest the presence of distinct checkpoints similar to that observed in lymphocyte development[43].

Table 4.5 Gene Expression in Ly6C$^-$CD115$^-$CD11c$^+$	
Gene	**Stage relevant function**
Ciita	MHCII transactivator; positive regulator of MHCII expression[44]
Stat5a/b	Inhibits pDC development[45]
Stat3	Maintains resting state; preventing spontaneous maturation[46]
Batf3	Required for development of migratory DCs[47]
Relb	Required for and highly expressed in CD11b$^+$ DCs[48]
Nfkb1	Critical for maintain resting state, preventing uncontrolled activation of inflammatory pathways[35]
Id2	Transcription factor which blocks pDC differentiation[34]
Irf4	Required for non-CD8α$^+$ DC development[49]
Cd34	Required for trafficking of mucosal DC subsets[39]

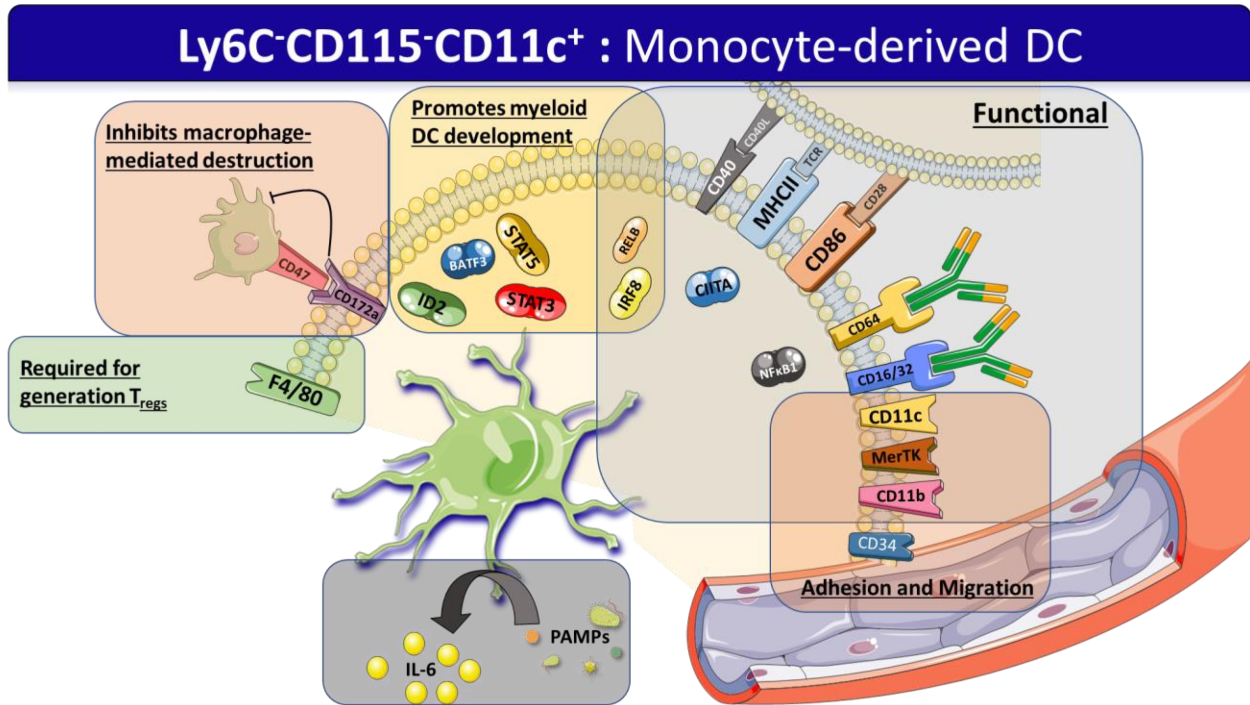

Figure 4.5 Characterization of Ly6C⁻CD115⁻CD11c⁺. This developmental stage can be most closely resembling the Monocyte-derived DC (moDC). This stage is fully differentiated and gives rise to no other cell types. Like moMac/moDPs, this stage maintains the expression of receptors critical in adhesion and migration, including CD34, a receptor which homes migratory cells to mucosal membranes. Additionally, there is a further increase in transcription factors that drive the final stages in DC development. These cells also maintain a high level of proteins involved in T cell activation, including an increased expression of the MHCII transactivator, CIITA.

Summary of Previously Published Models

Others have published protocols for isolating distinct stages in GM-CSF driven development, including strategies that rely on identifying cells by their proliferative capacity (such as the use of CFSE)[50] or alternative cell surface markers (such as CD31 and Ly6C)[51]. While such strategies are viable methods, we found them unsuitable for our goals.

When CFSE was used to identify proliferative cells (i.e. progenitors and precursor developmental stages), the brightness of CFSE resulted in significant bleed-over into adjacent channels. While the bleed-over could be reduced with compensation, we found the extent of manipulation required to be unsuitable. The brightness could also be reduced by staining with lower concentrations of CFSE. However, this resulted in poor resolution between proliferative levels.

Similarly, we tried a previously described strategy by Leenen's which used CD31 and Ly6C as a basis for identifying stages in GM-CSF driven development[51]. While Ly6C proved to be an excellent tool for differentiating myeloid stages, we found the combination of CD31 and Ly6C did not accomplish our goals. CD31 is expressed early in development and is absent after 2 days of culture in GM-CSF. Ly6C is expressed at intermediate stages and lost on highly differentiated cells.

While this combination is useful for identifying the early and intermediate stages, it did not allow for the identification of the late stages, which are the most poorly understood and most difficult to isolate. Furthermore, CD31 is expressed at very low levels, resulting in poor resolution between populations. Many sorting strategies take advantage of markers that have only modest changes between one stage and the next. We found this to be problematic because,

without significant experience, it is difficult to determine how liberal gating strategies need to be. Therefore, we sought cell surface markers that clearly resolved each stage.

Short-comings, Limitations, and Future Work

An important limitation of this work is that these stages represent heterogenous compositions of cells. For example, the $Ly6C^{+}CD115^{+}$ population has a clear monocyte phenotype, but it likely represents monocytes at various stages within their own developmental pathway or different monocyte subtypes, such as $Ly6G^{+}$ monocytes and $Ly6G^{-}$ monocytes. It is our hope that this work will provide a framework for isolating the major stages, and that these major stages can be characterized in their own focus.

Due to the heterogenicity of the transitional stages (CMPs, GMPs, and monocytes), we expected a proportion of each population to be developmentally stagnant in our model due to the lack of necessary growth factors. For example, because GMPs have the capacity to give rise to granulocytes when stimulated with granulocyte-colony stimulating factor (G-CSF) and monocyte lineages when stimulated with GM-CSF, we expected some of the GMPs to retain their GMP phenotypes in the presence of GM-CSF because they are primed to become granulocytes but lack G-CSF necessary to drive their development. Indeed, this trend is observed in GMPs and monocytes but not in CMPs. Long term cultural analysis of CMPs showed they fully progress through each stage, and by 16 days post isolation, all cells exhibited a terminally differentiated phenotype.

This is an important observation as it suggests certain progenitors (such as GMPs) are developmentally primed for a certain lineage (granulocytic or monocytic) regardless of growth factor stimulation. However, earlier progenitors (such as CMPs) can give rise to stages which

represent a linear development. While the variables that govern the differences between linear and branched development are outside the scope of this project, they are important avenues to pursue as they can aid in removing much of the innate ambiguity in studying overlapping developmental pathways.

Finally, a minor limitation of this strategy is the loss of CD115 upon an activation signal. $CD115^+$ populations (monocytes and moMacs) quickly and significantly downregulate CD115 expression in the presence of pathogen-associated molecular patterns (PAMPs). Because CD115 is a growth factor receptor, downregulation is likely an attempt to pull reservoir cells (cells with limited functional capacity but strong capacity to give rise to effector cells) from development and push toward immediate differentiation. In other words, the presence of PAMPs overrides the traditional developmental pathway in favor of immediately available effector cells with reduced or modified functional capacities.

Although loss of CD115 is certainly a limitation if one has based their dissertation upon using its expression as a strategy for identification, this may also present new ways to detect cellular activation. Much of our knowledge in functional capacity is limited to effector cells which often begin expression of an effector molecule upon activation. For example, naïve DCs and activated DCs can be differentiated by increased antigen presenting molecules (MHCII, CD86, CD40) on the latter. However, this presents a problem when studying progenitors whose major function is to produce effector cells rather than produce molecules. Therefore, it is difficult to quantitatively differentiate a naïve progenitor and activated progenitor. Identification of molecules that undergo significant changes in progenitors upon PAMP detection will aid in developing stricter, more homogenic models.

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