Development of functional capacity and bactericidal activity in myeloid cells during GM-CSF driven differentiation

by

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Abstract

Dendritic cells (DC) form an interface between the innate and adaptive immune systems. DC respond to infection or inflammatory signals by orchestrating other immune cells to perform protective functions. These include activation of innate immune cells, like natural killer cells, and induction of an adaptive T cell response. Since the discovery of DC in the early 1970s, a lot of research effort has gone into understanding their uptake mechanisms, the kinetics of proteolysis, and the efficiency through which DC process and present antigens to T cells. While these functional attributes have been well studied in terminally differentiated DC or DC precursors, the stage at which DC develop these functions is yet to be determined. In this study, we examined the ontogeny of antigen uptake, degradative activity, antigen presentation, and bactericidal activity in DC precursor cells during GM-CSF driven differentiation.

To determine the functional attributes of DC and DC precursor cells, bone marrow cultured in GM-CSF was isolated into five distinct populations: CMP, GMP, monocyte, moMac/moDP, and moDC that represent the stages of their development. The CMP population demonstrated the lowest uptake capacity, and proteolysis. Also, CMP were unable to process and present antigen to T cells. In addition, CMP were the least efficient at uptake of Listeria monocytogenes and did not support intracellular replication of the bacteria.

The GMP and monocyte populations demonstrated the highest uptake capacity of both large and small particles, moderate proteolytic activity, and were able to induce only modest T cell activation. These cell populations showed moderate uptake of L. monocytogenes, yet did not
provide a conducive niche for bacteria replication, an observation that was attributed to high levels of reactive oxygen and nitrogen intermediates produced by these cells.

Lastly, although the moMac/moDP and moDC populations appeared to be functionally similar, there were notable differences. First, both populations had moderate uptake capacity of bioparticles and dextran, strong proteolytic activity, and were potent inducers of T cell proliferation. Additionally, moMac/moDP and moDC demonstrated the highest level of uptake of *L. monocytogenes* when compared to other stages. This was attributed to high expression of uptake receptors in these cell populations. The moMac/moDP population supported *L. monocytogenes* intracellular replication while moDC did not support bacterial growth. In summary, through this project, we have determined the stages at which DC and DC precursor cells acquire different functional capacities. This allows researchers a more targeted approach to isolate their preferred cell types along the developmental spectrum.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>cMoP</td>
<td>Common Monocyte Progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosomal Antigen 1</td>
</tr>
<tr>
<td>Flt3L</td>
<td>FMS-like tyrosine kinase 3 Ligand</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophages Colony Stimulating Factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte Macrophages Progenitor</td>
</tr>
<tr>
<td>hip</td>
<td>Hour post infection</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
</tr>
<tr>
<td>Lm</td>
<td><em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte/Erythrocyte Progenitor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MHCI</td>
<td>Major Histocompatibility Molecule Class I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MHCII</td>
<td>Major Histocompatibility Molecule Class II</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived Dendritic Cell</td>
</tr>
<tr>
<td>moDP</td>
<td>Monocyte-derived Dendritic Cell Precursor</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>moMac</td>
<td>Monocyte-derived Macrophages</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent Progenitor</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
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Chapter 1: Introduction

1.1 Introduction

Dendritic cells (DC) are professional antigen presenting cells that link the innate and adaptive immune systems [1-3]. There are different subsets of DC. For instance, DC whose differentiation is dependent on Flt3L (FMS-like tyrosine kinase 3 ligand) represent conventional or migratory lymphoid resident DCs [1, 4]. In addition, inflammatory DC or monocyte-derived DC (moDC) are another subset of DC that differentiate from circulating monocytes in response to inflammatory or microbial signals, [1, 5]. The moDC can be generated in vitro through stimulation of bone marrow with granulocyte-macrophage colony stimulating factor (GM-CSF), a hematopoietic growth factor [5]. A classic example of inflammatory DC is tumor necrosis factor –α (TNF-α) and inducible nitric oxide synthase (iNOS) producing DC (referred to as TipDCs) found in spleen in response to *Listeria monocytogenes* infection [1, 5-7]. Through the advancement in cell culture, GM-CSF derived DC have become important for studying the cell biology of these cells, their functional attributes, as well as an important vaccine vehicle for clinical use [1, 8].

The developmental stages of myeloid cells during GM-CSF driven differentiation are well-characterized. These cells are known to go through sequential stages that include the common myeloid progenitor (CMP), the granulocyte/macrophage precursor (GMP), the macrophage/DC progenitor (MDP), the common monocyte progenitor (cMoP), and monocytes before differentiating into DC [9-12]. Most of what is known about functional attributes of myeloid cells, for instance their uptake capacity, proteolysis, antigen processing, and antigen
presentation, have been acquired from experiments that utilized terminally differentiated cells such as DC or macrophage [13-17]. Thus, comparatively little is known about when and how these functionalities develop. This dissertation work focuses on unraveling the stages at which these myeloid cells develop these functions during GM-CSF driven differentiation.

*L. monocytogenes* is a gram positive facultative intracellular bacterium that causes severe infection in immunocompromised individuals [18-20]. Early immune response against this pathogen is mediated by the innate immune cells. *In vivo* infection of mice with *L. monocytogenes* has been found to induce the proliferation and migration of monocytes and less developed myeloid cells from the bone marrow to the site of infection [21, 22]. In addition, the roles of DC and macrophages during *L. monocytogenes* infection have been studied [23-25]. It has been shown that DC provide a suboptimal condition for *L. monocytogenes* intracellular replication, whereas the bacterium was able to replicate readily within macrophages [23, 24]. In contrast, other studies have demonstrated that *L. monocytogenes* replicates effectively in CD11+ DC when compared with intestinal CD103+ DC [26]. While there is evidence in support of DC being able to control intracellular replication of this pathogen, the developmental stage at which this cell population acquires this function is not well defined. Also, the early developmental pathways of myeloid cells that mediate antibacterial defense or those that enhance the growth of the bacterium during the early stage of infection remains unknown.

### 1.1.1 Research Problem

The developmental stages of myeloid cells during GM-CSF driven differentiation have been well-characterized, resulting in the identification of all of the progenitor cells along the developmental spectrum of the cells becoming DCs [9, 27]. While there has been great advancement in understanding the ontogeny of cells within the myeloid lineage based on their
phenotypic profiles, there is still a need to characterize these cells based on their functional attributes. A vast majority of the functional properties of myeloid cells that include antigen recognition and uptake, antigen degradation, initiation of adaptive immune response, and susceptibility to infection have been mostly studied by utilizing more differentiated cells such as moDC and macrophages. Besides the studies on the differentiation potential of CMP and GMP [27], there are relatively few studies that have examined the functional characteristics of these early progenitor cells. Therefore, it is imperative to understand the stage at which these functions develop in myeloid cells. This will provide an avenue to compare how the differentiation potential of these cells influences their functional attributes. In addition, innate immune cells are the first responders in the event of microorganism invasion. As such, the role of some innate immune cells such as neutrophils, DC, and macrophages were extensively studied in relation of L. monocytogenes infection. However, it is yet to be determined how each of these cell populations handle bacterial infection.

1.1.2 Hypotheses and Objectives

This study hypothesized that the endocytic, proteolytic, and antigen presentation of myeloid cells change as cells develop from early progenitors into more differentiated precursors. Also, the rate of bacterial uptake and the antimicrobial activity of each cell population will determine their susceptibility to L. monocytogenes infection. The following objectives were pursued in testing these hypotheses.

**Objective 1**: To investigate the endocytic, the proteolytic, and antigen presentation of myeloid cells during GM-CSF driven differentiation. The purpose of this aim was to fully elucidate the antigen uptake mechanism present at different developmental stages. In addition,
the degradation potential and antigen presenting functions were also examined. The following questions were addressed in this first objective:

a. What type of receptors or antigen uptake mechanisms are present in each of these five distinct populations?

b. What antigen processing mechanisms are active in each developmental stage?

c. At what stage does antigen presentation function develop?

**Objective 2**: To determine the susceptibility and the rate at which *L. monocytogenes* grow in each population, and the bactericidal activity present at the distinct stages of development. The purpose of this aim was to fully understand how each population responds to *L. monocytogenes* infection with regard to bacterial containment and bactericidal activity. The rate of *L. monocytogenes* growth, the susceptibility, and the bactericidal activity in each population were examined in order to answer the following questions:

a. At what developmental stages are myeloid cells susceptible to *L. monocytogenes* infection, and at what rate does *L. monocytogenes* grow in each of these populations?

b. How does the uptake mechanism influence *L. monocytogenes* cytoplasmic entry in each population?

c. At what stage do myeloid cells develop bactericidal activity?

The results of this study enhance our understanding on how and when different functions develop in myeloid cells during GM-CSF driven differentiation, and how these functions influence their ability to process and present antigens to T cells. Also, this study further expatiates what is known about the role of myeloid cells in *L. monocytogenes* pathogenesis and the stage at which these cells develop bactericidal mechanisms to combat the infection.
1.1.3 Significance of the Study

The role of more differentiated myeloid cells in antigen uptake, proteolysis, and antigen presentation is well known. However, the stage during their development that these cells acquire these functions and the function/role of early progenitor cells is not well understood. Therefore, this study aimed to understand the functional capacities and bactericidal activities of myeloid cells along the developmental pathway of becoming fully differentiated DCs. This research is of significant importance because it helps to uncover the functional attributes of each myeloid cell along the developmental spectrum with regard to their antigen recognition, antigen processing and presentation, and how each population handles bacterial infection with regard to bacterial containment and bactericidal mechanisms utilized.

1.2 Innovation

This project is highly innovative because:

1. This is the first study that comparatively examined the uptake mechanisms, antigen processing and presentation, and bactericidal mechanisms at the developmental stages of GM-CSF driven myeloid cells from the early progenitor cells, CMP, to the more differentiated inflammatory DCs.

2. This study also provided a novel insight into how each myeloid cell controls *L. monocytogenes* infection by restricting intracellular replication of the bacterium and the antimicrobial properties of each myeloid cell population.

1.3 Organization of the Dissertation

This is written in five chapters. Chapter 1 is an introduction to the research problem and the research objectives. Chapter 2 is the literature review section. Chapter 3 and 4
address the research objectives 1 and 2, respectively. The summary of the project and future directions is presented in chapter 5.
References


Chapter 2: Literature Review

2.1 Introduction

The immune system is bifurcated into two major categories: the innate and the adaptive immune systems. The concept of innate immunity was first introduced by Elie Metchnikoff when he discovered the presence of some blood cells whose role was to recognize, internalize, and destroy invading pathogens [1, 2]. These cells, which include macrophages and neutrophils (previously known as microphages), were later referred to as phagocytic cells. Contemporaneously, Paul Ehrlich described the adaptive immune system as a system involving generation of antibodies against a particular antigen, thus providing long-lasting protection against the pathogen [1]. It is now clear that the adaptive immune system consists of both antibody producing B cells, and the T cells that mediate cellular immunity.

Besides comprising different cell types, the innate and adaptive immune systems also differ in: the mechanism of antigen recognition, the time it takes to initiate an immune response, and the effectiveness of the immune response. The innate immune cells are equipped with different receptors that allow for recognition of wide ranges of molecules expressed by pathogens, leading to rapid initiation of responses [3-5]. In contrast, the adaptive immune cells, specifically T lymphocytes, need to be educated by the innate immune cells about the nature of the pathogen. This process could take hours to days before generating an appropriate immune response. Although it takes more time to initiate the adaptive immune response, the antigen-specific immunity generated through this system could last for a lifetime. And while the immune
system is comprised of these two distinct groups that perform specific functions, these two groups need to work together as a coherent unit in order to generate protective immunity against any invading pathogen.

Dendritic cells (DC) are innate immune cells that serve as sentinels of the immune system. The discovery of DC in the early 1970s as a bridge between the innate and adaptive immune systems shed light on how these two systems communicate, and how the overall immune responses are coordinated in order to generate long-lasting, protective immunity against a specific pathogen [6]. DC are able to perform this role because they are localized in virtually all tissues, and they have exceptional migratory potentials that allow for sampling of the environment [7]. This leads to antigen presentation and initiation of an adaptive immune response by DC [8]. Thus, DC are regarded as the initiator and modulator of the adaptive immune response. The functional attributes of DC are well characterized but the stages at which DC acquire these functions during their development is not well understood. This review focuses on the known functions of DC that include antigen recognition, antigen processing, initiation of the adaptive immune response, as well as how they handle bacterial infection.

2.2 The Discovery and in vitro Generation of Dendritic cells

DC are now regarded as the ‘cornerstone’ of the immune system; however, the discovery of these cells appears to have been rather unintentional. Back in the early 1970s, while trying to study T cell clonal selection and the cells that initiate the adaptive immune response, Ralph Steinman observed novel cells within adherent mononuclear cell populations from the spleen of a mouse [6, 9]. Observing the morphology of these new cells, he quickly realized that these cells were different from the well-characterized adherent cells, such as macrophages, due to the unique stellate structure they possessed. In addition to their dendrite-like morphology, DC were
further characterized by their functional properties. The first functional assay used to categorize DC elucidated their ability to induce T cell proliferation in a “mixed-lymphocyte reaction” [10-12]. Additionally, DC have limited endocytic capacity when compared with macrophages, and are potent at antigen uptake, processing, and presentation to T cells [13].

One of the early challenges in the field of DC biology was the low frequency of these cells in vivo, which made isolating large numbers of DC for functional studies exceptionally burdensome [14]. However, there was a significant advancement in the field of DC biology in the early 1990s made by Inaba et. al. that led to the generation of large numbers of DC in vitro through stimulation of bone marrow with granulocyte/macrophage colony stimulating factor (GM-CSF) [15], revolutionizing the field by facilitating the proliferation of DC required for functional studies.

Although DC can be generated through Flt3L and GM-CSF driven differentiation, much of the insight on DC biology and functions have been drawn through GM-CSF culture system [16-18]. GM-CSF cell culture is heterogeneous, and consists of different cell types like granulocytes, macrophages, and DC. However, loosely adherent cells isolated on day 7 of cell culture with phenotypic profiles CD11c+MHC class II+ were initially considered a homogeneous population of DC [15]. It was recently demonstrated that within this CD11c+MHC class II+ cell population, there are at least two distinct cell types: DC and macrophages [19, 20]. It is therefore important to classify cells not only by their surface markers, but also by their functional attributes. This project focuses on correlating the features of DC and DC progenitors to their phenotypic profiles.
2.3 Features of Dendritic Cells

DC are essential components of the innate immune system that play significant roles in initiating the adaptive immune responses [21]. The major features of DC include constant sampling of the environment for antigens, pronounced mechanisms of antigen processing coupled with expression of high levels of MHC molecules and other membrane proteins, such as CD86 and CD40 that enhance their efficiency when interacting with T cells. The overview of DC functions that ranges from antigen uptake, antigen processing, and antigen presentation to T cells is illustrated in Figure 2.1 (Fig 2.1), and this section discusses these functional characteristics of DC in detail.
Figure 2.1. Different functional attributes of dendritic cells (DC). DC express different pathogen recognition receptors such as scavenger receptor (CD36), macrophage mannose receptor (MMR), dectin-1 receptor, and DC-SIGN that enhance antigen recognition and uptake. Antigen is processed and presented on MHC molecules to induce T cell activation.
2.3.1 Antigen uptake mechanisms utilized by Dendritic Cells

The recognition of pathogens with diverse molecular signatures is the first crucial step to mounting an appropriate immune response against invading pathogens. Moreover, because antigens come in different shapes and sizes, they require that phagocytes express a diverse array of receptors able to recognize and initiate phagocytosis of these different particles [22]. As such, DC, as well as other innate immune cells, are endowed with a plethora of pathogen recognition receptor (PRR) that allow for recognition of non-self antigens. Thus, expression of several PRRs that include mannose receptor, scavenger receptor, and DEC-205 by DC is a hallmark of their antigen uptake capacity [23-25].

The PRRs are strategically located on or within phagocytic cells in order to allow for recognition of a wide variety of pathogens. PPRs could be classified as secreted (pentraxins, collectins), plasma membrane bound (Toll-like receptor (TLR), C-type lectin), endosomal bound (TLR), and cytosolic (Nod-like receptor (NLR)) sensors (Figure 2.3) [26]. While some PRRs are mostly involved in the uptake of pathogens, others such as TLRs and NLRs, are important in recognizing bacterial ligands and inducing signaling cascades that lead to changes in expression of genes involved in production of pro-inflammatory cytokines (Fig 2.2).
Figure 2.2. The expression of different pattern recognition receptors on the cell surface, endosome, and cytoplasm. These receptors include Toll-like receptor (TLR), macrophage mannose receptor (MMR), scavenger receptor (CD36), NOD-like receptor (NLR), and Rig-like receptor (RLR).
The types of surface receptors expressed and the maturation status of DC influence their ability and mechanisms to take up pathogens. Fluid phase macropinocytosis, phagocytosis, and receptor-mediated endocytosis are some of the well-defined cellular uptake mechanisms [27]. Macropinocytosis involves internalization of large extracellular materials including nutrients, solute molecules, and antigens in a nonspecific uptake mechanism [24, 28, 29]. This process involves initial ruffling of the plasma membrane mediated by the Rho GTPase’s, termed cell division cycle 42 (Cdc42), to form a vesicle called a macropinosome [30]. This is subsequently followed by remodeling of the actin cytoskeleton around the plasma membrane mediated by the Rac protein [31]. Fluid/antigens internalized through macropinocytosis by the DC are then delivered to the MHC class II -positive compartment by macropinosomes, where they are processed and presented as Peptide-MHC class II complex on the surface of the DC [24].

Phagocytosis is a crucial anti-bacterial host defense mechanism vital to clearing invading pathogens, and it is an important uptake mechanism utilized by DC and macrophages [32]. This is triggered by PRRs, and involves ingestion of relatively large (≥ 0.5 µm) particles in a receptor-mediated process [33]. This process involves the recognition of specific moieties, such as oligosaccharides and/or pathogen associated molecular patterns (PAMPs) present on the invading pathogens by the cell surface receptors, such as Dectin-1, scavenger receptors, and Fc receptors. The recognition of these microbial particles induces actin polymerization and internalization of the pathogen by the phagocytic cells [33].

Receptor-mediated endocytosis is another mechanism utilized by DC to internalize antigens. As the name suggests, this involves the use of different receptors present on the surface of DC for antigen uptake [21]. DC express receptors such as DEC205 and mannose receptors
that detect carbohydrate residues present on pathogens. These receptors facilitate the internalization of the pathogen through phagocytosis or clathrin-dependent endocytosis.

### 2.3.2 Antigen processing and presentation capacity of Dendritic Cells

The recognition and uptake of a pathogen by DC is just the beginning of achieving the definitive goal of antigen clearance which includes antigen processing through phagosomal acidification and proteolysis, and induction of T cell activation through antigen presentation. The newly formed phagosome undergoes rapid transformation through phagosomal maturation, a process that involves formation of a hostile environment characterized by lower pH that will lead to degradation of the ingested pathogen. It is not surprising, however, that some pathogens have evolved several mechanisms to thwart this process [34, 35]. Notably, *L. monocytogenes* secretes a pore-forming hemolysin, listeriolysin O (LLO), to evade phagosomal degradation [34, 36, 37].

The maturation of the phagosome begins immediately after the scission of the compartment from the plasma membrane [38]. The first step in phagosomal maturation involves fusion of the nascent phagosome with the early endosomes. This allows for acquisition of early endosomal proteins, EEA1 and Rab5, that enhance the intracellular trafficking and interaction of this phagosome with other vesicles [39, 40] (Fig. 2.3). The early phagosome then transitions into the late phagosome phase by fusing with the late endosomes. This stage allows for the acquisition of proton-pump vATPase that gradually acidifies the phagosomal lumen, and the Rab7 protein allows this vesicle to interact with the lysosome [38, 39]. Finally, the late phagosome fuses with the lysosome to generate a phagolysosome, a compartment enriched with proteases that are crucial for degradation of the ingested antigen.
**Figure 2.3. Phagosome maturation.** Antigen within the phagosome becomes degraded as a result of interaction between the phagosome and the endocytic vesicles, which include early endosome, late endosome and lysosome. This leads to acquisition of proteins and enzymes necessary to induce phagosome maturation. Consequently, the phagosomal lumen becomes acidified, leading to degradation of the pathogen.
Antigen degradation through phagosomal acidification and proteolysis is cell type dependent [33]. While M2 macrophages are known to rapidly acidify their phagosome [41], the phagosomal pH within neutrophils and M1 macrophages tend to remain neutral for a lengthy period [41, 42]. In contrast, degradation of ingested cargo by DC is relatively slow because these cells have mechanisms through which they control lysosomal degradation, thereby preserving useful peptides for T cell recognition [43]. Thus, the rate of phagosomal maturation is tailored to the functional capability of the cells.

The route of antigen uptake determines the intracellular compartment into which the antigen is delivered, and the fate of the antigen during processing. Antigens taken up through macropinocytosis are translocated into the late endosome/lysosome compartment where they are then processed and presented as peptide-MHC class II complexes to CD4+ T cells [44]. On the other hand, distinct receptor-mediated endocytosis delivers antigen to different compartments within the cells. For instance, DEC205-mediated endocytosis of ovalbumin delivers the antigen to the late endosomal compartment, leading to efficient antigen processing and presentation to CD4+ T cells [44], while mannose-mediated endocytosis targets the antigen to early endosomal compartment, leading to poor antigen presentation to CD4+ T cells [45].

As a result, the route of antigen uptake determines the magnitude of T cell response [46, 47]. For instance, antigen uptake through receptor-mediated endocytosis such as DEC205 increases the efficiency through which DC induce naïve T cell activation by a 1,000 fold when compared to antigen capture through macropinocytosis [46]. Moreover, the origin of antigens determines the type of T cells that will be activated. Antigens of cytosolic origin are presented by MHC Class I molecules to the cytosolic T cells, while exogenous antigens, derived through phagocytosis or endocytosis, are preferentially presented by MHC class II molecules to CD4+ T
cells [48]. Under certain conditions, exogeneous antigens can gain access to the cytosol of DC. These antigens will be processed through the cytosolic pathway and presented by MHC class I in a process called cross presentation.

The aforementioned features have been extensively studied in DC, as well as in other innate immune cells, such as macrophages. While these are important features that aid in mounting an appropriate immune response, the stage during DC development when these cells acquire these capacities has yet to be determined. Therefore, this dissertation project focuses on elucidating the development of antigen uptake, antigen processing, and initiation of the adaptive immune responses in DC and DC precursor cells.

2.3.3 The functional attributes of Dendritic Cells depend on its maturation status

DC exist in two main states that are phenotypically and functionally distinct: immature and fully mature [49]. In most tissues, DC exist in an immature state and are unable to induce potent T cell activation due to lack or low expression of accessory molecules, such as MHC class II, CD86, CD80, and CD40 that are necessary for providing positive signals for T cell activation [50]. However, DC can undergo morphological, phenotypical, and functional changes after they encounter microbes, or become stimulated by inflammatory signals in a process called maturation. The maturation signals in DC allow the cells to increase and maximize their ability to induce T cell activation through upregulation of the expression of molecules crucial in T cell activation. DC maturation affects their antigen internalization, processing, and presentation capability, as well as MHC trafficking (Table 2.1).
### Table 2.1: Summary of the differences between immature and mature DC

<table>
<thead>
<tr>
<th>Features</th>
<th>Immature DC</th>
<th>Mature DC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigen uptake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macropinocytosis</td>
<td>High</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>High</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Receptor-mediated endocytosis</td>
<td>High</td>
<td>Relatively the same</td>
</tr>
<tr>
<td><strong>Antigen processing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Retain ingested cargo for longer time</td>
<td>Rapid degradation of antigen</td>
</tr>
<tr>
<td>Phagosomal pH</td>
<td>High pH (more basic)</td>
<td>Low pH (more acidic) due to increase in the number of vATPase proton pump</td>
</tr>
<tr>
<td>Degradation</td>
<td>Low proteolysis</td>
<td>High proteolysis due to increase in proteolytic enzymes e.g. cathepsins, proteases</td>
</tr>
<tr>
<td><strong>MHC trafficking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localization</td>
<td>Rapid internalization from the plasma membrane and degradation in the endo-lysosome compartment</td>
<td>Increase in peptide-MHC complexes on the plasma membrane</td>
</tr>
<tr>
<td>Half-life</td>
<td>&lt; 10 hours</td>
<td>&gt;100 hours</td>
</tr>
<tr>
<td><strong>Expression of accessory molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Costimulatory molecules (CD86, CD40)</td>
<td>Low expression</td>
<td>High expression</td>
</tr>
<tr>
<td>MHC class II molecule</td>
<td>Low expression</td>
<td>High expression</td>
</tr>
<tr>
<td><strong>T cell activation</strong></td>
<td>Poor at inducing T cell activation</td>
<td>Potent at inducing T cell activation</td>
</tr>
</tbody>
</table>
The first functional change that occurs upon DC maturation is downregulation of their ability to take up new antigens [24, 31, 51]. Immature DC are highly phagocytic cells that are efficient at capturing a wide range of antigens in receptor-mediated endocytosis or nonspecific uptake through macropinocytosis. Immediately after stimulation with inflammatory signal, there is a transient increase in macropinocytosis, followed by a drastic decrease in antigen internalization [52]. This process is thought to be mediated by a decrease in Rho GTPase proteins, specifically Cdc42, that promotes actin polymerization [31]. As such, overexpression of Cdc42 in mature DC was found to restore their ability to internalize antigens via macropinocytosis.

The downregulation of endocytosis in mature DC is an important mechanism that regulates the range of antigens presented by DC [21, 31], and is thought to prevent the cells from capturing and presenting newly encountered antigens after the initial activation signal. It is now known that although mature DC down-regulate antigen uptake through macropinocytosis and phagocytosis, these cells still have the ability to take up antigens through receptor-mediated endocytosis [45]. This new finding indicates that mature DC may continue to initiate an immune response against newly encountered antigens during infection.

Another function of DC that changes upon maturation is the rate at which internalized antigens are being degraded. The degradation of internalized proteins needs to be tightly regulated in order to prevent complete degradation of useful antigens that can be presented to T cells. The degradation of proteins by DC and other phagocytes is controlled by lysosomal proteases. The optimal proteolytic activity of many of these enzymes is between pH of 5.5 and 6.5 [43]. Immature DCs are able to retain ingested cargo for a longer period of time before degradation due to high lysosomal pH and attenuated proteolytic activities [33, 53-56]. Shortly
after DC activation, proteolytic enzymes, such as cathepsin S, B, L, and H, are recruited from the lysosomes to the late endosomal compartment [57]. These enzymes enhance antigen degradation and formation of antigenic peptide that could be loaded on MHC class II molecules [57]. In addition, DC maturation leads to an increase in accumulation of vATPase proton pumps on the late endosomal membrane, leading to acidification and degradation of the antigens within the compartment [58].

In immature DC, there is an increase in biosynthesis of MHC class I and class II molecules [21, 59]. Despite an increase in synthesis of these molecules, immature DC are incompetent at generating stable MHC-peptide complexes on the plasma membrane. This is partly because the majority of these molecules are ubiquitinated and diverted to the endo-lysosomal compartment where they are degraded [60, 61]. However, upon maturation, MHC class II are deubiquitinated, followed by re-localization of these molecules to the MHC class II-rich peptide loading compartment. This leads to formation of stable peptide-MHC II complexes that will be redistributed to the plasma membrane to enhance antigen presentation to T cells [62]. In addition, MHC class II on immature DC have remarkable short half-life of about 10 hours because these molecules are constantly being internalized and recycled [63]. However, as DC undergo maturation, there is an increase in the synthesis of MHC class II molecules, and their half-life increase to more than 100 hours. These changes increase the number of peptide-MHC class II complexes and enhance the ability of these cells to induce T cell activation. In summary, the activation status of DC determines their functional attributes, and the efficiency through which they activate the adaptive immune cells.
2.4 The role of Dendritic Cells during infection with *Listeria monocytogenes*

There is evidence that DC mediate early immune response against pathogens in addition to their role in initiating an adaptive immune response. The role of DC, as well as other innate immune cells such as macrophages, has been extensively studied during *L. monocytogenes* infection. This is because *L. monocytogenes* is one of the well-characterized microbes that can be easily manipulated to examine the initiation of different cascades of immune responses [64]. In addition, *L. monocytogenes* has a remarkable ability to survive intracellularly and reproduce in different innate immune cells. This intracellular lifestyle helps to understand the interaction between this pathogenic microorganism and host immune cells. The following section focuses on *L. monocytogenes* life cycle and the role played by innate immune cells, specifically DC and macrophages, in mediating the early immune response against this pathogen.

2.4.1 Overview of *Listeria monocytogenes* Life cycle

*L. monocytogenes* is a facultative intracellular, Gram-positive bacterium that causes serious diseases in immunocompromised individuals, such as pregnant women, elderly, and children [65-67]. *L. monocytogenes* infection in pregnant women can induce spontaneous abortion and cause meningoencephalitis in adult and children. *L. monocytogenes* is an opportunistic bacterium with a unique ability to thrive in several environments, such as within plants, animals, water, and soil [66, 67]. Due to the vast amount of information known about this microorganism, it has become a useful pathogen in studying cellular processes, such as endocytosis, and the activation of both the innate and the adaptive immune responses.
Figure 2.4. Overview of *L. monocytogenes* life cycle. *L. monocytogenes* enters phagocytic cells through phagocytosis and initially reside within the host phagosome. Listeriolysin O (LLO) and phospholipase A and B (PlcA and PlcB) induce lysing of the vacuole and the bacterium escape to the cytosol. Within the cytosol, *L. monocytogenes* replicates and recruits the host actin protein to facilitate cell-to-cell motility. In the secondary cell, the bacterium is enclosed in a double membrane, and the membrane is lysed by LLO, PlcA, and PlcB. This allows the bacterium to begin a new infection process.
The mechanism utilized by this pathogen to invade the host cells is widely known. *L. monocytogenes* pathogenic life cycle begins with its entry to the host cell (Figure 2.4). *L. monocytogenes* typically induces its internalization into non-phagocytic cells with the help of two virulence surface proteins called internalin A (InIA) and B (InIB) [68, 69]. The InIA targets the E-cadherin receptor expressed by the epithelial cells while the InIB binds to the growth factor receptor, c-Met receptors found, on the hepatocytes. The interaction of these bacterial surface proteins with the host receptors induces signaling cascades that facilitate the entry of the bacterium into non-phagocytic cells [70]. In contrast, macrophages and DC recognize this bacterium through their PRRs and internalize it in an actin-dependent phagocytosis.

After successful invasion, *L. monocytogenes* has a short window of time to escape from the host phagosome to avoid the hostile environment that could interfere with its life cycle. Inside the host phagosome, *L. monocytogenes* secretes a toxin called listeriolysin O (LLO, a hemolysin that forms pores in the host vacuoles, which enables the bacterium to escape from the internalized vacuole into the cytosol where it can replicate [65, 66].

In addition to cytosolic entry and replication, *L. monocytogenes* continues the infection process by invading the neighboring cells with the help of ActA protein. The ActA protein induces actin polymerization around the bacterium, propels the bacterium within the host cytosol, and allows it to navigate its way to neighboring/adjacent cells in order to mediate cell-to-cell infection [65]. Cell-to-cell spread encloses this bacterium in a double membrane in the secondary host cell. Thus, *L. monocytogenes* within the secondary vacuoles utilizes two phospholipases, PlcA and PlcB, together with LLO to lyse the vacuole and escape.
2.4.2 Differences in susceptibility of Dendritic Cells and Macrophages to Listeria monocytogenes infection

Several studies have demonstrated the differences in the rate at which L. monocytogenes replicates in DC and macrophages. Studies by Westcott et. al. have demonstrated that DC provide a suboptimal environment for L. monocytogenes replication when compared with macrophages [71]. In that study, L. monocytogenes’ intracellular doubling time was 45 minutes in macrophages and 70 minutes in DC. In addition, the bacteria within the DC increased 10-fold compared to a 70-fold increase in macrophages. A subsequent study showed that bacteria that failed to escape to the cytosol were restricted to a MHC class II-rich phagosome, providing a suboptimal intracellular niche for L. monocytogenes growth [72]. Another study found that fascin-1, an actin budding protein, plays an important role in the ability of DC to control L. monocytogenes infection [73]. Fascin-1 facilitates the fusion of the lysosome with the L. monocytogenes containing phagosome, enhancing the killing of the ingested bacteria.

While DC are less susceptible to L. monocytogenes infection when compared to macrophages, it is worth mentioning that different subsets of DC respond to L. monocytogenes infection differently. For instance, CD11c+ DC support bacterial growth, while CD103− and CD103+ conventional DC isolated from lamina propria and mesenteric lymph nodes of naïve mice does not provide a productive niche for L. monocytogenes growth [74]. Therefore, the ability of DC to restrict L. monocytogenes infection are dependent on several factors.

Moreover, recent studies have compared monocytes and macrophages to determine which cell type is more susceptible to L. monocytogenes intracellular growth [75]. To determine if monocytes provide a better intracellular niche for L. monocytogenes growth, bone marrow cells were stimulated with M-CSF, which allowed the isolation of three cell types: monocytes
(Ly6C^+CD64^{neg}), transitioning cells (Ly6C^+CD64^+), and macrophages (Ly6C^{neg}CD64^{hi}) [75]. Macrophages were found to have high expression of E-cadherin on the surface, but monocytes had no detectable expression of this protein [75]. These differences in the expression level of E-cadherin were found to correspond with the efficiency at which L. monocytogenes invade these cell types. Moreover, this study demonstrated that monocytes were limited in their ability to take up L. monocytogenes and restrict cytosolic invasion when compared to macrophages, and further supported the claim that macrophages provide a better intracellular niche that enhances L. monocytogenes growth [71].

### 2.5 When do Dendritic Cells develop their functional attributes?

Despite the well-characterized uptake mechanisms utilized by DC, there is a gap in knowledge on the specific stage during their development when DC acquire these functional attributes and how their developmental stages influence their uptake capacity and/or the overall immune function. Most of what are known about DC functions have come from studies that utilized terminally differentiated DC. In some cases, there are few comparative studies that examine these functions between terminally differentiated myeloid cells, such as macrophages and DC. Yet, there is little that is known about the progression of the development of these functions. It is therefore important to fully understand when and how these functions develop during DC development.

### 2.6 Dendritic Cells Development

#### 2.6.1 Hematopoiesis

There are more than 10 distinct, mature immune cells that originate from common progenitor cells called Hematopoietic Stem Cells (HSC). Not only are HSC pluripotent cells that
have the ability to differentiate into any blood cell, HSC are also self-renewing cells that have the ability to give rise to themselves without differentiation [76]. The HSC first give rise to multipotent progenitors (MPP), a population of cells that have lost the self renewing capacity that is typical of HSC but retain the pluripotency potential [76, 77]. The differentiation of the MPP to the first step in lineage commitment limits the cells to one of two lineages: the Common Lymphoid Progenitors (CLP) [78] and the Common Myeloid Progenitors (CMP) [79] (Fig 2.5). The CLP have the ability to differentiate into cells of the adaptive immune system that include B and T cells, as well other innate immune cells of lymphoid origin, such as natural killer cells and dendritic cells [78, 80]. The CMP lineage, as the name suggests, gives rise to the myeloid cell populations that include the megakaryocyte/erythrocyte progenitors (MEP), granulocyte/macrophage progenitors (GMP), monocytes, and DC [79]. It is important to note that DC can be derived from both CLP and CMP lineages through stimulation with Flt3L (FMS-like tyrosine kinase 3 ligand) or GM-CSF growth cytokine [81-87].
Figure 2.5. Overview of Hematopoietic stem cell (HSC) differentiation. HSC differentiation into MPP characterized with multipotency and limited self-renewing capacity. MPP differentiate into either common lymphoid progenitor (CLP) or common myeloid progenitor (CMP) lineage.
2.7 GM-CSF driven Myelopiesis towards becoming Dendritic Cells

GM-CSF represents a unique hematopoietic growth factor that is critical in myeloid cell differentiation because it induces bone marrow to give rise to not only cells of the granulocytic lineages, but also cells that represent the monocytes, macrophages, and DC lineages in a process called myelopoiesis [87]. Under steady state condition, the level of GM-CSF is relatively low; however, there is a surge in the level of this cytokine under inflammatory conditions [18, 88]. Stimulation of bone marrow myeloid precursor cells with GM-CSF gives rise to common myeloid progenitors (CMPs), granulocyte macrophage precursors (GMPs), monocyte-macrophage/DC progenitors (MDPs), and monocytes in a successive committed stages in development before becoming monocyte derived DC (moDC) (Fig 2.6) [89, 90].

2.7.1 Common Myeloid Progenitors (CMP):

Following the classification and isolation of the common lymphoid progenitors that give rise to all the lymphoid cells in late 1990s, the CMP populations were successfully isolated and classified in 2000 [79]. CMP are progenitor cells that give rise to all the cells of the myeloid lineage, but which have lost their lymphoid potential [79, 91]. They are identified \textit{in vivo} with the expression of c-kit, CD34, CD31, FcγR [79], and lack lineage markers such as CD3, NK1.1, and B220 [92]. CMP are heterogenous cell type that have the potential to differentiate into GMP, the first progenitor cells that give rise to all other cell type of myeloid lineage [93]. Based on the previous studies, the CMP represent the first developmental stage that GM-CSF stimulated bone marrow go through before ultimately differentiating into the terminally differentiated moDC [94, 95]. The CMP population is classified \textit{in vitro} with the phenotypic profile: Ly6C^−CD115^−CD11c^− as well as expression of the early progenitors’ surface markers mentioned above. In addition to
the phenotypic profile, CMP expresses the following transcripts: *Irf2, Kit, Pecam1, Cebpa, GFil*, and *Klf4* [94].
Figure 2.6. Overview of the stages of myeloid cells development towards dendritic cells. The CMP differentiate into megakaryocyte and erythroid progenitor cells (MEP) or the granulocyte/macrophage progenitor cells (GMP). The GMP populations differentiate into all the cells that represent the myeloid lineages that include the macrophage/dendritic cell progenitor (MDP), common monocyte progenitor (cMoP), monocyte, monocyte-derived macrophages/monocyte-derived dendritic cells precursor (moMac/moDP), and monocyte-derived dendritic cells (moDC).
2.7.2 Granulocyte Macrophage Precursor (GMP):

GMPs are the progeny of differentiated CMPs that have lost the potential to differentiate into platelets and erythrocytes [96], and they have limited self-renewing potential when compared with HSCs. The GMPs give rise to the granulocyte or monocyte lineages when stimulated with G-CSF or GM-CSF, respectively. These cells have a phenotypic profile of Ly6C\textsuperscript{hi} Sca-1\textsuperscript{hi} CD11b\textsuperscript{−} CD115\textsuperscript{−}.

2.7.3 Monocytes:

Monocytes originate from HSCs in the bone marrow that go through a series of developmental stages (CMP, GMP, MDP, and common monocyte progenitor (cMoP)) before becoming monocytes (Figure 1.1) [89, 90, 97, 98]. These cells constitute about 2–4 % of circulating leukocytes in mice. Monocytes are heterogeneous in size and shape which makes it difficult to distinguish them morphologically from DCs and NK cells [90]. However, they can be classified based on the phenotypic profile: CD115\textsuperscript{+} Ly6C\textsuperscript{+} Ly6G\textsuperscript{−} CD11b\textsuperscript{+} and low levels of chemokine receptor CX3CR1 [90, 99].

Monocytes have been thought to represent a pool of myeloid precursor cells endowed with the capability of giving rise to macrophages and DCs. For instance, several studies have shown that in vitro stimulation of bone marrow monocytes with GM-CSF gives rise to DC that phenotypically resemble inflammatory DC (iDC) or moDC in vivo [88, 100]. Two main subsets of monocytes, Ly6C\textsuperscript{lo} and Ly6C\textsuperscript{hi} monocytes, with different functional properties have been identified [99, 101]. The Ly6C\textsuperscript{hi} monocyte is considered to be the immediate precursor of moDC [101-103], while Ly6C\textsuperscript{lo} monocytes give rise to an anti-inflammatory or tolerogenic DC [102]. However, most of these studies were done in vivo with a level of difficulty in clearly identifying any potential intermediate precursor cell between Ly6C\textsuperscript{hi} monocytes and the moDC.
2.7.4 Monocyte-derived Macrophages (moMac)/monocyte-derived DC Precursor (moMac/moDP)

Monocytes, macrophages, and DC are more differentiated myeloid cells capable of phagocytosis and eliciting an immune response. These cells are thought to have MDP as the common precursor [89]. MDP are proliferating cells that have myeloid precursor activity with the ability to differentiate into monocytes, macrophages, and common DC precursor (CDP) \textit{in vivo} (Figure 1.1). The identification of CDP, a restricted progenitor cell that can only differentiate into DC downstream of MDP [83, 104], has led researchers to inquire if such a cell exists that could give rise to monocytes or moMac. Recently, cMoP, a restricted progenitor cell, was identified downstream of MDP. The cMoP has the tendency to differentiate into monocyte and moMac, but it has lost the capability of differentiating into DC \textit{in vivo} [98]. However, \textit{in vitro} stimulation of cMoP with GM-CSF was shown to give rise to DC [98].

Several studies have established that bone marrow cultured in GM-CSF could generate a large population of CD11c\(^+\)MHCII\(^+\) moDC. However the relatively high frequency of moMac in these cultures had not been appreciated [19, 20]. Recent progress in GM-CSF cell culture has led to identification of two cell populations that are distinguishable based on the expression level of CD11c and MHC class II molecules (MHCII) during GM-CSF driven differentiation. The moMac populations were identified as: CD11c\(^+\)MHCII\(^{lo}\)CD115\(^+\)MerTK\(^+\) while the moDC were identified as CD11c\(^+\)MHCII\(^{hi}\)CD115\(^-\)CD135\(^+\) [19]. A recent study by Rogers et. al. confirmed and extended these findings and identified an intermediate precursor cell, the monocyte-derived DC precursor (moDP), which shares many phenotypic characteristics with moMac, but are distinguished by higher MHCII expression and ability to give rise to moDC [94].
2.8 Conclusion

Over the past decades, there has been substantial research done on the role of DC in host immunity. Specifically, their role with regards to antigen uptake, antigen processing and activation of the adaptive immune cells through antigen presentation have been extensively studied. What remains to be determined is the stages during DC development that these cells acquire these functional properties. This project sets out to investigate the development of DC functional attributes during GM-CSF driven differentiation by researching on the following questions: (1) what type of antigen uptake receptors or antigen uptake mechanisms are present in DC and DC precursor cells? (2) How does antigen processing capacity changes during DC development? (3) At what stage during DC development do these cells acquire antigen presentation capacity? (4) How does the developmental stage of myeloid cells influence their susceptibility to *L. monocytogenes* infection? (5) At what stage do myeloid cells develop bactericidal activity? Answering the above questions will help to better understand the functional characteristics of each myeloid cell population.
References


Chapter 3

Development of endocytosis, degradative activity, and antigen processing capacity during GM-CSF driven differentiation of murine bone marrow

Citation: Olatunde AC, Abell LP, Landuyt AE, Schwartz EH. Development of endocytosis, degradative activity, and antigen processing capacity during GM-CSF driven differentiation of murine bone marrow. PloS one. 2018 May 10;13(5)
3.1 Abstract

Dendritic cells (DC) are sentinels of the immune system, alerting and enlisting T cells to clear pathogenic threats. As such, numerous studies have demonstrated their effective uptake and proteolytic activities coupled with antigen processing and presentation functions. Yet, less is known about how these cellular mechanisms change and develop as myeloid cells progress from progenitor cells to more differentiated cell types such as DC. Thus, our study comparatively examined these functions at different stages of myeloid cell development driven by the GM-CSF. To measure these activities at different stages of development, GM-CSF driven bone marrow cells were sorted based on expression of Ly6C, CD115, and CD11c. This strategy enables isolation of cells representing five distinct myeloid cell types: Common Myeloid Progenitor (CMP), Granulocyte/Macrophage Progenitor (GMP), monocytes, monocyte-derived Macrophage/monocyte-derived Dendritic cell Precursors (moMac/moDP), and monocyte-derived DC (moDC). We observed significant differences in the uptake capacity, proteolysis, and antigen processing and presentation functions between these myeloid cell populations. CMP showed minimal uptake capacity with no detectable antigen processing and presenting function. The GMP population showed higher uptake capacity, modest proteolytic activity, and little T cell stimulatory function. In the monocyte population, the uptake capacity reached its peak, yet this cell type had minimal antigen processing and presentation function. Finally, moMac/moDP and moDC had a modestly decreased uptake capacity, high degradative capacity and strong antigen processing and presentation functions. These insights into when antigen processing and presentation function develop in myeloid cells during GM-CSF driven differentiation are crucial to the development of vaccines, allowing targeting of the most qualified cells as an ideal vaccine vehicles.
3.2 Introduction

Dendritic cells (DC) are specialized immune cells that function in antigen uptake, processing and presentation, and induction of the adaptive immune response [1-3]. DC represent remarkable group of cells found in both lymphoid and non-lymphoid tissues under inflamed and/or steady state conditions. These cells have been classified into different subsets based on phenotypic and functional profiles [4, 5]. Phenotypically, expression of the integrin CD11c and high levels of MHC class II have been used to broadly identify DC. Subsets of DC are further separated based on expression of CD8, CD4, CD11b, and CD45R [6-8]. The functional attributes used in sub-setting DC include migration potential, antigen uptake capability, processing and presentation to the T cells [2, 9, 10]. Steady state DC, whose differentiation is dependent on Fms-like tyrosine kinase 3-ligand (Flt3L), represent conventional lymphoid resident or migratory DC [11-15]. The steady state DC that include both conventional DC (cDC) and plasmacytoid DC (pDC) differentiate from common DC precursor (CDP) and through an intermediate stage known as pre-DCs [16, 17]. Under inflammatory conditions however, monocyte-derived DC (moDC) development and differentiation is driven by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) [18-21].

Historically, it has been difficult to acquire sufficient numbers of DC directly ex vivo for functional analysis. DC are typically present in much lower numbers than lymphocytes in lymphoid organs and are of relatively low abundance in peripheral tissues as well [22]. Thus, for decades, GM-CSF has been a favorite cytokine used to generate large numbers of DC from mouse bone marrow in vitro [23-25]. Much of what we understand about the endocytic capacity, proteolytic activity, phagosomal maturation, and antigen processing and presenting function of GM-CSF-driven cells has come from studies on differentiated cells, DC and macrophages [26-
Thus, we know comparatively little about the developmental stage at which these functions develop. It is therefore important to investigate the development of these functions in order to identify the most qualified cells for therapeutic uses.

Recent studies have demonstrated the previously unrecognized heterogeneity of bone marrow cultured in GM-CSF [31, 32]. While the GM-CSF-driven culture method is known to generate a large population of CD11c^+MHCII^+ moDC, the relatively high frequency of monocyte-derived macrophages (moMac) in these cultures had not been appreciated [31]. These two cell types were distinguished based on the expression level of CD11c and MHC class II (moMac are MHCII^{lo/int}, moDC are MHCII^{hi}) [31]. Our recent study confirmed and extended these findings and identified an intermediate precursor cell, the monocyte-derived DC precursor (moDP), which shares many phenotypic characteristics with moMacs, but are distinguished by higher MHCII expression and ability to give rise to moDC [33]. Thus, this cell type is termed moDP.

To elucidate the functional capacity of each distinct myeloid cell population during GM-CSF driven differentiation, we have developed a sorting strategy that enables the isolation of five distinct myeloid cell populations based on the expression of Ly6C, CD115, and CD11c. The phenotypic profile of these developmental stages is as follows: common myeloid progenitor (CMP): Ly6C^-CD115^-CD11C^-, granulocyte-macrophage progenitor (GMP): Ly6C^-CD115^-CD11C^-, Monocytes: Ly6C^+CD115^-CD11C^-, monocyte-derived macrophages/monocyte-derived DC precursors (moMac/moDP): Ly6C^-CD115^+CD11C^+, and monocyte-derived DC (moDC): Ly6C^-CD115^+CD11C^+ [33]. Thus, by isolating each of these populations, we set out to clearly define how the cellular uptake mechanisms, phagosomal acidification, and proteolytic activity change during myeloid cell development, and how these functional mechanisms impact the
antigen processing and presentation function of these distinct myeloid cell populations along the developmental pathway.
3.3 Materials and methods

3.3.1 Mice

C57BL/6, OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J) and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice were obtained from Jackson Laboratory. T cells derived from OT-I mice recognize OVA_{257-264} peptide presented by MHC class I (H-2 K\textsuperscript{b}), while OT-II mice recognize OVA_{257-264} peptide presented by MHC class II (I-A\textsuperscript{b}). These mice serve as a source of large numbers of antigen-specific T cells for measuring presentation of that antigen (ovalbumin). All mice were housed under specific pathogen free conditions. All experiments were approved by the Auburn University Institutional Animal Care and Use Committee and were performed in accordance with the approved guidelines.

3.3.2 Antibodies and reagents

Primary antibodies specific for murine CD11c (clone N418), CD206 (MMR) (clone C068C2), CD115 (CSF-1R) (clone AFS98), Dectin-1 (CLEC7A) (clone RH1), MHC class I and MHC class II (I-A\textsuperscript{b}) (clone AF6-120) were from Biolegend (San Diego, CA). Ly6C (clone HK1.4) was purchased from Ebioscience and CD36 (clone CRF D2712), CD25 (clone PC61), CD4 (clone RM4-5) and CD8 (clone 53.6.7) were purchased from BD-Bioscience (San Jose, CA). DQ\textsuperscript{TM} ovalbumin, OVA-Alexa 647, Dextran Alexa Fluor® 488 (mw 10,000) Anionic Fixable, Staphylococcus aureus (wood strain without protein A) BioParticles®, Alexa Fluor® 488 conjugate pHrodo® Green Escherichia coli BioParticles® were purchased from Molecular Probe (Eugene, Oregon). Nigericin sodium salt was purchased from Enzo Life sciences (Farmingdale, New York). Monensin sodium salt was purchased from Amresco (Solo, Ohio).
3.3.3 Cells and sorting strategies

Myeloid cells were generated from C57BL/6 bone marrow (isolated from 8-24 week-old mice) as previously described [34]. In brief, bone marrow was harvested from mice and the red blood cells were lysed. The cells were cultured in RPMI media supplemented with 10% fetal calf serum (FCS), glutamine (2 mM) and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). The cultured cells were sorted into 5 distinct myeloid cell populations: CMP, GMP, monocyte, moMac/moDP, and moDC using Beckman Coulter Moflo XDP High Speed Cell Sorter at the Auburn University Flow cytometry facility as described previously [33]. Briefly, cultured cells were harvested on either day 3 (to isolate early progenitor cells) or day 5 (to isolate more differentiated cell types) and stained with antibodies to CD115 (CSF-1R), Ly6C and CD11c. CMP, GMP, Monocyte, moMac/moDP, and moDC were isolated based on the phenotypic profile: Ly6C^-CD115^-CD11c^-, Ly6C^+CD115^-CD11c^-, Ly6C^+CD115^+CD11c^-, Ly6C^-CD115^+CD11c^low, and Ly6C^-CD115^-CD11c^+, respectively. The five isolated cell types (from here on referred to as sorted populations) were re-suspended in growth media and were used for all experiments described below, except where indicated otherwise.

In order to differentiate between the moMac/moDP populations, day 5 cells were stained with antibodies to CD11b, CD11c, and MHC class II. Then, the CD11b^+CD11c^+ cells were isolated into MHCII^{hi}, MHCII^{int}, and MHCII^{hi} cell populations that correspond to moMac, moDP, and moDC.

3.3.4 Ex vivo cell isolation

Freshly harvested bone marrow from C57BL/6 mice after lysing of the red blood cells were stained with antibodies to CD115, Ly6C and CD11c. These cells were sorted into CMP,
GMP, and Monocytes based on the expression of these phenotypic markers and sorting strategies described above.

3.3.5 Cell surface receptor staining

Bone marrow cells cultured in GM-CSF were harvested and stained with antibodies to CD36 PE, Dectin-1 PE, CD206 PE, CD115, Ly6C and CD11c from day 3 to 7. About 2 x 10^5 cells were stained with each antibody at a final concentration of 2 µg/mL, and incubated at 4 °C for 30 min. Following the incubation period, cells were washed three times with ice cold FACS Buffer (PBS supplemented with 3% fatal Bovine serum). Unstained cells or cells stained with only one of the antibodies served as control in order to determine the level of auto fluorescence, compensate for fluorescence spillover, and set the gating boundary. The expression of these receptors was measured using flow cytometry.

3.3.6 Phagocytosis/Macropinocytosis Assay

Cells from each sorted cell population (2 x 10^5 cells/well) were seeded in a 96 well plates. Dextran (0.5 mg/mL) or BioParticles (ratio of 2 particles to 1 cell) were added to the wells. The cells were incubated at either 37°C or 4°C (as a negative control) for 1 hr and the uptake of these endocytic tracers was measured using flow cytometry. The uptake capacity of each cell population was calculated by subtracting the mean fluorescence intensity (MFI) of samples incubated at 4°C from the MFI of samples incubated at 37°C for 1 hr.

3.3.7 Phagosomal acidification/pH assay

Sorted cell populations were seeded at 2 x 10^5 cells/well in 96 well plate, and were pulsed with 10 µL pHrodo® Green Escherichia coli BioParticles® for 15 min at 37°C, and washed twice by adding cold growth media, centrifuge at 1200rpm for 5 min and the supernatant
was discarded. Cells were then resuspended in growth media and chased for additional 90 min at 37°C. At the end of the chase period, the cells were washed once with FACS buffer and analyzed using flow cytometry. The uptake of the BioParticles was measured using flow cytometry. To determine the actual pH of the pHrodo-containing phagosomes, pH calibration buffer containing 50 mM HEPES, 30 mM Ammonium Acetate, 10 µM nigericin, and 10 µM monensin was used. The pH of the calibration buffer was adjusted with HCl to create a standard curve of varying pHs, ranging from pH 2.5 to pH 7.5. After the 90 min chased period, cells from each sorted population were washed with FACS wash buffer and incubated with the pH calibration buffer for 15 min at 37°C and the samples were analyzed using flow cytometry. A pH standard curve was generated for each population by plotting the MFI of the pHrodo against the pH values. The standard curve equations generated from these graphs were used to predict the actual pH of the pHrodo-containing phagosome in each sorted population.

3.3.8 Proteolysis assay

The proteolytic capacity of each cell population was determined by incubating 2 x 10^5 cells from each sorted population with 10 µg/mL DQ™ ovalbumin (DQ-OVA) for 15 min at 37°C. After the initial incubation, cells were washed twice with cold growth media (as described above) and chased for additional time points. Degradation of DQ-OVA (increasing fluorescence) was measured using flow cytometry. To control for differences in uptake capacity, sorted cell populations were incubated with both DQ-OVA and OVA-Alexa 647 for 1 hr. DQ-OVA fluorescence was then measured specifically in cells containing OVA-Alexa 647.

3.3.9 In vitro T cell activation assay

CD8+ T cells were isolated from spleens of OT-I mice (C57BL/6-Tg(TcraTcrl)1100Mjb/J) and CD4+ T cells were isolated from spleens of OT-II mice (B6.Cg-
Tg(TcraTcrb)425Cbn/J). About $6 \times 10^7$ cells were labeled with 5 $\mu$M CFSE in Hanks’ Balanced Salt Solution at room temperature for 8 minutes while shaking gently every 2-3 mins. This was followed by adding 2 mL FCS for at least 1 min to stop the labelling, washed and counted before incubation with sorted populations. For OT-I cell stimulation, sorted cell populations were seeded in a 96 well plates and incubated with 300 $\mu$g/mL of OVA protein or with 10 nM OVA$_{257-264}$ Peptide for 2 hrs followed with direct co-culture with CFSE labeled OT-I T cells. For OT-II cell stimulation, sorted cell populations were seeded in 96 well plates and incubated with either OVA protein (100 $\mu$g/mL) or OVA$_{323-339}$ peptide (1 $\mu$M) for 2 hrs followed with direct co-culture with CFSE labeled OT-II T cells. About $5 \times 10^4$ of sorted cell populations and $2.5 \times 10^5$ T cells at a ratio of 1:5 was used for this experiment. T cell proliferation and activation was measured by flow cytometry after day 4 or 5 for OT-I and OT-II T cells, respectively.

3.3.10 Flow Cytometry

Where indicated, cells were stained with appropriate fluorophore-conjugated antibody in ice-cold FACS wash buffer (PBS + 3% FCS) on ice for 30 min. This was followed by washing twice with FACS wash buffer, centrifugation at 1200rpm for 5 min. Prior to acquisition, cells were resuspended in FACS wash buffer and filter through 35 $\mu$m. Unstained cells or cells stained with only one fluorophore were used to set the gates. In experiments where cells were loaded with fluorescence tracers, untreated samples serve as control and the gates were set on these untreated samples to determine the baseline fluorescence, and to set the compensation in that channel. Also, the forward and side scatter plots were used to exclude debris and doublets. All data were collected on an ACCURI C6 Flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).
3.3.11 Statistical Analysis

The statistical analysis was performed using either GraphPad Prism 6 or Statistical Analysis System (SAS) software. All graphs were plotted in GraphPad Prism 6. The effect of treatment between different cell populations was analyzed using one-way analysis of variance (ANOVA), the Tukey multiple comparison PROC GLM procedure. Where indicated, treatments were considered significantly different at p< 0.05.
3.4 Results

3.4.1 Expression of uptake receptors during GM-CSF driven differentiation

Myeloid cells are known to express a wide range of uptake receptors such as scavenger receptors [35, 36], mannose receptors, and Dectin-1 [37, 38]. These receptors play important roles in cell adhesion as well as optimizing uptake of different types of particles or antigens. However, the relative expression of these receptors at distinct stages of GM-CSF-driven cell development is not well defined. Thus, we measured the expression of these receptors during GM-CSF driven differentiation of murine bone marrow from days 3 through 7. The heterogeneous populations of bone marrow were stained with antibodies to scavenger receptor (CD36), mannose receptor (MMR, CD206) and Dectin-1 and expression was measured by flow cytometry. There was an increase in the expression of CD36 and CD206 from days three to seven of culture with the expression of these two receptors peaking on day seven (Fig 3.1A). The expression of Dectin-1 increased from day 3 to day 5, with a slightly decreased expression on day 6 and 7 (Fig 3.1A). This result suggested that CD36 and CD206 are expressed at later stages during myeloid cell development while Dectin-1 is most highly expressed earlier during development.
**Figure 3. 1. Differential expression of uptake receptors during GM-CSF driven differentiation.** Bone marrow was harvested from C57BL/6 mice and was cultured in GM-CSF and stained with antibodies to CD36, CD206, or Dectin-1. (A) Representative histograms showing the expression of CD36, CD206, and Dectin-1 from day 3 to 7. Data were compiled from at least three independent experiments showing mean fluorescence intensity (MFI) of (B) CD36 and (C) MMR expression in the five cell populations. Statistical analysis was conducted using one way ANOVA Tukey multiple comparison test. Letters over bars indicate statistically significant differences in means (p<0.05).
We then measured the expression of these receptors with respect to distinct developmental stages, based on Ly6C, CD115 and CD11c expression. Interestingly, moMac/moDP and moDC, which were the dominant cell types on day 6 and 7, had the highest expression of CD36; whereas, CMP and GMP, the most prevalent cell types on day 3, had low expression of CD36 (Fig 3.1B). These results are consistent with a previous study that found high expression of scavenger receptor in DC, and the expression of this receptor is up-regulated upon DC maturation [39]. In contrast, all of the cell types expressed CD206 at relatively the same level with the exception of moDC that had a slightly increased expression (Fig 3.1C). This result is consistent with another study that found high expression of CD206 in immature DC [40]. This study showed that early progenitor cells expresses low levels of uptake receptors.

3.4.2 Distinct uptake capacities exhibited during GM-CSF driven differentiation

Immature DCs are known to utilize both macropinocytosis and phagocytosis as mechanisms of uptake [40-43]. However, endocytic capacity is down-regulated as dendritic cells undergo maturation [41]. What remains to be determined is how uptake capacity changes during development. Thus, to determine the uptake capacity of each myeloid cell population along the developmental spectrum, the sorted cells were fed one of two different endocytic tracers: Alexa-488 dextran (mw 10,000) as a measure of fluid-phase uptake or pinocytosis or Alexa-488 tagged BioParticles (*Staphylococcus aureus* wood strain without protein A), as a measure of phagocytosis. As illustrated by representative histograms (Fig 3.2A), CMPs demonstrated a low level of dextran uptake above the ice control, at a low level. Pinocytic activity increased only moderately in the GMP population, yet reached its peak in monocytes and moMac/moDP (Fig 3.2A and 3.2B). Pinocytic activity of moDC was slightly reduced compared to monocytes and moMac/moDP (Fig 3.2A and 3.2B).
Figure 3. 2. The uptake capacity of GM-CSF driven myeloid cells increased as cell progress in development and modestly decreased in more differentiated cell types. Sorted cell populations were fed 0.5 mg/mL Dextran Alexa Flour 488 (10,000 MW) or S. aureus BioParticle Alexa flour 594 conjugate for 1 h at 37°C (red), or at 4°C (blue) as negative control. The uptake of the fluorescent tracers was measured by flow cytometry. (A) Representative histograms of Dextran uptake (top panels) and BioParticle uptake (bottom panels) by the five isolated myeloid cell populations. Uptake of dextran (B) and bioparticles (C). Uptake values were calculated by subtracting the MFI of the ice control for each population from the MFI of that population incubated at 37°C. These data are compiled from at least four independent experiments. Statistical analysis was performed using SAS, one-way ANOVA Tukey’s multiple comparison tests. Letters over bars indicate statistically significant differences in means (p<0.05).
The uptake of the larger cargo, BioParticles, followed a similar general trend as dextran uptake with some notable differences. CMP demonstrated virtually no phagocytic capacity (Fig 3.2A and 2C). Strikingly, GMP exhibited strong phagocytic activity, similar to that of monocytes (Fig 3.2A and 2C), and considerably stronger than their pinocytic activity (Fig 3.2B and 2C). Again, monocytes demonstrated the highest uptake capacity, with the highest phagocytic activity (Fig 3.2C). In contrast to dextran uptake, phagocytic activity also tended to decrease more significantly in moMac/moDP and moDC as demonstrated by decreased BioParticle uptake in these cell types (Fig 3.2C). These observed differences in uptake capacities of each myeloid cell type reflect significant functional diversity among these cell types.

3.4.3 Phagosomal acidification increases progressively during GM-CSF-driven development

Acidification of the phagosome is one of the key events of phagosome maturation, and it is important for both antimicrobial and antigen processing functions [44, 45]. To assess the phagosomal pH of each myeloid cell population, we used a pulse-chase approach with pH-sensitive fluorescent particles (pHrodo Green *E. coli* BioParticles, Molecular Probes/Invitrogen). Cells were loaded with the pHrodo particles for 15 min, washed, and chased for additional 90 mins. This allowed us to track the acidification of a synchronized cohort of particles taken up during the short pulse period. Representative histograms of each cell population following the pulse of pHrodo *E. coli* BioParticles are presented in Fig 3.3A (top panels). Each population had a low background fluorescence indicating minimal acidification at this early time point (Fig 3.3A and 3B). After the 90 min chase period, phagosomal acidification became evident to different degrees in the five populations. Fluorescence was especially high in the monocyte and moMac/moDP populations (Fig 3.3A (bottom) and 3.3B). CMP demonstrated the little to no
phagosomal acidification (Fig 3.3A and 3.3B). The percent of pHrodo *E. coli* BioParticle-
positive cells as well as the MFI of pHrodo gradually increased in GMP and monocytes and
peaked in moMac/moDP population, decreasing substantially in moDC (Fig 3.3B).
Figure 3. Phagosomal pH of each developmental stage. Sorted populations were pulsed with 10 µL pHrodo *E. coli* BioParticles for 15 min, washed and chased for 90 min. The uptake of the pHrodo *E. coli* BioParticles and the phagosomal acidification was measured by flow cytometry. (A) A representative histograms of pHrodo *E. coli* BioParticle uptake at 15 min (top) and phagosomal acidification at 90 min (bottom) in all the five isolated populations. (B) The MFI of pHrodo *E. coli* BioParticle uptake at 15 min and 90 min, in all the five isolated myeloid cell populations. Data are compiled from at least four independent experiments. (C) The phagosomal pH of each myeloid cell population was calculated vs a standard curve at varying pHs maintained in Hepes buffer containing nigericin and monensin. The statistical analysis was performed using SAS, one way ANOVA Tukey’s multiple comparison test. Letters over bars indicate statistically significant differences in means (p<0.05).
Our previous studies indicated that the *L. monocytogenes*-containing-phagosomes in DC had a slightly elevated pH compared to the same compartment in bone marrow derived macrophages [34]. To better understand phagosomal acidification across the developmental spectrum, we quantitatively compared the phagosomal pH of the five developmental stages driven by GM-CSF. To determine how fluorescence corresponded to actual pH values, standard curves were generated for each cell type in pH-normalized buffers using nigericin and monensin to equilibrate intracellular and extracellular pH. Phagosomal pH decreased progressively from the CMP stage (which was close to neutral pH 7.01) through the moMac/moDP stage which had the lowest pH at ~ pH 4.26. The pH of the moDC population was higher, ~ pH 5.89, which was very similar to monocytes (~5.87). GMP had an average pH of 6.52 (Fig 3.3C). Thus, these findings support our previous result indicating that moDC have a higher pH than moMac [34, 46].

**3.4.4 Proteolysis is a function exhibited later in the GM-CSF-driven developmental pathway**

To elucidate the proteolytic capacity of myeloid cells along the developmental spectrum, we utilized a fluorescence-quenched probe- DQ™ ovalbumin (DQ-OVA) that emits bright green fluorescence upon proteolysis. Each isolated cell population was pulsed with 10 µg/mL DQ-OVA for 15 min, washed and chased for additional 30 min, 1 hr, or 2 hr. As expected, there were no significant differences in the fluorescence of DQ-OVA across the populations after the initial 15 min pulse (Fig 3.4A and 3.4B) as this was likely too soon to observe degradation. After the 30 min chase, the moMac/moDP and moDC populations showed significant degradative activity evidence by increased fluorescence. This fluorescence was maintained at 1 hr chase, but decreased moderately by 2h, suggesting more complete degradation. In contrast, there was no
significant increase in the proteolysis of DQ-OVA by the CMP and GMP populations (Fig 3.4A and 3.4B).
Figure 3.4. Proteolytic activity increases as myeloid cells become more differentiated.

Sorted populations were pulsed with 10 µg/mL DQ™ ovalbumin (DQ-OVA) for 15 min, washed and chased for 30 min, 1hr or 2hr. Proteolysis (indicated by increased fluorescence) of DQ-OVA over time was measured by flow cytometry. (A) A representative histogram of DQ-OVA uptake at 15 min (blue) and degradation at 30 min (red). (B) The MFI of DQ-OVA at 15 min and degradation at 30 min, 1hr and 2hr in all the five isolated myeloid cell populations. (C) The MFI of DQ-OVA degradation only within OVA-Alexa positive cells measured at 1 hr post incubation. Compiled data come from a minimum of three independent experiments. Statistical analysis was conducted using one way ANOVA Tukey multiple comparison test. Asterisk indicate a p<0.05 when compared with the 15 min pulse.
To control for any effect that the different uptake capacities of the five populations might have on the degradative measurements (Fig 3.2), we utilized an additional pH-insensitive tracer, OVA-Alexa 647, in combination with DQ-OVA to track the degradation only within the cells that had taken up the OVA-Alexa 647. In support of the previous result, we found that moMac/moDP and moDC had the highest degradative potential while GMP and Monocytes were slightly less degradative, and there was little to no proteolysis detected in CMP (Fig 3.4C). The data thus suggest that proteolytic activity is acquired after the CMP stage of myeloid cell development and peaks in the moMac/moDP and moDC stages.

3.4.5 Antigen processing and presentation function is evident prior to myeloid cells differentiating into moDC

In order to assess the ability of each cell population to process and present antigen, we incubated them with OVA protein or peptide and analyzed their ability to stimulate proliferation and activation of naïve OVA-specific T cells (isolated from OT-1 (C57BL/6-Tg(TcraTcrob)1100Mjb/J) or OT-II (B6.Cg-Tg(TcraTcrob)425Cbn/J) mice). To measure proliferation, T cells were stained with CFSE prior to culture with each population and activation was measured based on CD25 expression.

To assess antigen-presenting capacity, OVA peptides were used as the antigen source. GMP and monocyte populations were unable to stimulate robust OT-II proliferation and CD25 up-regulation when loaded with OVA_{323-339} compared to the no antigen control (Fig 3.5A and 3.5B; S2A, B and C). However, GMP and monocytes were able to stimulate significant proliferation of OT-1 T cells when loaded with OVA_{257-264} (Fig 3.5 D and 5E; S2D, E and F). As a point of comparison, GMP and monocytes also had the lowest expression of MHC class II molecules, yet were similar in MHC class I expression (Fig 3.5I). Thus, the poor stimulatory
capacity of GMP and monocytes to activate CD4^+ T cells might be attributed to their lower expression of MHC class II molecules as well as reduced proteolytic activity (Fig 3.5B). In contrast, moMac/moDP and moDC were able to stimulate strong proliferation and activation of both OT-II and OT-I T cells when loaded with peptide (Fig 3.5A, 5B, 5D and 5E; S2A, B and C).

Antigen processing capacity was tested using soluble OVA protein as the antigen source. GMP and monocytes were able to induce minimal activation and proliferation of OT-II cells when loaded with OVA protein (Fig 3.5A and 5C). Again, moMac/moDP and moDC induced the strongest OT-II proliferation and activation when loaded with OVA protein, indicating their strong antigen processing and presentation capacities (Fig 3.5D and 5F). Interestingly, when we assessed their cross presentation potential using OVA protein to stimulate OT-I T cell activation, we observed that both moMac/moDP and moDC were potent at cross-presenting the protein antigen, while GMP and monocytes induced OVA-specific CD8^+ T cell proliferation but induced lower level up regulation of CD25 (Fig 3.5D and 5F).

CMP induced little CD4^+ or CD8^+ T cell proliferation or CD25 expression in the presence of OVA protein or OVA peptide (data not shown). This observation was not surprising given that CMP had minimal uptake capacity, proteolytic activity and phagosomal acidification.
**Figure 3.5.** moMac/moDP and moDC exhibited both antigen processing and presentation functions. The sorted cell populations were incubated with different concentrations of OVA peptide or OVA protein and co-cultured with CFSE labeled T cells. (A) The dot plots illustrate CD4⁺ OT-II T cells stimulated with 1 μM of OVA₃₂₃-₃₃₉ (top) or 100 μg/mL of OVA protein (bottom). T cell proliferation and activation was measured using CFSE fluorescence dilution at day 5 by flow cytometry. (B) and (C) Compiled data of CD25 expression by OT-II T cells in the presence of OVA peptide (1 μM) or OVA protein (100 μg/mL), respectively. (D) The dot plots of CD8⁺ OT-I T cells stimulated with 10 nM of OVA₂₅₇-₂₆₄ (top) or 300 μg/mL of OVA protein (bottom). T cell proliferation and activation was measured at day 4 by flow cytometry. (E) and (F) CD25 expression by OT-I T cells in the presence of OVA peptide (10 nM) or OVA protein (300 μg/mL), respectively. Compiled data are derived from a minimum of 3 independent experiments. Statistical analysis was conducted using one-way ANOVA Tukey multiple comparison test. Letters over bars indicate statistically significant differences in means (p<0.05).
Fig 3.S1. The expression of MHC class I and MHC class II molecules during GM-CSF driven differentiation. Sorted cells were stained with antibodies to Ly6C, CD115, and CD11c. (A) MFI of MHC class I and (B) MHC class II expression in the five cell populations.
**Fig3.S2. Histogram depiction of T cell activation from Fig 3. 5.** (A) The histogram depicts CFSE fluorescence dilution in CD4$^+$ OT-II T cells stimulated with 1 μM of OVA$_{323-339}$ (top) or 100 μg/mL of OVA protein (bottom). (B) and (C) Compiled data of absolute number of OT-II T cells in the presence of OVA peptide (1 μM) or OVA protein (100 μg/mL), respectively. (D) The histogram of CFSE fluorescence dilution in CD8$^+$ OT-I T cells stimulated with 10 nM of OVA$_{257-264}$ (top) or 300 μg/mL of OVA protein (bottom). (E) and (F) The absolute number of OT-I T cells in the presence of OVA peptide (10 nM) or OVA protein (300 μg/mL), respectively.
3.4.6 The moMac and moDP population have similar functional capacities

We had previously determined that moMac/moDP and moDC (all CD11c+) could further be sub-divided based on the expression of CD11b and MHC class II (MHCII), with MHCII\textsuperscript{lo}, MHCII\textsuperscript{int}, and MHCII\textsuperscript{hi} cell types corresponding to moMac, moDP and moDC, respectively [33]. In order to examine the functional differences between the moMac, moDP and moDC populations, cells were isolated into three populations based on the expression of these molecules as previously described [33]. We observed that MHCII\textsuperscript{lo} population had the highest phagocytic capability, with a slightly lower BioParticle uptake by MHCII\textsuperscript{int}, and the MHCII\textsuperscript{hi} cells had the least phagocytic activity (Fig 3.6A and 6B). The observed decrease in the uptake potential of MHCII\textsuperscript{hi} population especially when compared to the MHCII\textsuperscript{int} precursor cell, moDP, supports the concept that as DC become more differentiated toward maturation, they down-regulate their endocytic capacity [41].
Figure 3. 6. The MHCII\textsuperscript{lo} and MHCII\textsuperscript{int} cell populations are similar in uptake capacity and phagosomal acidification when compared with MHCII\textsuperscript{hi} cells. Cells were sorted into three populations on day 5 of culture: MHCII\textsuperscript{lo}, MHCII\textsuperscript{int} and MHCII\textsuperscript{hi} after first gating on CD11c\textsuperscript{+}, and CD11b\textsuperscript{+} cells. (A) Sorted cells were fed \textit{S. aureus} BioParticle-Alexa flour 594 conjugate for 1 h. Representative histograms of BioParticle uptake at 4°C (blue) or 37°C (red). (B) Compiled MFI of BioParticle uptake from three experiments. (C) Representative histograms of cells pulsed with pHrodo \textit{E. coli} BioParticles at 15 min (red) and chased for 90 min (brown) or at 4°C for 90 min (blue). (D) The compiled MFI of pHrodo \textit{E. coli} BioParticle at 15 min and 90 min. (E) Calculated phagosomal pH of each cell population based on standard curves. (F) Representative histograms of cells pulsed with 10 µg/mL DQ-OVA for 15 min (red) and chased for 30 min (brown) or at 4°C for 1 hr (blue). (G) Compiled MFI of DQ-OVA at 15 min and degradation at 30 min and 1hr. (H) The compiled MFI of DQ-OVA degradation within OVA-Alexa positive cells measured at 1 hr post incubation. Statistical analysis was performed using SAS, one-way ANOVA Tukey’s multiple comparison test. Letters over bars indicate statistically significant differences in means (p<0.05).
In addition, we measured the phagosomal pH in each population and found that MHCII\textsuperscript{lo} and MHCII\textsuperscript{int} had highly increased fluorescence at 90 min at a level higher than the background fluorescence observed at 15 min after the initial incubation with pHrodo (Fig 3. 6C and 6D). Interestingly, we found that MHCII\textsuperscript{lo} and MHCII\textsuperscript{int} had an average pH 4.76 and 3.82, respectively (Fig 3. 6E), a pH comparable to that observed in moMac/moDP (pH 4.26) (Fig 3. 3). However, MHCII\textsuperscript{hi} populations still demonstrated the highest phagosomal pH, with an average pH 5.68 (Fig 3. 6E), a pH closer to that observed in moDC (Fig 3. 3).

Finally, we assessed the proteolytic activity of these cells by utilizing DQ-OVA. There was a slight increase in the fluorescence of the DQ-OVA across all populations between the initial 15 min pulse and the 30 min chase (Fig 3.6F and 6G), with MHCII\textsuperscript{int} demonstrating the highest proteolysis at the 30 min chase period. However, when we utilized the OVA-Alexa 647 to control for differential levels of uptake, (examining only the cells that had taken up the OVA-Alexa 647), we observed that both MHCII\textsuperscript{int} and MHCII\textsuperscript{hi} had a slightly higher degradation potential when compared to the MHCII\textsuperscript{lo} population (Fig 3.6H). Taken together, these results indicate that the moMac population had the highest phagocytic activity, moderate pH and mild proteolysis while the moDP population was slightly less phagocytic and had the lowest phagosomal pH and highest degradative activity. Finally, the moDC population had greatly reduced phagocytosis, more neutral pH and moderate degradative activity.

### 3.4.7 Functional capacity of early progenitors isolated directly ex vivo

Finally, to ensure that our functional analyses were reflective of those observed \textit{in vivo}, we examined the functional capacity of early progenitor cell types CMP, GMP, and monocytes freshly isolated from bone marrow. We found that CMP had the lowest uptake capacity, followed with increased uptake of Bioparticles in GMP, and the monocyte population had the
highest uptake capacity (Fig 3. 7A and 7B). Consistent with our *in vitro* studies (Fig 3. 3), CMP isolated *ex vivo* had the highest phagosomal pH (pH ~7.14), with GMP having an average pH of 6.42, while monocytes had a slightly more acidic pH (pH ~5.92) (Fig 3.7C, D, and E). Lastly, we observed that CMP had the least proteolytic capacity when compared with GMP (Fig 3.7F). Thus, we conclude that our *in vitro* cultured cells: CMP, GMP and monocytes closely resemble their *ex vivo* counterparts.
Figure 3. 7. CMP, GMP and Monocyte derived ex-vivo are similar in functionality to in vitro GM-CSF derived counterpart. Freshly harvested ex-vivo bone marrow cells were sorted into 3 populations has previously described. (A) Sorted cells were fed E. coli BioParticle-Alexa flour 594 conjugate for 1 h. Representative histograms of BioParticle uptake at 4°C (blue) or 37°C (red). (B) Compiled MFI of BioParticle uptake from three experiments. (C) Representative histograms of cells pulsed with pHrodo E. coli BioParticles at 15 min (red) and chased for 90 min (brown) or at 4°C for 90 min (blue). (D) The compiled MFI of pHrodo E. coli BioParticle at 15 min and 90 min. (E) Calculated phagosomal pH of each cell population based on standard curves. (F) The compiled MFI of DQ-OVA degradation within OVA-Alexa positive cells measured at 1 hr post incubation.
3.5 Discussion

Most studies of DC function such as endocytosis, proteolysis, and antigen processing and presenting function have focused on fully differentiated DC. As a result, less is known about when these cellular mechanisms develop or how they change as myeloid cells progress from progenitor cells to more differentiated cell types. In this study, we have now determined that as the cells develop, they undergo significant changes in phenotypic and functional characteristics including: expression of uptake receptors, endocytic capacity, phagosomal pH, proteolytic activity, and antigen processing and presenting capacity.

CMPs, the earliest cell type examined demonstrated little endocytic or phagocytic activity, low proteolytic potential, and the highest phagosomal pH of all of the populations, at near neutral. CMPs are progenitor cells that have the ability to give rise to all the myeloid cells [47]. As such, CMPs have not yet developed all the necessary cellular machinery that could either enhance their antigen uptake capacity or degradative potential. In addition, CMPs had little ability to induce T cell proliferation or activation. CMPs showed the lowest response in all of the functional analyses in this study. However, this does not indicate that these cells are functionally incompetent. As such, a recent study illustrated the immune suppressive activity of CMP and GMP in the context of tumors as evidence in the ability of these cells to inhibit T cell proliferation [48]. Taken together, our study shows that CMPs are not potent at inducing T cell activation, but does not rule out a role for these cells in immune regulation.

GMP, the second stage along the GM-CSF-driven developmental spectrum, demonstrated high phagocytic capacity, moderate proteolysis and slightly more acidic phagosomal pH. Also, GMP demonstrated only slight antigen presentation function, evidenced in their ability to present OVA peptide to CD8+ T cells (Fig 3.5D). This observation might be attributed to the fact that
innate immune cells constitutively express MHC class I molecules, potentially accounting for the ability of GMP to induce CD8\(^+\) T cell proliferation. However, GMPs were only able to induce CD8\(^+\) T cell proliferation without activation, as evidenced in low expression of CD25. In addition, GMP had lower expression of MHC class II, which could explain the inability of these cells to induce CD4\(^+\) T cell proliferation.

Monocytes had the highest phagocytic capability, and high expression of the uptake receptors when compared with CMP and GMP. The high uptake potential observed in monocytes was expected because these cells are known to be highly phagocytic, and they are endowed with a large repertoire of uptake receptors [49]. In addition, monocytes demonstrated high proteolytic capacity and low phagosomal pH, two functional properties that also support the antimicrobial activities of this cell type [50]. There have been conflicting results about the role of monocytes in antigen processing and presentation. For instance, a recent study demonstrated that Ly6C\(^+\) monocytes can cross-present cell-associated antigen to CD8\(^+\) T cells in a manner similar to DC [51], while another showed that monocytes are poor at presenting antigen [10]. In this study, we observed that within this population, antigen processing and presentation functions started to develop as evidenced in their ability to induce minimal CD4\(^+\) T cell proliferation when given OVA protein. Notably, monocytes were poor at presenting OVA peptide to naïve CD4\(^+\) T cells when compared with moMac/moDP and moDC (Fig 3.5A). Perhaps the peptide was less stable in this population. However, we observed that monocytes could cross present OVA protein to CD8\(^+\) T cells and induce T cell proliferation.

moMac/moDP includes cell types that phenotypically resemble macrophages as well as precursor cells, moDP, that can further differentiate to moDC [33]. Thus, some of the functional characteristics of the moMac/moDP cell population closely resemble moDC. First, we observed
that moMac/moDP had high expression of uptake receptors and moderate uptake of BioParticles and dextran when compared with monocytes. In addition, the kinetics of proteolytic activities showed that moMac/moDP had high degradative capacity and the lowest phagosomal pH when compared with other cell types. Macrophages are known to have antimicrobial properties and are highly degradative, in line with their function in clearance of invading microbes [52].

While this is the first study examining the function of moDP, the functional characteristics of moMac have been more extensively studied. The moMac can acquire different functional attributes depending on the environmental cues, activation status, and inflammatory signals [53-55]. In addition, moMac can change their phenotype from a proinflammatory mediator to anti-inflammatory mediator and vice-versa, demonstrating their functional plasticity [56, 57]. To further determine the functional differences between the moMac and moDP, the mixed cell population was separated into three distinct populations based on the expression of MHCII. When we compared uptake capacity in MHCII<sub>low</sub> and MHCII<sub>int</sub> populations, corresponding to moMac and moDP respectively, we observed that the moMac cell population demonstrated higher uptake capacity than moDP, and moDC (MHCII<sub>hi</sub> population) (Fig 3. 6A). Although different moMac populations have been shown to differ to some extent in phagocytic capacity [53, 58] owing to the distinct array of uptake receptors they express [59], moMac generally have a higher uptake capacity when compared with DC [53]. This observation was consistent with our results.

Moreover, the phagosomal pH of different macrophage subtypes may also be distinct. For example, pro-inflammatory macrophages maintaining a pH close to neutral while the anti-inflammatory macrophages rapidly acidify their phagosomes [29]. When we examined the phagosome acidification in moMac and moDP, we observed that both cell populations had lower
phagosomal pH when compared with moDC (MHCII<sup>hi</sup> population) (Fig 3. 6). The highly efficient phagosomal acidification observed in moMac/moDP could make this cell better suited at controlling microbes, a function that will need to be further examined. Finally, moDP had a slightly higher proteolytic activity when compared with moMac.

Intriguingly, we found that antigen processing and presenting functions develop before GM-CSF driven myeloid cells became fully differentiated moDC. moMac/moDP had a strong ability to induce CD4<sup>+</sup> T cell proliferation and activation at a rate that is comparable with moDC. The ability of moMac/moDP to stimulate CD4<sup>+</sup> T cell activation was greater than that observed in GM-Macs, a cell type highlighted by Helft et.al (similar to moMac in our study) [31]. Our data demonstrated that moMac/moDP is a unique mixture of cell types including those with similarity to GM-Macs, but also potent at priming naïve CD4<sup>+</sup> T cells in a manner similar to moDC. This potent T cell activation capacity is likely mediated by the moDP population present in this complex population.

moDC functions are well characterized in the literature. It is well established that as DCs mature, they down-regulate their uptake mechanisms in favor of presenting the processed antigen via MHC on the cell surface [26, 41]. The observed decrease in uptake of BioParticles by moDC when compared with moDP (Fig 3. 3. 2C) could be a result of moDC being more differentiated and having less phagocytic activity. However, the uptake of dextran in both moMac/moDP and moDC reflects their ability to continuously take in bulk extracellular fluid, likely by macropinocytosis. In addition, moDC demonstrated a similar proteolytic activity and slightly higher phagosomal pH than moMac/moDP. These observed differences between moMac/moDP and moDC might be because moDC are more differentiated. In fact, several studies have indicated that DC have low degradative potential and high phagosomal pH, allowing for
preservation of antigen for immune recognition [52, 60, 61]. Interestingly, phagosomal pH is known to decrease at least initially upon DC maturation, attributed to full assembly of vATPase on the mature DC phagosomal membranes [26]. Future studies in this arena should thus include an examination of vATPase assembly and activity in developing cells. Among all cell types examined in this study, we found that moDC were most potent at inducing both CD4+ and CD8+ T cell proliferation and activation, reaffirming their status as professional antigen presenting cells.

In summary, we have been able to systematically show the function of distinct myeloid cell populations during GM-CSF driven differentiation and how these functions change as the cells progress through their development. Importantly, we have been able to demonstrate in this study that antigen processing and presenting capability is a function that is acquired prior to the cells becoming moDC. This insight is essential because it could enhance the utilization of most qualified cells to function as antigen presenting cells in the design of vaccines.

3.6 Acknowledgements

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References


34. Westcott MM, Henry CJ, Amis JE, Hiltbold EM. Dendritic cells inhibit the progression of *Listeria monocyctogenes* intracellular infection by retaining bacteria in major histocompatibility


Chapter 4

Development of bactericidal activity in myeloid cells during GM-CSF driven differentiation

4.1 Abstract
The relative susceptibility of dendritic cells (DC), macrophages, and neutrophils to intracellular pathogen, *Listeria monocytogenes*, have been extensively studied. Yet, little is known about how early progenitor cells handle bacterial infection. In this study, we examined how DC and DC precursor cells control *L. monocytogenes* infection. Bone marrow culture in GM-CSF were isolated into five distinct cell populations: CMP, GMP, monocytes, moMac/moDP, and moDC, that represent the stages of their development. The early progenitor cells, CMP and GMP, demonstrated the lowest uptake of *L. monocytogenes*; however, these cell populations were more susceptible to bacterial cytosolic entry. In addition, the GMP and monocytes populations did not provide favorable environment for *L. monocytogenes* intracellular replication. This was attributed to high production of reactive oxygen and nitrogen species by these cell populations. The moMac/moDP and moDC had high uptake of *L. monocytogenes*, but they were able to restrict bacterial cytosolic entry. The moMac/moDP population supported the highest intracellular replication of *L. monocytogenes*, an observation that was attributed to the presence of macrophages in this cell population. Finally, in support of previous studies, moDC cell population did not support bacterial intracellular replication. The results from this study also suggest the role for actin polymerization in *L. monocytogenes* phagosomal escape.
4.2 Introduction

*Listeria monocytogenes* is a Gram positive, facultative intracellular pathogen that shows little to no symptoms in immune competent individuals but causes serious disease in the immunocompromised. Invasion of mammalian cells, vacuolar escape, intracellular growth, and cell-to-cell spread constitute the sequential processes that shape *L. monocytogenes* pathogenesis [1, 2]. Early immune responses against this pathogen are mediated by the cells of the innate immune system [3-8]. Specifically, the role of dendritic cells (DC), macrophages, neutrophils, and natural killer cells in controlling the pathogen at each stage of its life cycle have been studied. For instance, DC have been shown to limit *L. monocytogenes* intracellular replication by restricting the ability of the bacterial to escape from the phagosome [9]. In contrast, macrophages provide good intracellular niche for *L. monocytogenes* replication. In addition, macrophages support *L. monocytogenes*’ cell-to-cell spread. It was recently demonstrated that the type I interferon receptor expressed by macrophage promotes actin-based motility of the bacterium and enhances cell-to-cell spread [10].

DC are crucial for complete clearance of *L. monocytogenes* because they present bacterium derived antigens to T cells and elicit *L. monocytogenes*-specific T cell response, leading to generation of protective immunity against this pathogen [11]. Successful initiation of *L. monocytogenes*-specific T cell response by DC requires that these cells survive the infection long enough to perform this function. While the specific mechanisms through which DC survive *L. monocytogenes* infection are not well defined, our previous studies have demonstrated that DC provide a suboptimal niche for bacterial replication [8, 9]. In addition, another study has suggested that fascin, an actin bundling protein, is important in DC clearance of *L. monocytogenes* [12].
While several studies have investigated the relative contributions of DC and macrophages in \textit{L. monocytogenes} clearance, there is now evidence that these cells utilize disparate mechanisms to handle the pathogen. Differences in the intracellular fate of \textit{L. monocytogenes} may depend on the cytokine that drives cellular differentiation, the developmental stage of the cell, and/or the activation or maturation status of the cell. For instance, a recent study showed that GM-CSF driven bone marrow derived CD11c+ DC support \textit{L. monocytogenes} replication when compared to Flt3L derived DC or DC isolated from mesenteric lymph node [13]. Additionally, while macrophages support \textit{L. monocytogenes} intracellular growth [9], macrophages activated through IFN-\(\gamma\) pretreatment prior to infection have enhanced antimicrobial properties and are efficient at killing intracellular \textit{L. monocytogenes} [14-16]. In contrast, neutrophils are efficient at killing \textit{L. monocytogenes} regardless of their activation status [17-20]. Also, it has also been noted that DC induced to mature before \textit{L. monocytogenes} infection do not support bacterial growth [9]. However, one important gap remains in our knowledge: how the developmental status of cells during GM-CSF driven differentiation influences their susceptibility to \textit{L. monocytogenes} intracellular growth. Thus, the goal of this study is to address this question.

Culture of murine bone marrow with GM-CSF is a well-established method for the generation of monocyte-derived DC \textit{in vitro} [21, 22]. More recently, we described a method that allows for isolation of five cell populations representing distinct stages of development of myeloid cells during GM-CSF driven differentiation. This method utilizes Ly6C, CD115, and CD11c to isolate distinct stages of development, and underscores the heterogeneity present in these cultures during the differentiation process [23]. The common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), Monocytes, monocyte-derived
macrophages/monocyte-derived DC precursors (moMac/moDP) and monocyte-derived DC (moDC) represent the distinct cell types along this developmental pathway [23]. In this study, we examined how cells at distinct stages of GM-CSF driven differentiation handle *L. monocytogenes* infection. We observed that the bacterial replicate at the same rate in all of the five cell populations. We found that while moMac/moDP and moDC were more resistant to *L. monocytogenes* cytosolic invasion, bacteria that made their way to the cytosol of these cells rapidly replicated. In addition, GMP and monocytes were more susceptible to *L. monocytogenes* cytoplasmic invasion; however, these cell populations did not serve as a good replicative niche for intracellular bacterial growth. This was attributed to high production of reactive oxygen species and nitrite by these cells. We also found that in the presence of an inhibitor of actin polymerization, *L. monocytogenes* was confined to the host phagosome. These findings indicate that nascent actin polymerization by the *L. monocytogenes* is important in facilitating bacterial cytoplasmic entry.

4.3 Materials and Methods

4.3.1 Mice

C57BL/6 mice were obtained from Jackson Laboratory. The mice were housed under specific pathogen free conditions. All experiments were performed in accordance with the Auburn university animal care and use guidelines.

4.3.2 Antibodies and reagents

Primary antibodies specific for murine CD115 (CSF-1R) (clone AFS98), CD11c (clone N418), MHC class I and MHC class II (I-A^b^) (clone AF6-120) were from Biolegend (San Diego, CA). Ly6C (clone HK1.4) was purchased from ebioscience. Texas Red\textsuperscript{Tm} conjugated goat anti-rabbit IgG was purchased from RockLand antibodies & assay. Polyclonal anti-*L. monocytogenes* antibody was from ThermoFisher Scientific. Cytochalasin D and Latrunculin B were purchased.
from Life technologies. Cytochalasin D was reconstituted to 5 mM in DMSO and latrunculin B was reconstituted to 3 mM in DMSO and both were stored at -20°C and diluted in growth media just before use. CellROX™ Deep Red reagent for oxidative stress detection was obtained from ThermoFisher Scientific. Griess reagent was purchased from Enzo life sciences.

### 4.3.3 Bacteria

The wild type *L. monocytogenes* stain 10403S was a gift from Dr. Daniel Portnoy. The *L. monocytogenes* strain DH-L1245 (actA::gfp Lm) was a gift from Dr. Darren Higgins. DH-L1245 expresses green fluorescent protein (GFP) under actA promoter, which is expressed only upon cytoplasmic penetration. Thus, the bacterium turns green upon cytosolic entry [9, 24]. All bacterial strains were grown in brain-heart infusion (BHI) broth to stationary phase at 30°C. Bacteria were washed three times in PBS and resuspended in growth medium and cells were infected at a known MOI (multiplicity of infection). In some experiments, the actA::gfp Lm was co-stained with Alexa fluor 647 dye to allow quantitation of cytoplasmic vs. phagosomal bacterial cells. Thus, bacterial cells stained only with Alexa fluor 647 were identified as phagosomal, while bacteria with both Alexa fluor 647 and GFP fluorescence were identified as cytosolic.

### 4.3.4 Cell sorting strategies

Bone marrow was isolated from 8-16 week old C57BL/6 mice as previously described [8]. Briefly, bone marrow was harvested from tibias and femurs, followed with lysing of the red blood cells. The cells were suspended in RPMI medium that was supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 10 ng/mL GM-CSF. The cells cultured were isolated into 5 distinct cell populations that represent the distinct developmental progression of myeloid cells during GM-CSF driven differentiation by using Beckman Coulter Moflo XDP High Speed Cell
Sorter at the Auburn University Flow cytometry as previously described [23]. In brief, day 3 (to generate early progenitor cells) or day 5 (to generate more differentiated cells) GM-CSF cultured bone marrow cells were stained with antibodies to Ly6C, CD115 and CD11c and the cells were isolated based on the following phenotypic profile: 1. Common myeloid progenitor (CMP) Ly6C⁻CD115⁻CD11c⁻, 2. Granulocyte/macrophage progenitor (GMP) Ly6C⁺CD115⁻CD11c⁻, 3. Monocytes- Ly6C⁺CD115⁺CD11c⁻, 4. monocyte-derived macrophages (moMac)/monocyte-derived DC precursor (moDP) (moMac/moDP), Ly6C⁻CD115⁻CD11c<sub>low</sub>, and 5. monocyte-derived DC (moDC) Ly6C⁻CD115⁻CD11c⁺.

4.3.5  *L. monocytogenes* cytoplasmic entry and intracellular growth in myeloid cells

Cells (~2 x 10⁵) from each sorted population were seeded in 96-well round bottom plates and were infected with actA::gfp *Lm* co-stained with Alexa fluor 674 dye at a MOI of 2, incubated at 37 °C for 4 hrs. After 4 hr post infection (hpi), cells were washed twice with FACS washed buffer (FWB- PBS + 3 % FBS) and the samples were analyzed by Accuri flow cytometer. The percent of cells with internalized or cell-associated bacteria were measured based on Alexa flour 674 fluorescence. To control for varying uptake capacities in different cell types, we determined the percentage of cells with cytosolic bacteria, by examining only Alexa⁺ cells for GFP expression. The intracellular growth curve of *L. monocytogenes* was performed by infecting the cells with actA::gfp *Lm* at MOI of 0.5. At 1 hpi post infection, samples were washed thrice with growth media and then incubated for additional time points. At the end of each time point, samples were examined by flow cytometry.

4.3.6  Measurement of reactive oxygen and nitrogen species

Cells (~2 x 10⁵) from each sorted population were plated in round bottom 96 well plate. The cells were then infected with or without 10403S *L. monocytogenes* at MOI of 5 for 2 hrs.
After 2 hpi, samples were treated with CellROX™ reagent at a final concentration of 5 µM and incubated at 37 °C for 30 minutes. The samples were then washed with ice-cold FACS wash buffer (PBS + 3% FCS) three times before running through flow cytometry. To quantify nitrite production, cells were infected with or without 10403S *L. monocytogenes* at MOI of 5 and incubated at 37 °C for 4 hrs. At 4 hpi, 10 µg/mL of gentamycin was added and the samples were incubated for additional 20 hr. At the end of the infection period, the supernatant was collected and treated with equal part of Griess reagent. The samples were incubated at room temperature for 30 minutes. Following the incubation period, the optical density was measured at 548 nm. The concentration of the nitrite was determined by adding Griess reagent to a solution with known concentration of sodium nitrite. The standard curve generated was used to predict nitrite production by each cell population.

### 4.3.7 Immunofluorescence microscopy

Sorted cells were seeded on coverslips followed with infection with *actA::gfp Lm* as described above. At the indicated time points, cells were fixed with 2% paraformaldehyde for 30 min, washed thrice with PBS that contains 3% normal goat serum (NGS) and permeabilized in PBS solution that contain 1% NP-40, 0.01% saponin and 3% NGS for 10 minutes. The samples were stained with rabbit anti-*L. monocytogenes*, for 30 min, followed with staining with goat-anti rabbit IgG for 30 min. The slides were washed in permWash solution (PBS, 3% NGS, 0.01% saponin) and mounted on microscope slides with ProLong Gold Antifade mounting media containing DAPI. All samples were visualized by Olympus inverted microscope with 100X objective lens. The images were captured with digital camera and analyzed using Olympus cellSens standard software.
4.3.8 Inhibition of *L. monocytogenes* uptake

To assess the effect of different inhibitors on phagocytosis of *L. monocytogenes* by each myeloid cell population, the sorted cell populations were pre-incubated with 3 µM latrunculin B or 5 µM cytochalasin D for 30 min. After 30 min incubation with each drug, the cells were infected with *actA::gfp Lm* co-stained with Alexa fluor 647 at MOI of 2 and incubated at 37 °C for 4 hrs. To control for the effect of the DMSO, cells were treated with 0.1 % DMSO, the final concentration of DMSO in samples treated with latrunculin B or cytochalasin D. The uptake of *L. monocytogenes* and the cytoplasmic entry of the bacterial was determined based on the gating method described above.

4.4 Results
4.4.1 Intracellular growth of *Listeria monocytogenes* in GM-CSF driven myeloid cells at distinct stages of development

Traditional methods which we and others have used for measuring intracellular growth of *L. monocytogenes* have relied on lysing infected cells, plating the lysate, and enumerating bacterial colony forming units [9, 25]. This method potentially counts both the phagosomal bacteria as well as bacteria replicating within the host cytosol without discrimination/exclusion of any phagosomal bacteria that were not actively replicating. Thus, we modified this assay to measure specifically cytosolic growth by utilizing bacteria that express GFP, but only upon cytosolic entry and determining fluorescence by flow cytometry. This allows for more precise measurement of the intracellular growth. Cells were infected *in vitro* with *actA::gfp Lm* at MOI of 0.5 for 1 hr. After 1 h, cells were washed to remove unbound bacteria and incubated for additional time points. Figure 4.1A is a representative histogram of GFP expression in each population over the periods of infection. Figures 4.1B and 4.1C represent compiled data from 4
experiments illustrating intracellular growth as a function of total fluorescence (Fig 4.1B) or as the percent of cells with cytosolic *L. monocytogenes* (Fig 4.1C). There were no significant differences in the MFI of GFP expression in all the populations at 1h or even 2 h (Fig 4.1). However, cytosolic fluorescence increased in moMac/moDP and moDC beginning at 4h. Cytoplasmic growth was evident in all populations by 6h, and peaked at 8h. The cell population with the highest total cytosolic burden by 8h was the moMac/moDP population with the majority of cells demonstrating high levels of fluorescence at 8h indicating a high bacterial burden per cell. A significant intracellular growth of *L. monocytogenes* was observed in the moDC population as well, but not to the same extent as that observed in moMac/moDP. While there were significant differences in the levels of GFP expression in each population, the rate at which bacteria replicate in all the populations were somewhat similar. We have previously demonstrated that macrophages support *L. monocytogenes* intracellular replication better than DC [9]. The high bacterial growth observed in moMac/moDP was not surprising because this cell population contains monocyte-derived macrophages, and the moDP, the DC precursor that may be susceptible to cytoplasmic invasion as well [23].
Figure 4.1. Intracellular growth of *Listeria monocytogenes* in GM-CSF driven myeloid cells at distinct stages of development. Sorted cell populations were infected with *actA::gfp Lm* at MOI of 0.5 for 1 hr. At 1 h, cells were washed three times with cold growth media and incubated for additional time points. (A) A representative histogram that showed GFP expression over time in each population. (B) The mean fluorescence intensity (MFI) of GFP expression of the intracellular replication of *actA::gfp Lm* in each population over time. (C) Mean percentage of cells with GFP *L. monocytogenes* at each time point post infection. Data are compiled from at least four independent experiments.
4.4.2 Relative susceptibilities to *L. monocytogenes* cytoplasmic invasion.

Having previously determined that GM-CSF-driven cells had vastly different uptake capacities of bioparticles and of dextran at each stage of development [26], we next wanted to measure the relative abilities of these cells to take up *L. monocytogenes*. Thus, to assess the relative *L. monocytogenes* uptake capacities of each stage, *L. monocytogenes* were stained with Alexa 647 and fed to each cell population for 4 h (Fig 4.2A). We observed that *L. monocytogenes* uptake capacity increased with each stage of development, with CMP and GMP having the lowest level of *L. monocytogenes* uptake, monocytes having an intermediate level, and uptake capacity reaching maximal levels in moMac/moDP and moDC (Fig 4.2A). Given these significant differences in uptake capacity, we wanted to control for these differences in our assay to determine susceptibility to cytosolic invasion.

To determine the relative susceptibility of each myeloid cell population to *L. monocytogenes* cytoplasmic invasion, we utilized *L. monocytogenes* strain DH-L1245, a derivative of strain 10403S that expresses enhanced green fluorescent protein (GFP) under the control of the *actA* promoter [9]. Since this *L. monocytogenes* strain, *actA::gfp Lm*, only expresses GFP upon cytosolic entry, the bacteria were co-stained with Alexa fluor 647 dye to quantify the total bacterial load (Alexa⁺ cells) vs. the percent of the cells with cytosolic bacteria (Alexa⁺GFP⁺ cells). Figure 4.2B is a representative gating strategy used to identify infected cells and the percent of the cells with bacteria in the cytoplasm. While only about 5% of CMP took up bacteria, we observed that about 60% of these bacteria invade the host cytosol (Fig 4.2C and 4.2D). Moreover, while only about 7% of GMP population took up bacteria, about 80% of these bacteria gained access to the cytosol (Fig 4.2D). In contrast, moMac/moDP and moDC that had the highest number of cells positive with Alexa⁺ bacteria (Fig 4.2B), only about half of these
infected cells have cytoplasmic bacteria as measured by GFP expression (Fig 4.2D). Taken together, these data indicate that while GMP and monocytes take up fewer *L. monocytogenes*, once inside the cell, the bacteria are readily able to invade the cytosol. In contrast, while moMac/moDP and moDC take up large numbers of bacteria, the bacteria have a lower likelihood to make it to the cytoplasm in these cells (Fig 4.2B and 4.2D). In addition, the higher fluorescence observed in moMac/moDP population (Fig 4.1A and 4.1B) could be attributed to high numbers of bacteria cells taken up this cell population.
Figure 4.2. *L. monocytogenes* cytosolic invasion is dependent on the stages of cell development. Sorted cell populations were infected with *actA::gfp* *Lm* co-stained with Alexa Fluor 674 dye at MOI of 2 for 4 hrs. The presence of Alexa+ cells indicate cells that were associated with or internalized bacteria while the presence of Alexa+GFP+ cells indicate cells with cytosolic bacteria. (A) A bar graph showing the percent of each cell population associated with or internalized bacteria. (B) The gating strategy used to determine the cells with cytosolic bacteria. (C) A representative histogram of Alexa+GFP+ cells (D) A bar graph showing the percent of infected cells with cytosolic *L. monocytogenes* (% Alexa+GFP+ cells). Data were compiled from at least four independent experiments. The letters over the bars indicate that the means are statistically different (p<0.05).
Surprisingly, GMP and monocytes that had the highest *L. monocytogenes* cytosolic invasion showed the slowest bacterial intracellular replication (Fig 4.1 and 4.2D). This discrepancy led us to examine the cells microscopically (Fig 4.3). We observed that at 4 hpi about 40% of GMP contained at least one GFP bacterium and the number increased to about 90% at 8 hpi (Fig 4.3 A and B). In the monocyte, moMac/moDP and moDC populations, nearly 60% of these cells had at least one internalized bacterium at 4 hpi and the number slightly increased at 8 hpi. In contrast, the moDC population had the least number of cells with cytosolic bacteria and the number slightly increased to about 50% at 8 hpi (Fig 4.3B). In addition, quantitative analysis of number of bacterial per cell revealed that although GMP had the highest number of cells with cytoplasmic bacteria, these cells only have about 1 to 5 bacteria per cell at 4 hpi and about 6 to 10 bacteria per cell at 8 hpi (Fig 4.3C). In contrast, moMac/moDP population that had relatively lower number of cells infected, the few infected cells had more than 10 bacteria per cell (Fig 4.3C). These findings demonstrate that the moMac/moDP population supports optimal *L. monocytogenes* intracellular replication.
Figure 4.3. Microscopic analysis of *L. monocytogenes* intracellular replication in each cell population at 8 hpi. Sorted cell populations were infected with *actA::gfp Lm* at MOI of 0.5 for 1 hr. At 1 hpi, cells were washed three times with growth media and incubated for additional time points. At 8 hpi, cells were fixed, and permeabilized. The nuclei were stained with DAPI (Blue channel) and the bacteria express GFP upon cytosolic entry. (A) A representative micrograph from each cell population that showed cells with cytosolic bacteria. (B) The percent of cells with cytosolic bacteria at 4 and 8 hpi. (C) The number of GFP *L. monocytogenes* per cells at 8 hpi.
4.4.3 Production of reactive oxygen/nitrogen intermediates at each developmental stage

Production of antimicrobial molecules such as reactive oxygen and nitrogen intermediates (ROI/RNI) are some of the best-defined host immune responses against *L. monocytogenes* [15]. We predicted that differential production of these molecules within our sorted cell populations may contribute to the observed differences in intracellular replication of *L. monocytogenes*. To assess this, cells were infected with *L. monocytogenes* and we utilized cellROX, a ROS-sensitive fluorescent dye, to detect increased production of ROS in cells upon stimulation with live bacteria. While moMac/moDP and moDC infected populations demonstrated no increased ROS production over the controls, upon infection, GMP and monocytes demonstrated the highest level of ROS activity (Fig 4.4A and B). In addition, we measured nitrite production in the supernatant of cells infected with *L. monocytogenes* as a readout for RNI. While there was an increase in nitrite concentration in the infected samples when compared with the controls in all cell populations, monocyte had the highest production of nitrite of about 8 µM when compared with moMac/moDP and moDC (Fig 4.4C). This result is consistent with GMP and monocytes limiting *L. monocytogenes* intracellular replication through production of these antimicrobial compounds.
Figure 4.4. The production of ROS and nitrite following infection with *L. monocytogenes*.

Sorted cell populations were infected with 10304S *L. monocytogenes* at MOI of 5 for 2 hr (ROS quantification) or 24 hr (Nitrite quantification). For ROS measurement, at 2 hpi, 5 µM of CellROX reagent was added to each sample and incubated for 30 min at 37 °C. (A) A representative histogram that showed ROS production as measured by cellROX. (B) The MFI of ROS production in each population. (C) The nitrite concentration measured from supernatant collected at 24 hpi using Griess reagent. The actual nitrite concentration was calculated via a standard curve with known sodium nitrite concentration.
4.4.4 *L. monocytogenes* cytoplasmic entry is dependent on actin polymerization

Based on the observation that moMac/moDP populations provided a good niche for *L. monocytogenes* intracellular replication, we predicted that inhibition of phagocytosis would limit their uptake capacity and subsequently limit the number of *L. monocytogenes* that escape to the cytosol. Since phagocytosis involves coordinated rearrangement of actin filaments [27], we examined the effect of inhibitors known to interfere with actin microfilament function on uptake and cytoplasmic invasion of *L. monocytogenes* in each cell type. Latrunculin B, an inhibitor of actin-dependent macropinocytosis or phagocytosis, that works by inhibiting the formation of F-actin by sequestering G-actin [28, 29] was used to determine if the uptake mechanism influences *L. monocytogenes* cytoplasmic entry.

When cells were treated with 3 µM of latrunculin B prior to infection with *L. monocytogenes*, we observed a slight inhibition of bacterial uptake in CMP and GMP with no inhibitory effect on the uptake of *L. monocytogenes* in other three cell populations (Fig 4.5A). This observation led us to test the effect of latrunculin B and cytochalasin D (an inhibitor of actin microfilament polymerization and elongation) [27, 30], on the uptake of an endocytic fluorescence’s tracers, Bioparticles, as a measure of phagocytosis. In the presence of 3 µM latrunculin B, we observed inhibition of BioParticle uptake with the greatest inhibitory effect found in GMPs and monocytes (Fig 4.5B). Interestingly, cytochalasin D had a similar effect as the latrunculin B with about 50% inhibitory effect on BioParticle uptake by GMP and monocytes (Fig 4.5B). The effect tended to be minimal in moMac/moDP and moDC uptake BioParticles. The results taken together showed that these inhibitors had a slight inhibitory effect on *L. monocytogenes* uptake, but it thus has more potent inhibitory effect on the uptake of Bioparticle.
We examined the effect of this inhibitor on *L. monocytogenes* cytosolic entry. Interestingly, we found that addition of 3 µM latrunculin B led to nearly 100% inhibition of bacterial cytoplasmic entry in all five populations (Fig 4.5C and 4.5D). In contrast, cytochalasin D only had a minimal inhibitory effect on *L. monocytogenes* cytosolic invasion (Fig 4.5D). These striking results of latrunculin B led us to investigate if the slight inhibitory effect observed in relation to bacterial uptake could have a profound effect on cytosolic invasion. Thus, we reexamined the effect of this inhibitor on *L. monocytogenes* cytoplasmic entry by first infecting the cells with *L. monocytogenes* for 30 minutes prior to treatment with latrunculin B. This method allows the cells to take up bacteria. In support of our previous results, we observed a similar inhibitory effect (data not shown). This observation further supports the results that latrunculin B only had slight effect in inhibiting *L. monocytogenes* uptake especially in more differentiated cells types, but it thus has a profound effect on limiting *L. monocytogenes* cytoplasmic invasion.

Due to the profound inhibitory effect of 3 µM of latrunculin B on *L. monocytogenes* cytosolic entry, a dose dependent treatment assay was performed in order to determine the lowest concentrate of this drug with optimum effect on bacteria cytoplasmic invasion. A dose dependent response in all the five populations was observed with latrunculin B having the most inhibitory effect on more differentiated cell types, moMac/moDP and moDC, even at the least concentration of 0.375 µM (Fig 4.5E). To further confirm that latrunculin B inhibits vacuolar escape of *L. monocytogenes*, cells infected with actA::gfp *Lm* in the presence or absences of latrunculin B were stained with anti-*L. monocytogenes* antibodies, and the cells were examined microscopically for the presences of GFP *L. monocytogenes* within the cell cytosol. In the cells that were incubated without latrunculin B, majority of the bacteria that stained red with anti-*L.
monocytogenes antibodies also expressed GFP (Fig 4.5F). However, in the samples treated with latrunculin B, we only observed red bacteria without any evidence of GFP L. monocytogenes (Fig 5F). This further suggests that L. monocytogenes cytoplasmic entry is dependent on nascent actin polymerization.
**Figure 4.5. Effects of inhibitors on myeloid cells uptake capacity.** The sorted populations were treated with the indicated concentration of latrunculin B (LatB), cytochalasin D (cytoD) or vehicle for 30 min prior to infection with actA::gfp Lm at MOI of 2. The samples were incubated at 37 °C for 4 hr. (A) A bar graph of percent of cell with Alexa bacteria with and without LatB. (B) A bar graph of percent inhibition of Bioparticle uptake in the presence of LatB or cytoD. (C) A representative histogram showing the expression of Alexa or GFP in samples treated with 3 μM latrunculin B or vehicle. (D) Percent inhibition of L. monocytogenes cytosolic entry in the presence of 3 μM LatB or 5 μM cytoD. (E) A dose dependent curve of samples treated with different concentration of LatB (F) Micrographs of samples treated with or without 3 μM of latrunculin B. Blue- DAPI, Red- anti-L. monocytogenes stain, and cytosolic L. monocytogenes expressing GFP.
4.5 Discussion

In this study, we demonstrated that the developmental stage of myeloid cells during GM-CSF driven differentiation determines their susceptibility to *L. monocytogenes* cytoplasmic invasion and/or intracellular replication. First, we observed that the early progenitor cells, the CMP, GMP, and monocytes, had relatively lower uptake of *L. monocytogenes* when compared to moMac/moDP and moDC. The relative lower number of bacteria associated with and internalized by monocytes correspond to the levels reported in previous studies [31, 32]. Drevets et. al. 2010 [32] demonstrated that monocytes isolated from either steady state or sublethal infected mice were unable to internalize *L. monocytogenes in vitro*, while monocytes isolated from lethally challenged mice internalized nearly all the cell-associated bacteria. This suggests that developmental status of a particular cell could influences their ability to take up bacteria. In addition, we have previously shown that CMP, GMP, and monocytes had relatively low expression of scavenger receptor and mannose receptor when compared with moMac/moDP and moDC [26]. Other studies have also shown that scavenger receptor enhances *L. monocytogenes* phagocytosis [33]. Thus, the relative lower uptake of *L. monocytogenes* observed in these early progenitor cells could be attributed to low expression of receptors that could trigger the process of phagocytosis.

Moreover, we observed that *L. monocytogenes* replicates at the same rate in all of the five populations but the total bacteria burden in each population is a function of their bacteria uptake capacity. While only a few bacteria were taken up by GMP and monocytes, a larger percentage of these bacteria invade the host cytosol. This result was not in agreement with the recent study conducted by Jones et al. 2017 [31] that demonstrated the inability of vacuolar *L. monocytogenes* to invade monocyte cytosol. While the cell types isolated from the mesenteric lymph node were classified as inflammatory monocytes, the authors pointed out that these cells do not resemble
monocytes generated from bone marrow through GM-CSF driven differentiation. The differences in the cytosolic invasion in different monocyte populations could be attributed to their developmental status and as well as external inflammatory signals in different environments. In addition, moMac/moDP and moDC were able to somewhat restrict *L. monocytogenes* cytosolic entry despite having the highest numbers of associated/internalized bacteria. The lower *L. monocytogenes* cytosolic invasion observed in moDC was similar to previous studies that attributed this to higher phagosomal pH that impaired the activity of pH-dependent, pore-forming listeriolysin O from lysing the phagosomal membrane [8, 9].

The growth curve as well as the microscopy data demonstrated that although GMP are more permissive to *L. monocytogenes* cytoplasmic invasion, these cell populations do not readily support intracellular replication. It is possible that the limited intracellular replication of the bacteria observed in GMP and monocytes could be due to high production of ROS and RNS in these cells. When we quantified the number of bacteria per cell in the GMP and monocytes populations, we observed that most of the infected cells had fewer than 10 bacteria per cell. This further supports the inability of *L. monocytogenes* to replicate in these cell populations.

In contrast, moMac/moDP that showed more limited cytoplasmic entry of *L. monocytogenes*, the few infected cells support intracellular replication as measured by higher number of bacteria per cell. The moMac/moDP is an heterogenous cell population that contains both macrophages-like cells, and DC precursor cells, moDP [23]. The results from this study is in supports of other previous studies have indicated that macrophages support unrestricted *L. monocytogenes* intracellular replication [9, 31].

Several studies have shown that different subset of DC response to *L. monocytogenes* differently [11, 13, 34, 35]. It is well-established that GM-CSF derived DC provide a suboptimal
niche for *L. monocytogenes* replication. This was attributed to the ability of DC to limit *L. monocytogenes* cytosolic entry by restricting the bacteria in MHC class II rich phagosome [8]. In addition, fascin, an actin-budding protein, has been suggested to play a significant role in the ability of DC to limit *L. monocytogenes* growth by promoting colocalization of *L. monocytogenes* with LC3 [12]. In this study, we observed that moDC were able to limit *L. monocytogenes* intracellular replication. Also, this cell population has high levels of LC3, suggesting that autophagy could play a role in their ability to control bacterial replication.

The virulence of *L. monocytogenes* is dependent on the ability of the bacterium to escape the phagosome and access the host cytosol where it can replicate. It was initially thought that *L. monocytogenes* phagosomal escape solely depends on the pore forming activity of LLO and phospholipases secreted by this microbe [36-38]. However, recent findings have implicated some host factors in this process. For instance, the pore forming activity of LLO is dependent on the activation of this hemolysin by host Gamma-interferon Inducible Lysosomal Thiolreductase (GILT) [39]. In addition, calpain and cystic fibrosis transmembrane conductance regulator (CFTR) are other host factors shown to enhance *L. monocytogenes* phagosomal escape [40, 41]. CFTR works by increasing the concentration of chloride in the phagosome, a process that will enhance LLO pore formation and lysing of the vacuolar-containing *L. monocytogenes* [40]. While the role of host actin protein in *L. monocytogenes* intracellular motility [42, 43] as well as actin polymerization in the ability of *L. monocytogenes* to escape detection by autophagy machinery [44] are well documented, it is yet to be determined if this protein play any role in *L. monocytogenes* phagosomal escape.

In this study, we observed that de-novo formation of actin filaments is required for this bacterium to escape the phagosome. When we utilized cytochalasin D, an inhibitor that binds to
the barbed ends of F-actin and prevents actin elongation [27, 30], we observed a minimal inhibition of *L. monocytogenes* cytoplasmic entry. However, the presence of latrunculin B, an inhibitor that binds to G monomers and prevents the assembly of F-actin [28, 29], there was a complete inhibition of *L. monocytogenes* cytosolic entry. This result suggests that nascent actin polymerization is important in bacterial cytosolic invasion. In addition, several lines of research have demonstrated the role of autophagy in host resistant to *L. monocytogenes* as well other intracellular pathogens [43-46]. It was recently demonstrated that actin polymerization prevents co-localization of *L. monocytogenes* with autophagic proteins [44]. Thus, it is also possible that actin polymerization by *L. monocytogenes* could enhances the ability of this bacterium to escape the phagosome.

Our initial study that comparatively examined the endocytosis, proteolysis, and antigen presenting capacity of myeloid cells found disparity in the stage that myeloid cells develop these functions during GM-CSF driven differentiation [26]. This new study has also enhanced our understanding on how distinct populations of myeloid cells handle bacterial infection. This study has also provided insights into the role of actin polymerization in phagosomal escape of *L. monocytogenes*. It is clearer now than ever that the functional attributes of cells are determine by several factors that include the cytokines that drives their differentiation, their developmental stage, and the activation status. It is therefore important to take all these into consideration when interpreting their functional attributes.
References


Chapter 5: Discussion and Conclusion

Previous studies that examined endocytosis [1, 2], antigen processing, and initiation of adaptive immune response in terminally differentiated DC [3] have provided invaluable information about the functional characteristics of these cells. Consequently, these functional attributes of DC have offered great advancement in understanding DC biology and their role in infection, vaccine design, and tumor immunotherapy [4-7]. While these functional attributes of immature and mature DC have been well-studied [1, 8], the development of these functions in DC prior to their terminal differentiation to DC is yet to be determined. This project attempted to trace the development of these functions in DC and DC precursor cells during GM-CSF driven differentiation.

5.1 Summary of the Research Findings

CMP, being the least developed cell, exhibited the least functional characteristics examined in this study (Fig 5.1). While previous studies indicated that CMP expressed cell surface markers, such as CD34, c-kit, typical of early progenitor cells [9], this cell population had low expression of antigen uptake receptors. This lower expression of antigen uptake receptors could explain why these cells demonstrated the least uptake of both soluble and particulate antigens (Figure 3.2, Figure 5.1). In addition to the expression of antigen uptake receptors, the internalization of antigens is another complex process that requires a lot of signaling molecules, kinases, and membrane trafficking regulators, all working together to orchestrate actin cytoskeleton rearrangement, plasma membrane protrusion, and engulfment [10].
Some of the molecules, such as Rho GTPase, Rac, Cdc42, Arp2/3, and phosphoinositide-3-kinase involved in the process of phagocytosis have been studied in more differentiated cell types such as DC and macrophages [1, 11]. While these proteins play an important role in other physiological functions of the cells, such as cell proliferation, survival, and migration [12, 13], it is yet to be determined if the presence of these proteins in CMP performs effector functions, such as phagocytosis. Thus, the low uptake of antigen observed in CMP could be attributed to a lack of necessary machineries required to facilitate the process of phagocytosis. Lastly, CMP had a neutral phagosomal pH and low degradative potential, suggesting the inability of this cell population to process and present antigens to T cells (Figure 5.1).
Figure 5.1 The functional attributes of CMP. CMP, being the progenitor cells, demonstrated the least features examined. They expressed very low levels of surface receptors which indicate their inability to take up *L. monocytogenes* (Lm). Also, this cell population has a neutral pH coupled with poor proteolytic activity which could explain the inability of this cell population to induce T cell activation. (CMP- common myeloid progenitor, ROS-reactive oxygen species, NO$_2^-$ nitrite, Ag-antigen, Lm-*L. monocytogenes*).
Previous studies demonstrated that CMP had a remarkable immunosuppressive activities [14, 15], and this feature was dependent on nitric oxide production [15]. While this project does not specifically examine the immunosuppressive function of CMP and GMP, stimulation of these cell populations with *L. monocytogenes* led to nitrite production (Figure 4.4). In addition, cytokine profiling following stimulation with different cocktail of PAMPs (pathogen associated molecular patterns) showed that CMP and GMP had the least production of proinflammatory cytokines, such as IL-12 and IL-6 [16]. Extrapolating from these findings, one future direction to take could be to determine if these cells produce anti-inflammatory cytokines, such as IL-10 in response to PAMPs, which could further substantiate their immune regulatory functions.

Moreover, CMP had the lowest uptake of *L. monocytogenes* in all the populations examined. This could stem from inadequate expression of molecules required for the process of phagocytosis. In addition to CMP low uptake and high bacteria cytoplasmic entry, this cell population did not provide a productive niche for *L. monocytogenes* intracellular replication (figure 5.1). CMP stimulated with *L. monocytogenes* produced significantly high levels of nitrite compared to control, while there was no difference in reactive oxygen species (ROS) production between the control and infected cells (Figure 4.4). Previous studies have demonstrated that activation of immature myeloid cells cause these cells to become myeloid-derived suppressive cells (MDSC) [17]. Thus, stimulation of the early progenitor cells (CMP and GMP) with PAMPs prior to their differentiation into mature cells might induce these cells to become MDSC, further enhancing their immunosuppressive functions [15].

GMP and monocytes represent the second and third respective stages along the developmental spectrum during GM-CSF driven differentiation, and their functions, to certain extent, are similar. GMP and monocytes exhibited the highest endocytic capacity, demonstrated
by their high uptake of Dextran, a measure of fluid-phase endocytosis, and Bioparticle, a measure of phagocytosis (Figure 5.2). In other more differentiated cell types, the moMac/moDP and moDC, the uptake of Bioparticle decreased significantly while only a modest decrease in Dextran uptake was observed, reflecting the ability of these more developed cell types to continuously take up antigen through pinocytosis.

In contrast, the GMP and monocytes had lower uptake of *L. monocytogenes* when compared with higher uptake of bacteria observed in moMac/moDP and moDC populations (Figure 4.1). A previous study showed that scavenger receptor enhances *L. monocytogenes* uptake [18]. As reported earlier, CMP, GMP, and monocytes express relatively low levels of scavenger receptor (Figure 3.1). Thus, their relatively lower uptake of *L. monocytogenes* could be attributed to low expression of receptors that could facilitate phagocytosis.

Additionally, *L. monocytogenes* expresses surface proteins internalin A (InIA) and InIB that interact with host E-cadherin and c-Met receptors, respectively, and these proteins promote bacteria invasion of non-phagocytic cells [19, 20]. Recent report also showed that macrophages express high levels of E-cadherin with no detectable level observed in monocytes, and the efficiency through which *L. monocytogenes* invade these cell types correlate with the levels of E-cadherin they express [21]. While this study does not specifically examine E-cadherin expression on each cell type, the early progenitor cells could have lower expression of this molecule, suggesting their inability to efficiently take up *L. monocytogenes*. 
Figure 5.2 The functional attributes of GMP and monocyte. The GMP and monocytes express moderate levels of uptake receptors. They also have the highest uptake of soluble and particulate antigens, but their uptake of *L. monocytogenes* was relatively low compared to moMac/moDP and moDC. Both cell populations had slightly acidic pH and moderate proteolytic activity that enhanced their antigen processing capability. Due to the low expression of MHCII, these cell populations were poor at inducing CD4$^+$ T cells activation. However, they have moderate expression of MHCI that suggest the ability of these populations to induce CD8$^+$ T cells activation. (GMP and monocytes had the highest production of ROS and NO$^-$ that suggests the ability of these populations to limit *L. monocytogenes* intracellular replication. GMP-granulocyte/macrophage progenitor, ROS-reactive oxygen species, NO$^-$ nitrite, Ag-antigen, Lm-*L. monocytogenes*).
The rate and kinetics of antigen degradation are defining factors in generating an appropriate immunogenic peptide capable of eliciting a strong T cell response [22]. Thus, proteolysis is an important feature in determining the efficiency through which an antigen presenting cell (APC) could initiate an immune response, and this functional attribute of APC needs to be taken into consideration in vaccine design. The results presented in Figure 3.4 showed that GMP had moderate proteolytic activity that was slightly higher than monocytes. However, the proteolytic activity of both GMP and monocytes was significantly less than that observed in moMac/moDP population. In addition, GMP and monocytes expressed low levels of MHCII (Figure 3S1), and the expression of these molecules did not change upon stimulation with PAMPs [16]. Therefore, low proteolytic activity coupled with low levels of MHCII could explain the inability of these two cell populations to induce potent CD4+ T cells activation.

In respect to the ability of these cell populations to control bacterial infection, the results showed that GMP and monocytes were more susceptible to *L. monocytogenes* cytosolic invasion. However, these cell populations do not provide a conducive intracellular environment for replication potentially due to the high production of ROS and nitrite by these two cell populations.

The moMac/moDP and moDC populations represent the most developed cell types examined in this study, and their features are in close resemblance. First, these cell populations expressed high levels of uptake receptors. Expression of antigen receptors is important in the overall immune cell functions, and these uptake receptors are being targeted in several cell-based immunotherapies. Notably, antigens are being targeted to specific cell surface receptor on DC in vaccine design [23, 24]. This allows efficient uptake of antigen and channeling of the antigen to the appropriate endocytic compartment where it can be processed and presented to T cells.
Receptors that have been targeted in the design of vaccine include scavenger receptor, DEC-205, DC-SIGN, and mannose receptor [24]. In this study, the early progenitor cells, the CMP and GMP, had little expression of uptake receptors when compared with moMac/moDP and moDC (Figure 3.1, Figure 5.3). Interestingly, high expression of these receptors begins in moMac/moDP, indicating that these cell population could function in design of vaccine because they express receptors that could be targeted with antigens.

The moMac/moDP and moDC have the highest proteolytic activity when compared with other populations (Figure 5.3). Studies have illustrated that DC, when compared with macrophages, have a higher phagosomal pH and lower lysosomal proteases, a mechanism that allows them to limit the rate of antigen degradation [8, 25]. Thus, moMac/moDP having the highest proteolytic activity could be attributed to the presence of macrophages in this cell population.
Figure 5.3 The functional attributes of moMac/moDP and moDC. The moMac/moDP and moDC expressed high levels of uptake receptors. They demonstrated moderate uptake of soluble and particulate antigens, but their uptake of *L. monocytogenes* was relatively high compared to other three populations. moMac/moDP had the lowest phagosomal pH and high proteolytic activity. Both cell populations expressed high levels of MHCI and MHCII that enhanced their ability to induce potent CD4\(^+\) and CD8\(^+\) T cell activation. The moMac/moDP support *L. monocytogenes* intracellular replication, a function attributed to their lower pH and moderate production of antimicrobial molecules (ROS and NO\(^-\)). (MoMac/moDP- monocyte-derived macrophages/monocyte-derived DC precursor (moMac/moDC), moDC- monocyte-derived DC (moDC), ROS-reactive oxygen species, NO\(^-\) nitrite, Ag-antigen, Lm-*L. monocytogenes*).
Moreover, the ability to induce T cell proliferation and activation starts to develop in monocyte population, with moMac/moDP and moDC having the strongest antigen presentation and T cell activation capacity. The moMac/moDP and moDC cell populations have expressed high levels of MHCII and MHCII molecules when compared to other cell types. This observation could explain why these two cell populations are highly potent at inducing T cell proliferation and activation. While many studies have noted that DC are potent inducers of naïve T cell activation [26], this present study suggests that antigen presentation function develops prior to full differentiation of DC. This is an important finding because it demonstrates that moMac/moDP population could be exploited for other function of DC in areas where it seems technically difficult to acquire large numbers of DC for this function.

Previous studies that compared macrophages and DC response to *L. monocytogenes* found that macrophages were more susceptible to infection with this pathogen [27, 28]. In support of those studies, moMac/moDP populations enhance *L. monocytogenes* intracellular replication when compared to other cell types. moMac/moDP had a very low phagosome pH, which could support the pore forming activity of listeriolysis O, allowing *L. monocytogenes* to have access to the cytosol. Within the moMac/moDP population, not all the cells were infected. However, the few infected cells supported uncontrolled bacteria growth. As mentioned earlier, the moMac/moDP population contained macrophages. It is therefore possible that the few heavily infected cells in moMac/moDP are the macrophages.

It has been demonstrated that moMac/moDP could be separated into macrophages and DC precursor cell based on the expression of MHCII, with MHCII\textsuperscript{lo}, MHCII\textsuperscript{int}, MHCII\textsuperscript{hi} represent moMac, moDP and moDC population, respectively [29]. Thus, isolation of these cells based on the expression of MHCII and infection of the cells with *L. monocytogenes* could
provide further narrow down on the cell population within the moMac/moDP that support the unrestricted bacteria growth. Lastly, the moDC populations were able to restrict bacteria cytoplasmic entry and limit intracellular replication of *L. monocytogenes*.

### 5.2 Originality of the study and the Implications of these findings

Activation of an appropriate immune response depends on the development of the essential features that are critical in immunity. For the first time, this study comparatively examined the development of the antigen processing and presentation functions, as well as bactericidal mechanisms of DC and DC progenitor cells during GM-CSF driven differentiation. The results from this study have two pivotal implications. First, the data illustrated that moMac/moDP induce both CD4⁺ and CD8⁺ T cells activation and proliferation in a manner similar to moDC. This finding is specifically important because a lot of effort has been made to generate DC with potent immunogenicity within a short period of time for cell-based immunotherapy [30-33]. moMac/moDP populations can be generated within three days from bone marrow cell culture and within 1-2 days from monocytes, and this moMac/moDP has a similar immune stimulatory capacity to DC generated in 7 days of cell culture. The utilization of moMac/moDP instead of moDC in vaccine development and tumor immunotherapy could cut down on cost associated with reagents needed to maintain DC in culture for extended period of time.

Second, the early progenitor cells, the GMP and monocytes are more resistant to *L. monocytogenes* intracellular replication, a function that could be attributed to high production of reactive oxygen and nitrogen species. In support of this observation, a previous study has shown
that memory CD8+ T cells enhance the antimicrobial properties of neutrophils and monocytes, and licensed these cells to kill *L. monocytogenes in vivo* through production of reactive oxygen and nitrogen species [34]. It is therefore important to explore the effector functions of these cells in different infection models.

### 5.3 Limitations outside the scope of the study and Recommendations for future study

While this dissertation project has demonstrated the functional capacities of each myeloid cell population along the developmental spectrum and their bactericidal mechanisms, there are many opportunities to explore other functionalities of these cell populations. The results presented in Figure 3.7 showed that *in vitro* generated CMP, GMP, and monocytes closely resembled their *ex vivo* counterpart. However, it is yet to be determined if GM-CSF derived moMac/moDP and moDC are closely related to the *ex vivo* isolates. Since these cell populations are rare under steady state condition, one way to generate large numbers for functional studies would be to stimulate mice with GM-CSF, and isolate these cell populations to determine if there are differences between *in vitro* and *ex vivo* generated moMac/moDP and moDC during GM-CSF driven differentiation. Thus, if there is any disparity in their functionality, it might be attributed to the effect of GM-CSF stimulation and not the inherent capacity of the cells.

There is evidence that suggests DC function as a double-edged sword, modulating a tolerogenic or an immunogenic T cell response against self and non-self antigens [35-37]. In addition, a recent study has shown the immunosuppressive activities of the early progenitor cells, CMP and GMP [15]. In this present study, CMP and GMP do not induce a potent T cell activation, which could explain their immune regulatory function. Also, the moMac/moDP and
moDC are strong inducer of T cell activation, but it is yet to be determined if moMac/moDP populations have any immune regulatory function. If moMac/moDP perform an immunoregulatory function, this population could be harnessed in treatment of immune dysfunction.

Moreover, infection or inflammatory signals induce phenotypic, morphological, and functional changes in DC. These changes include downregulation of endocytosis [2], upregulation in the expression of co-stimulatory molecules and MHC class II, and increased T cell stimulatory function [38]. Other study indicates that stimulation of CMP and GMP with PAMPs do not increase the levels of MHCII and CD86 expression, but an increase in the expression of these molecules was observed in moMac/moDP and moDC populations upon stimulation [16]. While the scope of this project is limited to understanding the functional attributes of these cell populations in steady state, it is important to explore how these functions change upon microbial stimulation. One would predict that microbial stimulations would likely decrease the uptake capacity of moMac/moDP and moDC populations, but enhance their already potent T cell stimulatory abilities. Additionally, how inflammatory signals affect the uptake of CMP and GMP also needs to be determined in the future. As mentioned earlier, activation of CMP and GMP through microbial stimulation could lead to differentiation of these cells to MDSC [17]. It is therefore important to examine the anti-inflammatory cytokines produced by these early progenitor cells in response to microbial stimuli.

In addition, previous results suggested that moDC have a high phagosomal pH that delay the ability L. monocytogenes to escape from the vacuolar, by inhibiting the activity of the pore forming listeriolysin O [27]. This present study provides information on how early progenitor cells respond to L. monocytogenes infection. Specifically, GMP and monocytes are more
resistant to bacterial intracellular replication possibly through the production of reactive oxygen and nitrogen species. It will be interesting to isolate infected and uninfected cells in each population and examine certain genes expression that could explain why some populations are more resistant to *L. monocytogenes* cytosolic invasion and replication.

Lastly, prolonged maintenance of cells in culture was shown to cause changes in cytokine gene expression that was time-dependent [39]. This was attributed to prolong feedback regulation provided by the cytokines in the culture media. In addition, study that examined the susceptibility of GM-CSF derived CD11+ DC and Flt3L derived conventional DC to *L. monocytogenes* infection observed that the during of time in which the cells were maintained in the cell culture correlate to how these cells support *L. monocytogenes* intracellular replication [40]. While all the cell populations used in this project were isolated on either day 3 or day 5 of cell culture, it is important to perform a time-dependent experiment to determine if the time of cell isolation influences the functional capacity of the cell.

### 5.4 Conclusion

While the remarkable features of DC have been extensively studied in fully differentiated DC, surprisingly little is known about when these functions develop before DC become terminally differentiated cell type. This study has now, for the first time, demystified the stages at which these functions develop in DC and DC progenitor cells. These findings could contribute greatly to DC biology and functions. In addition, the results from these findings could offer a targeted approach to isolate and utilize the most qualified cells along the developmental spectrum to perform specific functions.
References


Appendix

Differential expression of LC3 during GM-CSF driven differentiation. Bone marrow was harvested from C57BL/6 mice and was cultured in GM-CSF and stained with antibodies to Ly6C, CD115, CD11c, and LC3. (A) The MFI of LC3 expression in each population.